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Perspective

Neuronal Nicotinic Acetylcholine Receptors as Targets for Drug Discovery†

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Introduction

Until recently, research on (*S*)-(−)-nicotine (**1**, hereafter simply nicotine) and nicotinic acetylcholine receptors (nAChRs) emphasized two principal aspects. The first was the considerable medical and commercial interest in understanding the health consequences of smoking and the mechanisms involved with tobacco dependence.[1−3] Second, the ready isolation of the nAChR from the electric organ of *Torpedo* ray or *Electrophorus* fish and the existence of snake toxins with high affinity for this receptor account for its status as the archetypal member of a superfamily of ligand gated ion channels (LGIC).[4,5] Recently, more attention has been directed to the potential role of neuronal nAChRs in disease and therapy.[6−13] To maximize the potential of nAChRs as therapeutic targets, the central questions are: What nAChR subtypes exist *in vivo*, and which are responsible for the various effects observed with nicotine, such as cognition/attention enhancement, analgesia, neuroprotection, neurotransmitter release, addiction, and seizures, and which, if any, are altered in various disease states? Can selective agonists and antagonists be developed for use as pharmacological tools and therapeutic agents? Discussion of issues relevant to these questions forms the basis of this Perspective Article.

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† Abbreviations and conventions used in this article are: α-Bgt, α-bungarotoxin; AD, Alzheimer's disease; Atx, (+)-anatoxin-a (**19**); ACh, acetylcholine (**8**); Cyt, (−)-cytisine (**7**); DA, dopamine; DHβE, dihydro-β-erythroidine (**14**); DMPP, 1,1-dimethyl-4-phenylpiperazinium (**12**); *d*-TC, *d*-tubocurarine (**3**); GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine = serotonin; LGIC, ligand-gated ion channel; mAb, monoclonal antibody; MCC, *N*-methylcarbamylcholine (**9**); MLA, methyllycaconitine (**13**); nAChR, nicotinic acetylcholine receptor; n-Bgt, neuronal bungarotoxin; NE, norepinephrine; nicotine refers to (*S*)-(−)-nicotine (**1**), unless designated otherwise.

Therapeutic Potential. A recent review has summarized the rationale for targeting nAChRs to treat a variety of diseases.[14] The most compelling arguments cite observations of beneficial effects following administration of nicotine to humans, which have been observed for cognitive and attention deficits, Parkinson's disease, anxiety, Tourette's syndrome, ulcerative colitis, and smoking cessation. Results of recent clinical studies demonstrating efficacy of nicotine in treatment of adult attention deficit hyperacitivity disorder[15,16] and depression[17] have since appeared. Nicotine also normalizes an auditory gating deficit found in schizophrenic patients.[18] Antinociceptive effects of nicotine have been known for some time (see ref 19 for review), but the discovery of epibatidine (**2**) as a much more potent and broadly effective analgesic agent that acts via neuronal nAChRs[19,20] has stimulated renewed interest in targeting nAChRs for analgesia.

Furthermore, considerable *in vitro* and some *in vivo* evidence exists for the cytoprotective effects of nicotine and related agents against excitotoxic insult.[21,22] Relevance to possible prevention of neurodegeneration in Alzheimer's disease (AD) is further supported by recent findings that nicotine can attenuate the neurotoxic effects of β-amyloid[23] and inhibit *in vitro* formation of amyloid from β(1−42) peptide[24] (although the latter effect does not appear to be nAChR mediated). Also, an abnormal structural variant in a nAChR subtype may account for some forms of epilepsy,[25] suggesting the possibility of targeting this abnormal nAChR for treatment of these conditions.[14]

Despite the existence of proof-of-principle findings for the effectiveness of nicotine in numerous disorders, issues remain with respect to establishing long-term efficacy and minimizing side effects. Oral activity (i.e. intestinal absorption) also is a desirable attribute which

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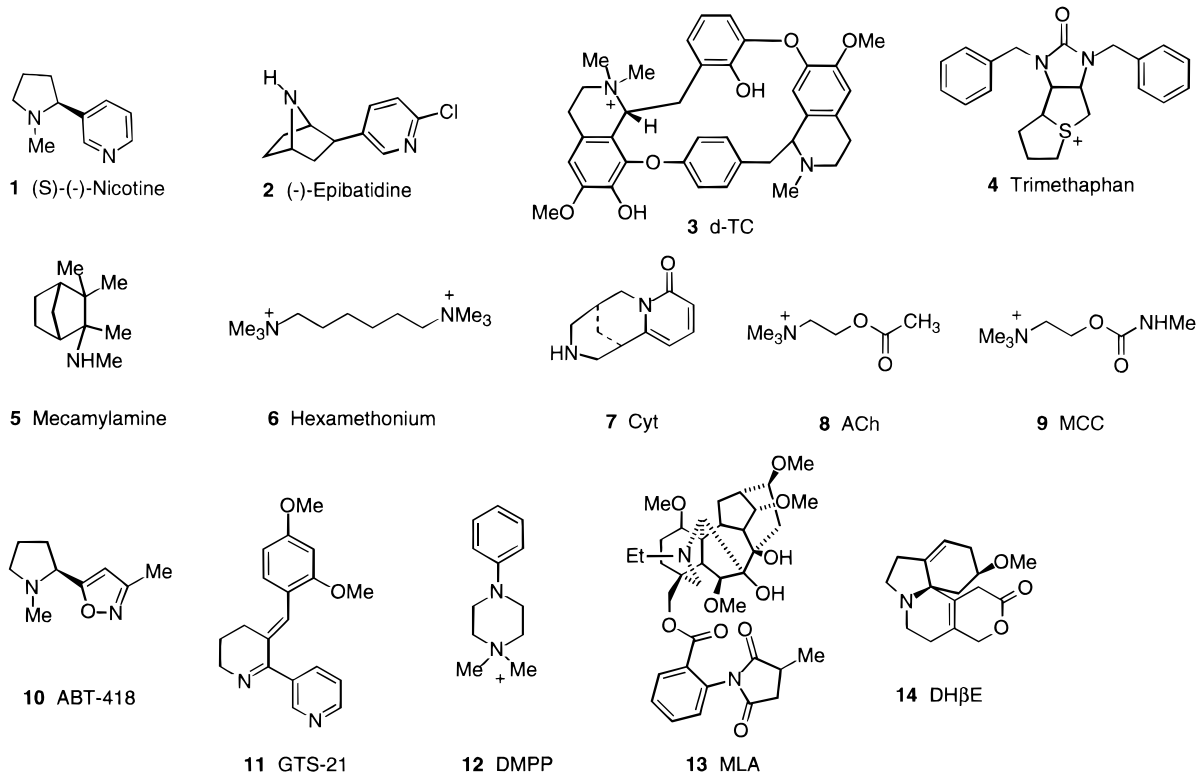

Figure 1. Structures of selected nAChR modulators.

is lacking in nicotine. Potential side effects include actions on the cardiovascular and gastrointestinal systems, dependence, sleep disturbance, and, at higher doses, neuromuscular effects and seizures. The possibility that these issues can be addressed with alternative agents that selectively, and perhaps only partially, activate specific subtypes of nAChRs represents an opportunity for drug discovery. Most of the current effort in this arena can be viewed as a search for agents that act via stimulation of nAChRs in the CNS, as do nicotine and epibatidine, but possess improved pharmacokinetic and side effect profiles. While much of this activity is quite recent, targeting nAChRs for therapy is not new. Thus, some agents that are blockers of nAChR function at the neuromuscular junction or in autonomic ganglia, such as *d*-tubocurarine (*d*-TC, **3**), trimethaphan (**4**), mecamylamine (**5**), hexamethonium (**6**), and related compounds, have been used clinically as muscle relaxants during surgery or as antihypertensive agents.[26]

Overview of Endogenous nAChR Subtypes: Localization, Structure, and Function

nAChRs are found on skeletal muscle at the neuromuscular junction, in autonomic ganglia of the peripheral nervous system, on sensory nerves and some peripheral nerve terminals, and at numerous sites in the spinal cord and brain. The nAChR found on mammalian skeletal muscle is analogous to the receptors from electric organ of *Torpedo* and *Electrophorus*, which have been extensively characterized.[27] This receptor consists of five protein subunits (two α and one each of β, γ, and δ) surrounding a central ion channel (Figure 2).

In neuronal tissues, nAChRs are believed to have a similar pentameric structure, but possess a considerable diversity of subunit combinations.[28−30] Thus, in neurons, nAChR subunits homologous to those in muscle have been found, which are designated α if they contain vicinal cysteine residues analogous to Cys192−Cys193 of the *Torpedo* receptor or β if they do not. Presently, nine α (α1−α9) and three β (β2−β4) subunits have been isolated and cloned from mammalian or avian neurons. Numerous combinations of α and β subunits, and in the case of α7−α9, the α subunits alone, can be expressed in oocytes or other expression systems resulting in functional ion channels with diverse pharmacological properties.[29,31−37] An important finding from the study of heterologous expression systems is that both α and β subunits influence the pharmacology of the derived channels.[38,39] The relationship of most of these channels to native receptors is an area of active investigation, but, with some important exceptions, many (particularly pairwise) combinations have properties that do not closely match those of nAChRs on neurons.[28,29,40,41] Current efforts to correlate heterologously expressed channels with native ones therefore are increasingly focused on combinations of three or more subunits.[29,42−44]

In the next several sections, the major known or putative *native* nAChR subtypes are discussed with respect to (1) structure and localization, (2) state of understanding with regard to function, and (3) common models used for pharmacological studies. The subsequent section will discuss ligands for each subtype with a focus on selectivity *vs* other nAChR subtypes. The information from these sections is summarized in Table 1 and Figure 2.

α4β2 nAChR Subtype: High-Affinity CNS Binding Site for Nicotine and Acetylcholine

Nicotine and acetylcholine (ACh) bind with high affinity to a population of receptors widely distributed in brain,[45] although the precise distribution of these receptors differs significantly among species.[6] It is

Table 1. Overview of Major Endogenous nAChR Subtypes

				typical assays				
				binding		function		
designation	localization	known subunits	possible functions	radioligand	tissue	technique	tissue	"selective" ligands
α4β2 nAChRs	CNS (widespread)	α4β2	cognition neurotransmitter release addiction	[^{3}H]nicotine [^{3}H]Cyt [^{3}H]MCC	rodent brain transfected cells (K177, M10)	ion flux ion currents	transfected cells (K177, M10, oocytes)	agonists:[b] A-84543 RJR-2403 ABT-418 compound **76**
non-α4β2/non-α7 nAChRs recognized by [^{125}I]n-Bgt and [^{3}H]epibatidine	CNS (limited)	α3?β2?β4?	DA release? (see text)			[^{3}H]DA release	rodent striatal tissue	agonists:[c] Cyt SIB-1765F antagonist: n-Bgt[d]
α7 nAChRs	CNS (widespread distinct from α4β2)	α7	cognition neuroprotection sensory gating	[^{125}I]-α-Bgt	rodent brain transfected cells (K28)	ion currents	transfected cells (oocytes)	agonists: DMAC (**23**) antagonists: α-Bgt[e] MLA
additional nAChR subtypes	CNS	not principally α7	neurotransmitter release			[^{3}H]ACh release [^{3}H]GABA release [^{3}H]NE release	rodent hippocampal tissue	
synaptic ganglionic nAChRs	PNS (at ganglionic synapse)	α3α5β4 α3α5β2β4 α3α7xyz[a] others likely	synaptic transmission in autonomic nervous system adrenal catecholamine release	[^{3}H]epibatidine		ion currents ion flux	neural crest cell lines (PC12, IMR-32, SH-SY5Y)	antagonist: hexamethonium n-Bgt[d]
ganglionic α7 nAChRs	PNS (near ganglionic synapse)	α7	neurite retraction cellular function					antagonists: α-Bgt[e] MLA
muscle nAChRs	skeletal muscle at neuromuscular junction	α1β1δγ(ϵ)	contraction of skeletal muscle					antagonists: α-Bgt[e]

[a] X, Y, and Z represent subunits of unknown identity. [b] Agonists selective for α4β2 function *vs* ganglionic function; not selective *vs* DA release. [c] Agonists selective for DA release *vs* α4β2 function. [d] n-Bgt is a potent antagonist of both DA release and synaptic ganglionic function. [e] α-Bgt is a potent antagonist at α7 and muscle nAChRs.

