Perspective

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Neuronal Nicotinic Acetylcholine Receptors: Structural Revelations, Target Identifications, and Therapeutic Inspirations

Anders A. Jensen,* Bente Frølund, Tommy Liljefors, and Povl Krogsgaard-Larsen

Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark

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1. Introduction

The neurotransmitter acetylcholine (ACh) exerts its effects on the central nervous system (CNS) and peripheral nervous system (PNS) through two distinct types of receptors: the muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively). The five mAChR subtypes m1−m5 belong to the superfamily of G-protein-coupled receptors and mediate the slow metabolic responses to ACh via coupling to second messenger cascades, whereas the nAChRs are ligand-gated ion channels mediating the fast synaptic transmission of the neurotransmitter.

The nAChRs are involved in a wide range of physiological and pathophysiological processes. The muscle-type nAChR is localized postsynaptically at the neuromuscular junction, where it is a key mediator of the electrical transmission creating the skeletal muscle tone, and thus, it is the target of several clinically used muscle relaxants.[1] The numerous neuronal nAChR subtypes are located at presynaptic and postsynaptic densities in autonomic ganglia and in cholinergic neurons throughout the CNS, where they are involved in a number of processes connected to cognitive functions, learning and memory, arousal, reward, motor control, and analgesia.[2−6] Equally important to the overall contribution of nAChRs to cholinergic neurotransmission are the roles of presynaptic and preterminal nAChRs as autoreceptors and heteroreceptors regulating the synaptic release of ACh and other important neurotransmitters such as dopamine (DA), norepinephrine (NE), serotonin (5-hydroxytryptamine, 5-HT), glutamate (Glu), and γ-aminobutyric acid (GABA).[3,5,7] It is primarily because of their modulatory input to these neurotransmitter systems that neuronal nAChRs have been proposed as potential therapeutic targets for the treatment of pain, epilepsy, and a wide range of neurodegenerative and psychiatric disorders such as Alzheimer's disease, Parkinson's disease, Tourette's syndrome, schizophrenia, anxiety, and depression.[2−4,8−10] Furthermore, somatic mutations in nAChRs have been linked to certain forms of epilepsy and schizophrenia.[3,9] Finally, nAChR ligands have also been suggested for the treatment of drug addiction, and systemic nicotine administration is the predominantly used smoking cessation aid today.[2,4]

The heterogeneity of the native nAChR populations in the CNS presents major possibilities as well as challenges in terms of developing therapeutics targeted at these receptors. The fact that several important physiological processes appear to be regulated by a single or a few nAChR subtypes makes it possible to target specific functions without affecting other aspects of cholinergic neurotransmission. Furthermore, since the well-documented cardiovascular and gastrointestinal side effects of nicotine and other nonselective nAChR agonists as well as the potential addiction liability of these compounds also seem to be mediated by specific nAChR subtypes, pharmaceutical agents acting on distinct subpopulations of nAChRs might be devoid of these side effects. The perspectives of rational design of these agents are, however, faced with at least two

* To whom correspondence should be addressed. Phone: (+45) 3530 6491. Fax: (+45) 3530 6040. E-mail: aaj@dfuni.dk.

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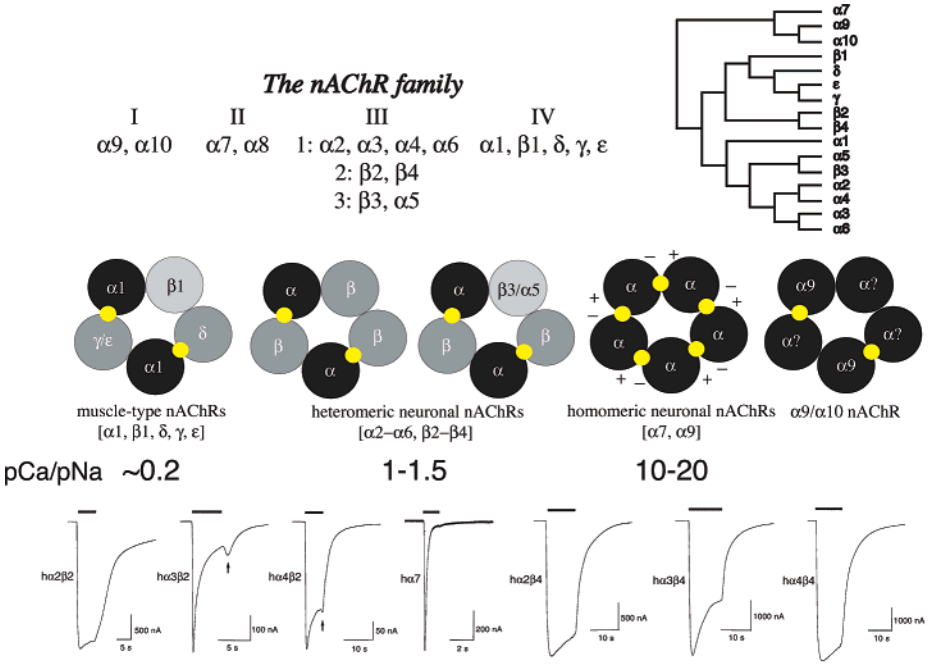

Figure 1. Diversity of human nAChR subtypes. Upper panel: The nAChR family with its four subfamilies of subunits and a cladogram of the human nAChR subunits. Middle panel: The various heteromeric and homomeric nAChR subtypes and their respective pCa/pNa ratios. Bottom panel: Electrophysiological traces recorded in *Xenopus* oocytes expressing human heteromeric α(2−4)/β(2,4) nAChRs and homomeric α7 nAChR upon application of maximal ACh concentrations. The oocyte traces are reproduced from a figure in *J. Pharmacol. Exp. Ther.* **1997**, *280*, 346−356, by permission from American Society for Pharmacology and Experimental Therapeutics, Copyright 1997.[17]

major obstacles. First, the design of subtype-selective nAChR ligands is complicated by the homologous nature of the receptor proteins, as especially the regions forming the orthosteric sites (the ACh binding sites) in the receptors are characterized by high degrees of amino acid sequence identities. Second, the identification of the exact molecular compositions of specific native nAChR subtypes modulating the synaptic release of different neurotransmitters in different CNS regions has been complicated by the staggering number of nAChR combinations that can be formed from the neuronal nAChR subunits.

A prerequisite for rational drug design is definition of one's target based on its physiological expression and function as well as a basic understanding of the molecular architecture of the particular protein. In this Perspective, the insights into neuronal nAChR structure and function obtained from recently published high-resolution X-ray structures of proteins pertinent to these receptors will be outlined, and the perspectives of rational design of nAChR selective ligands offered by these structures will be discussed. Furthermore, recent advances in the identification of native nAChR subtypes of potential therapeutic interest and examples of currently available subtype-selective nAChR ligands will be presented.

2. Cloned nAChRs

The nAChRs belong to a superfamily of ligand-gated ion channels (LGICs), the so-called "Cys-loop" receptors, which also include receptors for GABA, glycine, and 5-HT.[11−14] The LGICs are pentameric assemblies of subunits surrounding a central aqueous pore, and the receptors gate the flux of either the cations Na^+, K^+, and Ca^{2+} (nAChRs and 5-HT_3R) or anions such as Cl^- and HCO_3^- ($GABA_{A/C}$ and glycine receptors).

To date, 17 nAChR subunits have been cloned. The subunits have been divided into muscle-type (α1, β1, δ, γ, and ϵ) and neuronal (α2−α10 and β2−β4) subunits.[6] On the basis of gene structures and protein sequence similarities, the nAChR subunits have been grouped into four subfamilies (I−IV), with subfamily III further divided into three tribes (Figure 1). In contrast to the multiplicity at the gene level, the nAChR subunits do not exhibit the same degree of splice variation as other receptor families.

The muscle-type nAChR is the prototypic LGIC, which has been subjected to a substantial number of molecular pharmacology and structure−function studies. The receptor is composed of two α1 subunits and β1, δ, and γ (ϵ in the fetus) subunits organized in a clockwise α1γα1β1δ arrangement, and ACh binds to two orthosteric sites located at the α1−γ and α1−δ interfaces of the receptor complex (Figure 1). In contrast to the single muscle-type nAChR combination, the 12 neuronal nAChR subunits can form a plethora of different nAChR subtypes characterized by dramatically different characteristics in terms of ligand pharmacology, activation and desensitization kinetics, and cation permeability (Figure 1). The α(2−6) and β(2−4) subunits from subfamily III are involved in the formation of heteropentameric nAChR complexes. The prevalent subunit stoichiometry in the heteromeric nAChR is believed to be $(\alpha)_2(\beta)_3$, arranged as $\alpha\beta\alpha\beta\beta$ (Figure 1), although the formation of heteromeric nAChRs with $(\alpha)_3(\beta)_2$ and also other possible stoichiometries have been demonstrated in vitro.[15] ACh and other orthosteric ligands bind to a site positioned at the interface between

an α and a β subunit, thereby giving rise to two orthosteric sites in the heteromeric nAChR (Figure 1). When expressed in *Xenopus* oocytes or mammalian cell lines, the α2, α3, and α4 subunits have all been shown to form functional heteromeric receptors in combination with β2 or β4 subunits, whereas α6 is capable of forming a functional nAChR with β4 but not with β2.[6,16−20] The α subunits from tribe 1 of subfamily III can be paired into two groups based on their amino acid sequence similarities, α2/α4 and α3/α6 being highly homologous, and the tribe 2 β-subunits β2 and β4 are also quite homologous (Figure 1). Tribe 3 contains the "structural" subunits β3 and α5, which lack amino acid residues critical for agonist binding and thus cannot participate in the formation of binding sites. However, together with pairs of α(2−6) and β(2,4) subunits they can form more complex nAChRs, where they become the fifth subunit not involved in orthosteric ligand binding (Figure 1). The presence of either of these two subunits in a nAChR has been shown to have profound impact on its pharmacology, Ca^{2+} permeability, and desensitization kinetics.[21−23] Furthermore, the generally accepted notion that these complex nAChRs are formed by two pairs of α(2,3,4,6)/β(2,4) subunits and only one structural subunit has recently been challenged, as α6 and β4 subunits were shown to require coexpression of both α5 and β3 in order to obtain a functional nAChR, a putative (α6)2β4β3α5 subtype.[24] The "simple" heteromeric nAChR (1α/1β) is believed to be rare in native tissues, where most nAChRs are constituted by three, four, or five different subunits.[2,11]

The higher-order nAChR subunits α7, α8, and α9 form homopentameric receptor complexes characterized by dramatically larger Ca^{2+} permeabilities and faster desensitization rates than those of the muscle-type and heteromeric neuronal α/β nAChRs (Figure 1).[2] α7 has also been shown to be capable of forming heteromeric receptors with other nAChR subunits in vitro, and these receptors have displayed pharmacological properties significantly different from the pharmacological properties of the homomeric receptor.[25,26] However, although the currents elicited by α7-selective agonists in neuronal cultures can differ significantly from those observed at heterologously expressed homomeric α7 nAChRs, the existence of heteromeric α7-containing receptors in vivo has not been unequivocally proven.[2,25,26]

The α10 nAChR subunit is not able to form functional homomeric receptors, but it can form a functional nAChR together with α9. The exact stoichiometry and arrangement of this heteromeric α9/α10 nAChR is unknown (Figure 1). The α9 and α9/α10 nAChRs are characterized by mixed muscarinic/nicotinic pharmacological profiles significantly different from those of other nAChRs.[27,28] The α9 nAChR subunit is expressed in a wide range of tissues and organs, such as bone marrow, nasal epithelium, and embryonic blood cells, and it is coexpressed with α10 in cochlear outer hair cells and in a few ganglia.[27,28] Since α9 and α10 are not expressed in the CNS and since α8 exclusively is found in chicken, these subunits will not be discussed further in this Perspective.

3. Native Neuronal nAChRs

The identification of the molecular compositions and physiological functions of specific nAChR subtypes has for years been hampered by the heterologous nature of native nAChR populations and the lack of truly subtype-selective ligands. In the following, the physiological distribution of neuronal nAChR subunits in the CNS and the PNS will be outlined, and recent insights into the compositions and functions of native nAChR subtypes will be presented.

3.1. Distribution of Neuronal nAChRs. The expression levels of the nAChR subunits in the CNS are significantly lower than those of mAChRs.[29,30] The predominant nAChR subunits in the CNS are α4, β2, and α7, whereas α3 and β4 are the prevalent subunits in the periphery.[3] Hence, the majority of nAChRs in the CNS (∼90%) are α4β2* receptors characterized by high-affinity ACh binding, whereas the low-affinity ACh binding α7* nAChR is the other major CNS subtype. Historically, the two subtypes have been distinguished in binding assays using radiolabeled nicotine, cytisine, or epibatidine for α4β2* nAChRs and [^{125}I]bungarotoxin (a snake toxin) for α7* receptors.[2,4,11] The asterisks used in the receptor nomenclature indicate the possibility of additional neuronal nAChR subunits being present in the receptor complexes.

The nAChR subunits predominantly expressed in various CNS regions are depicted in Figure 2. It is stressed that expression levels and patterns determined for receptors in different studies often vary depending on the techniques used. Furthermore, nAChR subunits highly expressed in substructures within a particular region are not indicated in this map. Finally, species differences have been observed in the distribution patterns of nAChR subunits in the rodent and the mammalian CNS.[3,31,32] Hence, the map should only be seen as a crude representation of nAChR subunit expression in the CNS. The β2 subunit is expressed in almost all CNS regions, where its distribution overlaps with at least one of the α(2−4,6) subunits.[29,30] Although also abundantly expressed, α4 is less broadly distributed in the CNS than β2, with which α4 is colocalized in most regions. The highest concentrations of the two subunits are found in the hippocampus, thalamus, and cortex.[29,30,33] The α7 subunit is also expressed in the majority of the brain with highest expression levels in cortex and hippocampus, whereas the subunit is absent or expressed at low levels in thalamic regions and in the basal ganglia.[5,30,33]

The distribution of the other nAChR subunits in the CNS is much more limited. In addition to their wide expression in the PNS, α3 and β4 are localized in CNS regions such as the medial habenula, the dorsal habenula, the interpeduncular nucleus, and the locus coeruleus. The expression levels are significantly lower in cortical and hippocampal regions, and the two subunits are virtually absent from parts of the midbrain. The two subunits are colocalized in many but not all of these regions. For example, α3 is highly expressed in several thalamic regions, where no or low levels of β4 are found (Figure 2).[5,30,33] The expression of the α2 nAChR has not been studied in great detail, but this subunit appears to be expressed in very few brain regions with the highest expression in the interpeduncular nucleus, where it is believed to form a α2β4* nAChR.[29,33] The distribution of subunits α6 and β3 in the CNS is also very limited. The two subunits are highly colocalized

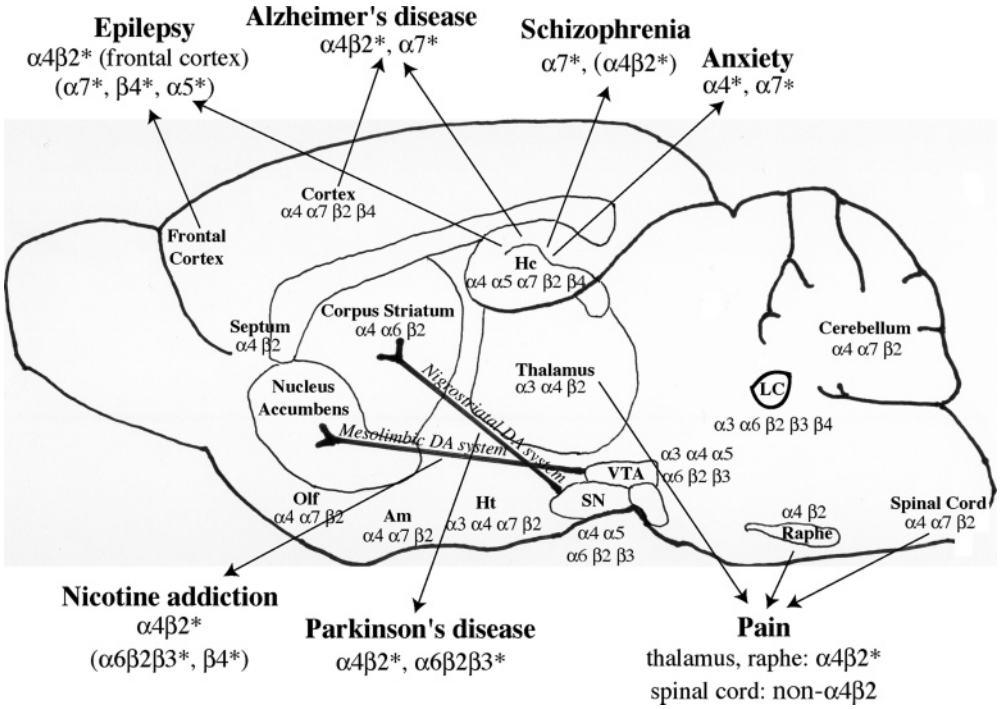

Figure 2. Distribution of nAChR subunits in the rodent brain. The nAChR subunits predominantly expressed in selected CNS regions are shown, and the nAChRs proposed as potential therapeutic targets in various disorders are indicated: Hc, hippocampus; Ht, hypothalamus; VTA, ventral tegmental area; SN, substantia nigra; Olf, olfactory region; Am, amygdala; LC, locus coeruleus. The figure is reprinted in part from *Pharmacology & Therapeutics*, Vol. 92, Picciotto et al., "Neuronal nicotinic acetylcholine receptor subunit knockout mice: physiological and behavioral phenotypes and possible clinical implications", pp 89−108, Copyright (2001), with permission from Elsevier.[33]

Table 1. nAChRs as Autoreceptors or Heteroreceptors: Selected nAChR Subtypes Involved in the Modulation of the Synaptic Release of ACh and Other Neurotransmitters in Various Rat CNS Regions[a]

	ACh	DA	NE	5-HT	GABA	Glu
cerebellum						α7*
hippocampus	α4β2*/αxβ4*		α3β4*, α6β2β3*	X	α3β2*,α4β2*, α7*	α7*
cortex	α4β2*/αxβ4*		α3β2*/(α4)α6β2β3*	X		α7*
olfactory bulb						α7*
striatum		α4β2*, α6β2β3*			α4β2*	α7*
thalamus		α4β2*			α4β2*	
IPN						
dorsal raphe nucleus	α3β4*					
spinal cord				non-α4β2, non-α7	αxβx*, α7*	α7*

[a] "X" indicates that the neurotransmitter release is under nAChR modulation by a not yet identified subtype. The table is primarily based on ref 5. IPN: interpeduncular nucleus.

and are found at high levels in catecholaminergic regions, such as the substantia nigra and the ventral tegmental area, and in locus coeruleus, interpeduncular nucleus, and medial habenula (Figure 2).[19,33,34] The α5 subunit is also found in relatively few CNS regions, displaying the highest expression levels in the substantia nigra, the ventral tegmental area, the medial habenula, and certain cortical regions.[30, 33]

In the autonomic ganglia, the predominant subunits α3 and β4 exhibit a substantial degree of colocalization with α5 and β2 subunits, and thus, the peripheral α3β4* nAChR appears to be a heterologous population of α3β4, α3β4α5, α3β2β4, and α3β2β4α5 subtypes, α3β4α5 being the major subtype.[30,35,36] α7 is also abundantly expressed in the ganglia, and thus, α7* receptors constitute another major ganglionic nAChR.[30]

3.2. Physiological Functions of Native nAChRs. Presynaptic and preterminal nAChRs modulate the release of ACh and several other important neurotransmitters throughout the CNS.[5,7] Stimulation of the presynaptic nAChR induces the influx of Ca^{2+} into the presynaptic terminal via voltage-dependent Ca^{2+} channels or via direct cation influx through the receptors, and this intracellular Ca^{2+} subsequently drives the neurotransmitter release. As can be seen from Table 1, the synaptic release of a particular neurotransmitter can be regulated by different nAChR subtypes in different CNS regions, with the notable exception of α7*, which appears to be the universal nAChR for regulation of glutamatergic neurotransmission. Because of the complexity of neuronal networks, several examples exist of neurotransmitters being released through the actions of nAChRs not actually located on the particular neuron.

Although most of the nAChRs in the CNS are found on presynaptic and preterminal densities, the presence of postsynaptic α7*, α4β2*, and α3β4* nAChRs has also been demonstrated in several regions, and other heteromeric nAChRs may function as postsynaptic receptors as well.[5] In addition to the acute effects of the

nAChR-mediated depolarization of the postsynaptic neuron, nAChR signaling also seems to have long-term effects on metabolic pathways and gene expression. The influx of Ca^{2+} into the neuron upon nAChR activation, either through the nAChRs or through the opening of voltage-gated calcium channels, stimulates Ca^{2+}-dependent kinases such as protein kinase C and MAPK/MEK kinases.[5,33] Still, it is as presynaptic/preterminal auto- and heteroreceptors that the nAChRs are believed to be of most significant physiological importance.

3.3. Identification of Native nAChR Subtypes. In 1998, Changeux and co-workers identified four general classes of CNS nAChR subtypes based on electrophysiological recording on neurons from wild-type and $\beta2$ knock-out mice: I ($\alpha7$* nAChRs), II ($\beta2$ nAChRs, predominantly $\alpha4\beta2$*), III ($\alpha3\beta4$* nAChRs), and IV (other $\beta4$ nAChRs).[37] Subsequently, the molecular compositions of several regional nAChR subtypes have been disclosed in even greater detail in studies combining single-cell reverse transcription-polymerase chain reaction (RT-PCR) techniques, patch clamp recordings, nAChR knock-out mice, and the few subtype-selective ligands available, including a couple of α-conotoxins (section 5.4).

The colocalization of multiple nAChR subunits throughout the CNS extends to the individual neuron. However, although a neuron may contain transcripts for most of the $\alpha2-\alpha7$ and $\beta2-\beta4$ subunits and thus has the potential to express a considerable number of distinct nAChRs, a single or a few receptor combinations are normally preferred.[35,36] Different subsets of neurons within a small, defined CNS region often express entirely different nAChRs, indicating that specific nAChR combination(s) expressed by the individual neuron is tightly controlled by its transcription/translation machinery. These interneuronal differences in nAChR expression appear to be important for the cholinergic control of the respective neurotransmitter systems and a key component of the sophisticated orchestration of the physiological nicotinergic tone.

