A Methyl Scan of the Pyrrolidinium Ring of Nicotine Reveals Significant Differences in Its Interactions with $\alpha 7$ and $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptors^[S]

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ABSTRACT

The two major nicotinic acetylcholine receptors (nAChRs) in the brain are the $\alpha 4\beta 2$ and $\alpha 7$ subtypes. A “methyl scan” of the pyrrolidinium ring was used to detect differences in nicotine’s interactions with these two receptors. Each methylnicotine was investigated using voltage-clamp and radioligand binding techniques. Methylation at each ring carbon elicited unique changes in nicotine’s receptor interactions. Replacing the 1’-N-methyl with an ethyl group or adding a second 1’-N-methyl group significantly reduced interaction with $\alpha 4\beta 2$ but not $\alpha 7$ receptors. The 2’-methylation uniquely enhanced binding and agonist potency at $\alpha 7$ receptors. Although 3’- and 5’-*trans*-methylations were much better tolerated by $\alpha 7$ receptors than $\alpha 4\beta 2$ receptors, 4’-methylation decreased potency and efficacy at $\alpha 7$ receptors much more than at $\alpha 4\beta 2$ receptors. Whereas *cis*-5’-methylnicotine lacked agonist activity and displayed a low affinity at both receptors, *trans*-5’-methylnicotine retained considerable $\alpha 7$ receptor activity. Differences between the two 5’-methylated analogs of the potent pyridyl oxymethylene-bridged nicotine analog A84543 were consistent with what was found for the

5’-methylnicotines. Computer docking of the methylnicotines to the *Lymnaea* acetylcholine binding protein crystal structure containing two persistent waters predicted most of the changes in receptor affinity that were observed with methylation, particularly the lower affinities of the *cis*-methylnicotines. The much smaller effects of 1’-, 3’-, and 5’-methylations and the greater effects of 2’- and 4’-methylations on nicotine $\alpha 7$ nAChR interaction might be exploited for the design of new drugs based on the nicotine scaffold.

SIGNIFICANCE STATEMENT

Using a comprehensive “methyl scan” approach, we show that the orthosteric binding sites for acetylcholine and nicotine in the two major brain nicotinic acetylcholine receptors interact differently with the pyrrolidinium ring of nicotine, and we suggest reasons for the higher affinity of nicotine for the heteromeric receptor. Potential sites for nicotine structure modification were identified that may be useful in the design of new drugs targeting these receptors.

Introduction

Nicotine activates a variety of ligand-gated acetylcholine receptors (nAChRs) that play important signaling roles in neuronal and some non-neuronal cells, and it has a number of potentially therapeutic effects. However, it is one of the most

addictive drugs when self-administered as a tobacco product. Nicotine is also widely used as a useful smoking-cessation drug in nonsmoked formulations (Prochaska and Benowitz, 2016). Several nicotine analogs have already been tested as potential treatments for neurodegenerative and psychiatric disorders.

Vertebrate nAChRs are cation permeant ion channels composed of five homologous subunits. Molecular biologic investigations have revealed genes for 17 different subunits, so a large number of subunit combinations are theoretically possible, and >15 pentameric subunit combinations have already been identified in mammalian tissues (Wu and Lukas, 2011). At least two α subunits occur in each nAChR pentamer. Agonist binding sites are located within a groove

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ABBREVIATIONS: ACh, acetylcholine; AChBP, acetylcholine binding protein; AChR, acetylcholine receptor; α -BTX, α -bungarotoxin; nAChR, nicotinic acetylcholine receptor.

Fig. 1. Structure and International Union of Pure and Applied Chemistry nomenclature for (S)-nicotine and A84543 monocations. The striated bond between the two rings indicates that the pyridyl substituent is situated below the pyrrolidinium ring in the S-configuration.

between the N-terminal extracellular domains of an α subunit and an adjacent subunit. The $\alpha 7$ and $\alpha 4\beta 2$ nAChRs are the most numerous and widely distributed receptor subtypes in the brain and have been demonstrated to participate in cognitive function. The $\alpha 7$ nAChRs are mainly homomeric complexes containing five $\alpha 7$ monomers, although small concentrations of $\alpha 7\beta 2$ nAChRs also occur in the brain (Moretti *et al.*, 2014; Nielsen *et al.*, 2018). All the other nAChRs contain two or three non- α subunits. Significant decreases in the concentrations of these two receptors have been found in the postmortem brains of patients with Alzheimer disease and Parkinson disease (Burghaus *et al.*, 2003). In many patients with schizophrenia, the $\alpha 7$ receptor is expressed at lower than normal levels, and this is thought to contribute to deficient sensory gating and cognition (Martin *et al.*, 2004). There is considerable pharmaceutical interest in developing selective nicotinic agonists and allosteric modulators that target either $\alpha 7$ or $\alpha 4\beta 2$ nAChRs for treating cognitive deficits in neurodegenerative diseases. Partial agonists for $\alpha 4\beta 2$ receptors like varenicline and cytisine are widely used as smoking-cessation drugs. In addition, $\alpha 7$ nAChRs occurring in macrophages (and their brain counterparts, microglia), lymphocytes, keratinocytes, lung epithelium, and certain cancer cells modulate a variety of signaling pathways and have become attractive therapeutic targets (Bertrand *et al.*, 2015; Bouzat *et al.*, 2018).