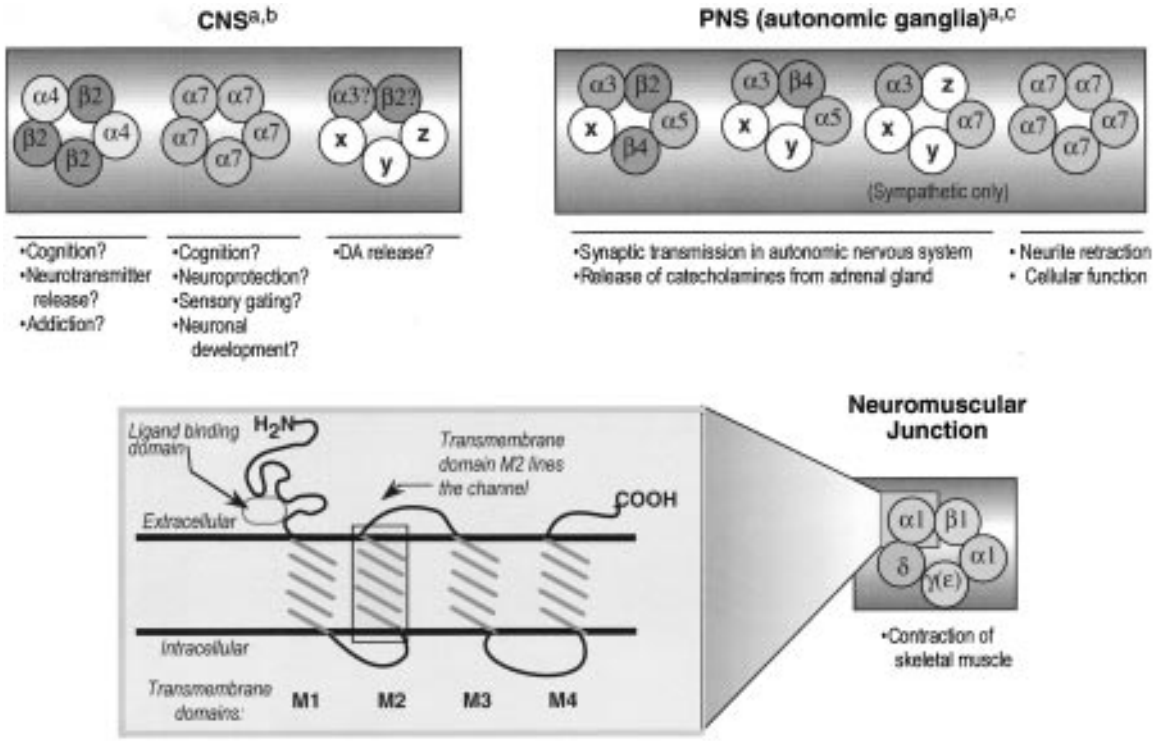

Figure 2. Major putative endogenous nAChR subtypes. *a*. X, Y, and Z represent subunits of unknown identity. *b*. Possibility exists for presence of as yet unidentified subunits in α4*β*2 and α7 subtypes. *c*. Possibility exists for additional combinations lacking the α5 subunit, e.g. α3*β*2, α3*β*4, α3*β*2*β*4, etc.

generally accepted that the predominant nAChR in the CNS which binds [^{3}H]nicotine with high affinity (K_d = 0.5−5 nM) is composed of α4 and *β*2 subunits (α4*β*2). This conclusion is based on immunoprecipitation studies,[46] comparison of cloned amino acid sequences to immunopurified receptor subunits,[47−52] and correlation of autoradiography data with results from *in situ* hybridization experiments.[53−55] Although this nAChR could conceivably contain other as yet unidentified subunits, the presence of α2, α3, α5, α7, *β*3, and *β*4 subunits has been specifically excluded.[46,56] Pharmacological correlations of native receptors with heterologously expressed channels composed of α4 and *β*2 subunits further support the hypothesis that the predominant α4*β*2 nAChR contains only these two subunits.[31,34,47] Heterologously expressed α4*β*2 nAChRs assemble with the stoichiometry two α4 subunits to three *β*2 subunits.[31,57,58] In addition to the predominant α4*β*2 nAChR, the presence of minor populations of α4- and *β*2-containing nAChRs combined with additional subunits is possible.

The role of α4*β*2 nAChRs in the CNS is not well understood.[59] A possible function for this subtype in associative memory is suggested by gene knockout experiments which have demonstrated that mice lacking the *β*2 subunit lose high-affinity CNS binding sites, do not produce electrophysiological responses to nicotine in thalamic neurons, and fail to exhibit nicotine-enhanced performance in a passive avoidance model of cognitive function.[60] On the other hand, a distinct lack of overt changes in behavior in these animals compared to controls also is noteworthy. There is a dramatically decreased number of these receptors in the human cortex associated with AD,[61,62] further supporting a possible role in cognitive function, although this finding must be viewed in the context of the loss of many different receptor types in AD. Evidence supports the location of some nAChRs in the CNS at presynaptic nerve terminals and their involvement in neurotransmitter release.[63] Initial studies comparing the pharmacology of ACh release in rat hippocampus with responses from recombinant chick α4*β*2 nAChRs suggested the possible involvement of this subtype in ACh release.[64] However, differences in pharmacology emerge when α4*β*2 receptors from rat are used in the comparison, and therefore this hypothesis may require revision.[65] Studies on the participation of nAChRs in developmental processes suggest that α4*β*2 nAChRs may have a role in neuronal migration.[66−68]

Whereas in many neurotransmitter systems prolonged or excessive agonist exposure leads to receptor down-regulation,[69] a unique feature of some neuronal nAChRs is their up-regulation following chronic treatment with nicotine and other nAChR agonists.[70−74] Although this effect is observed for other subtypes of nAChRs, including the α7 subtype (*vide infra*), only the α4*β*2 nAChRs appear to be upregulated by physiologically relevant concentrations of nicotine.[75] Furthermore, [^{3}H]nicotine binding sites are elevated in postmortem brains of smokers *vs* nonsmokers.[76] The extent of up-regulation of presumed α4*β*2 nAChRs is variable by brain region.[73] Evidence in favor of up-regulation mechanisms involving decreased receptor turnover,[77] increased recruitment from a reserve pool of nAChR,[78] and increased protein synthesis[74] have been presented.

Radioligand binding assays in rodent brain using [^{3}H]-nicotine are widely used as an initial probe of ligand interactions with CNS nAChRs.[79] Alternatively, tritium-labeled (−)-cytisine (Cyt, **7**),[80] acetylcholine (ACh, **8**),[81] or *N*-methylcarbamylcholine (MCC, **9**),[82,83] which bind with high affinity to the same sites as nicotine, have been used as radioligands. For the study of regulation and functional properties of α4*β*2 nAChRs, several *in vitro* expression systems have been used.

Transient expression of chick,[84] rat,[47] and human[85] α4β2 nAChRs in oocytes has been reported, as well as stable expression systems containing chick α4β2 in a mouse cell line (M10 cells) [31] and human α4β2 in a human cell line (K177 cells).[34,86] Rodent thalamic tissue also is used to study endogenous α4β2 nAChRs.[87,88] Responses in such systems have been measured electrophysiologically, as flux of radioactive ions (e.g. $^{86}Rb^+$ or $^{22}Na^+$), or as calcium influx detected with calcium-sensitive dyes.

Brain α7 nAChR Subtype: High-Affinity CNS Binding Site for α-Bungarotoxin (α-Bgt)

α-Bgt is a 75-amino acid peptide isolated from a species of East Asian snake (*Bungarus multicinctus*) and has been recognized historically for its high affinity to muscle-type nAChRs.[89] An additional class of nAChRs in the CNS is identified by propensity to bind [^{125}I]-α-Bgt with high affinity (K_d = 0.65−1.7 nM) and nicotine with relatively low (micromolar) affinity.[90] The overall population of these sites is similar to that for α4β2 nAChRs, but the localization is different and varies according to species.[13] The subunit composition of this nAChR subtype has not been firmly established, but evidence supports the hypothesis that most native mammalian α-Bgt receptors are α7 homooligomers. Thus, there is a correlation between [^{125}I]-α-Bgt binding and α7 mRNA distribution in brain.[53,56] Also, α7 subunits can assemble in heterologous expression systems as homooligomers that pharmacologically resemble native receptors.[33,37,91,92] Not all channel properties are identical, however, so the assignment of subunit composition remains provisional.[93] In brain and retina of chickens, α-Bgt-sensitive receptors composed solely of α7, solely of α8, or of a combination of α7 and α8 have been identified.[94]

The α7 nAChRs have an unusually high permeability to calcium compared to other subtypes[95] and exhibit exceptionally rapid desensitization following exposure to agonists.[96−98] Chronic exposure to nicotine causes an increase in [^{125}I]-α-Bgt binding,[37,99,100] but higher doses are required than are necessary for up-regulation of α4β2 nAChRs. Like α4β2, up-regulation of α7 is selective by brain region when elicited *in vivo*.[73]

The exact role of α7 nAChRs is still under investigation. Recently, a role in stimulating release of the excitatory neurotransmitter glutamate has been demonstrated in the hippocampus,[101] a brain region where modulation of glutamate receptors has been connected with learning and memory.[102−104] In this context, however, it is worthwhile to note that no loss of α-Bgt binding was observed in the cortex of AD patients.[105] A role in sensory processing and the pathophysiology of schizophrenia is suggested by molecular, biochemical, and genetic studies linking a deficiency of α7 nAChRs to sensory gating deficits experienced by patients with this disease.[18,106] α7 nAChRs also may play a role in neuroprotection. Thus, glutamate-induced neurotoxicity in primary rat cortical neurons was reduced by pretreament with nicotine and other nAChR agonists,[107−110] effects that were blocked by α7-selective antagonists. A role in neurite outgrowth is implied for α7 nAChRs by increased expression of nerve growth factor and brain-derived neurotrophic factor mRNA in rat hippocampus upon intraventricular administration of α-Bgt.[111,112] Expression of nAChRs prior to neuronal differentiation further supports the possible participation of these receptors in neuronal development.[113]

Binding studies to native receptors in rodent brain[90,114] or in stable expression systems[115] have been carried out using [^{125}I]-α-Bgt as the radioligand. Functional activities have been measured electrophysiologically in cultured brain cells,[92] in oocytes transiently expressing α7 nAChRs,[116] or in stable expression systems, including rat α7 in a rat cell line (GH_4C_1 cells)[37] and human α7 in a human cell line (K28 cells).[33] Detection of calcium influx in oocytes or K28 cells using calcium-sensitive dyes also has been reported.[95,117]

Brain nAChR Subtype(s) Identified by [^{125}I]Neuronal Bungarotoxin and [^{3}H]-(±)-Epibatidine and Subtype(s) Mediating [^{3}H]DA Release

Neuronal bungarotoxin (n-Bgt,[118] also referred to as κ-bungarotoxin,[119] toxin F,[120] and bungarotoxin 3.1[121]) is a minor component of *Bungarus multicinctus* venom from which α-Bgt is isolated. Originally characterized by its ability to block cholinergic transmission in peripheral autonomic ganglia, n-Bgt was subsequently found to potently antagonize several discrete responses in the CNS, most notably including nicotine-stimulated release of [^{3}H]dopamine (DA) from rat[122] and mouse[123] striatal tissues. Autoradiography studies in brain demonstrate that n-Bgt labels both α-Bgt-sensitive sites as well as a population of sites displaced by a high concentration of nicotine. However, the latter sites are distributed much less widely than are α4β2 receptors.[122] That these latter sites are distinct from α4β2 receptors is further supported by the finding that n-Bgt is weak in displacing [^{3}H]nicotine from mouse striatum (K_i value > 30 μM).[123]

More recently, autoradiography studies using [^{3}H]-(±)-epibatidine have shown that the distribution of sites labeled by this ligand in rat brain closely matches those labeled by [^{3}H]Cyt, but the binding density is greater for [^{3}H]-(±)-epibatidine in several regions.[124] Radioligand binding studies with [^{3}H]-(±)-epibatidine in homogenates from mouse,[125] rat,[126] and human[126] brains also reflect high affinity of the radioligand for a second site in addition to α4β2, whereas all other currently available radioligands for α4β2 receptors (including tritium labeled nicotine, Cyt, ACh, and ABT-418 (**10**)) bind to only a single class of high-affinity sites in rat brain.[80,90,127] In rat forebrain, the data fit a two-site model with K_d values of 15 and 360 pM, with each site comprising about 50% of the total number of binding sites.[126] In transfected mouse fibroblasts (M10 cells) that presumably only express α4β2 receptors,[31] [^{3}H]-(±)-epibatidine binding fits best to a one-site model (K_d = 4 pM).[126] Thus, based on the observed affinities, the higher affinity site in rat brain is likely the α4β2 subtype. The identity of the second site is presently unclear, but it is noteworthy that considerable overlap in the localization of the non-α4β2 receptors that bind radiolabeled n-Bgt and those that bind epibatidine can be discerned. It is therefore possible that these two probes label a common class of nAChRs.

The subunit composition of these nAChRs is uncertain. Among several pairwise combinations of α and β subunits, n-Bgt most potently inhibits α3β2,[128] and (±)-

epibatidine also interacts potently with α3-containing nAChRs, including α3β2, α3α5β2, and α3β4.[42,129] However, these ligands are not highly selective, and additional studies will be required to ascertain the constitution(s) of non-α4β2-nAChRs identified by [^{125}I]n-Bgt and [^{3}H]-(±)-epibatidine.

Possible roles for these nAChRs also are unclear. A population of human cortical receptors labeled by [^{125}I]n-Bgt was found to be decreased in AD patients relative to age-matched controls,[130] which could in principle be related to the decline in cognitive function associated with this disease. It is also tempting to hypothesize that this class of nAChRs may be involved in the release of [^{3}H]DA from striatal nerve terminals, a response commonly used in characterization of nAChR modulators. This hypothesis is consistent with the potent stimulation of DA release by (±)-epibatidine[131,132] and the potent inhibition of agonist-stimulated DA release by n-Bgt.[123,133] Also, mRNAs for α3, α4, α5, β2, and β3 have been detected in the substantia nigra, which projects to the striatum (reviewed in ref 41). On the other hand, aggregate data from several studies indicate that a group of agonists (epibatidine (**2**), ACh (**8**), nicotine, ABT-418 (**10**), GTS-21 (**11**)) exhibits similar rank order potencies in stimulation of striatal DA release in rat and activation of recombinant rat α4β2 nAChRs in oocytes,[134] suggesting the potential involvement of α4β2 in DA release. However, Cyt (**7**) does not fit this correlation. Thus, **7** is more potent than nicotine or DMPP (**12**) in stimulation of rat striatal DA release[135] but is substantially weaker than the other two agents at recombinant rat α4β2 nAChRs expressed in oocytes.[136] In addition, compound **7** exhibits substantial efficacy in DA release (*ca.* 65% of nicotine response) but only very low efficacy at rat α4β2 nAChRs.

Recent studies with the snail toxin α-conotoxin MII indicate that striatal DA release may be mediated by more than one nAChR subtype, one of which likely contains an α3β2 subunit combination.[137] Thus, this toxin is selective for blockade of the α3β2 combination in oocytes (*vs* α2β2, α2β4 α3β4, α4β2, α4β4, α1β1γδ, and α7), but is able to block only up to 49% of nicotine-stimulated DA release from striatum. Since α-conotoxin MII can exert its blocking action by interaction at a single α3/β2 interface and because the toxin's effect on more complex subunit combinations has not yet been determined, elucidation of the precise subunit composition of the conotoxin-sensitive nAChR that mediates DA release requires further investigation.