The identification of the nAChR subtypes modulating the synaptic release of DA in striatum is the most notable example of coupling of distinct nAChR subtypes to a specific physiological function. The impressive deductive reasoning underlying this multistep identification process is summarized in Figure 3A. In situ hybridization and single-cell RT-PCR experiments had demonstrated the presence of $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, $\beta2$, $\beta3$, and $\beta4$ subunits in the dopaminergic midbrain,[38] and the striatal DA release elicited by nicotine was shown to be composed of two components: one sensitive to the $\alpha3\beta2$*/$\alpha6\beta2$* nAChR-selective α-conotoxin MII (~30% of the DA release) and one insensitive to the toxin (~70%).[39,40] $\alpha7$* and $\alpha3\beta4$* nAChRs were demonstrated not to be directly involved in the modulation, since the nAChR-mediated DA release was not inhibited by α-conotoxins ImI or AuIB, selective antagonists of $\alpha7$* or $\alpha3\beta4$* nAChRs, respectively.[39,41] In an elegant study, the DA release elicited by (±)-UB-165, a very weak partial agonist of $\alpha4\beta2$ nAChRs displaying substantially higher efficacies at other nAChRs, was shown to be eliminated almost completely by α-conotoxin MII, indicating that $\alpha4\beta2$* is the subtype responsible for the MII-insensitive component.[42] Since [^{125}I]-α-conotoxin MII binding sites throughout the CNS, including the basal ganglia, were eliminated in $\alpha6$ knock-out mice and not in $\alpha3$ knock-out mice, the MII-sensitive component was attributed to a $\alpha6\beta2$* nAChR.[43,44] [^{125}I]-MII binding and the MII-sensitive DA release component were dramatically reduced in $\beta3$ nAChR knock-out mice, which indicated the presence of this subunit in the $\alpha6\beta2$* nAChRs.[34] This finding was in good agreement with the general colocalization of $\alpha6$ and $\beta3$ in the CNS and the required coexpression of $\beta3$ in order to get functional $\alpha6$* nAChRs in vitro.[19,24] In recent studies using several different knock-out mice, the $\alpha4\beta2$* nAChRs involved in DA release have been further subdivided into $\alpha4\beta2$ and $\alpha4\beta2\alpha5$ subtypes and the $\alpha6\beta2\beta3$* nAChRs into $\alpha6\beta2\beta3$ and $\alpha4\alpha6\beta2\beta3$ subtypes (Figure 3A).[45,46] Also, the distribution of the different nAChR subtypes at the level of the individual neuron has been investigated. $\alpha6\beta2\beta3/\alpha4\alpha6\beta2\beta3$ nAChRs and $\alpha4\beta2/\alpha4\beta2\alpha5$ nAChRs

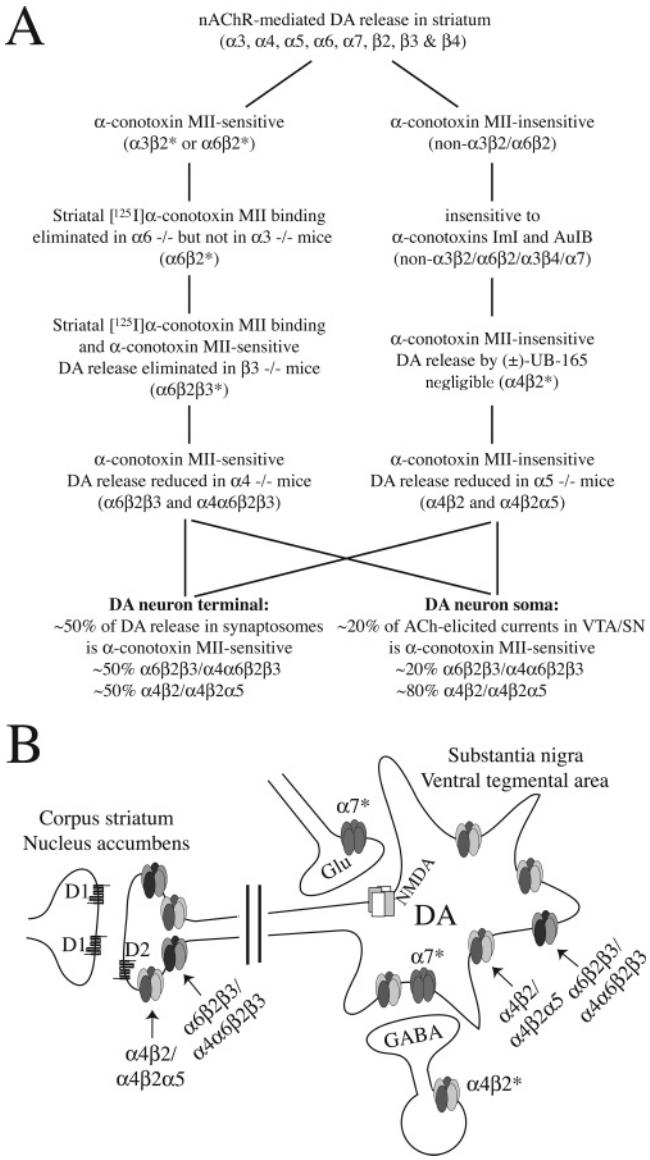

Figure 3. Native nAChRs modulating DA release in the rodent striatum: (A) stepwise identification of native nAChRs involved in striatal DA release; (B) DA neurons in the mesolimbic and nigrostriatal pathways and the nAChRs modulating striatal DA release directly and indirectly. Part B is reprinted in part with permission from *J. Neurosci.* **2003**, *23*, 7820−7829.[46] Copyright 2003 Society for Neuroscience.

located on the neuron terminal have been proposed to contribute equally to the nAChR-mediated DA release, whereas α4β2/α4β2α5 seem to be the major nAChR subtypes in the somato-dendritic part of the neuron (Figure 3).[46] In addition to the direct modulation of DA release exerted by nAChR agonists through these presynaptic and preterminal nAChRs, activation of α7* nAChRs on glutamatergic terminals has been shown to elicit release of Glu, which in turn stimulates ionotropic Glu receptors on dopaminergic terminals and causes striatal DA release (Figure 3B).[46,47] Furthermore, desensitization of a α4β2* nAChR located on GABAergic interneurons has been proposed to reduce the GABA-mediated inhibition of DA release, thereby indirectly eliciting DA release (Figure 3B).[46,48]

As if such investigations into nAChR-mediated regulation of neurotransmitter release in various CNS regions were not complex enough, extrapolation from studies of rodents to the human brain can be further complicated by species differences in nAChR expression patterns. For example, the relatively higher expression of β4 in the striatum of primates compared to rodents and the inefficient assembly of human α6β2β3 nAChRs in vitro suggest that β4 may in fact replace β2 in α6* receptors in the mammalian striatum.[19,24,31] Despite such uncertainties, the continuous identification of native nAChR subtypes in rodents remains crucial for a better understanding of the complex nicotinergic neurotransmission.

3.4. Therapeutic Prospects of Neuronal nAChRs. Lessons from Knock-Out and Knock-In Mice. An in-depth review of the etiologies and pathomechanisms of the disorders in which neuronal nAChRs have been proposed as therapeutic targets is beyond the scope of this Perspective, and thus, the reader is referred to recent reviews.[2−5,8−10] The nAChR subtypes currently considered being of greatest therapeutic interest in some of these disorders are given in Figure 2. It is hardly surprising that α4β2* and α7* nAChRs dominate the figure, considering the abundance of these two subtypes throughout the CNS. However, the lack of truly subtype-selective nAChR ligands capable of penetrating the blood−brain barrier (BBB) has complicated investigations of the physiological roles of specific nAChR subtypes, in particular those of the minor CNS subtypes. In recent years, studies of knock-out mice lacking α3, α4, α5, α6, α7, β2, β3, or β4 nAChR subunits and knock-in mice expressing hypersensitive α4 or α7 mutants have provided important insights into the physiological roles played by the different nAChR subtypes and their therapeutic potentials (Table 2).[33,49]

3.4.1. α3, β4, and α5. The most dramatic phenotype in all of the nAChR knock-out mice has been observed in the α3 −/− mouse. An amount of 40% of these mice died within 1 week after birth, and the surviving mice were severely growth impaired and displayed massive bladder dysfunction and lack of pupil contraction in response to light.[50] This phenotype was ascribed solely to the essential role of the α3β4* nAChR for the autonomic control of peripheral organs, since none of the deficiencies could be accredited to a CNS α3* subtype. In contrast, a β4 −/− mouse did not display any visible phenotypic abnormalities, although some dysfunctions in ganglionic transmission were observed.[51,52] The double knock-out mouse β2 −/− β4 −/−, on the other hand, displayed a phenotype very similar to that of α3 −/−, and consequently, it was proposed that the concurrent expression of the two β subunits in the PNS enabled β2 to compensate for the loss of β4 in the β4 −/− mouse (Table 2).[51] Interestingly, α5 −/− and in particular β4 −/− mice have been shown to be significantly more resistant to nicotine-induced seizures than wild-type mice (a phenotype even more pronounced in α5/β4 double knock-out mouse), and the two subunits were consequently proposed as candidate genes for epilepsy (Figure 2).[53,54] Finally, the β4 −/− mouse displayed decreased somatic signs of mecamylamine-induced nicotine withdrawal such as jumping, leg tremors, and cage scratching compared to wild-type mice, suggesting a role for the subunit in the creation of nicotine withdrawal symptoms.[55]

3.4.2. α4 and β2. The complete absence of the major CNS nAChR does not seem to lead to dramatic dysfunctions in the mouse. The β2 knock-out mouse has been reported to be viable without any apparent physical deficits, and although deletion of the α4 gene in two different mouse strains have resulted in somewhat different phenotypes, both have been relativity mild (Table 2).[56,57] In contrast to its positive effects on cognition in the wild-type mice, nicotine did not enhance the performance of the β2 −/− mouse in a passive avoidance test.[58] The importance of the subunit for cognitive functions was supported further by the significant degree of neurodegeneration of cortical and hippocampal structures observed in the aging β2 −/− mouse.[59] This degeneration was accompanied by significant deficits in spatial learning in the 2-year-old mouse compared to the 1-year-old mouse. The link between the neurodegeneration associated with Alzheimer's disease and α4β2* nAChRs is well-documented, and substantial reductions in α4β2* nAChR binding sites and in α4 and β2 mRNA levels have been observed in the postmortem Alzheimer brain.[2,3] Thus, on the basis of its anatomical and behavioral characteristics, the aging β2 −/− mouse was proposed to be useful as a model in studies of Alzheimer's disease and dementia.[33,59] Analogous studies of the aging α4 −/− mouse have not been published, but it will be important to clarify whether the cognitive dysfunctions in the β2 knock-out mouse can be ascribed solely to the lack of α4β2* receptors.

The deletion of either the β2 or the α4 gene have resulted in significant decreases in the antinociceptive effects of nicotine administration (Table 2).[56] Whereas the currents elicited by nicotine in raphe magnus and thalamus, two regions known to be important for the nAChR-mediated antinociceptive effects, were eliminated or significantly reduced in α4 −/− and β2 −/− mice compared to the wild-type animal, the nicotine-mediated augmentation of postsynaptic currents in neurons from the dorsal horn of the spinal cord did not differ significantly between mutant and wild-type mice.[56] Although the physiological mechanisms underlying the analgesic properties of nonselective nAChR agonists such as nicotine and epibatidine are not well understood,[60] α4β2* nAChRs in the raphe magnus and in the thalamus were proposed to be essential mediators of the nicotinic antinociception pathway, albeit with signifi-

Table 2. nAChR Knock-Out and Knock-In Mice: Pharmacological/Anatomical Abnormalities and Behavioral Phenotypes Observed in Knock-Out Mice Lacking Specific nAChR Subunits and Knock-In Mice Expressing Hypersensitive Mutants of Certain Subunits[a]

KO	pharmacological and anatomical abnormalities	behavioral phenotype
α7[b]	Complete loss of [^{125}I]-α-bungarotoxin binding Loss of fast nAChR currents in hippocampus Normal baseline responses to nicotine Normal growth, viability, and neuroanatomy)	No apparent abnormalities
L250T −α7[c]	Homozygous: Apoptotic cell death in somatosens cortex Heterozygous: Normal growth, anatomy, and general appearance Heterozygous: More slowly desensitizing α7 currents in hippocampal neurons compared to WT neurons	Homozygous: Lethal (1 day postnatal)
*β*2[d]	Loss of high-affinity [^{3}H]nicotine binding sites Loss of agonist currents in thalamus and striatum Loss of nicotine-mediated striatal DA release Anatomical and functional deficits in the visual system Changes in cortical structures in aged mice (2 years old)	Loss of effect of nicotine in a passive avoidance test (for associated memory) Significantly reduced self-adminstration of nicotine Reduced analgesic effects of nicotine Impairments in spatial memory in aged mice (2 years old)
α4[e]	Loss of [^{125}I]epibatidine binding sites Loss of nicotine responses in thalamus and raphe magnus Reduced agonist currents in SNc and VTA Twice as high basal DA levels in striatum as in WT Loss of nicotine-mediated striatal DA release	Reduced analgesic effects of nicotine No difference in baseline locomotor activity compared to WT Faster recovery from nicotine-mediated depression of locomotor activity Longer lasting increased locomotor activity upon cocaine admistration
α4[f]	Loss of high-affinity agonist binding sites	Increased spontaneous and nicotine-induced locomotor activity Increased basal level of anxiety in a novel environment
L9′S[g] −α4	Heterozygous: Severe loss of DA neurons in SN Heterozygous (neo): Loss of DA neurons in SN	Heterozygous: Lethal (1 day post-natal) Heterozygous (neo): Locomotor activity extremely sensitive to nicotine Heterozygous (neo): Higher baseline level of anxiety Heterozygous (neo): Increased sensitivity to nicotine-induced seizures
L9′A[h] −α4	No gross developmental abnormalities Hypersensitivity to nicotine in ventral midbrain cultures Functional up-regulation of nAChRs by 10 nM nicotine	Reinforcement response to 10 *μ*g/kg nicotine (conditioned place preference) Development of tolerance at several doses (nicotine-induced hypothermia) Sensitization: increasing locomotor activity to daily administration of nicotine (15 *μ*g/kg)
α3[i]	Retarded growth, dilated ocular pupils, mydriasis Lack of bladder contractility, enlarged bladder, megacystis	Lethal 1 week postnatal
*β*4[j,n]	Reduced nicotine currents in superior cervical ganglion Lack of bladder contractility Abnormalities in bladder tissue (histochemical analysis) Reduced ileum contractile responses to nAChR agonists Impaired heart rate response to cervical vagal stimulation	Resistant to nicotine-induced seizures Decreased symptoms of mecamylamine-induced nicotine withdrawal
*β*2/*β*4[j]	Retarded growth, dilated ocular pupils Absence of ACh currents in superior cervical ganglion Lack of bladder contractility, enlarged bladder Abnormalities in bladder tissue (histochemical analysis)	Lethal 1−3 weeks postnatal
α6[k]	Limited loss of high-affinity agonist binding sites Complete loss of striatal [^{125}I]-α-conotoxin MII binding	No apparent abnormalities
*β*3[l]	Loss of striatal [^{125}I]-α-conotoxin MII binding Striatal α-conotoxin MII-sensitive DA release eliminated	Increased locomotor activity Decreased prepulse inhibition of acoustic startle response
α5[m,n]	Impaired heart rate response to cervical vagal stimulation	Increased resistance to nicotine-induced seizures
α5/*β*4[n]		Highly resistant to nicotine-induced seizures

[a] Unless otherwise indicated, the observations are from studies of homozygous mice. [b] Reference 67. [c] Reference 68. [d] Reference 56, 58, 59, 65, and 262. [e] References 38, 56, and 263. [f] Reference 57. [g] References 61−63. [h] Reference 64. [i] Reference 50. [j] Reference 51, 52, and 55. [k] Reference 34. [l] Reference 43. [m] Reference 53. [n] Reference 54.

cant contributions from spinal non-α4β2 receptors (Figure 2).[33,56]

In knock-in mice expressing the hypersensitive α4 mutants L9′S or L9′A (where the "resting gate" Leu residue has been replaced by a Ser or an Ala residue, respectively (see section 4.1)), α4* nAChRs can be selectively activated at agonist concentrations not affecting other nAChRs. The phenotype of the heterozygous L9′S α4 mouse was lethal, and the massive atrophy of DA neurons in substantia nigra observed in the mouse was accredited to the chronic DA release in this region elicited by choline in the prenatal mouse (Table 2).[61] In a heterozygous L9′S α4 mouse with an intact neo cassette, the mutant α4 subunit was expressed at lower levels, and this mouse was viable and did not deviate significantly from its wild-type litter mates in size or general appearance.[62] Still, a significant loss of DA neurons in the substantia nigra (but not in the ventral tegmental area) and reduced striatal dopaminergic innervations were observed in this mouse as well.[63] Furthermore, the locomotor activity of the mutant mouse was significantly reduced upon administration of nicotine in concentrations not affecting locomotion in wild-type animals, and nicotine-induced seizures and increased EEG amplitude and θ rhythm in the mutant mouse occurred at 8-fold lower doses compared with wild-type mice (Table 2).[61,62] This epileptic phenotype was reminiscent of the seizures observed in autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), a rare form of epilepsy caused by genetic mutations in the M2 domains of α4 and β2, which originates from the frontal cortex and is characterized by frequent brief seizures occurring during light sleep.[62]

Both heterozygous and homozygous mice expressing the L9′A α4 mutant have recently been reported to be viable and not to exhibit any apparent physical deficits and thus seem to be better suited for studies of α4* nAChR signaling.[64] In a study of these mice, α4* nAChRs have been shown to be sufficient for the nicotine-induced reinforcement and reward behavior, tolerance, and sensitization, all important determinants in the development and maintenance of dependence (Table 2).[64] In agreement with the disclosed composition of striatal nAChRs in rodents (Figure 3), nicotine has been found to be completely incapable of evoking DA release in the mesolimbic DA system in the β2 −/− mouse, and self-adminstration of nicotine was significantly attenuated in β2 −/− mice compared to wild-type mice.[65] In contrast to the decreased nicotine withdrawal symptoms observed in β4 −/− mice, however, the β2 −/− mice have been reported to undergo nicotine withdrawal not significantly different from that observed for wild-type mice.[55] Hence, although β4* nAChRs appear to be important mediators of the negative-reinforcing properties of nicotine, a substantial amount of evidence still unequivocally pinpoints α4β2* as the principal nAChR for nicotine dependence and the primary target for nAChR-based smoking cessation aids.

Surprisingly, both mice with hypersensitized α4* nAChRs (L9′S) and mice lacking the subunit (α4 −/−) have been reported to be more anxious than wild-type mice (Table 2).[57,61] In contrast, the β2 −/− mice have not displayed altered anxiety baseline levels in any of the numerous model systems they have been tested in.[33,66] Thus, α4* nAChRs appear to be obvious candidates when it comes to the anxiolytic activity of nAChR agonists, whereas it remains to be seen whether β2 is present in these receptors.

3.4.3. α6 and β3. As mentioned above, the deletion of the β3 nAChR gene has been shown to eliminate [^{125}I]-α-conotoxin MII binding to the striatum almost completely, indicating that the structural subunit is an essential component of the α6* nAChR and that it apparently cannot be replaced by another nAChR subunit in the knock-out mouse.[34] The decreased prepulse inhibition of the acoustic startle response and the increased locomotion observed in the β3 −/− mouse were proposed to arise from elimination of the α-conotoxin MII-sensitive component of the striatal DA release, although the possibility that other mechanisms could cause the phenotype was not ruled out.[34] However, in addition to their normal general appearance, growth, and anatomy, the α6 −/− mice also displayed normal locomotor behavior.[43]

3.4.4. α7. The absence of apparent deficiencies in a α7 knock-out mouse can only be described as surprising, considering the abundance of the subunit in the CNS and the proposed importance of α7* nAChRs for brain development and for the adult brain.[67] The knock-out mouse has been subjected to a battery of behavioral tests, but the results have not been particularly striking. Analogous to the L9′S and L9′A α4 knock-in mice, a mouse expressing the Leu^{250}Thr α7 mutant (a Thr mutation of the "resting gate" Leu residue (section 4.1)) has been created.[68] The homozygous knock-in mouse died within hours after birth, and substantial apoptosis was observed in the somatosensory cortex, which was ascribed to the increased Ca^{2+} influx into the neurons through this nondesensitizing α7 mutant. Although electrophysiological recordings on cultured hippocampal neurons from the heterozygous Leu^{250}Thr α7 mouse revealed a more slowly desensitizing component of the agonist-elicited currents compared to wild-type mice neurons, the mixed population of various WT/Leu^{250}Thr-α7 nAChR combinations did not result in any apparent behavioral dysfunctions (Table 2).[68]

In conclusion, the results from the studies of nAChR knock-out and knock-in mice seem to support the therapeutic potential of nAChRs as targets in the disorders given in Figure 2. However, considering the important neurotransmitter systems under nicotinergic control, the behavioral phenotypes of these mice have in general been surprisingly moderate (Table 2). As always, when the results from studies of constitutive gene knock-outs are interpreted, the possible existence of compensatory mechanisms should be considered because these could mask the "true" phenotypes of the mice. One way to address the issue of developmental compensation, to circumvent the lethal phenotype of the α3 −/− mouse, and to study the role of nAChR signaling in discrete CNS regions could be the development of conditional nAChR alleles, which would enable spatial and time-specific deletions of the receptor genes.[69] Future studies of conditional knock-out mice and additional knock-in mice expressing nAChR mutants with altered properties in terms of trafficking, signaling, and regulation will hopefully shed more light on the physiological roles of the respective nAChRs.

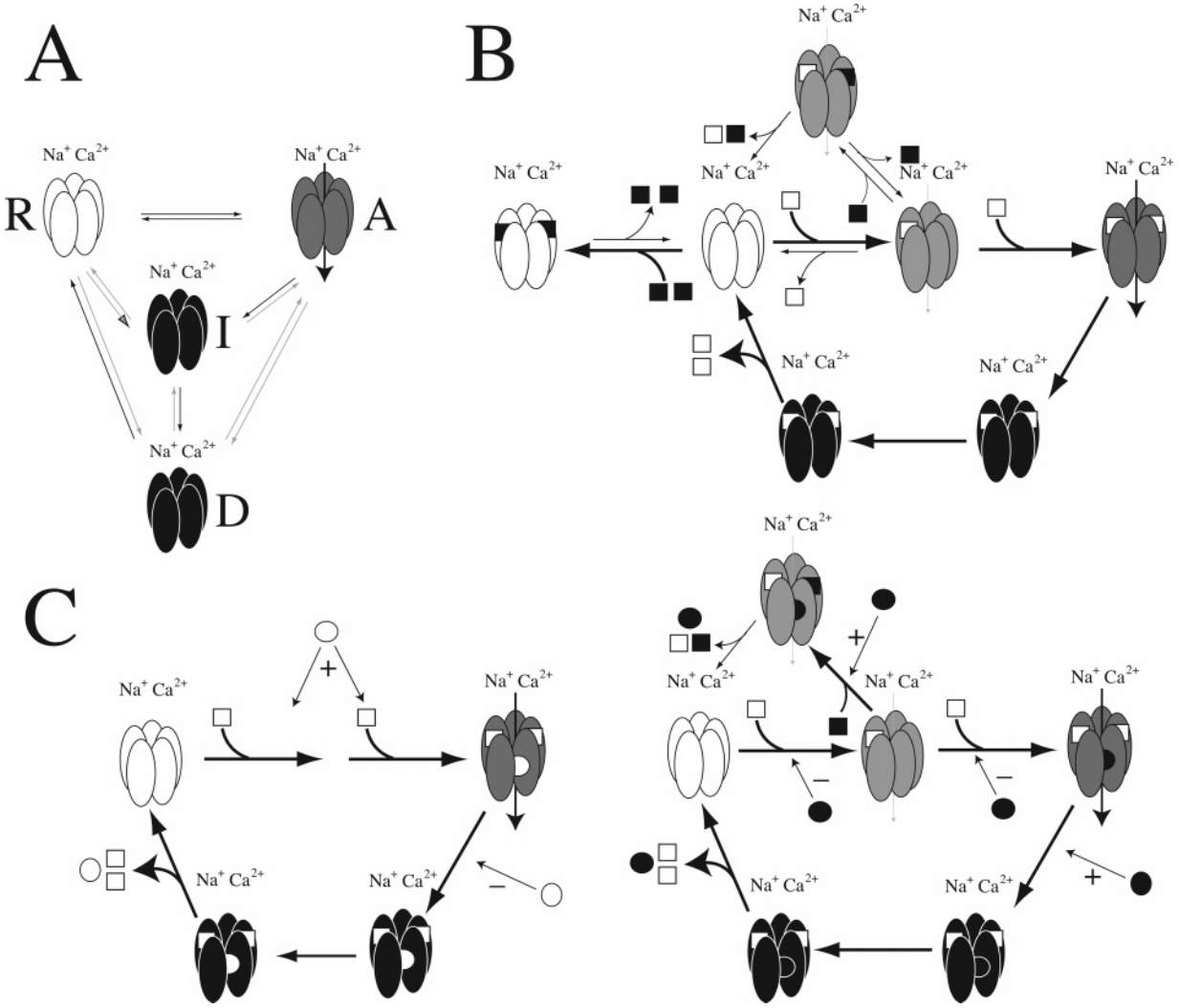

Figure 4. Allosteric nature of nAChR function. (A) The MWC model. Allosteric transitions between the resting (R, white complex), active (A, dark-gray complex), and fast-onset (I) and slow-onset (D) desensitized states (black complexes) of the nAChR. The predominant pathways in the model are given in black. (B) Allosteric transitions of the heteromeric nAChR in the presence of orthosteric ligands. The agonist and competitive antagonist are given as an open and a filled square, respectively. The coloring of the nAChR complexes is as in part A, with the addition of an intermediate active state with one agonist bound (light-gray). (C) Actions of an allosteric potentiator (open circle) and an allosteric inhibitor (filled circle) on the allosteric transitions of the heteromeric nAChR (left and right of the panel, respectively).

4. Structure and Function of the nAChR

In recent years, crystal structures of a mollusk ACh binding protein[70,71] and cryoelectron microscopy images of the *Torpedo* nAChR[72] have provided atomic-scale or near-atomic-scale models of the two domains constituting the nAChR. Combined with the results of the numerous biochemical studies of nAChRs and other LGICs performed over the years, these structures offer a unique insight into the molecular basis for the signaling of nAChRs and other Cys-loop LGICs.