Despite the widespread use of nicotine as an experimental nAChR probe and lead compound for the design of new drugs, the molecular basis for its preferential interactions with certain nAChRs is still poorly understood. Initial chemical modification and mutagenesis studies led to the identification of key aromatic amino acid side chains and peptide bonds constituting ACh binding sites (Changeux, 2012). A combination of cation- π , electrostatic, hydrogen, and van der Waals bonding forces are involved in ligand (agonist and competitive antagonist) recognition (Cashin *et al.*, 2005; Xiu *et al.*, 2009; Puskar *et al.*, 2011). High-resolution crystal structures of several homologous acetylcholine binding proteins (AChBPs) and their analogs mutated to better resemble the $\alpha 7$ orthosteric binding site have been available for some time (Celie *et al.*, 2004; Li *et al.*, 2011). Crystal structures of the extracellular portions of the adult skeletal muscle $\alpha 1$ subunit and the $\alpha 10$ subunit binding α -bungarotoxin (α -BTX) have been reported (Dellisanti *et al.*, 2007; Zouridakis *et al.*, 2014). Recently, a crystal structure of the nearly whole $\alpha 4\beta 2$ nAChR became available (Morales-Perez *et al.*, 2016). These structures have been very useful for developing homology models of various nAChRs and have enabled ligand docking studies.

In view of the multiplicity of nAChRs and the need to minimize the adverse effects of nicotinic drugs on unintended nAChRs, it is desirable to identify structural differences

between their orthosteric binding sites to enable the design of subtype-selective drugs. In the present study we employ a “methyl scan” (Black *et al.*, 1972) approach to assess the consequences of single methyl substitutions for each of the eight hydrogens located on the pyrrolidinium ring of nicotine. Methyl substitution was selected because of its relatively minimal effect (a 16% increase) in the mass of the pyrrolidinium moiety. The methyl group, like the four methylene groups in this ring, does not appreciably affect the lipophilic nature of the ring, introduces no new hydrogen-bonding atoms, and does not diminish ionization of the pyrrolidinium nitrogen, which has been shown to be essential for efficient nAChR binding by this compound (Barlow and Hamilton, 1962; Jeng and Cohen, 1980).

The actions of a few alkylated nictines on isolated tissues and organs have been reported (Glassco *et al.*, 1994; Dukat *et al.*, 1996). Two laboratories reported extensive rat brain nAChR (later shown to be largely $\alpha 4\beta 2$) binding data on the effect of individual methyl substitutions at four of the eight hydrogens on the pyrrolidinium ring (Lin *et al.*, 1994; Kim *et al.*, 1996; Wang *et al.*, 1998). In this paper, we report a comprehensive functional and radioligand binding analysis of all the possible chiral nicotine analogs generated by methyl replacement of each H atom attached to the pyrrolidinium ring of (S)-nicotine for $\alpha 7$ as well as $\alpha 4\beta 2$ nAChRs. In addition, we evaluate the effect of increasing the size of the pre-existing 1'-N-methyl group. To test the generality of our results with nicotine, we extended part of our methyl scan to a highly potent nicotine analog, A84543, whose two nicotine-like rings are connected through an oxymethylene bridge that preserves the chirality of the 2'-pyrrolidinium ring (Abreo *et al.*, 1996). Our results indicate that the pyrrolidinium moiety in these compounds has a similar binding mode in both nAChR binding sites but that the $\alpha 7$ site is less sensitive to most methyl substitutions.

Materials and Methods

Nicotine Analogs. Preparation of all known compounds followed methods already described in the literature [see Fig. 1 in Rouchaud and Kem (2012) for a summary of most methods we employed]. The compound ^1H and ^{13}C NMR spectral and mass spectrometric data of compounds whose syntheses were previously reported agreed with the published data. The chiral high pressure liquid chromatography conditions (Tang *et al.*, 1998) and identifying (Testa and Jenner, 1973) the individual enantiomers from the racemic 2'-, 3'-, and 5'-methylnicotines are provided in the Supplemental Data. Nicotine dihydrogen tartrate salt (MW 462) was obtained from Sigma-Aldrich (St. Louis, MO). 1'-Methylnicotinium iodide (MW 304) was purchased from Toronto Research Chemicals (Toronto, Canada), and its identity was checked by NMR spectroscopy. In this paper, the relative configurations (*cis*- or *trans*-) of the pyrrolidinium ring methyl substituents are always expressed with respect to the configuration

of the pyridyl substituent. All alkylnicotines in Tables 1–5 have the same 2'-(S)-pyrrolidinium ring configuration as in natural (S)-nicotine. Data for 3'-methyl and 5'-methyl-(R)-nicotines are presented in Table 6. Abbreviations: Me=methyl, Et=ethyl, N=nicotine, NorN=nornicotine.