Other nAChR Subtypes in the CNS

Numerous observations support the premise that a level of diversity of nAChR subtypes exists in the CNS beyond that specifically discussed above. The presence of multiple nAChR subunits that can assemble in various combinations to form functioning ion channels in heterologous expression systems constitutes one provacative clue.[29] Moreover, evaluation of different nAChR ligands in a variety of behavioral paradigms also suggests a diverse pharmacology that is most readily rationalized by the existence of multiple subtypes.[138] Similarly, a diverse pharmacology exists with regard to release of various neurotransmitters, such as DA release from rat striatum, and ACh, norepinephrine (NE), and serotonin (5-HT) release from rat hippocampus[132,135,139,140] (see ref 41 for a review). Since release of DA, ACh, and γ-aminobutyric acid (GABA) have been demonstrated to be insensitive to α-Bgt,[123,141,142] these responses apparently are not predominantly mediated by receptors composed principally of α7 subunits. Therefore, it would appear that at least several combinations selected from α2−α6 and β2−β4 are likely to be involved.

On the basis of studies with specific antibodies and pharmacological comparisons with reconstituted systems, a minor population of nAChRs immunopurified from rat cerebellum appears to be composed of the α4β2β3β4 combination.[143] A class of nAChRs characterized in chick habenula has channel properties similar to those of an α4α5β2 combination in oocytes.[29] A class of nAChRs in the spinal cord mediates release of excitatory amino acids and various behavioral effects, and on the basis of a unique pharmacological profile across a variety of agonists and antagonists, these nAChRs appear to represent a novel class.[144−147] In addition, nAChRs have been identified on the Edinger−Westphal nucleus of chick brain slices that are sensitive to low concentrations of methyllycaconitine (MLA, **13**) but not α-Bgt, representing a unique pharmacological profile[148] (see discussion on ligands for the α7 subtype, below).

It has been proposed that the nicotinic response in rat dorsolateral septal nucleus (DLSN) neurons may be mediated by a metabotropic nAChR coupled to a G-protein.[149] 1,1-Dimethyl-4-phenylpiperazinium (DMPP, **12**) induces a hyperpolarization response in DLSN neurons that is blocked by the nicotinic antagonists mecamylamine and n-Bgt, which is consistent with the pharmacology observed with ganglionic receptors. However, *d*-TC and dihydro-β-erythroidine (DHβE, **14**) are ineffective as antagonists at DLSN neurons, and the competitive ganglionic antagonist trimethaphan (**4**) displays agonist activity with a potency and efficacy similar to that of nicotine.

Synaptic Ganglionic nAChR Subtype(s): nAChRs Mediating Peripheral Autonomic Neurotransmission

The parasympathetic and sympathetic divisions of the peripheral autonomic nervous system are characterized (with minor exceptions) by nerves which release ACh and NE, respectively, onto target tissues, such as smooth muscle and glands. The cell bodies of these nerves are located in bundles (ganglia) and contain postsynaptic nAChRs that respond to ACh released by nerves arising in the spinal cord.[26] The adrenal gland is comprised in part of differentially developed postganglionic sympathetic neurons, such that activation of nAChRs on the adrenal medulla results in the release of NE and epinephrine into the bloodstream.[1] Thus, many of the cardiovascular and gastrointestinal side-effect liabilities associated with nicotine (increased heart rate, elevated blood pressure, increased motility of the gastrointestinal tract) are thought to be mediated by activation of these synaptic ganglionic nAChRs.[26]

Native autonomic ganglionic nAChRs have been investigated in tissues representative of both sympathetic and parasympathetic neurons. Sympathetic ganglionic nAChRs have been studied in dissected sympathetic ganglia and cultured bovine adrenal chromaffin cells,[44,150,151] as well as in cells lines having embryonic origins in common with sympathetic ganglia, including the PC12, SH-SY5Y, and IMR-32 cell lines.[152−155] SH-SY5Y cells express mRNAs for α3, α5, α7, β2, and β4 subunits,[153,156]

and IMR-32 and PC12 cells appear to show a similar pattern of subunit expression.[154] [^{3}H]-(±)-Epibatidine exhibits high affinity for nAChRs on IMR-32 cells[126] and SH-SY5Y cells,[42] and at least in the latter system, this binding appears to best fit a model of two binding sites (K_d = 0.15 and 7.4 nM). Correspondingly, two classes of receptors defined by the presence *vs* absence of the β2 subunit have been identified based on immunoprecipitation studies. One class potentially includes some combination of $\alpha3\beta2$, $\alpha3\alpha5\beta2$, $\alpha3\beta2\beta4$, and $\alpha3\alpha5\beta2\beta4$, and the other class could contain one or both of $\alpha3\beta4$ and $\alpha3\alpha5\beta4$. Immunodepletion experiments and pharmacological correlations with subunit combinations expressed in oocytes suggest that the β2-containing class includes nAChRs with higher affinity for [^{3}H]-(±)-epibatidine than those lacking β2. Since α5-specific monoclonal antibody 35 (mAb35) blocks catecholamine release from bovine adrenal gland, the presence of this subunit in nAChRs principally responsible for nAChR-mediated sympathetic outflow is indicated.[157] There is evidence to suggest that some synaptic nAChRs in sympathetic neurons may contain the α7 subunit.[158,159]

Native parasympathetic ganglionic nAChRs have been most extensively studied in chick ciliary ganglia.[43,160−164] Neurons in ciliary ganglia express five of the known nAChR gene products (α3, α5, α7, β2, and β4),[165] and experiments utilizing subunit-specific monoclonal antibodies confirm the presence of nAChRs containing $\alpha3\alpha5\beta4$ and $\alpha3\alpha5\beta2\beta4$, whereas the presence of a purely $\alpha3\beta4$ combination is still uncertain.[43,164] n-Bgt, which potently blocks ganglionic transmission, and α5-specific mAb35 bind to the same population of nAChRs, supporting the involvement of at least the above α5-containing nAChRs in synaptic ganglionic transmission.[121,166,167] Little if any of the α7 subunit is detected in the synaptic nAChRs in chick ciliary ganglia,[163] in contrast to the findings cited above for chick sympathetic ganglia. However, it is possible that other, as yet unknown, subunits are present. The α7 subunit does assemble into a separate class of predominantly nonsynaptic nAChRs recognized by α-Bgt (*vide infra*).

Synaptic ganglionic-like nAChRs have been probed with a number of radioligands in addition to [^{3}H]-(±)-epibatidine. [^{3}H]Nicotine binds to SH-SY5Y cell membranes, also according to a two-site model with K_d values 1.03 and 34 nM,[42] and to bovine adrenal chromaffin cells with a K_d of 8.9 nM.[168] [^{3}H]ACh binding in SH-SY5Y cells also best fits a two-site model (K_d = 1 and 100 nM),[153] and similar findings have been found in PC12[169] and IMR-32 cells.[154] Low levels of [^{3}H]-(±)-epibatidine binding are detected in rat adrenal gland membranes,[126] whereas [^{3}H]nicotine binding was not detected in ganglia from cynomolgus monkey,[170] and little or no [^{3}H]-Cyt binding was detected in the IMR-32 cell line.[126] [^{125}I]n-Bgt binds with high affinity (K_d = 5−6 nM) to ciliary ganglion neurons.[167] Micromolar concentrations of ACh or nicotine were required to displace [^{125}I]n-Bgt from ciliary ganglia, a finding which contrasts with the nanomolar affinities cited above. It is possible that, because of its large size, n-Bgt has multiple sites of interaction with the receptor, with which small molecules do not effectively compete at low concentrations.

Functional assays are more commonly employed than binding assays to characterize the interactions of new compounds with synaptic ganglionic-type nAChRs. The contrast of this practice with that most commonly employed with the $\alpha4\beta2$ and α7 subtypes may arise from the previous lack of suitable systems for measuring functional responses at $\alpha4\beta2$ and α7 nAChRs. Functional assays for synaptic ganglionic nAChRs using cell lines such as IMR-32 or PC12 (see above) commonly measure flux of radioactive ions ($^{86}Rb^+$ or $^{23}Na^+$)[153,154,171] or calcium influx.[172] Electrophysiological recordings of channel currents in cell lines and tissues containing native nAChRs also have been carried out.[150,173] Although SAR studies have been performed using heterologously expressed pairwise combinations, e.g. $\alpha3\beta2$ and $\alpha3\beta4$,[29,86,135,136,174] it is evident based on the foregoing discussion that the physiological relevance of these subunit combinations is still uncertain. Studies with more complex combinations of heterologously expressed subunits are presently hampered by the formation of heterogeneous populations of nAChRs following coexpression of more than two subunits in the same cell.[42,143]

Ganglionic α7 Subtype: High-Affinity PNS Binding Sites for α-Bungarotoxin. A prominent nAChR subtype in autonomic ganglia contains α7 subunits and potently interacts with α-Bgt. In the chick ciliary ganglion, these α-Bgt-sensitive receptors appear to be predominantly localized in perisynaptic clusters immediately adjacent to synaptic sites[175−177] and contain only α7 among the known nAChR gene products.[97,163] The [^{125}I]-α-Bgt binding affinities of heterologously expressed α7 homooligomers are in good agreement with values obtained from native receptors in human neuroblastoma IMR-32[33] and SH-SY5Y cells,[35,156] suggesting that the ganglionic α7 nAChRs may be homomeric. Thus it is possible, but not yet certain, that these nAChRs are structurally identical with the CNS α7 subtype.

In addition to their high affinity for α-Bgt, ganglionic α7 nAChRs exhibit many of the same characteristics as CNS α7 receptors, including a rapidly desensitizing response upon activation and a relatively high permeability to calcium ions.[117,164,172,178] Initial studies had found that α-Bgt was unable to block functional responses in ciliary ganglia,[179] suggesting that α7 receptors were not responsible for mediating ganglionic synaptic transmission. However, it has recently been shown that α-Bgt-sensitive nAChRs generate a large amount of the peak ganglionic synaptic current, despite their apparent perisynaptic location.[180] Activation of α-Bgt-sensitive receptors on chick ciliary ganglia neurons induces a calcium-dependent neurite retraction in cell culture[181] and leads to the release of arachidonic acid from intracellular stores.[182] Thus these α7 receptors may function not simply to regulate the membrane potential of the cell, but rather to regulate other cellular functions via second messenger systems.

Muscle-Type nAChRs. nAChRs on mature skeletal muscle are organized in clusters across the synapse from incoming motor neurons.[183] Activation of these receptors in skeletal muscle causes muscle endplate depolarization leading to muscle contraction.[184] However, prolonged exposure to depolarizing agents leads to desensitization and loss of muscle contractability (for an excellent discussion, see ref 185). The state of knowledge on the structure of the muscle nAChR has been extensively reviewed.[5,27,186−189] On the basis of

studies of the analogous nAChR from *Torpedo*, the five subunits of the receptor (two α1, one each of β1, δ, and γ) are proposed to each consist of four transmembrane domains, with the C- and N-termini projecting from the extracellular side of the membrane (Figure 1). The second transmembrane domain (M2) from the N-terminus of each subunit lines the pore of the ion channel.

Compounds under consideration as CNS therapeutic agents may be evaluated for activity at this subtype in order to judge the potential for side effects such as loss of motor function. Radioligand binding assays commonly utilize [^{125}I]-α-Bgt and nAChRs from *Torpedo* electric organ. Functional responses previously were assessed by measuring contraction of muscle tissue preparations from, for example, frog rectus abdominis or rat diaphragm.[190] More recently, cell-based assays such as radio-ion flux in TE671 (human medulloblastoma)[191] or BC_3H-1 (mouse muscle) cell lines have frequently been used.[152,154]

Other Peripheral nAChR Subtypes. Cholinergic receptors in cochlear hair cells may be involved in the modulation encoding of auditory signals. mRNA for the α9 nAChR subunit has been detected in rat cochlea, and α9 subunits form homooligomeric channels in oocytes with properties similar to those of the endogenous cochlear nAChRs.[32] Like α7 receptors, functional responses in these channels are blocked by α-Bgt. However, unlike other subtypes, α9 nAChRs are sensitive to nicotine only as an antagonist, and this subtype also is sensitive to blockade by the muscarinic antagonist atropine.

A minor subpopulation of αBgt binding sites in chick ciliary ganglia with nicotinic pharmacology has been detected which contains none of the presently known nAChR subunits.[192] These novel putative nAChRs bind both α-Bgt and mAb 35, but appear to lack all of the known neuronal nAChR gene products in ciliary ganglia including α3, α5, α7, β2, and β4. The inability to detect or identify subunits comprising the putative ciliary ganglion receptor defined by joint α-Bgt and mAb 35 binding raises the possibility that additional nAChR genes remain to be cloned.

Nicotine and ACh stimulate a number of types of sensory neurons, notably those responsible for pain transmission.[193,194] Recent evidence for the presence of nAChRs on certain sensory neurons includes the detection of functional nAChRs on chick dorsal root ganglion (DRG) cells, which contain transcripts for at least the α3 and α4 subunits,[195] and in a cell line derived from rat DRG.[196] Rat trigeminal ganglion neurons possess high-affinity binding sites for Cyt and epibatidine, and mRNA transcripts for several neuronal nAChR subunits have been detected.[129] The evidence suggests that α4β2 and α3β4 are two major nAChR subtypes in this tissue, although participation of other subunits in these receptors and the presence of other subtypes is possible. The function of nAChRs on sensory neurons is not clear, but the colocalization nAChRs in trigeminal ganglia with calcitonin gene related peptide (CGRP), a known pain neurotransmitter, and the potent activation of receptors in DRG-like cells by epibatidine support the possible role in modulating pain neurotransmission.[129,197]

Tobacco smoke and nicotine have been shown to suppress immune function in humans and experimental animals (see ref 198), and there is a body of evidence

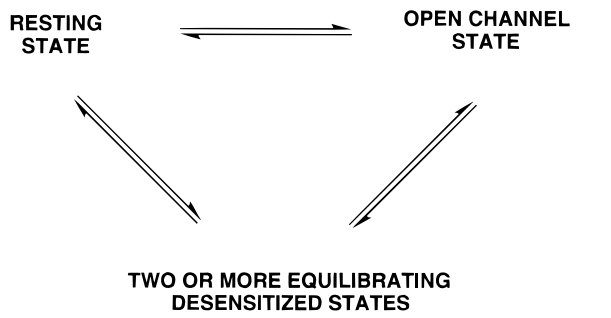

Figure 3. Simplified scheme of interconverting states of nAChRs (adapted from Lena and Changeux[338]). Ligands can independently interact with and thereby stabilize any of the states in a way that presumably varies according to ligand; ligand-bound receptor states can undergo analogous transitions to those shown. Assuming that the resting state predominates at equilibrium, rate constants for the transitions are such that exposure to a sufficient concentration of agonist can lead to a nonequilibrium transient increase in the ligand-bound open channel state, followed by reestablishment of an equilibrium in which the desensitized state(s) is often preferred for classical ligands.

indicating the presence of functional nAChRs on cells of the immune system. Thus, nicotine-stimulated enhancement of suppressor T-cell activity is blocked by *d*-TC,[199] and the antiproliferative effect of carbachol on human lymphocytes is blocked by *d*-TC and α-Bgt. Sites with high affinity (K_d < 10 nM) for [^{3}H]nicotine have been found on rat lymphocytes and thymocytes. On the basis of immunohistochemical evidence, some human thymus cells appear to express muscle-like receptors, whereas others contain transcripts for neuronal nAChR subunits α3, α5, and β4 (but not β2). The presence of nAChRs on immune cells suggests therapeutic opportunities in immunomodulation, but actions on the immune system also represent potential side effects for drugs targeting nAChRs for other indications.