The nAChR is an allosteric protein complex in that it is composed of multiple subunits and contains multiple orthosteric sites and allosteric sites through which its function can be modulated. In 1965, the Monod−Wyman−Changeux (MWC) model was formulated to describe the allosteric nature of nAChR signaling.[73,74] According to this model, the nAChR fluctuates between at least three functional states in the absence of agonist: a resting state (R), an active state (A), and a desensitized state (often divided into a fast-onset state I and a slow-onset state D) (Figure 4A). The equilibrium between two states is determined by the differences in the free energy of the states. The kinetic rate for the transition from one state to another is determined by the energy barrier between the two states, activation being a rapid transient process and desensitization occurring slowly (microsecond-to-millisecond and millisecond-to-second ranges, respectively). The rates of the activation and desensitization processes vary greatly among different nAChR subtypes, which contributes to the pharmacological diversity of this receptor class (Figure 1). Once the receptor has reached its desensitized state, it is believed to return to the resting state via the active state, a fast transition that does not elicit a "second" ion channel opening (Figure 4A).[74]

Binding of an agonist or a competitive antagonist to the orthosteric sites of the nAChR stabilizes its active and resting states, respectively (Figure 4B). Agonists are characterized by having higher affinity for the active state than for the resting state, and conversely antagonists have higher affinities for resting/inactive receptor states. Finally, high-affinity binding and very-high-

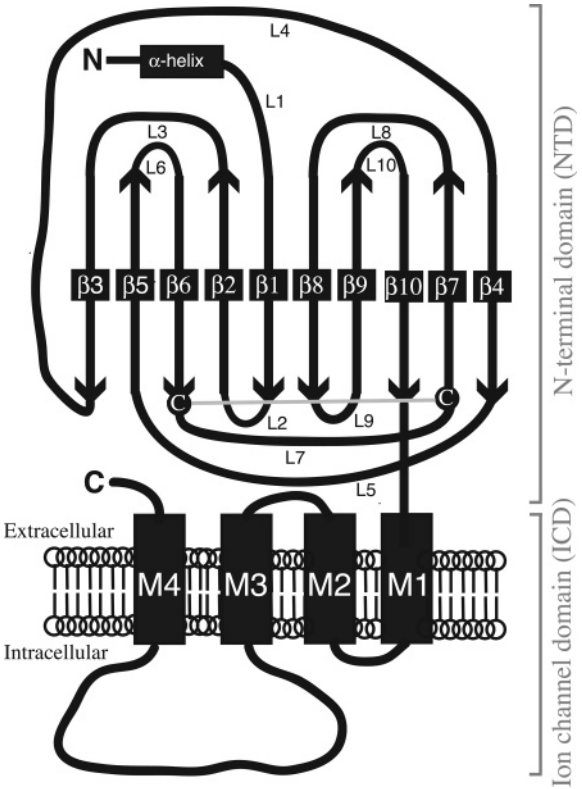

Figure 5. Topology of the nAChR subunit. The two regions involved in the formation of the NTD and ICD of the pentameric nAChR are indicated. The 10 β-strands and the loops between in the N-terminal domain are numbered according to the AChBP X-ray structure.[70] The disulfide bond between two cysteines in creating the hallmark "Cys-loop" between β6 and β7 is shown in gray.

affinity binding properties characterize the fast-onset (I) and slow-onset desensitization states (D) of the receptor, respectively. In concordance with the MWC model, spontaneous (constitutive) activity of nAChRs in the absence of agonist has been demonstrated.[75] However, the probability of channel opening increases upon agonist binding to one of the two orthosteric sites of the heteromeric nAChR, and it is further dramatically increased by agonist occupation of both binding sites (Figure 4B).[74] Binding of ligands to "allosteric" sites located at some distance from the orthosteric sites of the receptor complex can also modulate nAChR signaling via an effect on the equilibrium between the resting and active receptor states or on the desensitization kinetics (Figure 4C). The increasingly more sophisticated and sensitive electrophysiological recording techniques applied in studies of nAChR function have prompted the refinement of the MWC model into more complex models with additional functional states.[74] In the following, however, a basic understanding of the equilibria among resting, active, and desensitized nAChR states as outlined in Figure 4 will suffice.

The topology of the nAChR subunit is shown in Figure 5. The subunit is 500−600 amino acid residues long and can be divided into two regions: an extracellular N-terminal domain of ~210 amino acids and a transmembrane domain composed of 4 transmembrane α-helical segments of ~20 residues, M1−M4, separated by alternating intracellular and extracellular loops and a short extracellular carboxy terminal (Figure 5). Assembly of these two regions of the five subunits creates the N-terminal domain (NTD) and ion channel domain (ICD) of the pentameric nAChR complex.

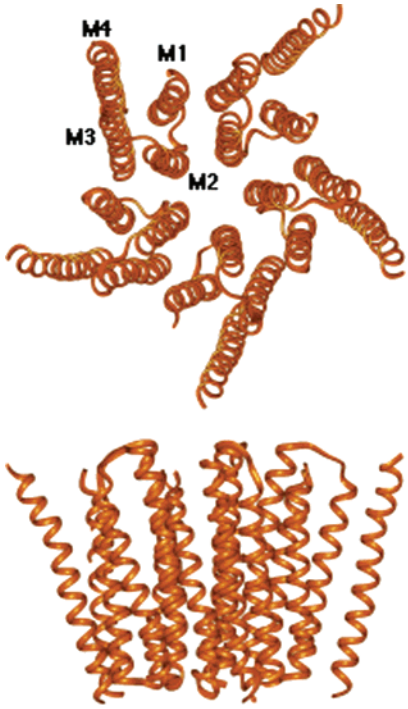

Figure 6. Atomic model of the ICD of the *Torpedo* nAChR (4 Å).[72] The ICD is viewed from the extracellular side (upper panel) and from the side (bottom panel). The four transmembrane α-helices M1−M4 are indicated for one of the subunits in the upper panel.

4.1. Structure of the nAChR Ion Channel Domain (ICD). Over the past 2 decades Unwin and coworkers have made remarkable contributions to the nAChR field with increasingly higher resolution cryoelectron microscopic images of the muscle-type nAChR [$(\alpha1)_2\beta1\gamma\delta$] using tubular crystals of postsynaptic membranes from the electric organ of *Torpedo marmorata*. The level of structural information to be gained from these structures has increased tremendously from the first structures with resolutions of ~18 Å to near-atomic-scale structures,[76−81] culminating with the recent publication of an atomic model based on a 4 Å density map.[72] Whereas most of the structures depict the receptor in its resting state,[72,77,79−81] a structure of the activated (open) nAChR (at 9 Å) has also been obtained.[78]

4.1.1. ICD Structure. The ICD of the *Torpedo* nAChR is 40 Å long and has a diameter of 80 Å.[72] The interface between the NTD and the ICD of the receptor is located 10 Å on the extracellular side of the cell membrane (Figure 5). The ion pore is formed by a bundle of the M2 α-helices from the five subunits arranged symmetrically around an axis perpendicular to the membrane, with the 10 M1 and M3 helices forming an outer circle behind them and the 5 M4 helices positioned at the periphery of the ion channel (Figure 6). In each of the subunits, the extracellular part of the M2 helix tilts radially inward toward its midpoint, where it is kinked in two positions (at Pro^{265} and near Leu^{251} in α1). In contrast, the other three helices tilt radially toward and tangentially around the central axis. The M2 helix appears to form minimal contacts with M1, M3, and M4, and thus, these helices constitute

an outer scaffold around the ion pore, sequestering it from the surrounding membrane. The water-filled cavities between the M2 helices and the outer helices are essential for the gating mechanism of the receptor because they provide the space needed for the agonist-induced conformational changes in the ion channel.[72]

4.1.2. Ion Conductance. The cations enter the ion pore of the nAChR through the extracellular vestibule formed by the NTD or through lateral openings, the largest of which are found at the subunit interfaces.[72] The segment of the ion channel embedded in the plasma membrane is composed of rows of predominantly nonpolar "rings" of aliphatic residues (and also Ser and Thr residues) presented into the lumen by the five M2 helices (Figure 7A).[72] At the extracellular wider third of the channel, residues from the five M1 helices also contribute to the lining of the channel.[72] The residues lining the ion pore according to the atomic model are in excellent agreement with those identified in studies using the substituted-cysteine accessibility method (SCAM) and in photolabeling studies (see references in refs 12 and 72). Whereas the relatively nonpolar channel lining of the ion pore does not present a particular attractive environment for cations to enter, negatively charged residues are found in both the intracellular and extracellular ends of M2 in α1, β1, δ, and γ (Figure 7A). Hence, the influx of cations into the *Torpedo* nAChR channel is facilitated by the presence of two rings of polar or negatively charged residues in the extracellular entrance to the channel (Ser^{266} and Glu^{262} in α1, aligning to Glu, Asp, or Gln residues in β1, γ, and δ), and an "intermediate" ring framing the intracellular entrance to the channel (Glu^{241} in α1, aligning to Glu and Gln residues in β1, γ, and δ) also influences the ion flow through the receptor (Figure 7A).[12,72,82] These residues are conserved in most of the neuronal nAChR subunits and in the 5-HT_3 receptor, and the fact that the anionic LGICs for glycine and GABA possess positively charged residues at aligned positions further supports the notion of these residues as key determinants of the ion conductance through the LGIC (Figure 7A).

4.1.3. Charge Selectivity Filter. The nAChR are cation-selective ion channels permeable to certain monovalent and divalent cations. The permeability for monovalent cations increases with their radius, whereas there is an inverse correlation between radius and nAChR permeability for divalent cations.[12,82] Thus, the nAChR ICD discriminates between ions based on both ion charge and size. Numerous studies have identified a small peptide sequence located at the intracellular border of M2 as the "charge selectivity filter" of the Cys-loop LGIC, i.e., the receptor region determining the characteristics and magnitude of ion-charge discrimination (Figure 7A).[82] Introduction of two mutations in this "constriction region" of the α7 nAChR concurrently with a Val-to-Thr mutation in the extracellular half of M2 has been shown to convert the ion selectivity of the receptors from cationic to anionic,[83] and the reciprocal mutations convert the α1 glycine receptor from an anionic to a cationic LGIC.[84] The charge selectivity filter also regulates the flux of divalent cations through the channel, as demonstrated by the complete abolishment of Ca^{2+} permeability in the α7 nAChR caused by a single Glu^{237}Ala mutation (corresponding to Glu^{241} in α1).[85] In subsequent studies of α7 nAChR and $GABA_A$ receptors, the specific molecular determinants of ion charge discrimination in anionic versus cationic LGICs have been found to be quite complex.[86,87] Furthermore, in a study of $(\alpha2)_2(\beta3)_2\gamma2$ $GABA_A$ receptors, the "constriction region" in the β3 subunit was found to be of key importance for the ion-charge discrimination of the anionic channel, indicating that the different subunits in the heteromeric LGIC may contribute differently to the selectivity filter of the receptor.[87]

4.1.4. The Gates. In the resting and desensitized states of the nAChR, the ion conductance through the ion channel is blocked by a "gate", i.e., a molecular barrier prohibiting the ions from passing through the channel. On the basis of SCAM experiments, Karlin and co-workers have proposed the "resting gate" in the muscle nAChR to be made up by a region of five residues in the region also containing the ion selectivity filter (Gly^{240}-Thr^{244} in α1, Figure 7A).[12,88] The atomic model of the *Torpedo* nAChR ion channel does not support the proposed localization of the resting gate, however, since the intracellular mouth of the channel does not seem to present a barrier to ion permeation.[72] The ion channel is at its most narrow in the middle section because of the presence of the two kinks and the bulky hydrophobic amino acid residues facing the pore lumen here, and comparisons between the structures of the resting and activated nAChR identify this region as the gate.[72,78,80,81] Hydrophobic intersubunit interactions between a Leu and a neighboring Ala/Ser residue (Leu^{251} and Ser^{252} in α1) and between a Phe and a neighboring Val/Ile residue (Phe^{256} and Val^{255} in α1) at all five subunit interfaces constitute a girdle around the middle section of the ion pore. Thus, the gate consists of a "leucine ring" formed by interactions between the side chains of five leucines and a "valine/isoleucine ring" one helix-turn above it (Leu^{251} and Val^{255} in α1, Figure 7A).[72,78] In the resting receptor state, the ion channel is only ~6 Å wide in this region, which makes permeation of hydrated monovalent ions and divalent cations impossible (Figure 7B). In the active receptor state, agonist binding to the NTD elicits a small (15°) axial rotation in the two α1-M2 helices, which disrupts or weakens the hydrophobic intersubunit interactions stabilizing the girdle around the ion channel. The relaxation of the structural constraints on the ion channel allows the five M2 helices to change orientation toward the outer helices and residues in the helices to form hydrophobic interactions with other residues in the receptor complex. As a result of this, the ion pore is widened by ~3 Å, thereby allowing the cations to pass through the channel and enter the cell (Figure 7B).[72,78,81,89] The proposed involvement of the leucine and valine/isoleucine rings in the resting gate of the nAChR is in good agreement with observations made in photolabeling and mutagenesis studies of the muscle-type nAChR (see references in refs 12 and 72).

The gates in the resting and desensitized nAChR are not necessarily identical.[12,90] In a SCAM study Wilson and Karlin have mapped the desensitization gate of the muscle-type nAChR to the Gly^{240}-Leu^{251} region (of α1) and thus to be an extension of the their proposed resting gate region (Figure 7B).[12,91] Considering the contrasting hypotheses regarding the localization of the resting gate

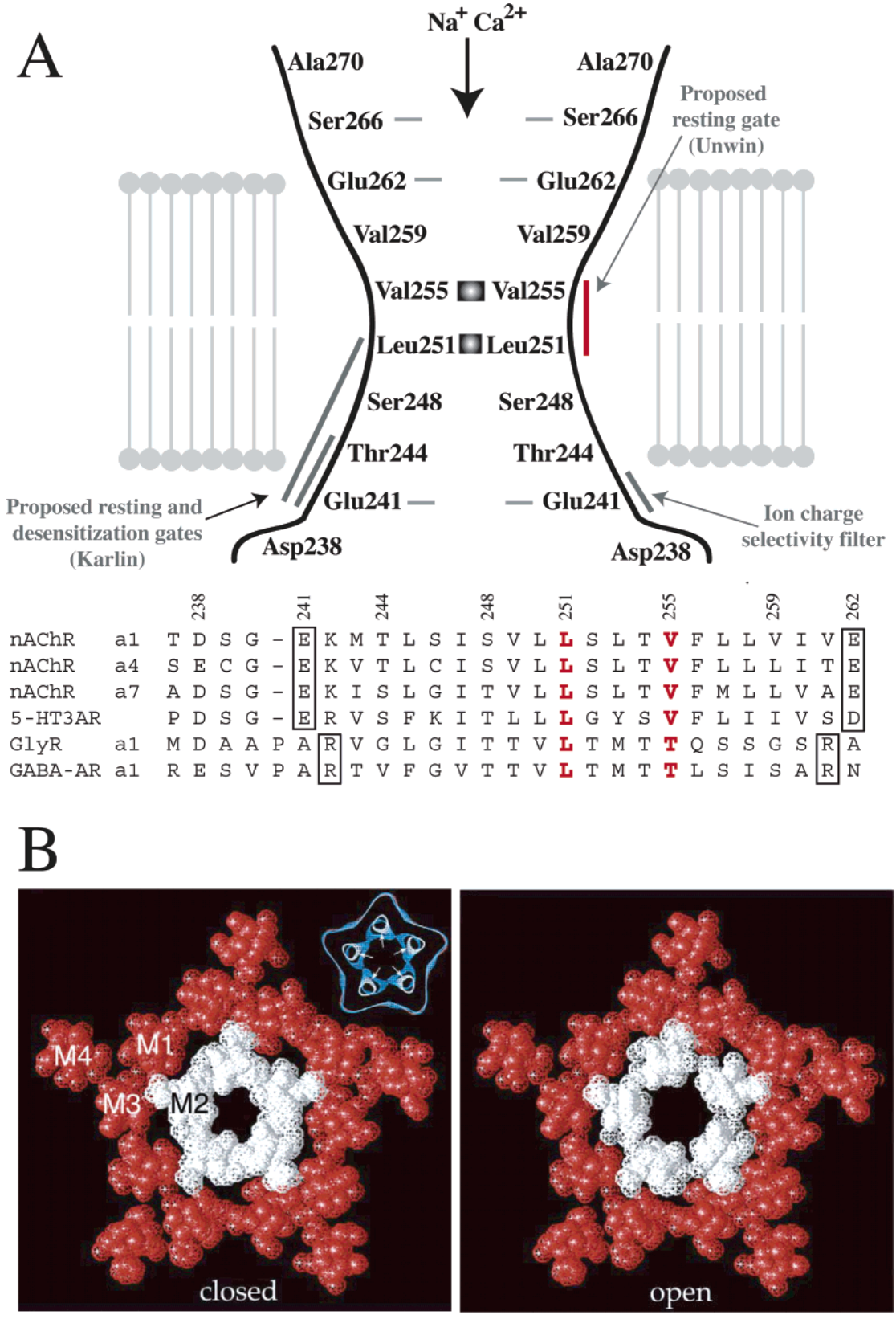

Figure 7. Ion pore of the Torpedo nAChR.[72] (A) α1 nAChR residues lining the ion pore. The localization of the negatively charged residues at the mouths of the ion channel, the ion charge selectivity filter, and the resting gates proposed by Unwin and Karlin are shown. The sequences of the M2's of α1, α4, and α7 nAChRs, 5-HT_{3A}R, α1 GlyR, and α1 $GABA_A$R are aligned with the residue numbers in the α1 nAChR indicated above. The charged residues at the intracellular and extracellular mouths of the receptors are boxed, and the resting gate residues are given in red. (B) Opening of the ion channel: cross-sections of the closed and open ion channels in the middle of the membrane. Reprinted by permission of Federation of the European Biochemical Societies from "Structure and action of the nicotinic acetylcholine receptor explored by electron microscopy" by Unwin, *FEBS Letters*, Vol. 555, 91−95.[89] Copyright 2003 Federation of the European Biochemical Societies.

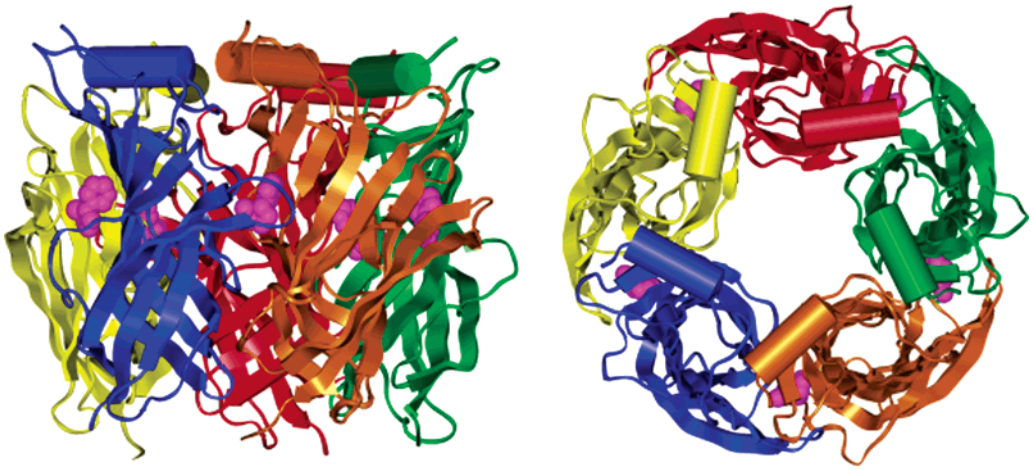

Figure 8. Crystal structure of the AChBP−nicotine complex (2.2 Å).[71] The structure of the AChBP pentamer complexed with nicotine (PDB 1UWG) is viewed from the side (left) and from above (right). Nicotine is shown in space-filling representation in magenta.

derived from SCAM studies and the electron-microscopic images, it is unfortunate that no cryoelectron microscopic images exist of the *Torpedo* nAChR in its desensitized state.

4.2. Structure of the nAChR N-Terminal Domain (NTD). The large, well-defined neurons of the freshwater snail *Lymnaea stagnalis* have been used extensively over the years as a model system for studies of synaptic transmission and neuronal networks. In 2001, the discovery and structure determination of an ACh binding protein (AChBP) in the snail not only exposed a novel concept of neurotransmission modulation but also brought remarkable insight into the structural architecture of the NTDs of nAChRs and other LGICs.[70,92]

4.2.1. AChBP Structure. The AChBP is produced and stored in the secretory pathways of the glial cells of *Lymnaea stagnalis*. Upon depolarization of the cell, the protein is released into the synaptic cleft, where it functions as a buffer of cholinergic neurotransmission through its binding of ACh, thereby rendering the neurotransmitter unable to activate postsynaptic receptors.[92,93] The mature AChBP is 210 amino acids long, and the protein displays low but significant amino acid sequence homology with the extracellular N-terminal regions of all members of the Cys-loop LGIC superfamily and in particular the nAChR α-subunits. Most prominently, the sequence identity between the AChBP and the corresponding region in the α7 nAChR is 24%. Almost all of the hallmark structural features of the nAChR NTD are found in the AChBP, including the residues forming the ACh binding pocket and the two cysteine residues forming the "Cys-loop" of the receptors. In concordance with these structural similarities, AChBP has been shown to form homopentameric complexes and to bind [^{125}I]-α-bungarotoxin and a wide range of cholinergic ligands with affinities comparable to those of the α7 nAChR.[70,92] This indicates that the protein is applicable as a model for the nAChR NTD, and compared to secondary structure predictions of the nAChR NTD,[11] the AChBP structures represent a major step forward in the understanding of the structure of this receptor region.

In the crystals used for X-ray analyses, AChBP is organized in a pentameric complex of 80 Å in diameter and a height of 62 Å, where the assembly of the five AChBPs creates a central axial channel of ∼18 Å (Figure 8).[70] These proportions are in good agreement with the size and shape of the NTD in the *Torpedo* nAChR structures.[80] The AChBP monomer is composed of an N-terminal α-helix, two short 3_{10} helices and 10 β-strands, termed β1−β10 (Figure 5). The protein exhibits a modified immunoglobulin topology in which two pairs of β-sheets (an inner sheet composed of β1, β2, β3, β5, β6, and β8 and an outer sheet of β4, β7, β9, and β10) are linked by the "Cys-loop" disulfide bridge between Cys^{123} and Cys^{136} and organized in a curled β-sandwich (Figures 5 and 8). Binding sites for orthosteric nAChR ligands are located at each of the five protomer interfaces in the AChBP pentamer, corresponding to the five orthosteric sites in homomeric nAChRs. These binding pockets are located close to the outside surface of the pentameric ring, where they are sequestered from the solvent by loop C regions (see below). Apart from the residues forming this binding pocket, the residues lining the interface between two AChBP protomers are very poorly conserved in the LGIC superfamily members, indicating that specific intermolecular interactions at the subunit interfaces are not essential for the pentameric assembly of the LGIC.[70,93] Another surprising lack of conservation between the AChBP and the LGIC family members is found in the Cys-loop, which is a highly conserved hydrophobic region of 15 residues (including the two cysteines) in the LGIC but a hydrophilic 14-residue region in the AChBP.

4.2.2. Orthosteric Site of the AChBP. The molecular composition of the orthosteric site of the prototypic LGIC, the muscle-type nAChR, has been delineated in great detail in an impressive number of studies spanning a period of more than 4 decades.[11,12,94] The two orthosteric sites in the muscle nAChR have been proposed to be located at the α−γ and α−δ subunit interfaces and to be formed by a principal and a complementary binding component represented by the α and the γ/δ subunits, respectively. Analogously, the two orthosteric sites in the heteromeric neuronal nAChR are composed of residues in the α and β subunits constituting the principal and complementary components, respectively, and the five orthosteric sites in the homomeric neuronal nAChR are composed of the (+)-

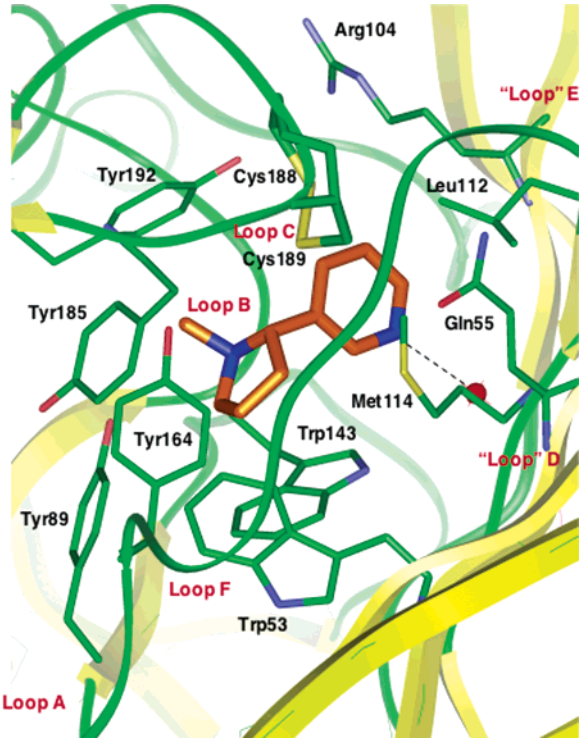

Figure 9. (*S*)-Nicotine binding to the AChBP. The orthosteric site of the AChBP pentamer in the nicotine−AChBP X-ray structure (2.2 Å, PDB 1UWG).[71] Loops A−F and the residues participating in the binding of (*S*)-nicotine to the AChBP are indicated.

and (−)-sides of the α-subunits (Figure 1). Agonists and competitive antagonists bind to these sites through interactions with residues in loops A, B, and C on the principal/(+)-side and in loops D, E, and F on the complementary/(−)-side of the binding pocket.[11,12,94]

The AChBP X-ray structures have largely confirmed the involvement of the proposed residues in loops A−F in ligand binding (Figure 9). The "loop" terminology will be used in the following, although loops D and E were found to be *β*-strand structures in AChBP and not loops (Figure 9).[70,71] In the original AChBP X-ray structure (2.7 Å), the protein was complexed with HEPES molecules present in the crystallization buffer.[70] This structure has recently been supplemented with another AChBP−HEPES structure (2.1 Å) and with structures of the protein complexed with nicotine (2.2 Å) and carbamoylcholine (CCh) (2.5 Å).[71] HEPES is a weak nAChR agonist, and since the HEPES molecules were located in the orthosteric sites at interfaces of the AChBP pentamer, the structure may not reflect the "ligand-free" nAChR NTD conformation. On the other hand, it is reasonable to assume that the crystal structures of the nicotine- and CCh-bound AChBP reflect the nAChR NTD in its high-affinity desensitized state.

All agonists and most of the small-molecule competitive antagonists of nAChRs possess a positively charged amino group, in the form of a quaternary ammonium group or a protonated tertiary or secondary amino group. In the AChBP, this amino group docks into an "aromatic box" formed by five aromatic residues (Tyr^{89}, Trp^{143}, Tyr^{185}, and Tyr^{192} from the (+)-side and Trp^{53} from the (−)-side) in proximity to two vicinal cysteines (Cys^{187} and Cys^{188}) forming a disulfide bond (Figure 9). The spatial orientations of nicotine and CCh in this cavity are very similar, whereas the interactions established with the surrounding residues seem to differ significantly for the two agonists.[71] In concordance with the findings in studies of the muscle-type nAChR, the ammonium group of CCh and the protonated amino group of nicotine form extensive cation−*π* interactions with the side chain of Trp^{143} and some with that of Tyr^{192} (Figure 9).[71,94,95] The backbone carbonyl group of Trp^{143} forms a hydrogen bond to the protonated pyrrolidine ring nitrogen in nicotine and an electrostatic interaction with the carbon adjacent to the quaternary amino group of CCh. Furthermore, the hydroxy group of Tyr^{89} makes contacts with the pyrrolidine ring of nicotine and the quaternary ammonium group of CCh. However, whereas nicotine interacts with Trp^{53} and Cys^{188} but does not coordinate to Tyr^{185} and Cys^{187}, the opposite is true for CCh. When it comes to the complementary binding component, the pyridine ring of nicotine and the carbamate group of CCh form hydrophobic contacts to the Arg^{104}, Leu^{112}, and Met^{114} residues (Figure 9). Finally, the pyridine nitrogen of nicotine coordinates to the peptide backbone of Leu^{102} and Met^{114} via a water molecule (Figure 9).[71] The aromatic residues and vicinal cysteines at the (+)-side of the AChBP binding site are conserved in all nAChR α-subunits except in the nonbinding structural α5 and *β*3 subunits, where Tyr^{185} is replaced by an Asp and a Glu residue, respectively (Table 3). In contrast, the residues making up the complementary binding component are much less conserved in the nAChRs, with the exception of the Trp^{53} residue conserved in the muscle *δ*, *γ*, and *ϵ* subunits as well as in the neuronal *β*2, *β*4, α7, α8, α9, and α10 subunits (Table 3).

4.3. Functional Coupling of the nAChR. The fragmentary information about the NTD and ICD of the nAChR gained from the AChBP and *Torpedo* nAChR structures has spawned several hypotheses about the mechanism underlying functional coupling of the LGIC, i.e., the translation of agonist binding to the NTD into ICD opening. This mechanism seems to be conserved throughout the LGIC superfamily, since a α7/5-HT_3 chimera consisting of the NTD of the α7 nAChR and the ICD of the 5-HT_3 receptor has been shown to possess the pharmacological profile of the α7 nAChR and the ion channel properties of the 5-HT_3 receptor.[96] Analogously, functional chimeras have been created by fusions of NTDs and ICDs of glycine and $GABA_C$ receptors.[97]

Comparisons of the AChBP−HEPES structure with the nicotine- and CCh-bound structures have revealed that the side chains of most of the agonist binding residues reorient slightly to accommodate agonist binding, the major difference being a movement of the loop C backbone.[70,71] This contraction of the binding pocket around the ligand has previously been proposed based on a SCAM study of the $GABA_A$ receptor, where the movement of loop C upon binding of agonists and allosteric modulators was suggested to trigger the ion channel opening.[98] Surprisingly, none of the regions in the AChBP corresponding to the nAChR NTD regions proposed to be in contact with the ICD of the nAChR (see Figure 5) appear to change orientations upon agonist binding to the AChBP. However, since the AChBP−HEPES structure is not necessarily a true representation of the ligand-free nAChR NTD but could

Table 3. Conservation of the Residues Making Up the Orthosteric Site in Neuronal NAChRs[a]

	A	B		C				D	E		
	Tyr[89]	Trp[143]	Thr[144]	Tyr[185]	Cys[187]	Cys[188]	Tyr[192]	Trp[53]	Arg[104]	Leu[112]	Met[114]
α2	Tyr	Trp	Thr	Tyr	Cys	Cys	Tyr	*Trp*	*His*	*His*	*Val*
α3	Tyr	Trp	Ser	Tyr	Cys	Cys	Tyr	*Trp*	*Leu*	*Thr*	*Ile*
α4	Tyr	Trp	Thr	Tyr	Cys	Cys	Tyr	*Trp*	*His*	*Gln*	*Thr*
α6	Tyr	Trp	Thr	Tyr	Cys	Cys	Tyr	*Trp*	*Leu*	*Thr*	*Thr*
*β*2	*Tyr*	*Trp*	*Thr*	*Asp*			*Tyr*	Trp	Val	Phe	Leu
*β*4	*Tyr*	*Trp*	*Thr*	*Gln*			*Tyr*	Trp	Ile	Gln	Leu
α7	Tyr	Trp	Ser	Tyr	Cys	Cys	Tyr	Trp	Leu	Gln	Leu
α5	*Phe*	*Trp*	*Thr*	*Asp*	*Cys*	*Cys*	*Tyr*	*Trp*	*Val*	*Thr*	*Thr*
*β*3	*Phe*	*Trp*	*Thr*	*Glu*	*Phe*	*Tyr*	*Tyr*	*Trp*	*Ile*	*Ser*	*Thr*

[a] The residues identified as participants in nicotine and CCh binding to AChBP[71] are listed together with the corresponding residues in rat nAChR subunits. Residues in loops D and E in α(2−4,6), in loops A, B, and C in *β*(2,4), and in α5 and *β*3 do not participate in orthosteric ligand binding and are indicated in italics.

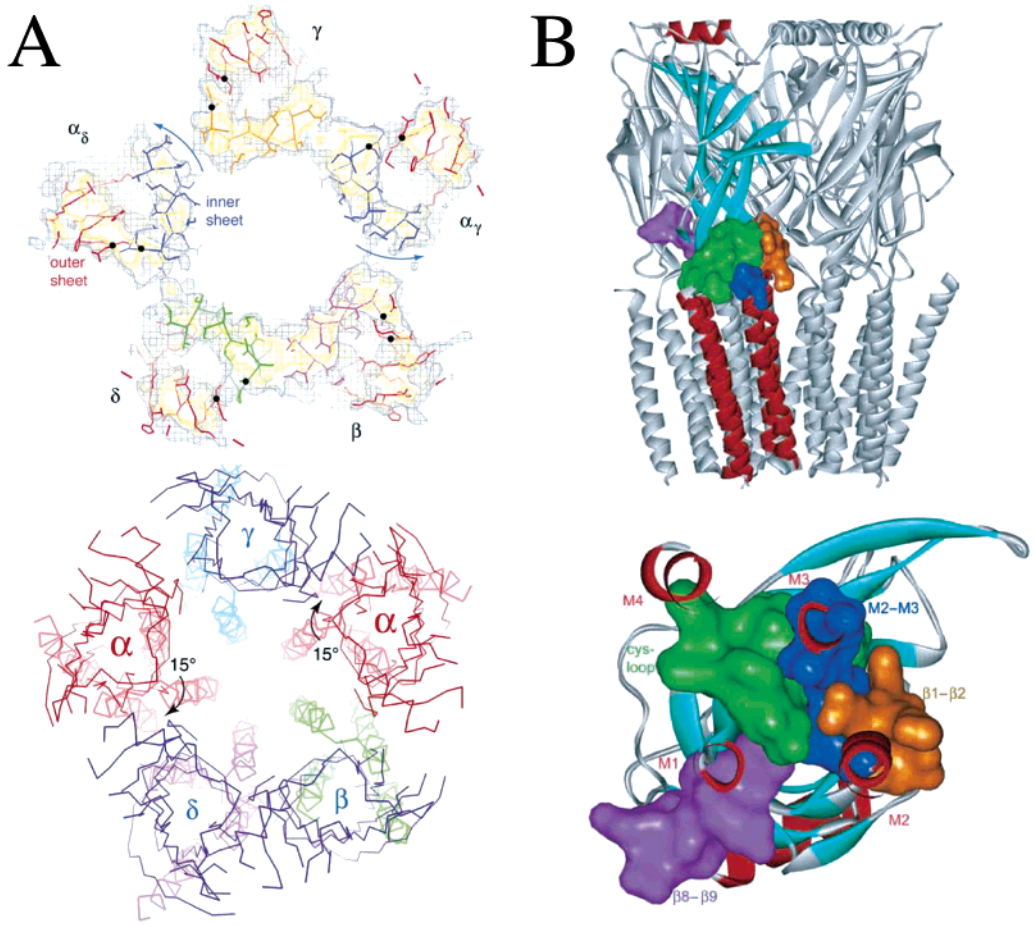

Figure 10. Functional coupling of the LGIC. (A) The crosstalk between the NTD and ICD in the *Torpedo* nAChR. Agonist binding elicits a relative rotation in the inner and outer *β*-sheets of the NTD, which subsequently leads to 15° rotations of the α1-M2 helices in the ICD. Upper figure of part A is reprinted in part from *J. Mol. Biol.*, Vol. 319, Unwin et al., "Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the α subunits", pp 1165−1176, Copyright (2002), with permission from Elsevier.[81] Bottom figure of part A is reprinted by permission of Federation of the European Biochemical Societies from "Structure and action of the nicotinic acetylcholine receptor explored by electron microscopy" by Unwin, *FEBS Letters*, Vol. 555, 91−95.[89] Copyright 2003 Federation of the European Biochemical Societies. (B) Model of the entire LGIC structure (an AChBP/5-HT_3 chimera) based on the AChBP and *Torpedo* nAChR structures. The NTD and ICD regions proposed to interact in the functional coupling of the LGIC are indicated. Reprinted by permission from *Nature* **2004**, *430*, 896−900 (http://www.nature.com/).[100] Copyright 2004 Macmillan Publishers Ltd.

be an intermediate conformation, most of the agonist-induced structural changes could already have occurred in this structure.[71] Unwin has proposed that agonist binding to the *Torpedo* nAChR NTD elicits a ∼15° rotation in the inner *β*-sheet relative to the outer *β*-sheet around the disulfide bridge forming the Cys-loop (Figure 10A).[78,81,89] Modeling of the AChBP structure onto the *Torpedo* nAChR structure has identified two loops in the AChBP/NTD, L2 and L7, in close contact with the extracellular regions of the ICD (Figure 5).[72] Hence, the agonist-induced rotation in the NTD was proposed to result in the formation of contacts between the L2 loop and the "M2−M3 linker" in the ICD (the top of M2 and the extracellular loop connecting M2 and M3) (Figure 5).[72] The close localization of loops L2 and L7 in AChBP and the conservation of L7 (the Cys-loop) in the NTDs

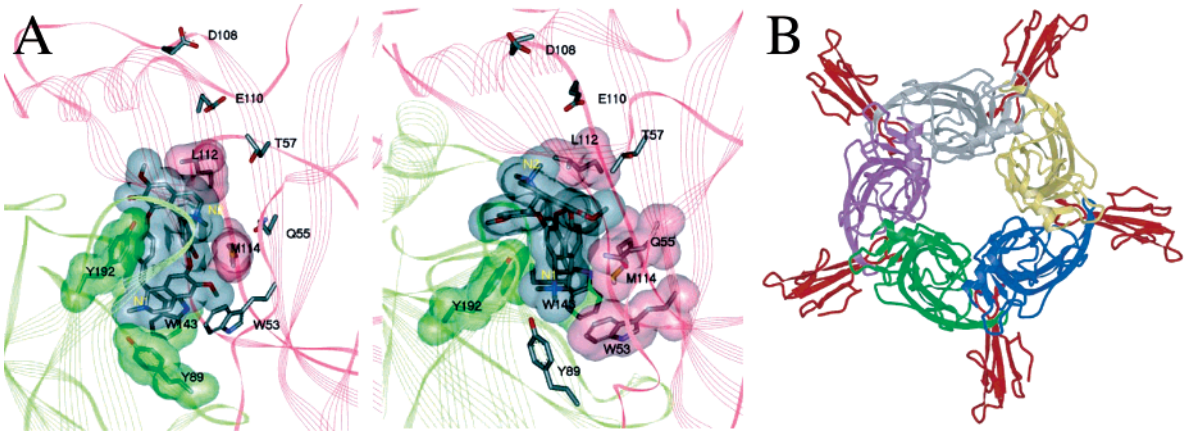

Figure 11. Competitive nAChR antagonist binding to the AChBP. (A) Proposed binding modes of competitive antagonists *d*-tubocurarine (left) and metocurine (right) to AChBP. Reprinted from *J. Biol. Chem.* **2003**, *278*, 23020−23026 with permission from The American Society for Biochemistry & Molecular Biology.[109] (B) Proposed binding mode of α-bungarotoxin to AChBP. Reprinted from *Neuron*, Vol. 32, Harel et al., "The binding site of acetylcholine receptor as visualized in the X-ray structure of a complex between α-bungarotoxin and a mimotope peptide", pp 265−275, Copyright (2001), with permission from Elsevier.[111]

of all LGIC family members have also suggested a role for this loop in gating,[72,94] As for the M2−M3 linker, a substantial number of experimental data support a role for this ICD region in the gating of nAChRs and other Cys-loop receptors. For example, naturally occurring and introduced mutations in this region of a wide range of LGICs have been shown to affect ion channel function without influencing the ligand binding characteristics, and the linker appears to undergo a conformational change during LGIC activation (see references in refs 94 and 99).

The proposed involvement of these NTD and ICD regions in LGIC gating has been verified in a recent elegant study of a series of AChBP/5-HT_{3A} chimeras, where the AChBP was fused onto the ICD of the 5-HT_{3A} receptor (Figure 10B).[100] When the loops L2, L7, and L9 in the AChBP part of the AChBP/5-HT_{3A} chimera were replaced by the corresponding 5-HT_{3A} regions, ACh-evoked currents through the chimera could be recorded. The fact that all three "5-HT_3 loops" had to be introduced into the NTD in order to obtain a functional receptor suggested a synergistic interaction between the three NTD loops and the M2−M3 linker. The L2 and L7 loops were proposed to straddle opposite sides of the M2−M3 linker, whereas the L9 loop appeared to mediate its effect on the ion pore more indirectly (Figure 10B).[100] Hence, the rotation in the NTD upon agonist binding is believed to cause a twisting motion in the M2−M3 linker through its interaction with the L2, L7, and L9 loops, which subsequently elicits the rotation of the M2 α-helices and facilitates ion permeation through the ion channel (Figure 10A).[72,78,81,94,100] Several negatively charged residues in L2 and L7 and a lysine residue in the M2−M3 linker of $GABA_A$ and glycine receptors have been shown to be crucial for proper gating of the anionic LGIC.[101,102] Only half of these residues are conserved in the cationic LGIC, however, so the proposed electrostatic interactions between L2 and L7 and the M2−M3 linker in the anionic LGIC may be replaced by hydrogen bonds in the cationic receptor.

4.4. Homology Models of the nAChR. Tools for Rational Ligand Design? 4.4.1. Homology Models of the nAChR−NTD. The original AChBP−HEPES structure[70] has been applied as template for several homology models of the NTDs of nAChRs[103−106] and other Cys-loop LGICs.[107,108] Judging from comparisons with the subsequently published AChBP−nicotine and AChBP−CCh structures,[71] the models appear to have been relatively successful in predicting the residues involved in the binding of agonists such as ACh, nicotine, epibatidine, and cytisine.[103−105] The rank orders of docking scores for ACh, nicotine, and cytisine to α3β4 and α4β2 models and the predicted binding energies of ACh and nicotine to α4β2, α3β2, α4β4, and α7 models have been found to be in reasonably good agreement with experimental determined binding affinities.[104,105] Furthermore, Schapira et al. predicted the localization of a water molecule coordinating the pyridine nitrogen of nicotine with the carbonyl group of Ser^{133} and amide group of Phe^{144} of β2 in their α4β2 model, which subsequently has been qualitatively confirmed by the AChBP−nicotine X-ray structure.[71,104] On the other hand, the presence of water molecules in the ACh-docked nAChR binding pocket has not been observed in the AChBP−CCh structure.[71,104,105]

The binding modes of the two competitive antagonists *d*-tubocurarine and its methylated analogue metocurine to the AChBP have been investigated via mutagenesis, ligand binding, and computational methods.[109] According to docking experiments, both curarine analogues coordinate to most of the residues implicated in agonist binding to AChBP.[71] However, despite their close structural similarity, the two antagonists appear to bind in different conformations to the AChBP (Figure 11A). This was supported by the observation that mutations in the orthosteric site had differential effects on the binding affinities of the two antagonists. Using a similar approach, *d*-tubocurarine and metocurine were shown to bind differently to the orthosteric site of the α1−ε interface of the fetal muscle-type nAChR receptor.[106] The different binding modes of the two antagonists in the receptor illustrate that minor changes in a molecule, even one with a rigid scaffold, can alter its orientation in the nAChR binding pocket significantly. The authors did not speculate on the structural determinants for the antagonism displayed by the compounds.[106,109] The classical competitive antagonists of nAChRs are gener-

ally bigger than agonists, and this bulkiness presumably prohibits the structural changes underlying the transition from the resting to the active receptor state from taking place. On the other hand, there are obviously limitations to just how big molecules can be and still be able to enter and fit in the orthosteric site of the nAChR. In concordance with the tight fit of agonists in the orthosteric site of the AChBP/nAChR,[71,103,104] introduction of very small substituents to the pyridine nitrogen of nicotine and to the pyridine ring of epibatidine has resulted in competitive antagonists (section 5.3). The spatial orientation of different antagonists in the orthosteric site may differ significantly, and thus, it will be interesting to study the binding modes of other small-molecule competitive antagonists to AChBP or nAChRs.

AChBP-based homology models have also been applied in studies of peptide toxins such as α-cobratoxin and α-bungarotoxin, two potent competitive antagonists of α7 and muscle-type nAChRs, isolated from the snakes *Naja kaouthia* and *Bungarus multicinctus*, respectively. The binding mode of α-cobratoxin at the α7 nAChR was elucidated via extensive mutagenesis work on both toxin and receptor in combination with docking of the toxin into a α7-NTD homology model.[103,110] According to the docking, the tip of the central loop in the toxin projects into the orthosteric site, and at least 13 residues in this tip were proposed to form interactions with the receptor, predominantly to loop C residues but also to residues in loops A, B, D, and F. The remaining portion of the toxin stabilizes its binding to the receptor via interactions with residues on the surface of the α7 NTD pentamer.[110] The delineation of the binding mode of α-bungarotoxin to the AChBP took its origin in a crystal structure (1.8 Å) of the complex between α-bungarotoxin and a high-affinity 13-mer peptide (HAP), which displays significant sequence similarity with loop C of the nAChRs.[111] The α-bungarotoxin/HAP complex was docked perpendicular into the AChBP−HEPES structure, superimposing the HAP peptide onto loop C of the binding site (Figure 11B). Although the specific interactions between the two toxins and the AChBP/nAChR were different in the two studies, the overall binding mode appeared to be shared by the two toxins.

AChBP-based homology models and the availability of comprehensive mutagenesis data have also made it possible to elucidate the binding modes of several well-known allosteric modulators of LGICs, for example, Zn^{2+} binding to the α1 glycine receptor and benzodiazepine binding to $GABA_A$ receptors.[107,108] At physiological concentrations, Ca^{2+} is an efficacious allosteric potentiator of several heteromeric α/β nAChRs and in particular of the α7 nAChR.[11] On the basis of mutagenesis and molecular modeling studies, Ca^{2+} has been proposed to coordinate to glutamate and aspartate residues distributed at both sides of the nAChR−NTD subunit interface.[103,112] The presence of five putative allosteric sites in the homomeric α7 nAChR and only two in the heteromeric nAChR was proposed as an explaination for the differences in the degree of potentiation exhibited by the cation at the respective nAChRs.[103] On the basis of their α3β4 nAChR−NTD model, Costa et al. have analogously proposed a binding mode for the allosteric nAChR potentiator physostigmine.[105]

4.4.2. Structure-Based Design of Orthosteric and Allosteric nAChR Ligands? It is reasonable to question the accuracy and usefulness of homology models in rational ligand design when the models are based on X-ray structures of a distantly related protein. In this connection, it is worthwhile to look at a success story from the mid-1990s, where homology models of the ligand-binding domains of ionotropic and metabotropic Glu receptors (iGluRs and mGluRs, respectively) were constructed on the basis of X-ray structures of distantly related bacterial periplasmic binding proteins (PBPs). Subsequently, X-ray crystal structures of the ligand-binding domains of iGluR2 and mGluR1 have revealed that the homology models were remarkably accurate in their prediction of the overall structures of these domains.[113,114] Analogous to the Glu receptor models, nAChR−NTD homology models will undoubtedly be valuable for the interpretation of results obtained in molecular pharmacology and SAR studies. Furthermore, when it comes to the applicability of homology models in rational design of orthosteric nAChRs ligands, the AChBP structure holds several advantages as a model template compared to the PBP structures. The protein actually binds the endogenous agonist and several other orthosteric ligands for nAChRs, which means that the molecular architecture in all regions of the binding pocket is designed to accommodate binding of these ligands. Furthermore, considerably higher degrees of homology exist between the orthosteric sites of AChBP and the nAChRs compared to that beween PBPs and Glu receptors, which is bound to result in more reliable models. Finally, all of the residues participating in orthosteric ligand binding to the nAChR have already been identified in mutagenesis studies, and this information can be built into the models. The availability of ligand-bound AChBP structures is also a major advantage. Not surprisingly, none of the first generation of homology models based on the AChBP−HEPES structure have been able to foresee the small structural changes observed in the nicotine- and CCh-bound structures, and thus, the new ligand-bound AChBP structures should constitute more accurate templates for future homology modeling efforts. Still, the nicotine- and CCh-bound AChBP structures represent the desensitized state of the nAChR, which raises an important point: how suited is a model of the desensitized nAChR conformation for the design of agonists targeted to the active receptor conformation? Is the molecular architecture of the binding pocket significantly different in the two states, or does the desensitization event predominantly consist of conformational changes in distant regions of the receptor complex? The fact that potent and highly subtype-selective nAChR agonists have been developed partly on the basis of homology models suggests that such models are indeed applicable for ligand design (section 5.1).

The AChBP structures also present some interesting possibilities with respect to rational design of allosteric nAChR modulators, although this clearly requires detailed insight into the structure of the allosteric site that is rarely available. Benzodiazepines modulate $GABA_A$ receptor function through binding to the α−γ subunit NTD interface of this receptor, and this allosteric site has been meticulously elucidated.[107] It is reasonable to

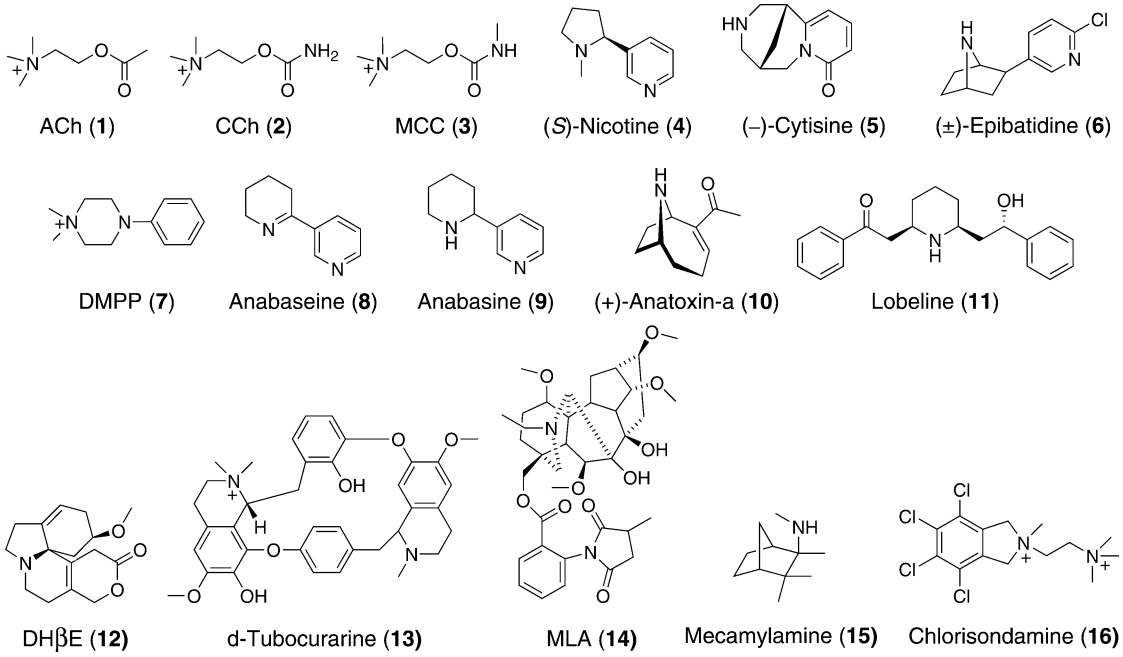

Figure 12. Structures of selected agonists (**1**−**10**), competitive antagonists (**12**−**14**), and noncompetitive antagonists (**15** and **16**) of neuronal nAChRs. Lobeline (**11**) is an atypical ligand able to displace orthosteric radioligands competitively, whereas it has not been unequivocally established whether it is an agonist or an antagonist.

assume that nAChR signaling analogously could be modulated allosterically through binding of the "right" ligand to the corresponding nAChR region. However, redesign of benzodiazepines or other $GABA_A$ modulators to create nAChR activity is clearly not trivial, and neither is in silico design of novel structures.