Nicotinic agonist-induced proliferation of neuroendocrine derived small cell lung carcinoma cells has been demonstrated, an effect which is blocked by α-Bgt.[200] These cells contain mRNA for α7 and α5 nAChR subunits.

Receptor Desensitization and Consequences for nAChR Pharmacology. nAChRs can exist in multiple states, each of which may have a unique interaction with ligands (Figure 3). Because the time scales on which binding studies are conducted (minutes to hours) are much greater than for functional studies (milliseconds to minutes), binding affinity normally reflects interaction with one or more desensitized states of the receptor, whereas functional assays measure interaction with the open channel state. One consequence of this distinction is reflected in considerations of subtype selectivity. Ideally, and most appropriately, subtype selectivity for a given agonist is determined by comparing functional responses at different receptor subtypes. However, on the basis of the assumption that binding affinities may predict functional potency, relative binding affinities have been used as a measure of subtype selectivity. The data in Table 2 suggest, albeit with a limited number of ligands, that this assumption should be used with caution when applied to some nAChRs. Compounds in Table 2 were chosen which possess at least 75% efficacy compared to nicotine (except where noted) to activate the nAChRs shown. The left half of Table 2 shows binding constants and functional EC_{50}

Table 2. Binding and Functional Potencies for Selected Ligands at nAChR Subtypes[a–c]

compound	binding K_i (nM)			function EC_{50} (nM)			function EC_{50}/binding K_i			binding selectivity	functional selectivity
	$\alpha4\beta2$	$\alpha7$	$\alpha1\beta1\delta\gamma$	$\alpha4\beta2$	$\alpha7$	$\alpha1\beta1\delta\gamma$	$\alpha4\beta2$	$\alpha7$	$\alpha1\beta1\delta\gamma$	$\alpha4\beta2$ *vs* $\alpha7$	$\alpha4\beta2$ *vs* $\alpha7$
nicotine (**1**)	1.05	4000	>10000	4000	54000	60000	3810	13.5	<6	3810	13.5
ABT-418 (**10**)	7.89	>10000	>100000	10000	264000	411000[d]	1267	<26	<4	>1267	26.4
A-85380 (**14**)	0.04	148	314	700	8900		17500	60		3700	12.7
(±)-epibatidine ((±)-**2**)	0.07	21	2.7	17	1300	200	243	62	74	300	76.5

[a] Binding data: ($\alpha4\beta2$) rat brain/[^{3}H]Cyt; ($\alpha7$) rat brain/[^{125}I]-α-Bgt; ($\alpha1\beta1\delta\gamma$)*Torpedo*/[^{125}I]-α-Bgt. [b] Functional data: ($\alpha4\beta2$) $^{86}Rb^+$ flux in recombinant nAChR in K177 cells; ($\alpha7$) ion currents in recombinant nAChR in oocytes; ($\alpha1\beta1\gamma\delta$) $^{86}Rb^+$ flux in TE671 cells. [c] Binding and functional data at $\alpha4\beta2$ for nicotine, ABT-418, and (±)-epibatidine are from ref 34. Other data for nicotine and (±)-epibatidine are from ref 115, and other data for ABT-418 are from ref 208. All data for A-85380 are from ref 218. [d] Partial agonist relative to nicotine.

values for several ligands from different structural classes. The right half of Table 2 presents selected ratios of these values in order to demonstrate (1) the disparities between binding K_i values and functional EC_{50} values for these compounds at the selected nAChR subtypes and (2) the differences in apparent selectivity when compounds are compared with respect to binding *vs* with respect to function. The first set of ratios indicates that the EC_{50} and binding K_i values for $\alpha4\beta2$ differ by 2−3 orders of magnitude, whereas, at least with these ligands, the corresponding differences are much smaller for the $\alpha7$ and $\alpha1\beta1\delta\gamma$ subtypes. The consequence, illustrated for $\alpha4\beta2$ *vs* $\alpha7$ on the right side of Table 2, is that selectivity for $\alpha4\beta2$ appears much greater when determined from binding data than when determined from functional data. It can also be noted that nicotine is more selective than (±)-epibatidine for $\alpha4\beta2$ *vs* $\alpha7$ with respect to binding affinity, whereas the reverse is true according to the functional data.

A further important consequence of receptor desensitization is that a single compound may be able to act as both an activator and an inhibitor of receptor function, depending on conditions. Furthermore, it has been clearly demonstrated that full receptor activation is not a prerequisite for receptor desensitization, since desensitization can be induced at agonist concentrations lower than those required to elicit a detectable agonist response.[73,87,88,201] Different compounds can have differential effects on receptor activation *vs* desensitization,[202] although similar rank orders of agonist potencies for the two effects also have been observed.[88] In many cases, it is not known whether the physiological effects of nicotine and other nAChR modulators are a consequence of nAChR activation, inhibition, or both.

Ligands for Major Endogenous nAChR Subtypes

$\alpha4\beta2$ nAChRs. In addition to nicotine, ACh, Cyt, and MCC, discussed above as high-affinity radioligands, there is an ever increasing and diverse range of compounds that bind with high affinity to $\alpha4\beta2$ nAChRs.[185,203–207] However, the number of compounds with selectivity for $\alpha4\beta2$ receptors is far fewer. ABT-418 (**10**),[208] RJR-2403 (**15**, Figure 4),[209] and A-84543 (**16**),[210] compounds described in more detail below, each possess some improvement in selectivity for $\alpha4\beta2$ *vs* ganglionic nAChRs compared to nicotine (Table 3). These compounds also stimulate rat striatal DA release with potencies less than or comparable to those for $\alpha4\beta2$ activation. Like nicotine, **10** and **15** are selective for $\alpha4\beta2$ *vs* muscle-type nAChRs, with **15** showing little or no activity at this subtype. DHβE (**14**) is a competitive antagonist at the $\alpha4\beta2$ subtype.[34,211,212] On the basis of experiments in PC12 and TE671 cells, **14** is a comparatively weak blocker of synaptic ganglionic-like and muscle-type nAChRs.[152] However, DHβE potently blocks striatal DA release in rat[139] and mouse,[123] as well as responses at a number of heterologously expressed rat and human nAChRs.[116,212,213]

nAChRs Mediating DA Release. As mentioned above, n-Bgt potently blocks and epibatidine potently stimulates [^{3}H]DA release in rodent striatal preparations, consistent with the possible mediation of this response by the class(es) of non-$\alpha4\beta2$/non-$\alpha7$ nAChRs recognized by these ligands. Most of the nAChR modulators in Table 3 also potently stimulate DA release. In general, these compounds also are potent activators of $\alpha4\beta2$ nAChRs, but several ligands appear to exhibit distinct responses in the two systems. As noted earlier, cytisine (**7**) is a potent partial agonist in stimulation of DA release in rat but only weakly activates rat $\alpha4\beta2$ nAChRs.[135,136] GTS-21 (**11**) is less potent than cytisine, but is also a partial (~70%) agonist relative to nicotine in DA release, while showing low efficacy in recombinant rat $\alpha4\beta2$ nAChRs in oocytes. For other compounds, data for both DA release and $\alpha4\beta2$ function are not always available from the same species, and as cited in more detail subsequently, comparisons of responses across species must be interpreted with caution. Nevertheless, intriguing recent data show that SIB-1765F (**17**) stimulates DA release from rat striatum with efficacy greater than that of nicotine (*cf.* SIB-1508Y below), albeit with *ca.* 25-fold lower potency,[214] whereas this compound is less efficacious than nicotine in stimulation of calcium flux in human $\alpha4\beta2$ nAChRs.[86] ABT-089 (**18**) is a partial (~70%) agonist relative to nicotine in dopamine release from rat tissue, but shows low efficacy in human recombinant $\alpha4\beta2$ and ganglionic-like nAChR in IMR-32 cells.[215] Anatoxin-a (Atx, **19**)[216,217] and A-85380 (**20**)[218] are highly potent but nonselective stimulators of DA release.

In addition to n-Bgt, DA release from rat striatum is potently blocked by DHβE with $IC_{50} = 0.03\ \mu M$[139] *vs* blockade of responses in rat recombinant $\alpha4\beta2$ nAChR ($IC_{50} = 0.37\ \mu M$).[212] A series of pyridine *N*-substituted nicotine analogues has been evaluated in binding assays and for ability to inhibit neurotransmitter release.[219] The *N*-*n*-octyl-substituted pyridine (**21**, NONI) is the most potent analogue in this series at blocking nicotine-evoked [^{3}H]DA release from rat striatal slices, with potency similar to that of mecamylamine and DHβE. Compound **21** is approximately 10000-fold less potent than nicotine in its ability to displace [^{3}H]nicotine

Table 3. Comparisons of Functional Potencies and Efficacies of Selected Ligands in Common Assays of Activity at nAChR Subtypes[a]

	human				rat		
agonists	recombinant $\alpha4\beta2$	recombinant $\alpha7$	IMR-32 (ganglionic)	TE671 (muscle)	recombinant[v] $\alpha4\beta2$	PC12 (ganglionic)	DA release
nicotine (**1**)	2−4 (100)[b,c]	40−83 (100)[h,i,j,k]	21 (100)[f,n]	60−160 (100)[o,t]	0.59 (100)[v,w] to ≈ACh[x]	20 (100)[t]	0.04−3.7 (100)[ff,gg]
ACh (**8**)		79−155 (100)[h,i,k]	30(167)[o]	5 (222)[t]	197−270 (∼100)[y,z]	40 (154)[t]	≈Nic[hh]
(±)-epibatidine ((±)-**2**)	0.017 (156)[b]	1.3−3.5 (∼80)[j,k]	0.007 (156)[f,n]	0.2 (∼140)[n]	0.016 (∼50)[y,aa]	∼0.1 (100)[dd]	0.0004−0.037 (100−140)[ii,jj]
Cyt (**7**)	38 (40)[b]	71 (100)[i]	26 (∼100)[p,q]	100 (222)[t]	low efficacy[x]	10 (100)[t]	0.06 (65)[gg]
DMPP (**12**)	2.5 (72)[b]	25−95 (100)[h,i,k]			weaker than ACh[x]		4.3 (104)[gg]
ABT-418 (**10**)	10.6 (93)[b]	264 (∼75)[j]	64 (85)[r]	411 (38)[u]	6 (∼50)[w,y]	214 (100)[ee]	0.38 (83)[ff]
ABT-089 (**18**)	low efficacy[d]	low efficacy[d]	low efficacy[d]	30 (60)[d]			1.1 (70)[d]
RJR-2403 (**15**)	4.3 (83)[e]		46 (42)[e]	>1000 (0)[v]	0.73 (91)[v,w]	>1000 (0)[v]	0.94 (82)[v]
A-85380 (**20**)	0.7 (163)[f]	8.9 (∼120)[l]	0.7 (113)[f]				0.003 (150)[l]
A-84543 (**16**)	0.75 (100)[f]		19 (73)[s]				(55% at 1 μM; 90% at 10 μM)[kk]
SIB-1765F (**17**)	2.6 (47)[c]						99 (143)[c,ll]
GTS-21 (**11**)	low efficacy[g]	low efficacy[j]	low efficacy[g]		low efficacy[bb]		10 (70)[g]
isoarecolone methiodide (**28**)				0.1 (267)[t]		100 (154)[t]	
Atx (**19**)					0.048 (∼100)[cc]		0.11[mm]
antagonists							
DHβE (**14**)	1.87[b]	19.6[m]		2000[t]	0.37[z]	1000[t]	0.03[nn]
mecamylamine (**5**)				30[t]		0.2[t]	

[a] Data are expressed as EC_{50} values in μM (% maximal response relative to nicotine) for agonists and as IC_{50} in μM for antagonists. [b] Reference 34. [c] Reference 86. [d] Reference 215. [e] Reference 248. [f] Reference 210. [g] Reference 242. [h] Reference 33. [i] Reference 156. [j] Reference 244. [k] Reference 95. [l] Reference 218. [m] Reference 213. [n] Reference 115. [o] Reference 154. [p] Reference 138. [q] Reference 134. [r] Reference 249. [s] Reference 283. [t] Reference 152. [u] Reference 208. [v] Reference 209. [w] Some data as indicated are from native $\alpha4\beta2$ nAChRs from rat brain. [x] Reference 136. [y] Reference 350. [z] Reference 212. [aa] Maximal response relative to nicotine inferred by comparison of ACh and nicotine responses in ref 136. [bb] Reference 107. [cc] Data for recombinant chick $\alpha4\beta2$ from ref 216. [dd] Reference 171. [ee] Reference 173. [ff] Reference 351. [gg] Reference 135. [hh] Reference 352. [ii] Reference 131. [jj] Reference 132. [kk] Abreo, M. A.; Sullivan, J. P., *et al.* Unpublished data. [ll] Reference 214. [mm] Reference 217. [nn] Reference 139.

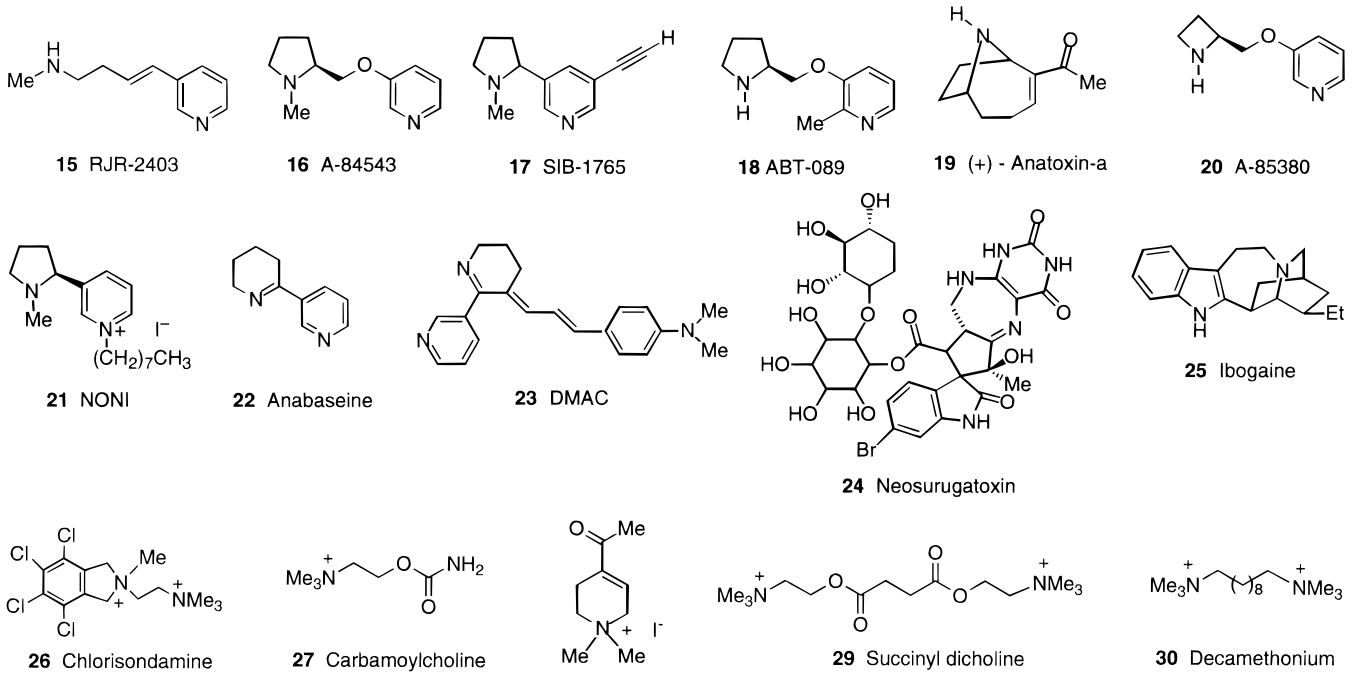

Figure 4. Structures of selected nAChR modulators.

binding in rat striatal membranes. Thus, **21** could be a useful probe for distinguishing the $\alpha4\beta2$ subtype from the subtype(s) responsible for striatal DA release. However, such utility awaits assessment of **21** as an inhibitor of $\alpha4\beta2$ function, since it is conceivable that the inhibitory properties in DA release arise from interactions with a site distinct from the agonist binding site, e.g. channel blockade.

Brain and Ganglionic α7 nAChRs. α-Bungarotoxin interacts potently with putative α7 AChRs in brain and ganglia as well as with muscle-type nAChRs. Another competitive antagonist that is commonly used to identify these sites is the tertiary diterpenoid MLA (**13**), a natural product isolated from a poisonous plant found in western Canada, *Delphinium brownii*.[220] In chicken, MLA has been demonstrated to be selective for putative α7 nAChRs over muscle-type nAChRs (*ca.* 200-fold), synaptic ganglionic nAChRs (*ca.* 50−100-fold), $\alpha4\beta2$ nAChRs, and the $\alpha3\beta2$ combination expressed in oocytes.[117,148,221] In rat, MLA is about 1000-fold selective for α7-type nAChRs over $\alpha4\beta2$ nAChRs[37,222] and does not potently block DA release from striatal synapto-

somes.[221] The alkaloid anabaseine (**22**) binds *ca.* 5-fold more selectively to rat α4β2 than rat α7-type nAChRs but is somewhat more efficacious at α7.[107] DMAC (**23**), an anabaseine derivative, possesses *ca.* 10-fold higher affinity for rat α7 than α4β2, and is also functionally selective for α7 as a partial agonist (75% efficacy relative to ACh, compared to <5% efficacy at α4β2).[107]

Synaptic Ganglionic nAChRs. Nicotine, cytisine (**7**), and DMPP (**12**) are classical ganglionic-stimulating drugs,[26,223] but on the basis of the data in Table 3, do not appear to be highly selective for this class *vs* one or more other nAChR subtypes. Epibatidine (**2**) and A-85380 (**20**) are highly potent but nonselective stimulators of synaptic ganglionic nAChRs (Table 3). Atx (**19**) is a potent ($EC_{50} = 1-2\ \mu M$) stimulator of catecholamine release in bovine adrenal chromaffin cells,[151] but has potent activity at most other nAChR subtypes as well.[216,217,224,225]

With respect to antagonists, many bis-quaternary ammonium compounds block ganglionic transmission. Hexamethonium (**6**) is the most potent and is essentially devoid of neuromuscular blocking activity. Compound **6** is believed to act noncompetitively by blocking the ion channel[226] and accordingly is weak at displacing [^{3}H]-nicotine from membranes of cultured bovine adrenal chromaffin cells.[168] Because of its charged structure, peripherally administered **6** is presumed not to enter the brain, and therefore is commonly used to distinguish peripheral ganglionic responses *in vivo* from those that arise centrally. n-Bgt potently blocks nicotinic activity in both chick and rat autonomic ganglia with IC_{50} values of <100 nM.[119,121,227] Although n-Bgt is very poor at blocking nAChRs at the neuromuscular junction, it also binds to sites in the CNS (*vide supra*) and is a potent blocker of DA release from striatal tissue.[123,133] Neosurugatoxin (**24**)[128] and ibogaine (**25**)[228,229] also block both ganglionic neurotransmission and central DA release. Other ganglionic blockers include chlorisondamine (**26**), a channel blocker which also causes persistent blockade of central nicotinic effects after peripheral administration,[230] and trimethaphan (**4**), which also interacts with the muscle-type nAChR.[152]

As mentioned previously, several radioligands ([^{3}H]-(±)-epibatidine, [^{3}H]nicotine, [^{3}H]ACh) label high-affinity sites in ganglionic preparations. The reported affinities for these sites are similar to those observed for α4β2 nAChRs.[34] On the other hand, the high affinity of [^{125}I]n-Bgt binding to ciliary ganglion neurons (K_d = 5−6 nM) contrasts with its reported low affinity for α4β2 nAChRs.[122,123]

Muscle-Type nAChRs. α-Bgt binds with exceptionally high affinity (picomolar range) to muscle nAChRs.[231,232] (±)-Epibatidine[115] and ACh[233] displace [^{125}I]-α-Bgt from *Torpedo* membranes in the low nanomolar range, whereas nicotine,[234] carbamoylcholine (**27**),[233,234] *d*-TC (**3**),[235] Atx (**19**),[234,236] and isoarecolone methiodide (**28**),[233,234] show affinities in the mid-nanomolar to low micromolar range. Ion flux in TE671 cells is potently stimulated by compound **28** ($EC_{50} \sim 100$ nM), with efficacy 120% that of ACh, whereas ACh ($EC_{50} \sim 5000$ nM) and succinyl dicholine (**29**) ($EC_{50} \sim 30\ 000$ nM) are less potent full agonists.[152] Nicotine is a partial agonist (45%, $EC_{50} \sim 100\ 000$ nM) relative to ACh,[152] and (±)-epibatidine (EC_{50} = 200 nM) has efficacy equal to *ca.* 140% that of nicotine.[115] *d*-TC (**3**) and decamethonium (**30**) appear to act as competitive antagonists, whereas mecamylamine (**5**) is a noncompetitive antagonist.[152] Most of the above agents show substantial activity at other subtypes of nAChRs. Thus, α-Bgt has high affinity for neuronal α7-containing receptors (see above), and *d*-TC and decamethonium are functionally nonselective *vs* synaptic autonomic ganglionic-like nAChRs in PC12 cells.[152] Nicotine activates synaptic ganglionic, α7, and α4β2 nAChRs with potency equivalent to or greater than at muscle nAChRs (see ref 152 and Tables 1 and 3), and isoarecolone methiodide exhibits high affinity for CNS α4β2 nAChRs.[237]

Medicinal Chemistry of nAChR Modulators

Several recent reviews[185,203−207] have summarized structure−activity relationships for both classical and some more recently discovered nAChR ligands. In this Perspective Article, new information is summarized for selected benchmark agents that have undergone advanced characterization as potential therapeutic agents or serve as important lead compounds. New information on several nAChR antagonists also is summarized. In an overview of other medicinal chemistry advances since the appearance of the previous reviews, new series of nAChR ligands and an update of structure−activity relationships on existing series are described.

Update on Development Compounds and Other Leads. ABT-418 (**10**), an isoxazole isostere of nicotine, is a full agonist at α4β2 receptors, with improved selectivity compared to nicotine to stimulate α4β2 *vs* synaptic ganglionic-like nAChRs and [^{3}H]DA release. ABT-418 is effective in animal models assessing anxiolysis and cognition with a reduced propensity for side effects compared to nicotine.[208] Neuroprotective properties of ABT-418 have been described,[109] supporting the hypothesis that the compound could potentially slow the progression of neurodegenerative diseases. Blockade of the neuroprotective effect by MLA (**13**) suggests that an α7 subtype may be involved in the neuroprotective actions. Results from a pilot trial in human AD patients demonstrate that ABT-418 given acutely is effective in a selective reminding task.[14]

GTS-21 (**11**, also known as DMXB) is being evaluated clinically for the treatment of AD.[238] Preclinical findings supporting the possible utility of GTS-21 as a cognition enhancing agent[239−242] have been extended to show that GTS-21 also is effective following chronic administration in several assays of learning and memory.[243] GTS-21 also has been shown to demonstrate protective effects against Aβ-induced neurotoxicity.[110] On the basis of studies of rat receptors expressed in oocytes, GTS-21 has been characterized as an α7 selective agent, behaving as a partial agonist (*ca.* 28% of ACh response) at α7 homomers, while showing negligible activity at the α4β2 subtype.[107] However, in studies of human α7 nAChRs expressed in oocytes, GTS-21 appeared to be less efficacious than in rat.[244] Interestingly, in this system, GTS-21 can inhibit the response elicited by ACh at concentrations at which it stimulates little activation on its own, i.e. it is more potent as an inhibitor than as an activator. Activation of human α4β2 receptors by GTS-21 was negligible.[242]

RJR-2403 (*trans*-metanicotine, **15**) has been identified as a cognition-enhancing agent with reduced potential for side effects as assessed by a variety of *in vitro* and

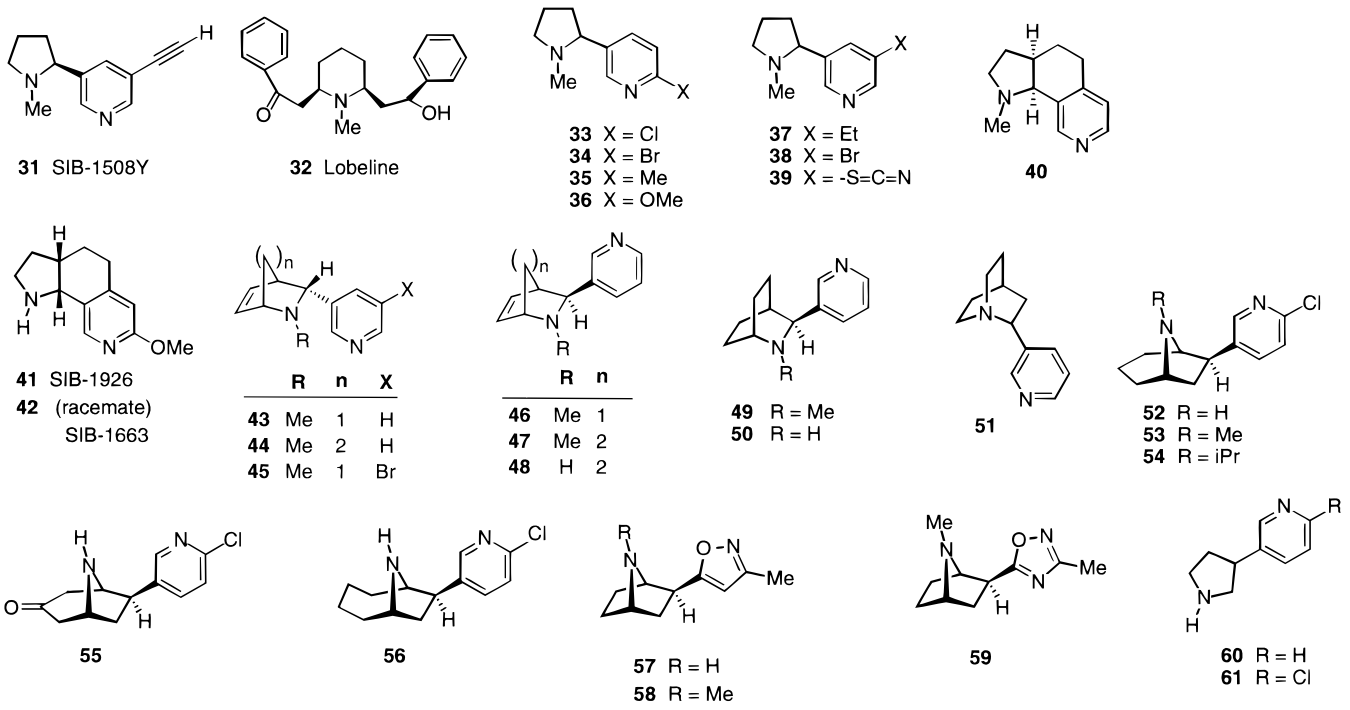

Figure 5. Structures of selected nAChR modulators.