Homology models based on the atomic model of the *Torpedo* nAChR[72] have not yet been published. The lower resolution of this structure (4 Å) compared to the AChBP structures (2.1−2.7 Å) and the fact that allosteric sites in the nAChR ICD are considerably less well-characterized than the orthosteric sites in the NTD complicate rational design of allosteric ligands targeted to this domain. However, the *Torpedo* nAChR structure offers important information about ion channel dimensions, the residues lining the ion pore, and physicochemical properties of local environments, which could be used to generate hypotheses regarding the binding modes of already identified allosteric ligands. Recently, a model of the muscle-type nAChR ICD has been used to elucidate the binding mode of philanthotoxins, a family of polyamines acting as noncompetitive antagonists of the muscle-type nAChR.[115]

5. Ligands for Neuronal nAChRs

Medicinal chemistry efforts in the nAChR field over the years primarily comprise a series of nicotine and epibatidine analogues, whereas other standard nAChR ligands have attracted limited attention as leads. In Figure 12, some classical agonists and antagonists of neuronal nAChRs are depicted, and selected strategies applied in nAChR ligand design are exemplified in Figure 13.

In terms of overall selectivity, the development of nAChR ligands devoid of activities at other receptors has typically not been a problem. This can primarily be ascribed to the substantial number of unique structures with inherent nAChR selectivities isolated from natural sources over the years. The use of compounds such as **4**−**6** and **8**−**14** as leads for new generations of nAChR ligands have meant that nAChR ligands with cross-activities at mAChRs or other receptors are fairly rare. In contrast, the vast majority of nAChR ligands published to date do not display any significant *functional* preference between the many neuronal nAChR subtypes, as illustrated for standard ligands **1**, **4**−**7**, **12**, **13**, and **15** in Table 4. The search for subtype-selective nAChR ligands has been severely hampered by the fact that most of the compounds synthesized for the receptors over the years only have been subjected to limited pharmacological characterization. Typically the compounds have been characterized in radioligand binding assays using native tissues, and thus, only their binding affinities to native $\alpha4\beta2^*$ nAChRs, and in fewer studies to native $\alpha7$, $\alpha3\beta4^*$ or muscle-type nAChRs, have been determined. Furthermore, as will be illustrated repeatedly in the following, the binding affinity of a ligand reflects its affinity for the desensitized receptor and is not necessarily representative of its functional properties. Generally, potency differences for a ligand at various nAChR subtypes are considerably less significant than the differences in its binding affinities, so selectivity displayed by a compound in a binding assay rarely translates into the same degree of functional selectivity.

The following will not be a systematic review of the plethora of published nAChR agonists and antagonists because this has been done in recent elaborate reviews to which the reader is referred.[116,117] Instead, the characteristics of selected key nAChR ligands and recent advances in the design of *functionally* selective agonists (sections 5.1 and 5.2), competitive antagonists (sections 5.3 and 5.4), and allosteric modulators (section 5.5) of nAChRs will be outlined. It will be discussed how the in vitro and in vivo properties of some of these ligands can be ascribed to the complexity of nAChR signaling

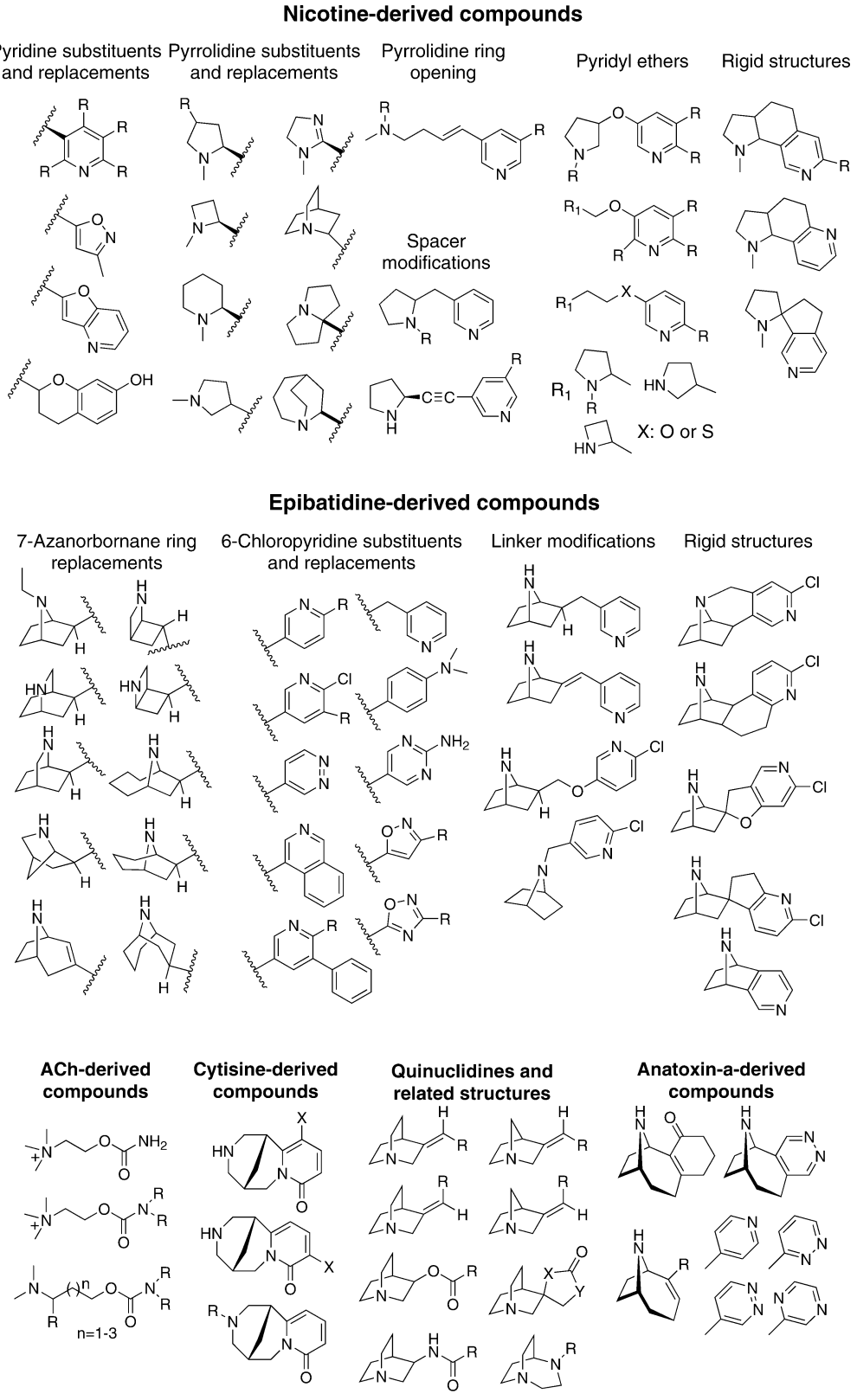

Figure 13. Selected strategies applied in orthosteric nAChR ligand design.

and the heterogeneity of the nAChR populations in the CNS.

5.1. Agonists for Heteromeric α/β nAChRs. Most of the nAChR agonists evaluated in clinical trials to date have been derived from (*S*)-nicotine (**4**). In this section, the in vitro and in vivo profiles of nicotine analogues developed by three major drug companies in the nAChR field will be outlined, and selected agonists from compound series not based on nicotine will be presented.

5.1.1. Abbott Compounds. Modifications of the linker between the pyridine and pyrrolidine rings in nicotine have resulted in several high-affinity and potent nAChR ligands (Figure 14).[117] A-85380 (**17**) and 5-I-A-85380 (**18**) bind to native α4β2* nAChRs with

Table 4. Functional Characteristics of Selected nAChR Ligands at Neuronal nAChRs[a]

	α3β2	α4β2	α2β4	α3β4	α4β4	α7
agonists						
ACh (**1**)	443,[g] 126/150%[h] 26/100%[m]	68,[g] 100/172%[h]	83[g]	203,[g] 163/100%[m]	20[g]	180,[g] 21/97%[k] 177/105%[l]
(*S*)-nicotine (**4**)	132,[g] 7.7/100%[h] 6.6/85%[i]	5.5,[g] 3.5/100%[h] 0.83/73%[c]	21,[g] 10/100%[f] 2.0/114%[i]	80,[g] 40/100%[f] 7.5/98%,[i] 2.9/40%[c]	5.0,[g] 6.9/100%[f] 0.9/85%,[c] 0.74/108%[i]	113,[g] 91/74%[l] 2.6/97%[c]
(−)-cytisine (**5**)	67,[g] 72/55%[h] 71/23%[m]	2.7,[g] 2.0/42%[h] 30/11%,[x] 2/2%[cc]	39,[g] 0.44/58%[f]	72,[g] 26/89%[f] 76/56%,[m] 67/77%[x]	0.9,[g] 0.52/51%[f] 0.11/70%,[c] 1/90%[cc]	71,[g] 14/90%[k] 86/105%[cc]
(±)-epibatidine (**6**)	0.443/163%[h]	0.043/117%[h] 0.030/73%[c]	0.010/115%[f]	0.15/267%[f] 0.014/40%[c]	0.038/99%[f] 0.003/85%[c]	0.072/97%[c]
DMPP (**7**)	56,[g] 4.3/124%[h] 2.1/100%[m]	18,[g] 6.7/90%[h]	23,[g] 12/58%[f]	19,[g] 12/101%[f] 10/107%[m]	19,[g] 18/46%[c]	31,[g] 26/54%[l]
A-85380 (**17**)		0.7/185%[s]		0.8/∼100%[s]		8.9/∼100%[s]
tebanicline (**19**)		0.27/130%[u] 0.062/102%[hh]		0.20/116%[u] 0.204/78%[hh]		56/83%[u]
A-98593 (**20**)		0.06/161%[u]		0.13/166%[u]		27/85%[u]
ABT-089 (**21**)		nd/∼0%[t]		nd/<15%[t]		>1000/∼2%[t]
ABT-418 (22)	119/∼35%[p]	6/∼55%[p]		288/∼50%[p]		155/∼50%[p]
altinicline (**23**)	3.2[j]	0.32,[j] 1.8/49%[n]	3.2,[j] 5/49%[n]	10,[j] 23/52%[n]	2,[j] 9/26%[n]	>10[j]
SIB-1553A (**26**)	nd/9%[i]	nd/13%[i]	0.71/102%[i]	1.10/71%[i]	0.59/62%[i]	>30[i]
SIB-1663 (**27**)		3/8%[y]	87/22%[y]	81/5%[y]	32/44%[y]	>300[y]
TC-2403 (**29**)	150/38%[d]	16/100%[d]		nd/20%[d]	50/55%[d]	240/16%[d]
TC-2559 (**30**)	>100[b]	0.18/33%,[b] 0.4/60%[c]	14/56%[b]	>30/∼24%,[b] >10[c]	13/42%,[b] >10[c]	>100,[b] >10[c]
SSR591813 (**33**)	>100[o]	1.3/19%[o]		>100[o]		
NEP (**38**)	−	3.6/112%[ee]		0.70/61%[ee]	0.98/147%[ee]	
NFEP (**39**)	−	0.25/41%[ee]		0.32/40%[ee]	0.24/131%[ee]	
40	−	0.013[w]		0.32[w]		25[w]
(±)-UB-165 (**41**)	3.9/∼50%[v]	nd/∼15%[v]	0.05/∼50%[v]	0.27/∼50%[v]	0.05/∼70%[v]	6.9/∼55%[v]
44		0.2/23%[cc]			0.010/70%[cc]	5.3/100%[cc]
45		0.086/47%[cc]			0.008/105%[cc]	1.5/107%[cc]
46		12/3%[x]		16/11%[x]		>100 (ant.)[x]
GTS-21 (**47**)		>10,[c] nd/0%[z]		>10[c]	>10[c]	6.0/23%[k] 26/28%[y]
DMACA (**48**)		nd/0%[z]				4.2/76%[z]
AR-R17779 (**49**)	>1000[aa] >10[c]	>2000[aa] >10[c]	>10[c]	>1000[aa] 0.42/93%[c]		10/78%[aa]
PSAB-OFP (**50**)	>100[e]	>100[e]	>100[e]	>100[e]	>100[e]	2.2/∼85%[e]
51		>10[c]		>10[c]	>10[c]	0.014/∼100%[c]
52		>10[c]		>10[c]	>10[c]	0.037/∼100%[c]
TC-1698 (**55**)	nd/5%[q]	nd/3%[q]		nd/2%[q]		0.44[q]
choline (**56**)	−					982/90%[l] 565/100%[k]
tropisetron (**57**)	>1000[aa]	>1000[aa]		>1000[aa]		0.38/38%[aa] 1.3/36%[bb]
antagonists						
α-bungarotoxin	>1[c]	>1[c]	>1[c]	>1[c]	>1[c]	0.005[c]
DHβE (**12**)	1.6,[g] 87[c]	0.11,[g] 2.7,[c] 33[hh]	3.6,[g] 5.1,[f] 57[c]	14,[g] 43,[f] 240[c]	0.01,[g] 60,[f] 4.8[c]	20,[g] 98[c]
d-tubocurarine (**13**)	2.4,[g] 0.23[c]	3.2,[g] 7.6[c]	4.2,[g] 2.3,[f] 4.0[c]	2.2,[g] 0.73,[f] 4.1[c]	0.21,[g] 0.86,[f] 2.9[c]	3.1,[g] 14.7[c]
MLA (**14**)	>1[c]	>1[c]	>1[c]	>1[c]	>1[c]	0.0017[c]
A-186253(**63**)		0.96[r]		9.2[r]		8.5[r]
MG624 (**66**)		3.2[dd]				0.11[dd]
allosteric inhibitors						
mecamylamine (**15**)	0.28[c]	0.78,[c] 3.1[hh]	3.1,[f] 16.0[c]	1.4,[f] 3.8,[c] 3.4[hh]	0.56,[f] 1.2[c]	12.3[c]
bupropion (**73**)	∼1[ff]	∼10,[ff] 12[gg]		1.8[gg]	14[gg]	∼50[ff]

[a] EC_{50} and R_{max} values are given for the agonists (in μM and in % of the R_{max} of ACh or (*S*)-nicotine) and K_i or IC_{50} values are given for the antagonists (in μM). The data have been obtained in electrophysiological recordings on nAChRs expressed in *Xenopus* oocytes or in mammalian cells by Ca^{2+} measurements using fluorescent dyes or in $^{86}Rb^+$ efflux assays. The data are primarily from recombinant human nAChRs, although data from studies using recombinant rat or chick nAChRs or cell lines expressing native nAChRs have also been included. nd: not determinable. [b] Reference 153. [c] Reference 172. [d] Reference 149. [e] Reference 179. [f] Reference 16. [g] Reference 17. [h] Reference 18. [i] Reference 264. [j] Reference 137. [k] Reference 265. [l] Reference 185. [m] Reference 23. [n] Reference 136. [o] Reference 159. [p] Reference 132. [q] Reference 183. [r] Reference 201. [s] Reference 119. [t] Reference 129. [u] Reference 123. [v] Reference 42. [w] Reference 165. [x] Reference 169. [y] Reference 146. [z] Reference 170. [aa] Reference 178. [bb] Reference 186. [cc] Reference 168. [dd] Reference 205. [ee] Reference 164. [ff] Reference 249. [gg] Reference 250.

affinities comparable to that of (±)-epibatidine and have displayed 800- to 1000-fold and 5000- to 25000-fold lower affinities to native α3β4* and α7 nAChRs, respectively.[118−120] The apparent high degrees of α4β2* specificities of these compounds prompted the development of ^{123}I, ^{125}I, and ^{18}F 5-analogues of A-85380 used in PET and SPECT in vivo imaging studies of neuronal nAChRs.[120] At recombinant nAChRs, A-85380 and 5-I-A-85380 have displayed similar binding affinities to α2β2, α3β2, and α4β2 and 50- to 400-fold and 400- to 1300-fold weaker affinities to the corresponding β4 nAChRs, respectively.[121] Furthermore, [^{125}I]-5-I-A-85380 has been shown to bind to α-conotoxin MII-labeled α6β2β3* nAChRs in striatum.[122] In a $^{86}Rb^+$ efflux assay, A-85380 has been shown to be equipotent as an agonist at a α4β2 nAChR-expressing cell line and at the native ganglionic α3β4* subtype in human IMR32 cells (Table 4).[118,119] Hence, A-85380 and 5-I-A-85380 can only be claimed to be β2 nAChR selective and only in terms of binding affinities.

The (*R*)- and (*S*)-enantiomers of the 2-chloropyridine analogue of A-85380, tebanicline (previously ABT-594,

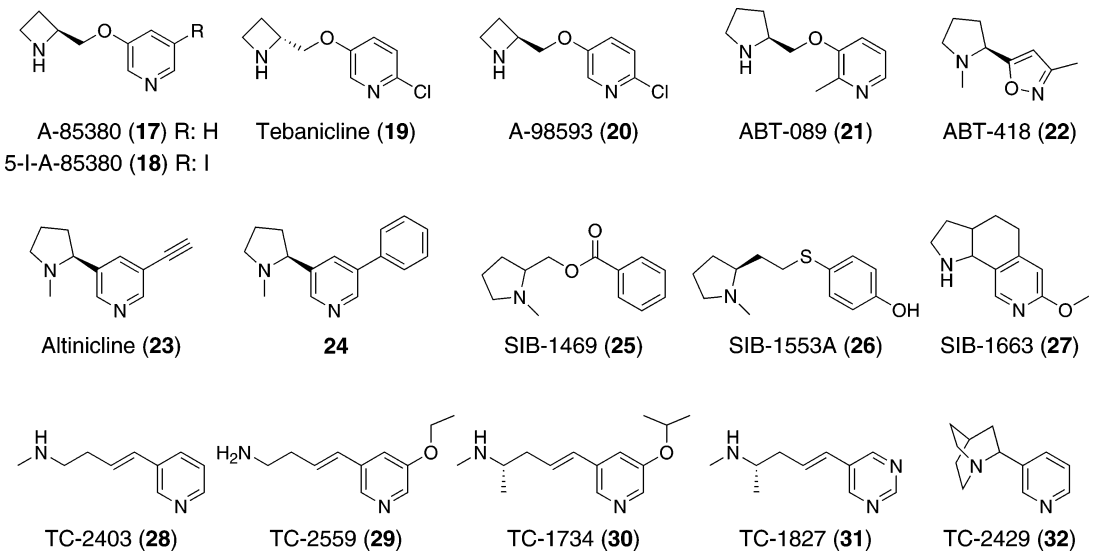

Figure 14. Agonists of heteromeric α/β nAChRs.

19) and A-98593 (**20**), are potent agonists of α4β2 and α3β4* nAChRs (Table 4).[123,124] The two enantiomers displayed very similar properties in binding and functional assays and in animal pain models, while the pronounced cardiovascular side effects of A-98593 compared to tebanicline was attributed to its higher efficacy at the ganglionic α3β4* nAChR.[123,124] Tebanicline was found to be an orally effective, broad-spectrum analgesic in animal models for acute, persistent, and neuropathic pain, whereas it did not elicit the withdrawal symptoms, physical dependence, or decreased gastrointestinal motility associated with opioids.[125,126] The analgesic activity of tebanicline could be antagonized by mecamylamine and was in good agreement with the well-documented antinociceptive properties of nicotine and epibatidine.[60,125−127] In contrast to marked side effects such as hypertension and neuromuscular paralysis observed upon epibatidine administration, tebanicline did not evoke these side effects at the same level of severity, which in part was accredited to its lack of muscle-type nAChR activity.[125,126] Nevertheless, the clinical development of tebanicline has recently been discontinued after phase II trials because of gastrointestinal adverse effects presumably caused by the α3β4* nAChR component of the compound. New generations of nAChR agonists from Abbott have entered phase I clinical trials for chronic inflammatory and nociceptive pain and for neuropathic pain, and they have displayed improvements in efficacy and safety compared to tebanicline.[60]

In contrast to the full agonism displayed by the azetidine pyridyl ethers at the α4β2 and α3β4* nAChRs, the pyrrolidine analogue ABT-089 (**21**) has exhibited remarkable low efficacies at these receptors as well as at the α7 nAChR (Table 4).[128,129] This was quite surprising considering the low nanomolar binding affinity of the compound to native α4β2* receptors and its ability to stimulate cation efflux through native α4β2* nAChRs in mouse thalamic synaptosomes.[128,129] The authors suggested that ABT-089 could exert its effects in this region through a α4β2* receptor incorporating α3 and/or α5 subunits, since these subunits are also expressed in the thalamus (Figure 2). In neurotransmitter release experiments, ABT-089 was as potent and efficacious as nicotine in stimulating ACh release from hippocampal synaptosomes, whereas it displayed a 25-fold lower potency compared to nicotine in stimulation of DA release in striatal slices.[129] In animal models, ABT-089 has exhibited significant positive effects for anxiety and cognitive functions and a significantly improved safety profile compared to nicotine in terms of cardiovascular and gastrointestinal side effects.[128−131] The compound is currently in phase II clinical trials for cognitive disorders such as Alzheimer's disease and attention-deficit/hyperactivity disorder (ADHD), although the molecular basis for its effects has not been completely resolved.[131]

The 3-methyl-5-isoxazole analogue of nicotine, ABT-418 (**22**), has become a standard nAChR ligand. It is a partial agonist at α2β2 and α4β2 nAChRs displaying ∼30-fold lower potencies at α3β2, α3β4, and α7 receptors (Table 4).[132] ABT-418 has displayed anxiolytic and cognition-enhancing effects and a significantly improved therapeutic window compared to nicotine in numerous rodent models.[133−135] On the basis of these studies, the agonist entered clinical trials for Alzheimer's disease, which recently have been discontinued.

5.1.2. SIBIA/Merck Compounds. The 5-position in the pyridine ring of nicotine seems to be an important determinant of β2/β4 nAChR selectivity. The 5-ethynyl analogue of nicotine, altinicline (formerly SIB-1508Y, **23**), is a partial agonist characterized by a modest functional preference for the α4β2 nAChR over β4-containing subtypes, while exhibiting no measurable activity at α7 or muscle-type nAChRs (Table 4).[136−138] Altinicline was found to be as efficacious as nicotine in evoking NE release in thalamic and cortical regions, whereas it only elicited a weak increase in hippocampal NE release.[138] Based on the basis of these observations, the authors speculated that NE release in the different CNS regions is modulated by different nAChRs, and the proposed involvement of a α3β4* subtype in NE release in the hippocampus has subsequently been supported by studies using α-conotoxin AuIB.[41] More importantly, altinicline was a highly efficacious stimulant of DA release in striatum and nucleus accumbens, and this gave rise to positive effects in animal models of Parkinson's disease.[138−140] Altinicline has been evaluated

in clinical trials for this disorder but has not progressed further.

Replacement of the 5-ethynyl group in altinicline with a phenyl group (**24**) switches the preference of the agonist toward β4 nAChRs, and further development has yielded phenyl esters and phenol thioethers such as SIB-1469 (**25**) and SIB-1553A (**26**).[141] SIB-1553A is distantly related to the pyridyl ethers **17**−**21** but possesses a completely different functional profile. Although not significantly more potent at β4 nAChRs than at β2 receptors, SIB-1553A is nevertheless a selective β4 nAChR agonist due to its low efficacies at β2 nAChRs (Table 4).[141] In microdialysis studies it has been shown to be far more efficacious than both altinicline and nicotine in stimulating ACh release in the hippocampus and prefrontal cortex, whereas the compound only induces minor increases in striatal dopamine release.[142] This modulatory action of SIB-1553A on cholinergic neurotransmission may underlie its promising effects in models of Alzheimer's disease and other cognitive disorders.[143−145] The compound was entered into clinical trials for these indications, but these have been discontinued.