in vivo measures.[209,245,246] RJR-2403 possesses activity in cognitive enhancement assays in rats that is equivalent to or better than that of nicotine, and was shown by *in vivo* microdialysis experiments to have effectiveness similar to that of nicotine to stimulate the release of ACh, DA, NE, and 5-HT.[247] On the other hand, RJR-2403 was 10−30-fold less potent than nicotine in eliciting changes in blood pressure, heart rate, temperature and locomotion in rats. RJR-2403 binds with high affinity (K_i = 26 nM) to α4β2 nAChRs in rat cortex but possesses weak affinity (36 000 nM) for rat brain α7 nAChRs. *In vitro* functional assays indicate that RJR-2403 is nearly as effective as nicotine at stimulating ion flux in rat thalamus, used as a model for activation of α4β2 receptors, but is about 9-fold less potent and slightly less efficacious than nicotine at stimulating DA release from rat striatum. RJR-2403 does not stimulate ion flux in models of rat autonomic ganglia (PC12 cells) or human muscle receptors (TE671 cells). At human nAChR subtypes, RJR-2403 also shows potency and efficacy similar to nicotine at α4β2 receptors, but in contrast to studies with rat tissue, was found to possess partial agonist activity in IMR-32 cells, a model of human autonomic ganglia.[248]

Among a recently reported class of potent 3-pyridyl ether nAChR ligands,[210] ABT-089 (**18**) was selected for advanced evaluation on the basis of its cognitive enhancing properties in rodents and monkeys, oral bioavailability, reduced activity at ganglionic-like receptors, and neuroprotective properties against glutamate and Aβ-mediated neurotoxicity.[21,108,215,249,250] *In vitro*, ABT-089 possesses high affinity (K_i = 19 nM) for α4β2 nAChRs in rat brain and low affinity (>10 000 nM) for α7 receptors. ABT-089 is as efficacious and nearly as potent as nicotine in stimulating ACh release from hippocampal tissue, but is both less potent (25-fold) and less efficacious (70% efficacy compared to nicotine) at stimulating DA release from striatal tissue. ABT-089 is a partial agonist of ion flux at the putative α4β2 nAChR in mouse thalamus. Interestingly, ABT-089 shows significantly lower efficacy at human α4β2 receptors,[215] which may reflect species differences in the pharmacology of human and rodent α4β2 receptors. ABT-089 also shows low efficacy at recombinant human α7 nAChRs in oocytes.

SIB-1508Y (**31**, Figure 5), the more active enantiomer of racemate SIB-1765F (**17**), has recently been disclosed as an agent for the potential treatment of Parkinson's disease.[86,206] SIB-1508Y possesses high affinity (K_i = 3 nM) for [^{3}H]nicotine sites in rat brain and stimulates release of DA from rat striatum with efficacy 163% that of nicotine. SIB-1508Y is also reported to be efficacious in various animal models of Parkinson's disease. Interestingly, in contrast to its DA releasing properties, SIB-1508Y appears to be no more than a partial agonist compared to nicotine[213] at several recombinant human nAChR subtypes expressed in oocytes or mammalian cell lines.

Lobeline (**32**) is under development as an aid for smoking cessation.[251] A pilot phase III clinical trial has shown that sublingual tablets of 7.5 mg of lobeline sulfate (NicErase-SL) taken nine times a day for 6 weeks resulted in approximately twice as many subjects ceasing to smoke as in the placebo group. A larger phase III clinical trial is being conducted in the United States and Europe. A novel sustained release system for delivery of lobeline for cessation of nicotine use also has been described.[252]

Epibatidine (**2**) remains the most potent naturally occurring nAChR ligand reported to date, with potency in many pharmacological and behavioral assays several 100-fold greater than that of nicotine (reviewed in ref 115). The numerous synthetic efforts toward epibatidine and various analogues have been reviewed.[253,254] Both enantiomers of epibatidine show similar effects in a number of nAChR binding, functional, and *in vivo* pharmacological assays.[19] The activity of epibatidine as a potent nonopioid analgesic agent has continued to attract considerable interest. It is not known which subtype(s) mediate the antinociceptive effects of epiba-

tidine, but central action appears to be at least partly responsible.[20,255] In contrast to nicotine, the antinociceptive effects of epibatidine are generally maintained after repeated administration,[256,257] although small differences were discerned in the propensity of the different enantiomers of epibatidine to develop tolerance in the tail flick response.[257] Epibatidine is not likely to be developed clinically as an analgesic agent, since antinociceptive responses are observed at doses only slightly lower than those causing severe hypertension, convulsions, and respiratory depression.[131,258] Unlike nicotine, epibatidine is ineffective in models of cognitive performance.[256,259]

Recent Studies of nAChR Antagonists. Mecamylamine (**5**), a noncompetitive nAChR antagonist selective for neuronal *vs* muscle subtypes, is commonly used in behavioral studies to establish an nAChR-mediated mechanism of action *in vivo*. Because of its prominent role in such studies, it is worthwhile to note mecamylamine's activity at specific subtypes of nAChRs. Mecamylamine blocks the activation of ACh-stimulated currents in human α7 homomers expressed in oocytes (IC_{50} = 1.8 μM).[260] This level of activity is comparable within an order of magnitude with that at numerous other neuronal subtypes,[260,261] whereas mecamylamine is less active at α3β4[261] and muscle-like receptors.[152]

As described previously, MLA (**13**) is a selective antagonist of heterologously expressed α7 homomeric nAChRs and of native receptors corresponding to CNS [^{125}I]-α-Bgt binding sites. It has recently been demonstrated that MLA enters the CNS following peripheral administration, which will permit its use to deduce what, if any, behavioral actions are mediated by central α-Bgt-sensitive nAChRs.[262] On the basis of studies with MLA, neither the nicotine discriminative stimulus effect[263] nor the tail flick analgesia response[264] appears to be mediated by nAChRs containing the α7 subunit.

d-TC (**3**) has been characterized recently in a panel of human nAChR subunit combinations (α2β2, α3β2, α4β2, α2β4, α3β4, α4β4, α7) expressed in oocytes.[213] The inhibition of responses by *d*-TC was within a *ca.* 20-fold potency range for all of the subunit combinations examined, with α4β4 being the most sensitive, followed by a *ca.* 6-fold lower sensitivity of α2β2. In the same study, DHβE (**14**) was found to be *ca.* 10-fold selective for human α4β4 *vs* α4β2, the next most sensitive subtype. While identification of highly selective antagonists to non α-Bgt-sensitive receptors remains elusive, rank order potencies determined by such studies may still prove useful to identifying correlations with responses at endogenous receptors.

Recent SAR and New Lead Series

Nicotinoids. The binding affinity and functional activity of racemic 6-substituted nicotine analogues have been reported.[265] Introduction of a chloro (**33**), bromo (**34**), or methyl (**35**) substituent at the 6-position of (±)-nicotine affords analogues that display affinity roughly equal to that of nicotine for α4β2 nAChRs in rat brain. In contrast, the 6-methoxy compound (**36**) is 17-fold less potent than nicotine. Compounds **33** and **34** are 15-fold more potent than nicotine in the mouse tail flick analgesia assay, and they also produce hypoactivity and nicotine-like responding in drug discrimination. The *in vivo* effects are sensitive to mecamylamine blockade. In addition to SIB-1508Y (**31**), several 5-substituted nicotine compounds have recently been prepared. Racemic 5-ethyl- (**37**) and 5-bromonicotine (**38**) exhibit low nanomolar affinity for α4β2 nAChRs, but are less efficacious than either nicotine or SIB-1508Y in stimulating DA release from rat striatal slices.[86] 5-Isothiocyanonicotine (**39**) is reportedly a high-affinity irreversible ligand for brain nAChRs.[266]

Some conformationally restricted isoquinoline analogues exhibit interesting pharmacological properties.[267−269] The (+)-enantiomer (**40**) produces antinociceptive effects in the tail flick assay and a reduction in spontaneous activity with a potency comparable to nicotine, but fails to displace [^{3}H]nicotine binding at concentrations >10000 nM. The corresponding (−)-enantiomer is 12-fold less potent *in vivo*, but binds with modest affinity (K_i = 605 nM). The antinociceptive and locomotor effects of each enantiomer are not blocked by mecamylamine, DHβE, naloxone, or atropine. In addition, neither enantiomer produces nicotine-like responding in drug discrimination. The racemic *N*-desmethyl derivative of **40** binds with higher affinity (K_i = 167 nM).[268] Analogues with larger substituents such as *N*-propyl and *N*-allyl exhibit decreased binding affinity, but maintain potency in the tail-flick assay and measures of locomotor activity.[269]

SIB-1926 (**41**) possesses lower affinity than nicotine for native rat α4β2 nAChRs (IC_{50} of racemate *vs* [^{3}H]-nicotine in the micromolar range), but shows greater efficacy than nicotine in striatal DA release and activation of β4-containing nAChRs.[206] The corresponding racemate, SIB-1663 (**42**), produces ipsilateral turning in unilaterally 6-hydroxydopamine-lesioned rats, an animal model of Parkinson's disease. This compound also has antinociceptive properties in the rat tail flick assay.[270]

Azabicyclo[2.2.1]heptene and azabicyclo[2.2.2]octene analogues have been described in the patent literature.[271] The *endo*-*N*-methyl-substituted azabicyclo-[2.2.1]heptene analogue **43** binds to rat native α4β2 with affinity comparable to that of nicotine and possesses similar potency, but lower efficacy (*ca.* 50%), compared to nicotine in stimulating DA release from rat striatum. The maximal effect of **43** on synaptic ganglionic (PC12) and muscle (TE671) nAChR subtypes also is substantially reduced compared to nicotine. The corresponding azabicyclo[2.2.2]octene analogue **44** and the 5-bromopyridine derivative **45** are much weaker than **43** in all of the above assays. The effect of the 5-bromo substituent of **45** (700-fold lower α4β2 affinity) is in contrast to the effect of the analogous change to nicotine, which led to only a *ca.* 5-fold decrease (*cf.* compound **38**). Larger nitrogen substituents (Et, Bn) also are not well tolerated. The *exo* analogue **46** has activity roughly comparable to that of **43** in these assays. Unlike the case in the *endo* series, the *exo*-azabicyclo[2.2.1]octene derivative **47** retains moderate binding affinity to α4β2 nAChRs (K_i = 33 nM), but still has low efficacy in the functional assays. The desmethyl analogue of **47** (compound **48**) has α4β2 binding affinity and effects on the muscle nAChR comparable to that of nicotine, with reduced efficacy in DA release and on ganglionic nAChRs. The saturated analogue **49** binds with 17-fold lower affinity than nicotine and exhibits only 4% efficacy in the DA release assay. The isomeric quinuclidine ana-

logue **51** (racemic mixture of C-2 epimers) has rat α4β2 binding affinity similar to that of nicotine, but is an exceptionally potent and efficacious agonist at the putative human muscle-type receptor in TE671 cells (EC_{50} = 55 nM; 130% of nicotine response).[272]

A versatile new method for preparation of racemic nicotinoids in the nicotine, anabasine, and related series has been reported,[273] and a general NMR method for determining enantiomeric purity of nicotinoids has been described.[274]

Epibatidine SAR. *N*-Methylation of (+)- and (−)-epibatidine (**2**) results in slightly reduced affinities for rat α4β2 nAChRs (*ca.* 6-fold and 2-fold, respectively).[19] In functional assays (activation of rat ganglionic-like nAChRs in PC12 cells or human muscle-like nAChRs in TE671 cells), *N*-methylation has small but differential effects on the activities of the two enantiomers, such that modest enantioselectivities can be seen in the *N*-methyl products.[19] *N*-Methyl-(±)-epibatidine shows analgesic activity in the mouse tail flick test similar to that of (±)-epibatidine.[275] The deschloro analogue of epibatidine possesses affinity comparable to that of epibatidine for α4β2 nAChRs,[19,269] and similar functional potency in models of ganglionic (PC12) and muscle (TE671) nAChR function,[255] but exhibits markedly reduced analgesic potency.[276,277]

Several racemic 8-azabicyclo[3.2.1]octane homoepibatidine derivatives have been reported to have analgesic activity in the hot plate assay.[278,279] Analogue **52** exhibits efficacy equal to that of epibatidine at doses only 4-fold higher, and the antinociceptive responses were blocked by pretreatment with mecamylamine. *N*-Methyl compound **53** shows similar activity, whereas the *N*-isopropyl derivative **54** is 15-fold less potent than epibatidine. The tropinone derivative **55**, however, failed to produce any analgesic response at high doses. A bis-homoepibatidine analogue **56** has been prepared, but no pharmacological data has been reported for this compound.[280]

(±)-Epiboxidine (**57**) is a potent nAChR agonist in which methylisoxazole has been incorporated as a replacement for the chloropyridyl ring of (±)-epibatidine.[281] (±)-Epiboxidine displaces [^{3}H]nicotine from rat brain with *ca.* 10-fold lower affinity than (−)-epibatidine, is approximately 10-fold less potent than (−)-epibatidine in the mouse hot-plate assay, and is at least 10-fold less lethal in mice. Compound **57** is also a potent agonist at ganglionic-like nAChRs in PC12 cells. The *N*-methyl analogue of (±)-epiboxidine (**58**) shows similar potency and efficacy to **57** in the hot-plate assay (M. J. Dart, A. W. Bannon, *et al.*, unpublished data). An oxadiazole analogue (**59**) elicited antinociception in the tail flick assay but is at least 30-fold less potent than epibatidine.[277]

Isonicotine analogues can be considered as epibatidine derivatives lacking one ethano bridge. Norisonicotine (**60**) displays low nanomolar affinity for native mouse α4β2 receptors but lacks antinociceptive properties (up to 270 μmol/kg) and has been reported to antagonize the analgesic and hypothermic effects of nicotine.[269,282] In contrast, 6-chloronorisonicotine (**61**) is active in the hot plate assay, although high doses are required compared to epibatidine.[278]

3-Pyridyl Ethers. A-85380 (**20**) and A-84543 (**16**) are members of a recently disclosed class of 3-pyridyl ether nAChR ligands.[210] A-85380 possesses high affinity (50 pM) for α4β2 nAChRs comparable to that of epibatidine and is a full agonist relative to nicotine at recombinant human α4β2 receptors. This compound also is a potent stimulator of DA release, is a full agonist relative to nicotine at recombinant human α7 nAChRs,[218] and has potent activity at ganglionic-like nAChRs. A-84543 (K_i = 150 pM) is a full agonist at human α4β2, but 32-fold less potent and also less efficacious (77% of nicotine response) at human sympathetic ganglionic nAChRs.[210,283] Structure−activity studies in this series show that, unlike the *ca.* 20-fold difference in binding affinities between nicotine and (*S*)-nornicotine, **16** and its *N*-desmethyl analogue **62** (Figure 6) possess roughly equivalent affinities for native rat α4β2 nAChRs, i.e., the *N*-methyl substitution has a differential effect in the two series.[210] Compound **63**, the (*R*)-enantiomer of **62**, has similar affinity to **16** and **62**, whereas the (*R*)-enantiomer (**64**) of A-84543 is substantially weaker. At human α4β2 and ganglionic-like receptors, **62** and **63** are full agonists but, unlike **16**, show little selectivity between the subtypes, whereas **64** acts as an antagonist at both subtypes. The SAR is similar for the corresponding azetidine analogues, with a notable exception that, unlike the corresponding pyrrolidine (**64**), the (*R*)-*N*-methyl azetidine analogue is a full agonist at recombinant human α4β2 receptors. ABT-089 is the pyridine 2-methyl analogue of **62**. Relative to **16**, **62**, and **63**, the corresponding pyridine 2-methyl congeners have 100−300-fold lower affinity for native rat α4β2 nAChRs and much reduced efficacy at both human recombinant α4β2 and ganglionic-like receptors in IMR-32 cells.[215,249,284] It is noteworthy that the secondary amine function of ABT-089 serves to enhance the oral bioavailability relative to the corresponding *N*-methyl analogue, possibly by enhancing stability to liver metabolism.[249] In structure−activity studies on the methyleneoxy moiety of **16**, replacement of the oxygen atom with −S− (**65**), $-CH_2-$ (**66**), or $-CH_2O-$ (**67**) results in analogues with rat α4β2 affinities reduced by *ca.* 2000-, 150-, and 120-fold, respectively.[285] Replacement of the pyridine ring of **16** with phenyl results in a compound (**68**) with 280-fold reduced α4β2 affinity,[286] but the corresponding *m*-fluorophenyl analogue (**69**, K_i = 5 nM) is *ca.* 8-fold more potent than **68**. Both **68** and **69** are less efficacious than **16** (<40% of nicotine response) at human recombinant α4β2 and ganglionic-like receptors in IMR-32 cells (R. E. Elliott, D. L. Donnelly-Roberts, *et al.*, unpublished data). Comparison of structure−activity patterns with respect to aryl substitution in the above phenyl ether series with those observed following replacement of the pyridine ring of nicotine with substituted phenyl moieties[287] indicates that the two series have different structural requirements.

RJR-2403 SAR. Structure−activity studies around RJR-2403 (**15**) have been reported in the patent literature.[288−290] Compounds were tested for displacement of [^{3}H]nicotine from rat brain, stimulation of DA release from rat striatal synaptosomes, activation of human muscle-type nAChRs, and activation of rat synaptic ganglionic nAChRs, and compared to nicotine (K_i = 2 nM, EC_{50} (DA) = 115 nM) and **15** (K_i = 16 nM, EC_{50} (DA) = 1470 nM). The already low activity of **15** at rat synaptic ganglionic nAChRs and human muscle-like nAChRs was little influenced by the modifications

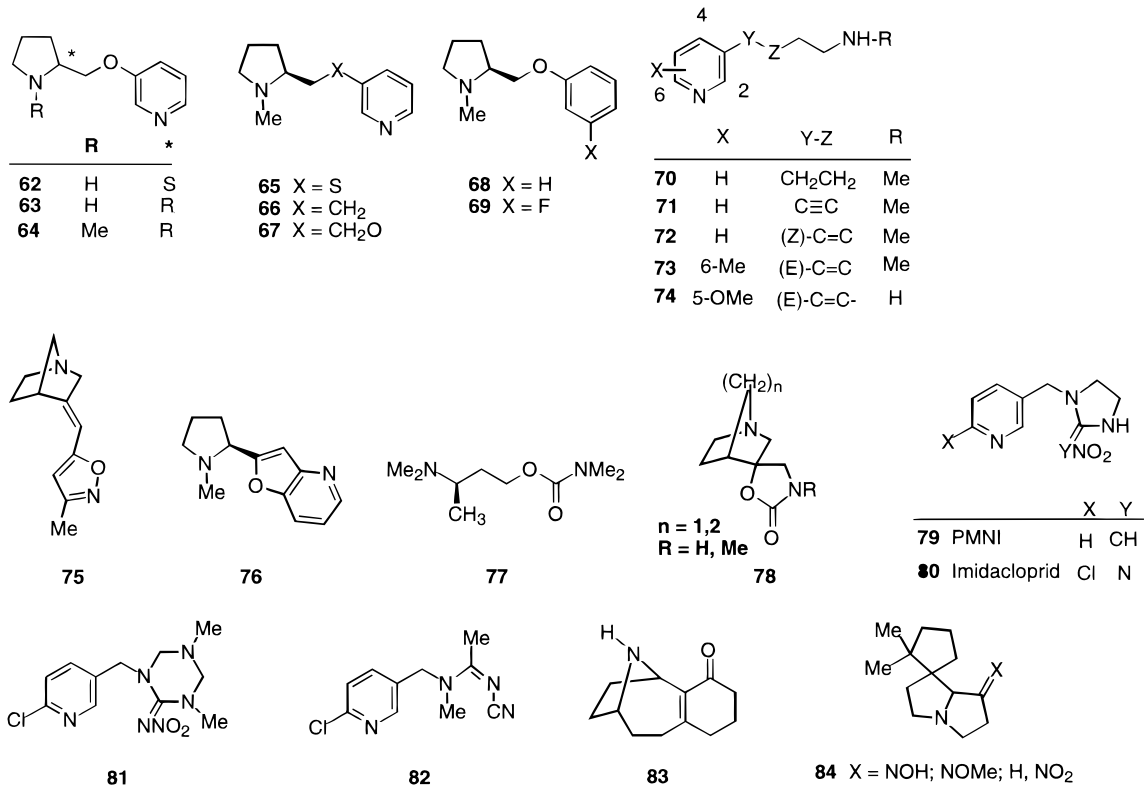

Figure 6. Structures of selected nAChR modulators.

examined, except for increased ganglionic efficacy for the saturated double bond (**70**) and acetylene (**71**) compounds. Compound **70** possesses significantly lower α4β2 binding affinity (K_i = 910 nM) than that of **15**, while acetylene **71** has a less substantial loss in α4β2 binding (K_i = 58 nM) together with a decrease in potency for DA release (EC_{50} = 8350 nM). Conversion of the olefin of **15** to the *cis* geometry (**72**) results in a modest loss in binding affinity to K_i = 77 nM but a dramatic loss in potency for DA release (EC_{50} = 11339 nM). A methyl group at the 6-position of the pyridine ring (**73**) causes reduced binding affinity (K_i =176 nM) but increased potency for DA release (EC_{50} = 219 nM). An analogue that both lacks the *N*-methyl group of **15** and also contains a pyridine 5-methoxy substituent (**74**) has binding affinity (K_i = 22 nM) similar to that of **15**, and comparable efficacy with only slightly reduced potency (EC_{50} = 4000 nM) in DA release. Replacement of the pyridine ring with pyrimidine moderately decreases both binding affinity (K_i = 86 nM) and potency in DA release (EC_{50} = 5800 nM).

MLA SAR. Recent structure−activity studies on MLA (**13**) and related analogues have demonstrated that the methylsuccinimidobenzoyl portion of the molecule is important for high-affinity interaction with brain α7 nAChRs. Thus, removal of the methyl group from the succinimide moiety results in a 20-fold decrease in binding affinity.[291] Removal of the methylsuccinimido moiety results in >1000-fold reduction in affinity,[292] and removal of the entire substituted benzoyl moiety results in a >2000-fold reduction in affinity[293] for this site. On the other hand, several simultaneous changes in the diterpenoid portion of the molecule results in an analogue with potency equivalent to that of MLA.[293]

Other Series. A series of compounds, exemplified by compound **75**, showing moderate selectivity for nicotinic receptors over muscarinic receptors has been claimed for treatment of CNS diseases caused by malfunctioning of the nicotinic cholinergic system.[294] Among a number of close analogues, **75** exhibited the best selectivity, measured by competitive binding against [^{3}H]MCC *vs* [^{3}H]oxotremorine (muscarinic agonist) in rat brain (IC_{50} (MCC) = 20 nM; Oxo/MCC = 55).

A novel series of furopyridine compounds typified by compound **76** has been disclosed with low nanomolar affinity for native rat α4β2 nAChRs as measured by [^{3}H]-Cyt binding.[295] Compound **76** is selective for activity at recombinant human α4β2 (79% of nicotine response) *vs* human ganglionic-like nAChRs in IMR-32 cells (7% of nicotine response). Structural elements discernible in the furopyridine moiety include an oxygen atom vinylogously adjacent to a nitrogen atom (*cf.* the adjacent nitrogen and oxygen atoms in the isoxazole moiety of ABT-418, **10**) or, alternatively, a conformationally restricted 3-pyridyl ether (*cf.* A-84543, **16**). Structure−activity studies with respect to stereochemistry and pyrrolidine *N*-substitution suggest a greater correspondence with **10** than with **16**.

A series of substituted (carbamyloxy)propylamine or (carbamyloxy)ethylamine derivatives has been claimed for use in the treatment of cognitive, neurological, and mental disorders in which nAChR dysfunction is involved.[296] Displacement of [^{3}H]nicotine in homogenized rat brain tissue was demonstrated for a number of derivatives, among which compound **77** was the most potent (IC_{50} = 3 nM).

A series of spiroazabicyclooxazolidinones (**78**) has been claimed for CNS disorders related to the nicotinic system.[297] This set of compounds is stated to demonstrate a preference for native α7 receptors over α4β2 nAChRs based on binding affinities to sites labeled by [^{125}I]-α-Bgt *vs* [^{3}H]nicotine.

1-(Pyridin-3-ylmethyl)-2-(nitromethylene)imidazolidine (PMNI, **79**) and imidacloprid (**80**) were developed based on insecticidal activity.[298] Related compounds **81** and **82** bind to native rat α4β2 receptors in the micromolar range and are claimed for use in CNS disorders.[299]

ACh Binding Site. A thorough understanding of the site on nAChRs at which ACh, nicotine, and other direct-acting nAChR modulators exert their actions could be beneficial to the design of novel ligands and, moreover, is the subject of considerable theoretical interest. Most of the investigations on the ACh binding site have been directed to muscle-type nAChRs from *Torpedo* or embryonic muscle tissue. Kinetic studies support the existence of two ACh binding sites per receptor molecule, whereby the simultaneous occupancy of the two sites increases the probability of channel opening, accelerates the rate of opening, and increases the channel open time.[300−302] Fluorescent resonance energy transfer (FRET) measurements indicate that the sites are approximately 30 Å above the cell membrane,[303] a finding supported by electron microscopic observations.[27,304,305] This position 30 Å above the cell membrane is well below the maximum extracellular protrusion of the receptor (55−65 Å), and binding of ACh is believed to induce a conformational change that is translated approximately 50 Å down to the gate of the receptor.[305]

Affinity labeling studies indicate that ACh interacts predominantly with the α-subunit. Thus, agonist protected, photoaffinity labeling by the competitive antagonist [^{3}H]-*p*-(dimethylamino)benzenediazonium tetrafluoroborate (DDF) has demonstrated that α-Cys-192, α-Cys-193, α-Tyr-93, α-Trp-149, and α-Tyr-190 are in the vicinity of the ACh binding site.[306−308] Correspondingly, an analogue of the coral toxin lophotoxin affinity labeled α-Tyr-190,[309] and [^{3}H]acetylcholine mustard labeled α-Tyr-93.[310] Sulfhydryl-directed affinity labeling of reduced receptor with [^{3}H]-4-(*N*-maleimido)benzyl trimethlammonium iodide (MBTA) also identified α-Cys-192 and possibly α-Cys-193 as being close to the binding site.[311] Furthermore, [^{3}H]nicotine irradiated at 254 nm for 10 min labeled an additional aromatic residue, α-Tyr-198.[312] These labeled amino acids are contained within the amino terminus of the α-subunit and are conserved at homologous positions in all known vertebrate neuronal α subunits except α5.