A series of conformationally restricted nicotine analogues has yielded SIB-1663 (**27**).[146] SIB-1663 was found to be a selective agonist at recombinant $\alpha2\beta4$ and $\alpha4\beta4$ nAChRs mainly due to its low efficacies at $\alpha3\beta2$, $\alpha4\beta2$, and $\alpha3\beta4$ nAChRs.[146] The reduced $\alpha3\beta4$ component of SIB-1663 compared to SIB-1553A could be attractive from a therapeutic perspective, considering the link of this subtype to several of the adverse effects of nAChR agonists. Interestingly, the increase in DA release in rat striatal slices observed upon SIB-1663 adminstration could not be suppressed with the nonselective nAChR antagonists DHβE (**12**) and mecamylamine (**15**). Furthermore, SIB-1663 appeared to exert its effect on DA release through an nAChR subtype distinct from those targeted by nicotine, since coapplication of saturation concentrations of the two agonists increased the DA release in an additive manner.[147] As altinicline, SIB-1663 displayed varying effects on the NE release in the prefrontal cortical, hippocampal, and thalamic slices, being considerably less efficacious than nicotine in the last two tissues. Analogous to the DA release experiments, the SIB-1663-mediated NE release in hippocampal slices could not be antagonized with mecamylamine.[147] These atypical in vitro characteristics of the compound were mirrored in rodent models for pain, cognitive functions, and locomotor activities. Thus, although the in vivo behavior of SIB-1663 primarily seemed attributable to its nAChR activity, the involvement of other mechanisms could not be excluded.[147]

5.1.3. Targacept Compounds. The company Targacept has in recent years published several interesting subtype-selective nAChR agonists. The compounds have been developed using in silico drug discovery tools such as quantum chemistry techniques, pharmacophore models, QSAR methodologies, and molecular modeling combined with an archive of biological data from several years of nAChR research. In addition to the following examples, other nAChR agonists have entered phase II clinical trials for pain and ADHD.

Opening of the pyrrolidine ring in nicotine has resulted in compounds such as TC-2403 (formerly RJR-2403), TC-2559, TC-1734, and TC-1827 (Figure 14). TC-2403 (or *trans*-metanicotine, **28**), a metabolite of (*S*)-nicotine, is a moderately potent agonist exhibiting some functional preference for $\alpha4\beta2$ over other α/β, $\alpha7$, and muscle-type nAChRs (Table 4).[148,149] In microdialysis studies of cortical neurotransmitter release, systemic adminstration of TC-2403 has been demonstrated to give rise to the same degree of increases in the extracellular levels of ACh, DA, NE, and 5-HT as those elicited by nicotine.[150] TC-2403 has displayed analgesic effects in several animal models and is currently in phase II clinical trials for ulcerative colitis.[151]

Strikingly, two minor modifications of the TC-2403 molecule have resulted in a potent and completely $\alpha4\beta2$-selective agonist, TC-2559 (**29**).[152] The agonist displayed at least 70-fold higher potencies at $\alpha4\beta2$ than at $\alpha3\beta2$, $\alpha2\beta4$, $\alpha3\beta4$, $\alpha4\beta4$, and $\alpha7$ nAChRs expressed in mammalian cell lines, whereas the potency differences at $\alpha4\beta2$ and $\alpha4\beta4$ nAChRs expressed in oocytes were less pronounced (~20-fold).[153] TC-2559 was a partial agonist at $\alpha4\beta2$ and the three $\beta4$ nAChRs, whereas it displayed very low efficacies at $\alpha3\beta2$ and $\alpha7$ (Table 4).[153] Since α-conotoxin MII failed to block in the vivo responses of TC-2559, the activity of the compound at $\alpha6\beta2$* nAChRs also appeared to be negligible.[153] In agreement with its in vitro selectivity profile, TC-2559 has been shown to potently increase the firing rate of DA cells in midbrain slices, and the performance of the compound in models of cognitive functions has been promising.[152,153] Furthermore, compared to nicotine, both TC-2403 and TC-2559 have exhibited significantly reduced degrees of side effects mediated by the ganglionic $\alpha3$* and the muscle-type nAChRs, such as changes in blood pressure and heart rate and hypothermia.[152,154] Being one of the first truly $\alpha4\beta2$-specific agonists, TC-2559 could be a valuable tool in future investigations of the physiological functions of $\alpha4\beta2$* subtypes.

The 5-isopropoxy-3-pyridinyl and the pyrimidinyl analogues of (*S*)-α-methylmetanicotine, TC-1734 (**30**) and TC-1827 (**31**), have not been subjected to detailed pharmacological characterizations at recombinant nAChRs. However, both compounds have been shown to be $\alpha4\beta2$* nAChR agonists with negligible activities at ganglionic $\alpha3$*, $\alpha7$*, and muscle-type nAChRs.[155,156] Although the agonists displayed binding affinities similar to those of native $\alpha4\beta2$* nAChRs, they exhibited markedly different activities in an in vitro assay for striatal DA release, where TC-1734 was a partial agonist with an EC_{50} value of 106 nM and where TC-1827 was a full agonist with a 70-fold lower potency.[155,156] Oral administration of both TC-1734 and TC-1827 have been shown to enhance cortical ACh release, and both agonists have improved short- and long-term memory in a battery of rodent models.[155−157] TC-1734 has been shown to reduce the neurotoxicity induced by Glu in primary cultures and by hypoxia/glucose deprivation in hippocampal slices, and based on its long-lasting positive effects on memory and attention in phase I trials, the compound has entered phase II clinical trials for the treatment of cognitive impairment and memory disorders.[155]

Quinuclidine (1-azabicyclo[2.2.2]octane) constitutes the scaffold in a number of nAChR agonists. Introduction of a 3-pyridinyl group in the 2-position of this cyclic amine has provided the atypical nAChR agonist

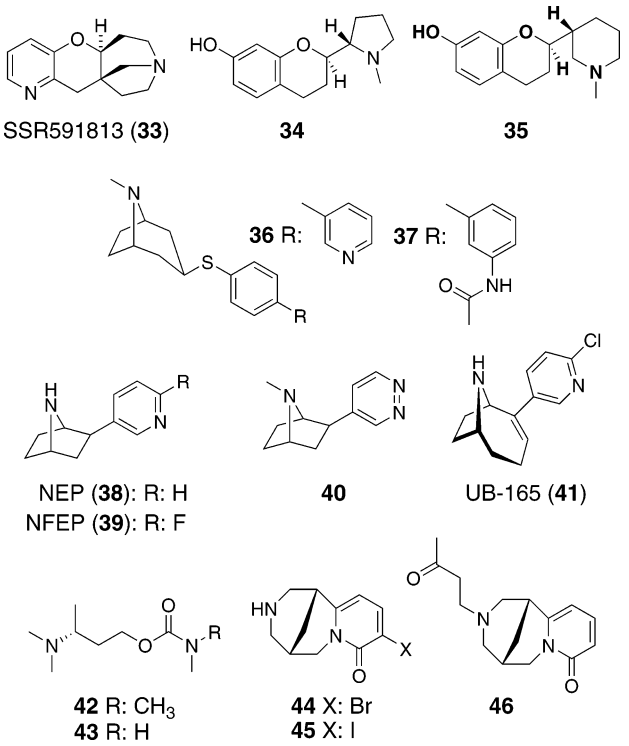

Figure 15. Agonists of heteromeric α/β nAChRs.

TC-2429 (**32**).[158] TC-2429 has been shown to stimulate DA release from rat striatal synaptosomes at low nanomolar concentrations, whereas it displayed >100-fold lower potencies at ganglionic nAChRs in PC12 cells and ileum tissue. Strikingly, the compound inhibited $^{86}Rb^+$ efflux from rat thalamic synaptosomes at ∼100-fold higher concentrations than its actions at striatal nAChRs. Originally, TC-2429 was proposed to discriminate not only between striatal α4β2* and ganglionic α3* nAChRs but also between striatal and thalamic α4β2* nAChRs.[158] Since TC-2429 is an antagonist at the recombinant α4β2 nAChR, the authors proposed that the striatal nAChR targeted by TC-2429 could be an α4α3β2* subtype and the thalamic nAChR another α4β2* subtype.[158] Subsequently, however, TC-2429 has displayed similar and 10-fold lower binding affinities to a α6β4β3α5 nAChR cell line and to native α7* nAChRs, respectively, than that displayed to α4β2* nAChRs.[24] Based on these findings, TC-2429 has been proposed to release DA in striatum via its activity at striatal α6* nAChRs.[24] Although this is not as exotic an explanation as the previously proposed dual agonistic/antagonistic properties at two different α4β2* nAChR subpopulations, the pharmacological profile of TC-2429 is still highly interesting as it appears to be able to discriminate between the highly homologous rat α3β4* and human α6β4β3α5 receptors. Limiting its use in vivo, TC-2429 displayed an EC_{50} of 60 nM at the muscle-type nAChR, making it the most potent agonist at this receptor published to date.[158]

5.1.4. Other Nicotine-Derived Agonists. The conformationally restricted pyridylamine SSR591813 (**33**) has recently been shown to be a functionally selective partial agonist at α4β2 displaying >100-fold lower potencies at α3β2 and α3β4 nAChRs (Table 4 and Figure 15).[159] The compound has exhibited promising antiaddictive activities in models for nicotine and amphetamine discrimination, and it has been shown to be able to prevent nicotine withdrawal symptoms and reduced nicotine self-administration in rodents, where it also appeared to be devoid of hypothermia and cardiovascular side effects.[159]

The 2-(2-pyrrolidine)chroman **34** (or chromaperidine) appears to possess a highly unique pharmacological profile.[160] The compound was found to be a full agonist in a striatal DA release assay, whereas it was unable to enhance extracellular ACh levels in cortex significantly above basal levels.[160] In contrast, the closely related chromaproline (**35**), a conformationally constrained analogue of ABT-089 (**21**), stimulated striatal DA release and cortical ACh release with efficacies comparable to those of nicotine. Both **34** and **35** were moderately potent agonists at recombinant α3β2 and α7 nAChRs, whereas **34** appeared to be an α4β2 antagonist.[160] The pharmacology of **34** ought to be characterized at additional recombinant nAChR subtypes, since it is one of the few agonists published to date showing functional preference for α3β2/α6β2 over α4β2 nAChRs.

Recently, an Eli Lilly group has published a series of (biarylthio)tropanes somewhat similar to SIB-1553A (**26**), which also display high degrees of selectivity for β4-containing nAChRs (exemplified by **36** and **37** in Figure 15). The effects of variations of the cationic amino group, the length and composition of the sulfur linker, and the two aromatic groups on the selectivity profiles of the compounds have been investigated in elaborate SAR studies.[161,162]

5.1.5. Epibatidine-Derived Agonists. (±)-Epibatidine (**6**) is an extremely potent but nonselective nAChR agonist[127] that has been subjected to numerous SAR studies (Figure 13). In contrast to the parent compound, the deschloroepibatidine analogues NEP (**38**) and NFEP (**39**), where the 2′-chloro atom at the pyridine ring of **6** has been replaced by hydrogen or fluorine, respectively, have displayed considerably higher binding affinities to α2β2, α3β2, and α4β2 nAChRs than to the corresponding β4 nAChRs, the differences being between 50- and 3000-fold.[163,164] However, both compounds were found to be equipotent agonists at α4β2, α3β4, and α4β4 nAChRs expressed in oocytes.[164] In contrast, replacement of the 2-chloropyridine ring in **6** with a 2-(pyridazin-4-yl) ring and methylation of the basic amino group have resulted in **40**. Besides being a submicromolar agonist of the muscle-type nAChR, **40** is the epibatidine-derived agonist published to date exhibiting the highest degree of functional preference for α4β2 over α3β4 nAChRs (Table 4).[165]

(±)-UB-165 (**41**) is a hybrid compound between epibatidine (**6**) and anatoxin-a (**10**) and bears some structural resemblance to TC-2429 (**32**). The potencies of (±)-UB-165 at α2β4, α3β4, and α4β4 have been reported to be at least 10-fold higher than at α3β2 and α7 nAChRs, and analogous to SIB-1553A, (±)-UB-165 elicits very low maximal responses through α2β2 and α4β2 (Table 4).[42]

5.1.6. ACh-Derived Agonists. The carbamate analogue of ACh, CCh (**2**), is an equipotent agonist at mAChRs and nAChRs. However, introduction of one or two methyl groups at the carbamate nitrogen yields MCC (**3**) and the dimethylated analogue DMCC, which are completely devoid of mAChR activity.[2,4] Recently,

the CCh SAR studies were supplemented with a series of one-carbon homologues of DMCC and its corresponding tertiary amine.[166,167] In contrast to DMCC, high-affinity nAChR binding of the one-carbon homologue of CCh required its basic amino group to be a tertiary amine. The 3-methyl CCh homologue (**42**) displayed binding affinities to recombinant α/β nAChRs in the nanomolar range. Furthermore, the sizes of the alkyl substituents at the carbamate nitrogen appeared to be important determinants of β2/β4 nAChR selectivities of the compounds as compound **43** displayed significantly decreased affinities for β4-containing nAChRs compared to **42**, whereas the binding characteristics of the two compounds at β2 nAChRs were similar. The affinity differences translated into a 20-fold lower potency of **43** at the α3β4 nAChR compared to **42**. The functional properties of **42**, **43**, and other compounds in the series are currently being characterized at recombinant nAChRs and in neurotransmitter release assays.

5.1.7. Cytisine-Derived Agonists. Cytisine (**5**) is a potentially interesting lead for the development of subtype-selective nAChR ligands. First, the agonist is characterized by an inherent preference for neuronal nAChRs over the muscle-type nAChR not observed for ACh, nicotine, or epibatidine. Second, it displays significantly lower efficacies at β2-containing nAChRs than at β4 nAChRs (Table 4). Finally, cytisine has displayed preference for α4- over α3-containing nAChRs. Despite these interesting properties, cytisine has only been used as a lead in a few SAR studies (Figure 13).[116,117] Halogenation in the 3-position of the pyridone ring of cytisine has resulted in significant increases in the potency and efficacy at several nAChR subtypes. The 3-Br analogue **44** has displayed 10-, 100-, and 16-fold lower EC_{50} values than cytisine at α4β2, α4β4, and α7 nAChRs, respectively, and the 3-I analogue **45** was even more potent (Table 4).[168] Furthermore, the efficacies of **44** and **45** at the α4β2 nAChR were 10- and 23-fold enhanced compared to that of cytisine.[168] In contrast, the 5-halogenation of cytisine did not result in improved potencies or efficacies at the three nAChRs.[168] In another series, aliphatic, alicyclic, arylalkylic, or heteroarylic groups have been introduced at the amino group of cytisine.[169] Compound **46** was an equipotent α4β2 and α3β4 agonist with a considerably reduced efficacy at α3β4 compared to that of cytisine, and other compounds in this series were found to be competitive antagonists (Table 4). Hence, the N-substituent appears to be another important determinant of the nAChR efficacy for cytisine. Although none of the cytisine analogues have displayed significant functional selectivity between α/β nAChR subtypes, the dramatically altered pharmacologies of the 3-halogenated analogues compared to the parent compound seem to warrant additional SAR investigations of the cytisine structure.

5.2. α7 nAChR Agonists. In contrast to the challenges associated with the design of ligands targeting specific neuronal α/β nAChR subtypes, several α7 nAChR-selective agonists have been published (Figure 16). In fact, because of the high homology between the orthosteric sites in α7 and 5-HT_3 receptors, α7 agonists more often exhibit cross-activity with this LGIC than with other nAChRs.

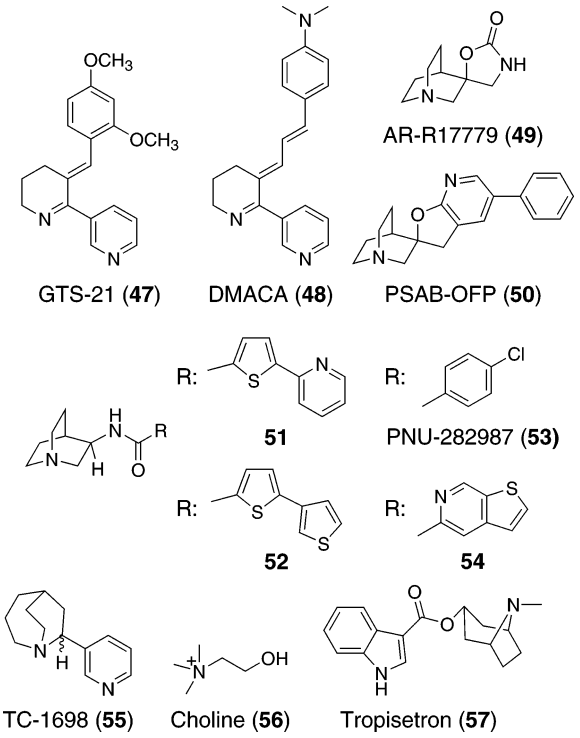

Figure 16. Agonists of the α7 nAChR.

5.2.1. Anabaseine Analogues. The toxin anabaseine (**8**) is found in a marine worm and in certain ant species. The toxin is a rather nonselective nAChR agonist but exhibits a significantly higher efficacy at α7 nAChR than at the heteromeric subtypes.[170,171] Introduction of conjugated aryl substituents in the 3-position of the pyridine ring of anabaseine have resulted in overall reduced activities at nAChRs. However, since the reduction in activity was less pronounced for α7 than for the heteromeric nAChRs, compounds such as DMXBA (**47**, also termed GTS-21) and DMACA (**48**) have turned out to be weak but selective partial agonists of the receptor (Figure 16 and Table 4).[170] Although DMACA is more potent and has higher efficacy at the α7 nAChR, GTS-21 has become the prototypic compound in this series. GTS-21 has recently been claimed to be considerably more potent as an antagonist at β2- and β4-containing nAChRs than as an α7 agonist, an antagonism ascribed to GTS-21-induced desensitization of the heteromeric nAChRs.[172] The compound has been consistently more efficacious at the rat α7 nAChR than at the human receptor, a difference attributed to four nonconserved residues in loops C, E, and F in the orthosteric sites of the two receptors.[173,174] The promising effects of GTS-21 in humans have thus been accredited to the three major metabolites generated by O-dealkylation of GTS-21.[173,175] However, in a recent study the metabolites were found not to penetrate the BBB to the extent that GTS-21 does, indicating that their contributions to the in vivo effects of GTS-21 could be negligible.[175] GTS-21 and its metabolites have also been shown to be antagonists of the human 5-HT_3R.[175,176] Whether the plasma concentrations of GTS-21 or its metabolites reach levels at which this 5-HT_3 component could be of physiological relevance has not been addressed.

5.2.2. Quinuclidine Structures. The quinuclidine (1-azabicyclo[2.2.2]octane) ring system forms the scaf-

fold in several α7 nAChR-selective ligands. The incorporation of the basic nitrogen into this bicyclic structure has been proposed to constrain the orientation of the protonation of the nitrogen in a manner that facilitates α7 nAChR binding and activation.[117] The spirooxazolidinone AR-R17779 (**49**), a conformationally restricted analogue of CCh (**2**), is a full agonist at the α7 nAChR, displaying pronounced selectivity against numerous heteromeric α/β nAChR combinations (Figure 16 and Table 4).[172,177,178] The α7 activity of the agonist has been found to be sensitive to even minor structural alterations, such as methylation of the nitrogen in the spirocycle and changes in the carbamate function.[177] However, further developments in this series have provided compounds such as PSAB-OFP (**50**), which is equipotent with AR-R17779 at the α7 nAChR and furthermore is a potent 5-HT_3R agonist.[179] This 5-HT_3R component has been eliminated by substitution of the phenyl group at the 5′-position of the furopyridine ring in PSAB-OFP with a pyridylmethylamine group.[172] In new generations of quinuclidine structures the bicyclic ring system has been coupled directly to arylamide, bisarylamide, or heteroarylamide groups, and judging from the patent literature, the quinuclidine amide appears to be the scaffold of choice in several α7 nAChR programs in the pharmaceutical industry.[117,172] These series include agonists such as **51**−**54**, which exhibit nanomolar potencies at the α7 nAChR and high degrees of selectivities against other nAChRs (Figure 16).[180−182] The Eli Lilly compounds **51** and **52** are potent full α7 agonists devoid of activities at other nAChRs and the 5-HT_3R (Figure 4).[117,181] The simple *p*-chlorbenzamide analogue PNU-282987 (**53**) has been shown to stimulate signaling through the α7/5-HT_3 chimera and through native α7* nAChRs in cultured hippocampal neurons with EC_{50} values of 128 nM and ∼3 μM, respectively.[180] Furthermore, the compound has displayed >500-fold lower (antagonistic) potencies at α1$\beta\gamma\delta$ and α3β4 nAChRs, a >100-fold lower binding affinity for native α4β2* nAChRs in rat brain homogenate, and a 30-fold higher IC_{50} value as a competitive 5-HT_3R antagonist than its potency at the α7/5-HT_3 chimera.[180]

5.2.3. Miscellaneous α7 nAChR Agonists. Substitution of the pyrrolidine group of nicotine with seven- and eight-membered ring analogues has generally resulted in ligands with dramatically decreased binding affinities and potencies.[117] In contrast, introduction of an azabicyclo[3.2.2]nonane ring has yielded TC-1698 (**55**), a full agonist at the α7 nAChR with insignificant activities at α3β2, α3β4, and α4β2 receptors (Table 4).[183] Surprisingly, the compound is also a partial agonist at the muscle-type nAChR with a 20-fold lower potency than at α7. TC-1698 has been shown to be neuroprotective through its α7-mediated activation of a JAK2/PI-3K cascade.[183]

Choline (**56**), the endogenous precursor for ACh and the metabolic product of ACh hydrolysis, has been shown to be a full agonist at the native and recombinant α7 nAChRs with EC_{50} values around 1 mM, whereas the compound has little or no activity at α4β2, α3β4, α3β2, and muscle-type nAChRs.[184,185] Thus, choline appears to be an endogenous α7 nAChR agonist. So far, this finding has not sparked synthesis and pharmacological characterization of a compound series based on choline possibly because of the inherent low potency of this lead.

The 5-HT_3R antagonist tropisetron (**57**) is a partial agonist of the α7 nAChR with an EC_{50} around 1 μM.[178,186] The compound has been reported to possess >1000-fold higher binding affinities at 5-HT_3 and at α7 receptors than at α4β2, α3*, and muscle-type nAChRs.[186] However, in a recent study tropisetron has been shown to antagonize α3β4 nAChR signaling in the same low micromolar concentrations where it activates the α7 nAChR, and thus, it cannot be claimed to be selective for the α7 nAChR.[178]

The results from in vivo studies of α7-selective agonists have not been particularly promising. **51** and **52** have failed to show significant effects in a wide range of animal models,[117,187] and AR-R17779 (**49**) has displayed varying degrees of effects in cognition models.[188−192] The shortcomings of AR-R17779 in vivo have been attributed to poor pharmacokinetic characteristics.[172] Based on observations made in vitro where rapid desensitization of the α7 nAChR occurs at agonist concentrations significantly lower than those required for activation of the receptor, it has been proposed that α7 agonists may in fact display functional antagonism in vivo.[117,187,193] This could mean that the true therapeutic prospects in α7 nAChR stimulation may not have been fully disclosed in preclinical and clinical studies of α7 agonists. However, in vivo studies supporting the therapeutic prospects in α7 agonists do exist. In contrast to several of the "clean" α7 agonists, GTS-21 (**47**) has consistently displayed beneficial effects in models for cognition enhancement and neuroprotection, and the agonist has entered clinical trials for Alzheimer's disease.[194] Considering its activities on the 5-HT_3R and possibly also other nAChR subtypes, it remains to be determined whether its interesting in vivo properties can be ascribed solely to its α7 component.

There is a substantial amount of evidence for the α7* nAChR as a highly interesting target for the treatment of schizophrenia.[8,9] In concordance with this, administration of GTS-21 has been shown to normalize the inherent impaired auditory gating in the DBA/2 mice strain and in isolation-reared rats,[195,196] and in a recent study GTS-21 and PNU-282987 (**53**) were shown to be capable of restoring the amphetamine-induced sensory gating deficit in anesthetized rats.[197] In the hippocampus, α7* nAChRs are predominantly located on GABAergic interneurons, where the receptors modulate the inhibitory synaptic activity. In agreement with this, PNU-282987 was found to produce a long-lasting potentiation of the GABAergic synaptic activity in hippocampal slices and to enhance hippocampal oscillatory activity, and the authors ascribed the beneficial effects of α7 agonists on cognitive deficits in rodent models to this enhancement.[197]

5.3. Competitive nAChR Antagonists. Analogous to nAChR agonists, most of the standard antagonists for the receptors have been obtained from natural sources. The peptide toxin α-bungarotoxin from the Taiwan banded krait (*Bungarus multicinctus)* and methyllycaconitine (MLA, **14**), isolated from *Delphinum* and *Consolida* species, are highly selective competitive antagonists of the α7 nAChR (Table 4). This selectivity only applies to the CNS, however, since both antagonists

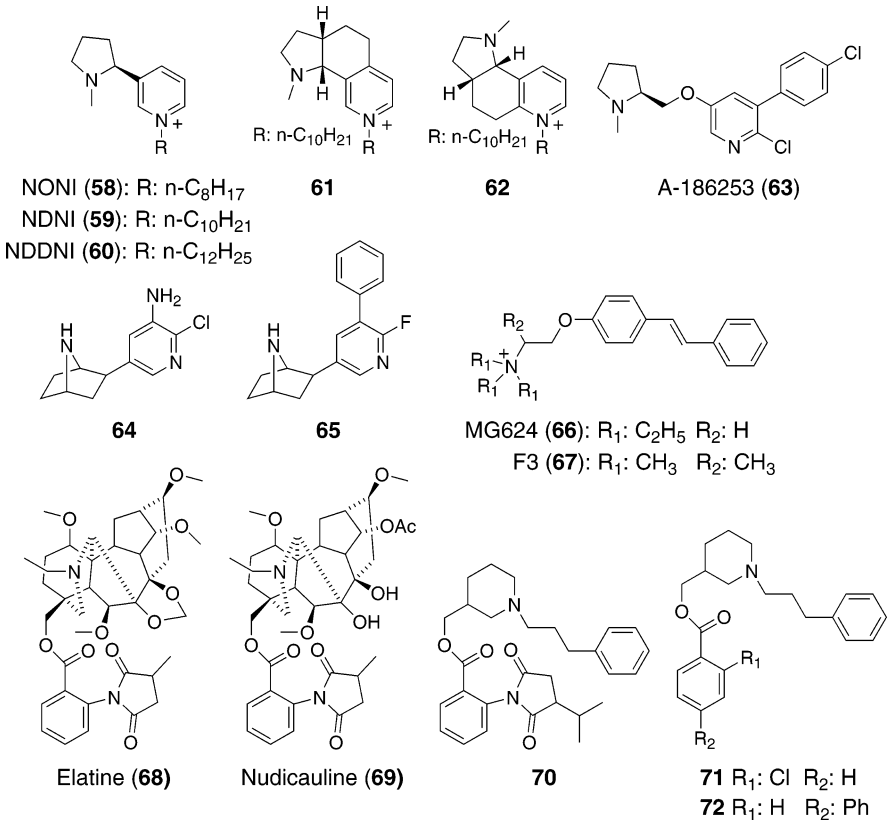

Figure 17. Competitive antagonists of nAChRs.

target α9 and α9α10 nAChRs as well, and α-bungarotoxin also is a potent antagonist of the muscle-type nAChR. In contrast to the selectivity of these two antagonists, other classical competitive nAChR antagonists such as dihydro-*β*-erythroidine (DH*β*E, **12**) and *d*-tubocurarine **(13**) are far less discriminative between different nAChR subtypes (Figure 12 and Table 4).

Introduction of *n*-alkyl groups ranging from methyl (CH_3) to dodecyl ($C_{12}H_{25}$) at the pyridine nitrogen of (*S*)-nicotine has produced several potent nAChR antagonists (exemplified by **58**−**60** in Figure 17). The compounds displayed binding affinities ranging from high-nanomolar to mid-micromolar concentrations to the native α4*β*2* nAChRs in the rat brain, whereas they all were unable to displace [^{3}H]MLA from α7* nAChRs at 50 μM.[198] There was a clear correlation between the lengths of the respective *n*-alkyl groups of the compounds and their binding affinities at α4*β*2*, with the exception of the *n*-octyl-analogue NONI (**58**), which displayed weak binding to the receptor.[198] Furthermore, a correlation also existed between *n*-alkyl lengths and the antagonistic potencies of the compounds when it came to inhibition of nicotine-elicited DA release in striatal synaptosomes.[199] Interestingly, the *n*-decyl analogue NDNI (**59**), which exhibited the highest α4*β*2* affinity, was unable to inhibit the DA release, whereas NONI (**58**), despite its weak α4*β*2* affinity, was a potent inhibitor.[199] Since these observations suggest that the two antagonists target different neuronal nAChR subtypes, it would be interesting to characterize the antagonistic profiles of the *n*-alkylnicotine analogues at recombinant nAChRs. Compounds in the "syn" and "anti" series of conformationally restricted *N*-*n*-octyl, *N*-*n*-nonyl-, *N*-*n*-decyl-, *N*-*n*-undecyl-, and *N*-*n*-dodecylnicotine analogues (exemplified by **61** and **62**) were also found to be devoid of α4*β*2* binding affinity, although they inhibited striatal DA release.[200]

The pyridyl ether A-186253 (**63**) has displayed high selectivity for native α4*β*2* versus α3*β*4* and α7* receptors in binding assays.[201] The functional selectivity displayed by the compound was far less impressive, however, as the α4*β*2*/α7* ratio of >200000 observed in binding only translated into 10-fold differences in antagonistic potencies at α4*β*2, α3*β*4, and α7 nAChRs (Table 4). Interestingly, A-186253 also displayed partial agonism at human and rat α4*β*2 and α3*β*4 nAChRs. Whereas the efficacies of this compound at the two α4*β*2 nAChRs and at the rat α3*β*4 nAChR were insignificant (∼5% of R_{max} of ACh), its maximal response at the human α3*β*4 was 40% of the R_{max} of ACh. The authors proposed that the activity at the ganglionic α3* nAChRs in humans could produce side effects not seen in mice and rats and focused on the compound's potential with respect to the development of α4*β*2*-selective PET ligands.[201]

The most potent competitive nAChR antagonists have, not surprisingly, come from SAR studies of epibatidine.[202−204] With a K_i value of 1 pM in [^{3}H]epibatidine binding assay to rat brain (α4*β*2*) nAChRs, **64** is the nAChR ligand with the highest binding affinity published to date.[203] Furthermore, the compound has exhibited a dual agonist/antagonist profile in the pain models, showing antinociceptive effects at doses 100-fold higher than those needed to inhibit the effects elicited by nicotine.[203] The authors proposed that these characteristics could arise from activity of **64** at different nAChR subtypes, but this remains to be verified. Introduction of a 3′-phenyl group in the 2′-fluoropyridine

ring of NFEP (**39**) results in the antagonist **65**, which blocks the antinociceptive effects of nicotine.[202] Introduction of electron-withdrawing or electron-donating substituents in the 3′ and 4′ positions of this phenyl group has further increased binding affinities to α4β2* receptors and the antagonistic properties in the pain models.[204]

The 4-oxystilbene derivative MG624 (**66**) has been shown to be a 30-fold more potent antagonist at the chick α7 nAChR than at α4β2 and α1β1γδ nAChRs (Table 4).[205] Furthermore, MG624 and its analogue F3 (**67**) appear to be considerably more potent as antagonists at rat and human α7 receptors.[206] However, since F3 is also a potent inhibitor of the α3β4* nAChR in rat chromaffin cells, it remains to be seen whether the 4-oxystilbene derivatives are truly selective antagonists of mammalian and rodent α7* nAChRs.[206]

The complex molecule of MLA (**14**) has also been subjected to derivatization. Small modifications of this norditerpenoid alkaloid has resulted in elatine (**68**) and nudicauline (**69**), which are equipotent to and slightly more potent than MLA as α7 antagonists, respectively.[207] In contrast, analogues where the 2-((*S*)-methylsuccinimidobenzoyl) moiety has been replaced with methoxy groups or other ring systems have displayed significantly reduced α7 activities.[207] Analogously, the so-called "E ring" analogues of MLA (exemplified by **70** in Figure 17) have displayed negligible activities at α7, indicating that both moieties of MLA are important for its α7 activity.[208] Besides its α7 component, MLA also competitively antagonizes heteromeric nAChRs such as α3β4* nAChRs, albeit with potencies ∼1000-fold lower than at α7. In contrast to the loss of α7 activity, the "E ring" analogues of MLA and compounds where the succinimide group has been replaced with other substituents have retained the antagonistic potency at the α3β4* nAChR (exemplified by **70**−**72** in Figure 17).[208,209] Although it is interesting that α3β4*-preferring antagonists can arise from MLA, neither the E ring analogues nor the compound series derived from them have been characterized at other heteromeric nAChR subtypes.

5.4. α-Conotoxins. The venoms used by predatory cone snails to capture prey are rich sources of pharmacologically active peptides.[210] The entire mollusk family has been estimated to contain ∼50000 neuropharmacologically active toxins,[210] and a considerable fraction of these *Conus* peptides are the so-called "conotoxins". These small peptides of 12−20 amino acid residues have been demonstrated to act on ligand-gated ion channels such as NMDA and 5-HT_3 receptors and voltage-gated ion channels for Ca^{2+}, K^+, and Na^+, often with a high degree of selectivity for a particular ion channel.[211] Furthermore, conotoxins targeting G-protein-coupled receptors for vasopressin, neurotensin, and norepinephrine have also been identified.[211,212] In addition to the numerous conotoxins acting on the muscle-type nAChR, α-conotoxins targeted to neuronal nAChRs have, during the past decade, brought considerable insight into the molecular composition of heterologous native nAChR populations (see section 3.3) and could potentially be interesting templates in the design of new nAChR ligands.[211,212]

5.4.1. α-Conotoxin Family. The conotoxins have been divided into subfamilies A, M, S, and O based on their respective disulfide connectivities. The majority of conotoxins targeted to nAChRs belong to superfamily A, which is divided into the α, αA, and κA subfamilies.[211] So far, only α-conotoxins have been shown to act on neuronal nAChRs. All α-conotoxins possess four cysteine residues, and disulfide bonds are formed between the first and third and between the second and fourth cysteine (Figure 18A). Most of the identified α-conotoxins are organized in one of the three arrangements: CCX_3CX_5C ($\alpha_{3/5}$-conotoxins), CCX_4CX_3C ($\alpha_{4/3}$-conotoxins) or CCX_4CX_7C ($\alpha_{4/7}$-conotoxins). Only toxins with the last two disulfide connectivities seem to target neuronal nAChRs. The amino acid sequences and pharmacological properties of selected α-conotoxins are given in Table 5.

The $\alpha_{4/3}$-conotoxin ImI was the first α-conotoxin reported to inhibit neuronal nAChR signaling.[213] The peptide has been an important tool in studies of α7 nAChRs for years in part because it, unlike the classical α7 antagonist α-bungarotoxin, does not antagonize the muscle-type nAChR.[211,212] Recently, α-conotoxin ImII has been shown to inhibit α7 nAChR activity as well. However, in contrast to ImI, ImII does not displace [^{125}I]-α-bungarotoxin binding to the receptor, indicating that the two highly homologous toxins bind to different sites or at least to different microdomains of the receptor.[214]

The $\alpha_{4/7}$-conotoxin MII was originally proposed to be a potent and selective α3β2* nAChR antagonist.[215] MII has been used in combination with ImI and the α3β4-selective α-conotoxin AuIB to distinguish different subpopulations of nAChRs in the CNS and the periphery,[211,212,215] and a ^{125}I-labeled MII analogue has been used extensively in binding and autoradiography studies.[212] Some years ago, interpretations of these studies became more complicated when MII was shown to be an equipotent antagonist of α6-containing nAChR subtypes.[19,20] Recently, the $\alpha_{4/7}$-conotoxin PIA has displayed significant preference for α6-containing nAChRs compared to the corresponding α3-subtypes.[20] The $\alpha_{4/7}$-conotoxins AnIB and GIC have also exhibited pronounced selectivities for the α3β2 nAChR compared to α4β2, α3β4, α7, and muscle-type nAChRs, but their activities at α6-containing nAChRs have not been investigated.[216,217] The $\alpha_{4/7}$-conotoxin GID is an equipotent antagonist at α3β2 and α7 nAChRs, but more interestingly it is the first α-conotoxin to display a nanomolar IC_{50} value at the α4β2 nAChR.[218]

Interestingly, the two α-conotoxins with disulfide connectivities other than $\alpha_{4/7}$ and $\alpha_{4/3}$ display markedly different selectivity profiles from the other toxins in Table 5.[41,219] AuIB with its unusual CCX_4CX_6C arrangement is the only α3β4-selective α-conotoxin identified to date, although it remains to be characterized at the α6β4 nAChR (Table 5).[41] The recently isolated $\alpha_{4/4}$-conotoxin BuIA displays significant selectivity for α3- and α6-containing nAChRs over α2- and α4-subtypes and α7, whereas the toxin is equipotent at β2 and β4 nAChRs containing the same α-subunit (Table 5).[219] Interestingly, the dissociation kinetics for BuIA are highly dependent on the β-subunits present in the various nAChR subtypes, as the off-rates of the toxin at β4 nAChRs are considerably slower than at β2 nAChRs. Accordingly, a slow recovery of heteromeric

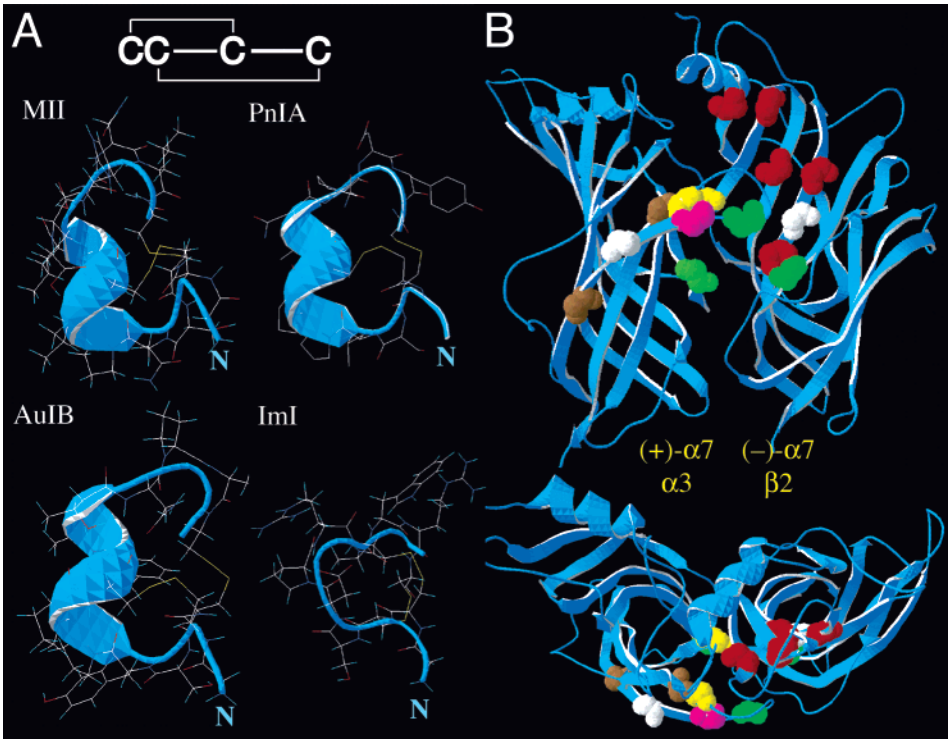

Figure 18. α-Conotoxins: molecular structures and binding sites at nAChRs. (A) Three-dimensional structures of α-conotoxins. The resolution of the X-ray structure of PnIA[258] is 1.1 Å, and the rmsd values of the NMR structures of MII,[259] ImI[260] and AuIB[261] are 0.07, 0.34, and 0.27, respectively. The position of the N terminal of the toxin is indicated for each of the structures. The general disulfide bond connectivities in α-conotoxins are depicted above the structures. (B) Residues in α3β2 and α7 nAChRs involved in α-conotoxin binding. The residues in the α7 nAChR shown to interact with ImI (red), PnIB (green), and both (yellow) and the residues in the α3β2 nAChR shown to interact with PnIA (brown) and MII (white) are indicated in the AChBP−HEPES structure.[71] An aligned residue in α7 and α3β2 involved in the binding of PnIB (to α7) and PnIA and MII (to α3β2) is given in purple. For clarity, only two of the monomers in the AChBP pentamer are shown and the HEPES molecule has been omitted.

nAChR function upon BuIA exposure will indicate the presence of β4 in one or both of the orthosteric binding sites of the receptor.[219] Hence, in contrast to other α-conotoxins that inhibit the signaling through all nAChRs containing one orthosteric site of a particular composition, BuIA appears to provide information about the compositions of both orthosteric sites in the heteromeric nAChR and could thus be a valuable tool in future studies of complex native nAChRs.

5.4.2. Molecular Determinants of α-Conotoxin Binding to nAChRs. The structures of several α-conotoxins have been determined by X-ray crystallography and/or NMR techniques, and four of these are depicted in Figure 18A. Since the peptide backbone structures of α-conotoxins having the same disulfide connectivities are almost identical, the pronounced differences in nAChR subtype selectivities displayed by related toxins arise from the side chains of nonconserved residues. This is supported by the observations made from surface diagrams of three-dimensional structures of α-conotoxins ImI and PnIA, where the nonconserved residues important for the nAChR activities are located on the solvent-accessible faces of the toxins, whereas the cysteines residues are buried.[220]

On the basis of the k_{on} and k_{off} characteristics of α-conotoxin MII binding to the α3β2 nAChR, Olivera and colleagues have termed the α-conotoxin a "Janus ligand" after the Roman two-faced god and proposed a "dock-and-lock" model for the toxin. According to the model, a "docking face" of the toxin establishes the initial binding to a complementary "docking site" in the β2 subunit, after which a distinct "locking face" forms bonds to a "locking site" on the α3 subunit.[211,221] Several residues in α7 and α3β2 nAChRs involved in the binding of α-conotoxins ImI, PnIB, PnIA, and MII have been identified (Table 6 and Figure 18B).[222−227] Conversely, the molecular determinants of binding of the four toxins to their respective nAChRs have also been delineated (Table 6).[224,225,228−230] In elaborate double mutant cycle studies of ImI and PnIB binding to α7, Sine and co-workers have identified several pairwise toxin−receptor interactions (Table 6).[223,224] Both toxins were found to coordinate to residues on both sides of the subunit interface, but the binding modes of $\alpha_{4/3}$ and $\alpha_{4/7}$-conotoxins were different. The N-terminal triad Asp[5]-Pro[6]-Arg[7] of ImI was proposed to establish contacts with aromatic residues in loops B and C in the (+)-side of the orthosteric site, whereas C-terminal residues of the toxin coordinate to (−)-side residues.[223] The principal ImI−α7 interaction seems to be a π−cation interaction between Arg[7] in the toxin and Tyr[195] in the aromatic box of the receptor.[223] Compared to ImI binding, the binding site of PnIB appears to be located lower in the orthosteric site of α7 (Figure 18B). The rigid scaffold of the $\alpha_{4/7}$-conotoxin has been proposed to present a hydrophobic spiral (Pro[6], Pro[7], and Leu[10]) to Tyr[93], Trp[149], and Tyr[151] located at the (+)-side of the subunit interface.[224] The toxin is anchored mainly by a direct interaction between its Leu[10] residue and the α7 Trp[149] residue also implicated in π−cation interactions with

Table 5. Pharmacological Characteristics of α-Conotoxins at Recombinant Neuronal nAChRs[a]

α-Conotoxin / species		sequence	Subfamily	Selectivity profile	IC_{50} range [nM]
AnIB	*C. anemone*	GGCCSHPACAANNQDYC*	$\alpha_{4/7}$	α3β2 >> α7$^{(250)}$ > α3β4, α4β2	0.3
AuIA	*C. aulicus*	GCCSYPPCFATNSDYC*	$\alpha_{4/7}$	α3β4	> 750
AuIC	*C. aulicus*	GCCSYPPCFATNSGYC*	$\alpha_{4/7}$	α3β4	> 750
EpI	*C. episcopatus*	GCCSDRPCNMNNPDYC*	$\alpha_{4/7}$	α7 > α3β2, α3β4$^{[>100]}$	30
GIC	*C. geographus*	GCCSHPACAGNNQHIC*	$\alpha_{4/7}$	α3β2 >> α4β2$^{[300]}$, α7$^{[700]}$	1.1
GID	*C. geographus*	IRDγCCSNPACRVNNOHVC	$\alpha_{4/7}$	α3β2 ~ α7 > α4β2$^{[30]}$ >> α3β4, α4β4$^{[>1000]}$	3 – 5
MII	*C. magus*	GCCSNPVCHLEHSNLC*	$\alpha_{4/7}$	α3β2, α6/α3β2β3 > hα6/α4β4$^{[20]}$ > α7$^{[100]}$ > α4β2$^{[300]}$	0.5 - 8
PIA	*C. purpurascens*	DPCCSNPVCTVHNPQIC*	$\alpha_{4/7}$	α6/α3β2, α6/α3β2β3 > α6β4, α6/α3β2$^{[30-70]}$ > α3β4$^{[500]}$	0.7 - 1
PnIA	*C. pennaceus*	GCCSLPPCAANNPDYC*	$\alpha_{4/7}$	α3β2 > α7 $^{[25]}$>> α2β2, α4β2	9.6
PnIB	*C. pennaceus*	GCCSLPPCALSNPDYC*	$\alpha_{4/7}$	α7 > α3β2$^{[30]}$	61
ImI	*C. imperialis*	GCCSDPRCAWR----C*	$\alpha_{4/3}$	α7 > α9$^{[10]}$, α3β4$^{[>10]}$	100-200
ImII	*C. imperialis*	AGGSDRRCRWR----C*	$\alpha_{4/3}$	α7	441
AuIB	*C. aulicus*	GCCSYPPCFATNPD-C*	$\alpha_{4/6}$	α3β4 > α7$^{(10)}$ > α4β2, α3β2, α4β4, α2β2, α2β4$^{(100)}$	0.75
BuIA	*C. bullatus*	GCCSTPPCAVLY---CGRRR*	$\alpha_{4/4}$	α6/α3β2, α6/α3β4 > α3β2$^{[30]}$, α3β4$^{[100]}$ α4β4$^{[300]}$> α7 > α2β4 > α2β2 > α4β2$^{[40,000]}$	0.3 – 1.5

[a] The approximate ratio of the IC_{50} value of the α-conotoxin at a certain specific subtype to its IC_{50} value at the subtype primarily targeted is given in brackets in superscript next to the data. The IC_{50} value at the nAChR subtype predominantly targeted by the α-conotoxin is given at the right end of the table. The activities of α-conotoxins at α6 nAChRs have been characterized using a α6/α3 chimera. The references for the pharmacological data are as follows: GIC,[217] AnIB,[216] PIA,[20] PnIA and PnIB,[226,232] GID,[218] AuIA/AuIB/AuIC,[41] ImI and ImII,[214,266] MII,[20,215] EpI,[267] and BuIA[219]. In the sequence column, ∗ represents an amidated C-terminal, γ is γ-carboxyglutamate, and O is hydroxyproline.

Table 6. α-Conotoxin−nAChR Interactions[a]

	α-conotoxin	nAChR
ImI−α7	Asp^5, Pro^6, Arg^7, Trp^{10}	(+): Trp^{149}, Tyr^{195} (−): Trp^{55}, Ser^{59}, Thr^{77}, Asn^{111}, Gln^{117}
	pairwise interactions: [Asp^5−Trp^{149}], [Arg^7−Tyr^{195}], [Trp^{10}− Thr^{77}] and [Trp^{10}−Asn^{111}]	
PnIB−α7	Ser^4, Pro^6, Pro^7, Ala^9, Leu^{10}	(+): Tyr^{93}, Trp^{149}, Arg^{186}, Tyr^{188}, Tyr^{195} (−): Ser^{34}
	pairwise interactions: [Pro^6−Trp^{149}], [Pro^7−Tyr^{93}], and [Leu^{10}−Trp^{149}]	
PnIA−α3β2	Ala^{10}, Asn^{11}	α3: Pro^{182}, Ile^{188}, Gln^{198}
MII−α3β2	Asn^5, Pro^6, His^{12}	α3: Lys^{185}, Ile^{188} β2: Thr^{59}

[a] Residues in four α-conotoxins and α7 and α3β2 nAChRs demonstrated to be important for binding of the α-conotoxins to the receptors. The table is adapted from a similar table in ref 268.

the positively charged amino groups of ACh and other nAChR agonists.[224] Hence, binding of both the $\alpha_{4/3}$-conotoxin and the $\alpha_{4/7}$-conotoxin to the nAChR appears to originate from a single anchoring interaction in the aromatic box supported by multiple weak interactions to residues at both sites of the subunit interface. Docking of the α-conotoxin into the AChBP structure suggests that the overall binding mode of the toxin is similar to that of α-bungarotoxin and α-cobratoxin (section 4.4 and Figure 11B).[231]

The molecular bases for the dramatically different nAChR selectivity profiles displayed by various α-conotoxins have also been elucidated in mutagenesis studies. Mutation of Ala^{10} in the α3β2-selective PnIA to a Leu

residue (the corresponding residue in the α7-selective PnIB) results in a selectivity switch from α3β2 toward α7,[232] and Glu[11]Ala and His[9]Ala/Leu[15]Ala mutants of α-conotoxin MII have exhibited significantly increased selectivities for α6 nAChRs over α3 nAChRs compared to the native toxin.[233] Furthermore, a general correlation between the length of the aliphatic side chain at position 10 of the $\alpha_{4/7}$-conotoxin and its nAChR selectivity has also been proposed, where increases in size result in increasing selectivity for α7.[220] Finally, the α4β2 activity of GID has been eliminated by truncation of the four "extra" residues in the unusual long N-terminal segment of the toxin as well as by a Arg[12]Ala mutation in the peptide.[218]

5.4.3. α-Conotoxin as a Ligand Template. The α-conotoxins currently available will undoubtedly continue to be important pharmacological tools. However, isolation of new generations of α-conotoxins could provide selective antagonists for nAChR combinations other than those targeted by the known toxins. Furthermore, the insight into the toxin−nAChR interactions gained from mutagenesis studies and the availability of homology models of nAChR NTDs could facilitate rational design of novel toxin analogues or peptidomimetics for nAChRs. The fact that all α-conotoxins identified to date display pronounced selectivities for α3-/α6-containing or α7 nAChRs indicates that there might be a inherent structural obstacle to α2/α4 nAChR binding built into the α-conotoxin structure, and thus, it remains to be seen whether selective ligands for these nAChR subtypes can be developed from the toxins. α-Conotoxin GID with its α4β2 activity would be an obvious starting template in these efforts.[218] The results from medicinal chemistry exploration into the α-conotoxin structure seem quite promising, since drastic alterations can be made in the peptide without compromising its nAChR activity. Ribbon-AuIB, an AuIB analogue with a non-native disulfide bond connectivity (disulfide bonds between the first and forth and between the second and third cysteine), is a 10-fold more potent antagonist at native rat α3β4* nAChRs than AuIB itself, despite a significantly more disordered overall structure.[234] Furthermore, elimination of one of the two disulfide bonds in ImI has been shown not to influence the overall structure of the toxin or its nAChR activity significantly.[229]

From a therapeutic perspective, the α-conotoxin shares the challenges of most peptides in terms of bioavailability and penetration of physiological membranes. Furthermore, the use of competitive antagonists in the form of 12-mer to 20-mer peptides as templates probably also holds inherent limitations in terms of developing nAChR agonists. However, the $\alpha_{4/7}$-conotoxin Vc1.1 has been shown to suppress vascular responses to unmyelinated sensory nerve C-fiber activation in vivo, and the 16-mer peptide is presently about to enter phase I clinical trials for chronic neuropathic pain.[235] Moreover, other α-conotoxins appear in the patent literature for a wide range of indications.