The preponderance of labeled aromatic amino acids near the active site has led to the postulate that the AChR binding site may be very similar to the binding site of acetylcholinesterase (AChE) or the combining site of the phosphorylcholine-specific antibody Fab McPC603. X-ray crystallographic studies indicate that the AChE binding site is a deep narrow gorge lined by aromatic residues. Thus, the aromatic side chains may provide a hydrophobic environment for the methyl groups of the ammonium moiety and may stablize the positive charge through cation−π interactions.[313−315] Similarly, at the combining site of Fab McPC603 with phosphorylcholine, the trimethylammonium group is surrounded by one tryptophan and two tyrosine residues to form an aromatic solvation shell with anionic residues appearing to act as a second solvation layer.[316,317] In addition, a synthetic receptor that binds ACh with 50 μM affinity is comprised primarily of aromatic rings.[318] On the other hand, some studies support the participation of an anionic residue which could either interact directly with the tetramethylammonium moiety through Coulombic interactions or, alternatively, enhance the negatively charged character of one or more nearby aromatic residues.[319,320]

Two- and three-dimensional models representing possible arrangements of the amino acid residues in the binding site have been proposed.[321−323] However, these remain highly speculative. It is worth noting that some studies suggest that the two binding sites are not identical.[302,324,325] This could arise either because the binding sites are at the interface of an α-subunit and a neighboring subunit[319,321] or because the binding sites on the α subunits are differentially influenced by unlike neighboring subunits.[27]

Molecular Modeling. Relevant background and several pertinent issues with respect to molecular modeling of the nicotinic system are presented in a recent review.[207] To summarize, it is likely, given the number of nAChR subtypes and the differential effects of different ligands on various subtypes, that there are more than one, and perhaps several, different nicotinic pharmacophores. Future efforts need to be precise in specifying which system is being modeled. It is further clear that, for a given receptor, definition both of relevant distances between putative pharmacophoric elements as well as a better understanding of the receptor tolerance for steric bulk are needed. With regard to distances, the recent identification of ligands like epibatidine and A-85380 having distances between putative pharmacophoric elements that are longer than those of previously modeled ligands has prompted reexaminations of the requirements for the distance parameters.[210,326]

Some further issues merit additional consideration. For one, in studies attempting to superimpose different ligands, the choice of pharmacophore elements to superimpose is an important consideration. Most studies to date have selected (1) a basic or quaternized nitrogen atom, (2) a less basic nitrogen (e.g. the pyridine nitrogen of nicotine) or a carbonyl oxygen (e.g. the carbonyl oxygen of ACh), and (3) a "dummy" point which imposes directionality requirements on element 2, namely, the carbonyl carbon or the pyridine centroid, or alternatively, lone pair electrons of the carbonyl oxygen or pyridine nitrogen.[327−330] We have recently argued that an additional point (4) which places a directionality requirement on element 1 also should be considered.[210] While it is not certain that a single locus of interaction for such a point 4 exists, it also does not appear warranted to exclude this possibility. We have studied a 4-point model, whereby the protein sites with which ligand atoms 1 and 2 would interact[331] provide pharmacophoric elements 3 and 4 (Figure 7). Although a 4-point superposition model is more restrictive than a 3-point model, it was found that reasonable overlaps of nicotine (**1**), (−)-epibatidine (**2**), and A-85380 (**20**),[210] as well as a diverse group of other α4β2 agonists[285] could be derived.

A further issue regards consideration of the differential interactions of ligands with different states of the receptor. Since it has been determined that binding affinities generally reflect interaction with a desensitized state of the receptor (see discussion above), phar-

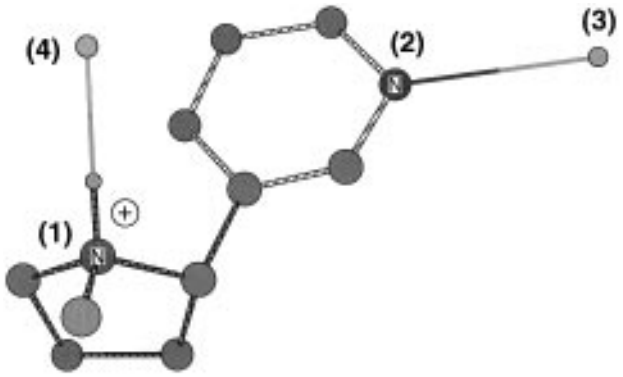

Figure 7. An example of pharmacophoric element selection for molecular modeling of nicotine. In this example, pharmacophoric elements (1) and (2) are the nitrogen atoms, and elements (3) and (4) are points on the nAChR protein with which elements (1) and (2) would optimally interact. Carbon is green, nitrogen is dark blue, the proton on the basic nitrogen is light blue, and putative protein binding sites are red.

macophore models based on this information may not accurately reflect interactions relevant to potency and efficacy in stimulating receptor function. Thus the decision to include, for example, Cyt (**7**) in the modeling of the $\alpha4\beta2$ pharmacophore may depend on whether one wishes to model interactions with the open channel state (with which Cyt interacts apparently only weakly, since it does not potently activate this subtype) or the desensitized state, with which Cyt presumably interacts strongly based on its high affinity in binding assays.

To aid in the development of nicotinic pharmacophores, there continues to be a need for highly active conformationally constrained ligands. Atx (**19**) is an attractive ligand to use for modeling studies because of its potent binding affinity, its potent agonist actions at a number of receptor subtypes, and its stereospecificity.[329] However, this molecule has a rotatable bond between the two putative pharmacophoric elements, and it remains uncertain which orientation of the acetyl side chain is the bioactive one.[332−334] The conformationally constrained analogue **83** of the *s-trans* conformation was synthesized by Hernandez and Rapoport to explore this issue.[332] Recent binding studies on this compound, reported here for the first time,[335] indicate that the K_i value for displacement of [^{3}H]Cyt from rat brain membranes is 4.6 ± 1.1 nM (n = 3), which is *ca.* 10-fold weaker than Atx under analogous conditions. Thus, **83** has been confirmed as a conformationally constrained analogue of Atx which possesses high affinity for native rat $\alpha4\beta2$ receptors and therefore can serve as a useful tool in efforts to develop pharmacophore models at this nAChR subtype.

Noncompetitive (Allosteric) Modulation. In analogy to the benzodiazepine sites on GABA-gated chloride channels[336] and the glycine and polyamine sites on the NMDA receptors,[337] muscle and neuronal nAChRs also contain loci distinct from the ACh site through which ligands can either negatively or positively modulate the function of the ion channels. Two prominent mechanisms of action for noncompetitive inhibitors are blockade of the channel (mecamylamine (**5**), anesthetics such as lidocaine, phencyclidine) or stabilization of a desensitized state of the receptor (steroids, receptor phosphorylation).[321,338] Members of a spiropyrrolizidine series (**84**) have been reported to be noncompetitive blockers of both ganglionic nicotinic receptors in rat PC12 cells and muscle-type nicotinic receptors in TE671 cells.[339] Activation of nAChRs through allosteric mechanisms (including enhancement of the action of direct-acting agonists) has been discovered for compounds such as the acetylcholinesterase inhibitors physostigmine and galanthamine, the opiate codeine, as well as the endogenous neurotransmitter 5-HT.[93,340,341] Many of these latter compounds are also channel blockers at higher concentrations,[341] and this latter effect may predominate in some circumstances. Thus, in DA release experiments, only blocking effects could be observed for physostigmine.[342] Elaboration on the potential usefulness of noncompetitive activators for their nAChR modulating properties probably will need to await the identification of compounds lacking the polypharmic actions of the currently known agents.

Future Directions

The availablility of the nicotine transdermal patch has offered new opportunities to directly evaluate in a clinical setting the effects of nAChR modulation on human diseases. Such studies will continue to provide valuable proof-of-principle information as a basis for continuing efforts to improve upon the safety and pharmacokinetic properties of nicotine. Recent nAChR-targeted drug discovery programs have focused most prominently on (1) cognitive enhancing agents, leading to identification of ABT-418, GTS-21, RJR-2403, and ABT-089; (2) compounds stimulating nAChR-mediated DA release for Parkinson's disease, leading to SIB-1508Y; and (3) analgesic agents based on findings with epibatidine. These therapeutic targets have in common the existence of established behavioral models which have doubtless played an early central role in the characterization of compounds as potential therapeutic agents. As should be clear from the foregoing discussions, this is in large part because the state of understanding with regard to what nAChR subtypes mediate functions of interest is not sufficiently well defined to confidently provide predictive pharmacological screens at the receptor level. Evaluation of most potential CNS-mediated side effects (for example, locomotor and temperature effects, seizure activity, lethality, and rewarding behavior) also rely heavily on behavioral models. Behavioral evaluation of analogues at an early stage in the compound screening program has the advantage of identifying agents at a more advanced level of preclinical characterization and also provides preliminary feedback on pharmacokinetic properties. However, behavioral assays are compound intensive, suffer from modest throughput, and present difficulties with interpreting SAR because there may be several simultaneously variable parameters (intrinsic activity, pharmacokinetics, metabolism, and in some cases, behavioral effects of animal handling, changes in testing environment, etc.). An alternative strategy is to rely more heavily on neurotransmitter release assays, whereby possible therpeutic utility can be hypothesized based on the state of understanding of the function(s) of the respective neurotransmitters (e.g. the known associations of 5-HT with depression and appetite, DA with Parkinsonism and psychosis, NE with analgesia and attention, ACh with cognition, etc.) However, neurotransmitter release assays also are comparatively labor intensive.

Use of the modern tools of drug discovery, particularly high throughput screening of diverse compound libraries in recombinant receptor systems, promises to lead to novel selective agents unlikely to be discovered by traditional methods.[206] Since there is a dissociation of binding affinities and functional activity in many nAChR systems, screening in high throughput functional assays will be of particular interest. The large number of possible nAChR subunit combinations and the uncertain status of given combinations with respect to function *in situ* raises issues with regard to strategy for implementation of this approach. One option is to screen against many subunit combinations, some of which may not exist *in vivo* or may not be related to functions of interest. On the basis of results in several neuronal tissues, it appears that combinations of at least up to four different subunits should be considered.[43,143,162] A key question under this scenario is the degree of avidity with which hits will be pursued with respect to secondary biological evaluation or further SAR, since it can be presumed that this avidity will in part be a function of the level of confidence that identified compounds interact with endogenous nAChRs of interest.

Ultimately, it would be desirable to correlate responses at recombinant receptors with those from endogenous systems in order to permit more focused screening. Preceding sections have suggested likely subunit combinations and possible therapeutic or toxicological endpoints for several known endogenous nAChRs. For example, since relevant physiological functions at peripheral nAChRs are comparatively well understood, *in vitro* functional screens at the receptor level are useful for evaluation of potential effects on the cardiovascular, gastrointestinal, or skeletal muscle systems mediated by peripheral nAChRs. However, subtypes responsible for many other endpoints of potential interest, including release of certain neurotransmitters, anxiolysis, appetite suppression, analgesia, addiction, anti-depressant action, attention, etc., are highly uncertain or completely unknown.

From the foregoing discussions, several important issues can be identified. A key consideration regards the sometimes marked species differences observed for functional responses at a given subunit combination. Such differences are perhaps not surprising when comparing chick and mammalian nAChRs,[29,150,156] but differences also are apparent between responses at analogous rodent and human subunit combinations.[134,213,242,244] Whereas ultimate utility for human therapeutics argues for the use of human recombinant receptors, efforts to correlate responses at these receptors with behavioral or other functional responses in nonhuman systems are complicated by the possibility of species variablility. Consequently, a comprehensive approach might reasonably encompass both human and appropriate nonhuman recombinant receptors. Whereas the ability to correlate specific nAChRs with the myriad of behavioral responses attributed to nAChRs would be highly informative, it can be presumed that correlations with responses in whole animals will be more difficult to extract than those measured *in vitro*. As a further consideration, it is reasonable to suspect that more than one subtype of nAChR may be involved in a given physiological response. For example, evidence has been presented to suggest that nicotine-evoked DA release may be mediated by more than one class of nAChRs.[137,343]

The characterization of heterologous systems expressing more than two subunits will require determination of which and how many of the possible combinations have actually assembled into functioning receptors, since recent results demonstrate that coexpression of more than two subunits yields a heterogeneous population of nAChRs.[42,143,344] Pharmacological analysis in such systems will be aided by the availability of selective antagonists of pairwise and triplex combinations. While most of the presently known antagonists show little selectivity among various combinations of neuronal subunits $\alpha2-\alpha4$ and $\beta2-\beta4$, certain snail-derived conotoxins offer some promise in this regard.[345] Elegant methods have been developed which will help to identify which combinations have been expressed. For example, a reporter epitope technique whereby subunit termini are tagged with amino acid sequences recognized by specific antibodies has been demonstrated.[42]

The possibility exists that differences in pharmacological responses of nAChRs in native cells *vs* those in heterologous expression systems could arise, not because of differences in subunit composition, but from differences in membrane composition or posttranslational processing.[44,65,93] Heterologous expression of nAChR subunits from a given species in cells from the same species is most likely to minimize such issues.

Development of correlates for nAChRs in the CNS clearly will be aided by the determination of subunit composition for minor subtypes of nAChRs using biophysical and immunological tools cited in preceding sections. The possible contributions of presently unknown subunits and the existence of more than one nAChR subtype in the same tissue continues to present challenges to the definitive assignment of native nAChR subunit composition.

nAChRs as Models for Other LGICs. nAChRs are the prototypical LGICs. They belong to a superfamily of LGICs that includes channels gated by glycine, GABA ($GABA_A$), and 5-HT ($5\text{-}HT_3$).[346] It has been determined that, like nAChRs, the $GABA_A$ and $5\text{-}HT_3$ receptors are pentamers of subunits possessing four transmembrane domains. Other superfamilies have been classified separately based on the number of transmembrane domains in the component subunits. Channels gated by glutamate (subunits have three transmembrane domains) and ATP (P2X subunits have two transmembrane domains) are members of different superfamilies. Most members of the nAChR-related, glutamate, and P2X superfamilies are believed to be heterooligomeric.[337,347,348] Splice variants of individual subunits have been found.[337] Furthermore, like nAChRs, other major types of LGICs, e.g. $GABA_A$ and NMDA receptors, possess multiple allosteric modulatory sites.[336,349] Thus, many of the challenges that have been addressed and still face the study of nAChRs apply also to other LGICs. A recent review[337] on NMDA receptors was organized around several key issues, which, together with questions about the precise function of receptor subtypes, would appear to concisely summarize the challenges for most LGICs: What is the subunit stoichiometry? What is the molecular basis for the difference between recombinant and native receptors? Are all subunits known?

Do individual cells express more than one type of receptor gated by the same transmitter?

It can be expected that the methods developed and lessons learned during study of nAChRs will be applicable to the study of other LGICs. A broader understanding of this class of receptors promises to open new possibilities for discovery of therapeutic agents.

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