5.5. Allosteric Modulators of nAChRs. A wide range of structurally diverse ligands modulate nAChR signaling through their binding to allosteric sites at the receptor complex.[236,237] In addition to the allosteric effects of numerous endogenous ions, proteins, and fatty lipids, several ligands with preferential activity on other ion channels also possess nAChR activities. These "privileged structures" includes ligands such as memantine, PCP, and MK-801 (channel blockers of NMDA receptors), strychnine (the prototypic competitive glycine receptor antagonist), 5-HT (endogenous agonist of 5-HT_3R), nimodipine and nifedipine (voltage-gated calcium channel antagonists), ivermectin (allosteric modulator of glycine and $GABA_A$ receptors), and various *n*-alcohols, barbiturates, and steroids (allosteric $GABA_A$ receptor modulators).[236,237] In contrast, relatively few novel allosteric modulators of nAChR function have been identified, and the effects of most of these on nAChR signaling are quite subtle. In the following a few examples of important and efficacious allosteric nAChR modulators will be presented.

5.5.1. Endogenous Allosteric Modulators. In addition to the allosteric potentiation of several neuronal nAChRs exerted by Ca^{2+} (section 4.4), nAChRs are also modulated by another divalent cation, Zn^{2+}. Zn^{2+} is an endogenous allosteric modulator of a wide range of neurotransmitter receptors and transporters, and at α2β2, α4β2, α2β4, α3β4, and α4β4 nAChRs the metal ion has displayed biphasic modulation, potentiating agonist responses at concentrations up to 100 μM and inhibiting nAChR function at higher concentrations.[238] In agreement with its complex pharmacology, it has been suggested that Zn^{2+} binds to multiple sites in both the NTD and the ICD of the heteromeric nAChR and coordinates to residues in both α and β subunits.[238] In contrast to the biphasic modulation on these nAChRs, Zn^{2+} only displays inhibition of α3β2 and α7 nAChRs signaling, which could suggest that only some of the allosteric sites are conserved in these subtypes.[238,239]

The formation of amyloid plaques in limbic and cortical regions is one of the hallmarks of Alzheimer's disease. The plaques are aggregates of the β-amyloid peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ formed by the proteolytic cleavage of the amyloid precurser protein by β- and γ-secretases.[2,3] Interestingly, $A\beta_{1-42}$ was recently found to be colocalized with the α7 nAChR subunit in these plaques and in neurons from AD patients, and the two proteins were shown to form a stable complex due to a high-affinity interaction.[240] In subsequent studies, $A\beta_{1-42}$ has displayed low picomolar binding affinities to native α7* and recombinant α7 nAChRs and ∼1000-fold lower binding affinities to other nAChRs.[241] $A\beta_{1-42}$ has been shown to elicit currents in oocytes expressing rat α7 nAChR,[242] but this direct activation has not been reproducible in other recombinant expression systems or in neuronal cultures, where $A\beta_{1-42}$ has been shown to antagonize α7 signaling.[243,244] Although $A\beta_{1-42}$ is able to displace [^{125}I]-α-bungarotoxin and [^{3}H]MLA binding from α7, it appears to antagonize the receptor in a noncompetitive manner by reducing its open channel probability.[241,243,244] $A\beta_{1-42}$ is completely inactive at the 5-HT_3R but retains its antagonistic effect at an α7/5-HT_3 chimera (consisting of the α7-NTD and the 5-HT_3−ICD), indicating that the peptide exerts its effect on α7 signaling through binding to the NTD of the receptor.[243] Conversely, the molecular determinants of the α7 activity of $A\beta_{1-42}$ have been identified to reside in a 17-residue fragment of the peptide, the $A\beta_{12-28}$ peptide.[241,244] The pathophysiological implications of the α7

modulation by Aβ_{1-42} are still under investigation, but the interaction has been proposed to constitute a contributing factor to the impairment of cognitive functions observed at all stages of Alzheimer's disease.[241,244]

The recent demonstration of allosteric potentiation of native chick α7 nAChR signaling mediated by bovine serum albumin (BSA) is highly interesting.[245] A direct interaction between BSA and the receptor was shown to increase the steady-state opening probability of the ion channel via increases in both the frequency of channel opening and the average opening time duration. In contrast to its actions of α7, BSA did not potentiate the signaling of other native nAChR subtypes in ciliary ganglion neuron, just as $GABA_A$, NMDA, and AMPA receptor signaling were unaffected by BSA exposure. Interestingly, serum albumins from 11 different species displayed dramatic variations in their potentiation of chick α7 nAChR signaling. For example, pig albumin elicited a 12-fold increase in agonist efficacy, whereas human, rat, and chick albumins were virtually inactive. Hence, albumin does not appear to be an endogenous allosteric modulator of α7, although this will have to be confirmed in studies of cognate pairs of receptors and albumins from other species.[245] The significantly different α7 activities displayed by different species of albumin could facilitate the identification of the molecular determinants for the interaction of the protein with α7, which in turn could enable the design of novel allosteric modulators targeted at this site.

5.5.2. Synthetic Allosteric Modulators. Mecamylamine (**15**) is a rather nonselective nAChR antagonist with a slight preference for α3-containing subtypes (Table 4). Mecamylamine binds to the ICD of the nAChR, and its antagonistic potency at the α3β4 nAChR has been shown to decrease significantly upon replacement of four amino acid residues in the TM2 of β4 with the corresponding four residues in the muscle-β1 subunit.[246] Mecamylamine was originally marketed (as Inversine) on the U.S. market as an antihypertensive drug in the 1950s, but its use was limited by the pronounced side effects arising from the inhibition of parasympathic α3* nAChRs at the doses required for its antihypertensive effects.[247] In recent years, however, it has been reintroduced into the U.S. market for use in the treatment of symptoms of Tourette's syndrome. Furthermore, mecamylamine has been proposed as a potential therapeutic for treatment of cocaine abuse, and it is currently undergoing phase II testing for ADHD at doses below the antihypertensive dose.[247] Finally, mecamylamine administration has been shown to reduce self-administration of nicotine in animal models, and administration of it in combination with nicotine patches has been shown to increase the effectiveness of the patches.[247]

Bupropion (**73**) is used clinically as an antidepressant and as a smoking cessation aid (marketed as Zyban) (Figure 19). Although the pharmacological effects of the compound primarily have been ascribed to its inhibition of NE and DA transporters, it has recently been shown to be a moderately potent noncompetitive antagonist of nAChRs (Table 4).[248−250] Since the potencies displayed by bupropion at the nAChRs are in the same concentration ranges as those required to inhibit the monoamine transporters, the nAChR component of the compound

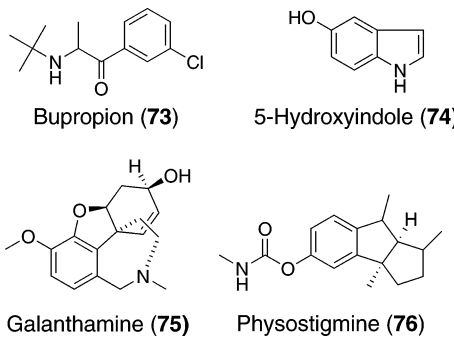

Figure 19. Allosteric modulators of nAChRs.

has been proposed to contribute to its positive effects. Interestingly, several other inhibitors of NE, DA, and 5-HT transporters have been shown to be nAChR antagonists as well.[10,248] These findings have prompted interest in nAChR antagonists as potential antidepressants and smoking cessation aids.[10]

5-Hydroxyindole (**74**) has been shown to be an allosteric potentiator of recombinant and native α7 nAChRs and to enhance α7*-mediated Glu release in cerebellar slices.[251] At a concentration of 1 mM, 5-hydroxyindole increased the potency and the maximal response of ACh at the receptor by 4- and 2-fold, respectively, and the EC_{10} response of ACh was enhanced 12-fold upon coapplication with 10 mM allosteric potentiator.[251] The ACh-elicited currents in oocytes expressing human α4β2 and α4β4 nAChRs were slightly reduced upon coapplication of 1 mM 5-hydroxyindole, and although it also potentitated 5-HT_3R signaling,[252] its effect on α7 nAChR signaling was considerably more significant.[251] 5-Hydroxyindole analogues appear in the patent literature.[172]

The most frequently prescribed treatment of Alzheimer's disease in the clinic today is acetylcholinesterase inhibitors. It has been known for some time that some of these inhibitors also are allosteric potentiators of nAChRs (Figure 19).[253,254] Galantamine (**75**) and physostigmine (**76**) are believed to bind to an allosteric site located in the NTD of the α-subunit in the nAChR complex, thereby increasing the receptors' affinity for the orthosteric agonist and/or the probability of ion channel opening.[105,236,237,253−255] Both physostigmine and galantamine potentiate the ACh-mediated responses through several nAChRs.[253,256] Although the potentiation elicited by galantamine at recombinant nAChRs is not dramatic, galantamine has recently been shown to potentiate the effects of nAChR agonists on NE, DA, and GABA release in selected brain regions.[237,255,257] The significance of the nAChR component of these compounds for their beneficial effects in Alzheimer patients still remains to be established.

6. Future Perspectives

The complexity of nAChR signaling at the molecular level and the intricate organization of the multiple native receptor subtypes with specialized functions in the CNS and PNS makes rational ligand design in this area highly challenging. The detailed insight into the molecular architecture of the orthosteric site of the nAChR obtained from the AChBP structures will undoubtedly be essential for future medicinal chemistry efforts. However, the AChBP structures also emphasize

the major problem facing the medicinal chemist trying to design subtype-selective ligands: the high degrees of homology between the orthosteric sites of the nAChRs. The residues constituting the primary binding component are identical in the α-subunits participating in orthosteric ligand binding, whereas the residues in β2, β4, and α7 making up the complementary binding component are considerably more diverse. This is reflected in the fact that selective α7, β2, and β4 nAChR agonists exist, whereas α2/α4 and α3/α6 nAChR selective agonists are rare. Furthermore, to our knowledge no agonist has unequivocally been demonstrated to discriminate between α2 and α4 nAChRs or between α3 and α6 nAChRs, although TC-2429 (**32**) seems to possess some preference for α6* over α3* subtypes.[24,158]

Functionally selective orthosteric agonists seem to arise in two different ways. Agonists such as TC-2559 and SSR591813 are selective because of their significantly higher potency at one subtype (α4β2) compared to others, whereas the selectivities of SIB-1553A, SIB-1663, and (±)-UB-165 originate from their higher efficacies at β4 nAChRs than at β2 nAChRs. These efficacy differences can be caused by different degrees, frequencies, or duration times of ion channel opening induced by agonist binding to the orthosteric sites. Thus, in addition to the insight into the static molecular composition of the orthosteric site of the nAChR, it will be important to understand the mechanisms underlying the functional coupling of the nAChR and to identify the molecular triggers translating agonist binding into ion channel opening.

As mentioned in section 5.2, nAChRs, and in particular the α7 subtype, exhibit rapid desensitization when exposed to low agonist concentrations. Hence, an alternative way to obtain efficacious nAChR activitation would be through the actions of an agonist characterized by slow desensitization kinetics. In this connection, it is worth mentioning that orthosteric agonists characterized by highly different desensitization kinetics are known from another class of LGICs, the iGluRs. Whereas the full agonists Glu and AMPA elicit fast responses through the AMPA receptors that desensitize over a millisecond scale, the partial agonist kainate evokes profoundly slower-desensitizing currents. The structural basis for these differences has been elucidated by crystal structures of the ligand binding domain of iGluR2.[113] Analogously, an increased insight into the molecular rearrangements underlying the transition from the active to the desensitized nAChR would be valuable for the understanding of the desensitization process. However, so far it remains a postulate that binding of different orthosteric agonists to the nAChR could elicit responses with significantly different desensitization kinetics, and rational design of nondesensitizing or slowly desensitizing orthosteric nAChR agonists will clearly not be feasible in the immediate future.

Some of the challenges associated with the use of homology models based on X-ray structures of a homologous protein in rational ligand design are discussed in section 4.4. The orthosteric sites in the AChBP−nicotine and AChBP−CCh structures reflect the structure of the desensitized nAChR conformation, which is not nessassarily identical to the active conformation.[71] Information about the conformational fluctations in the orthosteric site of the nAChR upon ligand binding obtained in dynamic molecular modeling studies or from X-ray structures of the AChBP in its nonliganded form or complexed with partial agonists and competitive antagonists would shed light on the differences between these states. Furthermore, molecular pharmacology studies of the events and the intermolecular interactions underlying the functional coupling of the LGIC will hopefully continue to "breathe life" into the AChBP and *Torpedo* nAChR structures.

In general, the therapeutic prospects for nAChR agonists are much greater than for nAChR antagonists, which is reflected in the vast number of agonists and the relatively few antagonists published to date. However, besides the growing interest in α-conotoxins and other subtype-selective antagonists as pharmacological tools in nAChR studies, recent studies have underlined the therapeutic potential in nAChR antagonists as antidepressants and analgesics as well as in smoking cessation. Hence, it will be interesting to see whether the medicinal chemistry exploration into nAChR antagonists will increase in the years to come.

It is rather surprising that so few allosteric modulators of nAChRs have been published, considering the susceptibility of other LGICs to allosteric modulation. Allosteric nAChR ligands seem to have an advantage over orthosteric ligands in several respects. Since receptor regions targeted by allosteric ligands typically are less conserved than their orthosteric sites, development of allosteric ligands could be a way to circumvent the subtype-selectivity problems connected with orthosteric ligands. Furthermore, the effects of allosteric modulators are closely linked to the physiological pulse of signaling, since the modulator only amplifies or reduces the neural signal when the endogenous agonist is present in the synaptic cleft. Considering the rapid desensitization of α7, allosteric potentiators exerting their effects through a reduction of the desensitization rate of this receptor could be particularly effective in vivo. In any case, it will be highly interesting to see in vivo data on allosteric potentiators of nAChRs. Since the molecular compositions of allosteric sites in the nAChR have not been identified in sufficient detail to enable design of allosteric ligands using homology models based on the AChBP and *Torpedo* nAChR structures, future progress in this area will most likely arise from optimization of already identified modulators or from novel leads identified in high-throughput screenings.

The correlation between the pharmacological profile of a ligand at heterologously expressed nAChRs and in neurotransmitter release assays or in animal models is not always evident. For example, the interesting in vivo properties of ABT-089 are not easily explained by its characteristics at recombinant nAChRs, and examples of ligands with interesting in vitro characteristics performing poorly in vivo also exist. There may be many reasons for such in vitro/in vivo discrepancies. For example, the pharmacologies of a compound may differ at the recombinant and native nAChRs because of different assay conditions or different desensitization kinetics of the receptors, and additional receptor subtypes may contribute to the effects of the compound in native tissues. Furthermore, it is worth considering

whether an nAChR agonist can be *too* selective because selective targeting of subsets of nAChRs might be desirable for some indications but not nessaccarily for others. Activation of the heteromeric α/*β* nAChR requires both of the orthosteric sites in the receptor complex to be occupied by agonists (Figure 4). Hence, an agonist specifically acting on a specific "simple" nAChR subtype in vitro will only target the subset of native nAChRs comprising this particular subunit combination in both orthosteric sites, and consequently it will be highly discriminating among the complex native nAChR populations. For example, *β*4-selective agonists will act on α3*β*4α3*β*4α5 and α4*β*4α2*β*4*β*4 combinations but not on α3*β*2α3*β*4α5 or α4*β*4α2*β*2*β*2 subtypes, and α4*β*2-selective agonists will elicit signaling through α4*β*2α4*β*2*β*2 and α4*β*2α4*β*2α5 subtypes but not through α4*β*2α6*β*2*β*3 and α4*β*4α4*β*2*β*2 subtypes. Still, the latter agonist will activate such a vast number of receptors throughout the brain that use of the term "selectivity" almost becomes absurd. The allosteric nature of the nAChR function also opens up to complex contributions to the overall pharmacological properties from the subunit not directly involved in orthosteric ligand binding to the heteromeric nAChR. The presence of a structural subunit in the nAChR complex can alter the agonist pharmacology of the receptor significantly,[21−23] and analogously the interaction of a ligand with one orthosteric site could be influenced by the subunit composition of the other orthosteric site in the receptor. Thus, an α4*β*2 agonist may not necessarily be equipotent at α4*β*2α4*β*2*β*2 and α4*β*2α4*β*2α5 receptors, and an agonist equipotent at "simple" α3*β*2 and α4*β*2 nAChRs may even exhibit a significantly different potency at an α3*β*2α4*β*2*β*2 subtype. These deliberations may be academic, but it is valid to question how well "simple" nAChR combinations expressed in oocytes or mammalian cell lines can predict the properties of a ligand at heterogeneous native nAChR populations. These in vitro/in vivo discrepancies clearly advocate for an extensive characterization of the pharmacological properties of an nAChR ligand at heterologously expressed nAChRs in mammalian cell lines, at native nAChRs in in vitro assays, and in animal models.

The combination of single-cell RT-PCR, patch clamp physiology, knock-out mice, and subtype-selective nAChR ligands has turned out to be quite effective in elucidating the molecular compositions and physiological functions of native nAChR subtypes in various CNS regions (sections 3.3). The identities and roles of additional nAChR subtypes will undoubtedly be disclosed in the years to come. The α-conotoxins have been important pharmacological tools in these studies, and since several groups are working on isolating new native α-conotoxins and developing new toxin analogues, it is not unlikely that the currently available α3*β*2/α6*β*2-, α3*β*4-, and α7-selective α-conotoxins in the near future will be supplemented with peptides selectively acting on other nAChR subtypes. A α4*β*2-specific toxin would be a particular useful pharmacological tool because it would facilitate an even more precise deciphering of the roles played by heteromeric nAChR subtypes, major as well as minor. The distinct pharmacological profiles of the atypical α-conotoxins AuIB and BuIA suggest that changes in the disulfide connectivities of the $\alpha_{4/7}$-conotoxins could be a way to redirect the actions of a toxin toward different nAChR subtypes.[41,219]

The deciphering of the compositions of the native nAChRs has highlighted the important roles played by the minor nAChR subtypes in selected CNS regions. Most strikingly, the α6 nAChR has been catapulted from its status as an enigmatic subunit characterized by limited CNS expression and poor in vitro function to its current status as a component of the α6* nAChR in substantia nigra, ventral tegmental area, and striatum, shown to be a potential target in Parkinson's disease, Tourette's syndrome, and possibly also smoking cessation. It will be interesting to compare the pharmacological properties of selective α6* or α3*/α6* agonists such as TC-2429 and chromaperidine to those of α4*β*2-selective and -nonselective nAChR agonists in striatal DA release experiments and in animal models.

Although the peripheral α3*β*4* nAChR mediates several of the side effects caused by nonselective nAChR agonists, the subtype also controls the release of important neurotransmitters in various CNS regions, and thus, it should not be ignored as a potential drug target (Table 1). Studies of *β*4 knock-out mice have suggested a role for *β*4* nAChRs in epilepsy and for the negative-reinforcing properties of nicotine,[54,55] and future clinical trials of agonists with pronounced *β*4 nAChR selectivities will hint at the potential of *β*4* nAChRs as therapeutic targets in other disorders. SIB-1663 with its negligible *β*2, α7, and α3*β*4 nAChR activities could be a valuable tool in investigations of the physiological roles of the minor α2*β*4* and α4*β*4* subtypes. In view of the α6 fairy tale, it would be premature to dismiss α2-containing nAChRs as potential therapeutic targets despite the limited CNS expression of the subunit.

Even if the minor nAChR subtypes have been found to be important contributors to the overall nicotinergic neurotransmission in the CNS, α4*β*2* and α7* remain the major neuronal CNS nAChRs (Figure 2). Whereas the α4 and *β*2 knock-out mice and the α4 mutant knock-in mice have confirmed the therapeutic prospects in α4*β*2* receptors, the α7 knock-out and knock-in mice have not shed much light on this receptor as a drug target candidate. Although the performances of α7 agonists in animal models for various disorders may not have been all that convincing either, the abundant expression of the α7* nAChR in the CNS, its apparent universal role as a heteroreceptor mediating Glu release, and its high Ca^{2+} permeability alone strongly support the case for the receptor as an interesting target. A number of α4*β*2-selective agonists and the α7-agonist GTS-21 are currently undergoing clinical trials, and it will be highly interesting to follow the progress of these compounds.

In conclusion, these are truly exciting years for the nAChR community. While the therapeutic prospects in nAChR ligands have never been questioned, results from studies of nAChR knock-out and knock-in mice and from clinical trials of novel nAChR agonists have underlined the possibilities in pharmaceutical agents targeted at the neuronal nAChRs. Furthermore, the recently obtained insight into the molecular structure of the nAChR and the identification of several native receptor subtypes has facilitated rational design of ligands targeted at specific subtypes mediating distinct

physiological functions. It will be interesting to see whether the structural information about the nAChRs will lead to a greater assortment of subtype-selective ligands and whether these will display improved in vivo properties and beneficial therapeutic effects compared to previous generations of nAChR ligands.

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Biographies

Anders A. Jensen achieved his Ph.D. degree in 2001 from The Danish University of Pharmaceutical Sciences. His thesis was based on molecular pharmacology studies of family C G-protein-coupled receptors, for which he received the Danish Academy of Natural Sciences Ph.D. Award in 2001. Since then he has worked as an Assistant Professor at the Department of Medicinal Chemistry at the same university. He has spent two 6-month periods as a Visiting Scientist at ACADIA Pharmaceuticals (San Diego, CA) and at the University of Basel (Switzerland). His current research is predominantly focused on ligand-gated ion channel receptors and neurotransmitter transporters.

Bente Frølund obtained her Ph.D in Medicinal Chemistry from The Danish University of Pharmaceutical Sciences in 1992. After 1 year as a Visiting Scientist at Merz & Co., Germany, she did postdoctoral studies at The Danish University of Pharmaceutical Sciences, primarily on the design of partial $GABA_A$ receptor agonists. From 1999 she has worked as an Associate Professor at the Department of Medicinal Chemistry at the same university. Her research is on the design and synthesis of selective ligands for studying ligand−receptor interactions focusing on the ionotropic $GABA_A$ and nicotinic acetylcholine receptors.

Tommy Liljefors was born in Malmö, Sweden in 1941. After graduation from Lund University, Sweden, in 1969 he obtained his Ph.D. degree in Organic Chemistry in 1973 from the same university. In 1994 he was appointed Research Professor in Computational Chemistry and in 1999 Full Professor in Computational Chemistry/Molecular Modeling at The Danish University of Pharmaceutical Sciences. He is a member of The Danish Academy of Natural Sciences and The Danish Academy of Technical Sciences. His research focuses on studies of molecular recognition, ligand−protein interactions, structure−activity relationships, and computer-aided molecular design by the use of computational chemistry techniques. The major part of these studies concerns ligand−receptor interactions for agonist and antagonists at neurotransmitter receptors. He has published 150 research articles and coedited 3 books.

Povl Krogsgaard-Larsen, Professor of Medicinal Chemistry, achieved his Ph.D. degree in 1970 in Natural Products Chemistry and his D.Sc. degree in 1980. His second thesis describes the design of specific $GABA_A$ agonists and GABA uptake inhibitors. One of these $GABA_A$ agonists, THIP (trade name: Gaboxadol), is now in phase III clinical trials. He was awarded Honorary Doctor degrees at University of Strasbourg in 1992 and at University of Uppsala in 2000. He has received a number of research awards and prizes for his design of specific GABA, glutamate (Glu), and muscarinic and nicotinic acetylcholine receptor ligands. The group of Glu receptor ligands includes AMPA, which gave name to the AMPA receptors. He is chairman of the Carlsberg Foundation, a member of the Board of Directors of the Alfred Benzon Foundation, and a member of the Royal Danish Academy of Science and Letters.

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