Functional Experiments with *Xenopus* Oocytes. Frogs were purchased, maintained, and used under a University of Florida Institutional Animal Care and Use Committee approval for the Kem laboratory. Mature female frogs (*Xenopus laevis*) were anesthetized by immersion in a 1.5 g/l solution of ethyl-3-aminobenzoate methanesulfonate (MP Biomedicals, Solon, OH) for 30 minutes. After the frog was completely unresponsive and immobile, it was decapitated with a guillotine, and its spinal cord was pithed before excision of the ovary, which was placed into calcium-free Barth saline [88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO₃, 0.82 mM MgSO₄, 15 mM HEPES, 0.012 mg/ml tetracycline hydrochloride (Sigma-Aldrich), pH 7.3 ± 0.1], opened with forceps, and washed three times with saline. Then, 50 ml of 1.25 mg/ml type 1 collagenase (Worthington Biochemical Corporation, Freehold, NJ) solution dissolved in Ca²⁺-free Barth saline was added, and the oocyte-containing mass was gently shaken for 2 hours at room temperature. After washing three times with calcium-free saline and then three times with Barth saline containing 0.7 mM Ca²⁺, healthy stage 5 oocytes were transferred into dishes and incubated at 17°C overnight. Oocytes were routinely injected with 50 nl of a solution containing 20 ng α7 mRNA (Peng et al., 1999) or 50 nl of a mixture of α4 and β2 mRNA (10 ng each) using a Drummond Nanoject II Auto-Injector. In some pilot experiments, oocytes were injected with 4:1 or 1:4 ratios (20 ng mRNA, total) of the two mRNAs to determine whether nicotine action under our experimental conditions was sensitive to subunit stoichiometry (Nelson et al., 2003). Finally, in one final set of experiments intended to investigate only the high agonist sensitivity α4β2₃ nAChR, an equal weight (5 ng) of RNA for the human β2AGSα4 concatemer was injected with 5 ng β2 RNA in each oocyte, since this ratio has been shown to minimize expression of alternative stoichiometries of this receptor (Kuryatov et al., 2005). Injected oocytes were cultured in Barth's saline for 5–10 days at 17°C with daily changes in the saline prior to recordings.

Individual oocytes were placed into a 20-μl oocyte perfusion chamber (model OPC-1 connected to a ValveLink8.2 system; Automated Scientific, Berkeley, CA) and perfused at a rate of 2.0 ml/min at room temperature with frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM CaCl₂, pH 7.3) containing 1 μM atropine sulfate (Sigma-Aldrich) to block potential muscarinic responses. The two-microelectrode voltage-clamp technique was used to measure current responses at a constant holding potential (−60 mV for α7 and −50 mV for α4β2 nAChRs). The voltage-measuring microelectrode (filled with 3 M KCl solution) resistance was 0.5–3.0 MΩ, and the current-passing electrode (containing 250 mM CsCl and 100 μM EGTA) resistance was 0.5–2.0 MΩ. Membrane currents were recorded with an AxoClamp-2 (Axon Instruments, Union City, CA). Sampling rates were between 5 and 10 Hz. Compounds were transiently applied (2 seconds for α7 and 5 seconds for α4β2 receptors) to avoid a cumulative desensitization. A rapid flow rate (2.0 ml/s) permitted complete replacement of the fluid bathing the oocyte at approximately 3× per second. Initially, each oocyte received two control applications of a near-maximal stimulatory concentration of ACh (1 mM ACh for α7 and 100 μM ACh for α4β2 receptors) to obtain a consistent response. ACh control applications alternated with test compound applications every 5 minutes. The peak current response for a given compound concentration was then normalized with respect to the mean current response, obtained by averaging the responses for the ACh applications before and after that test concentration. Clampfit 8.1 (Axon Instruments) was used for data acquisition, and Prism 3.0 (GraphPad, San Diego, CA) was used for analysis. The concentration-response curve for each compound was calculated by fitting the data using the following modified Hill equation:

$$\text{Response } I = I_{\max}(\text{Agonist}) / [(\text{Agonist})^n + (\text{EC}_{50})^n],$$

where each response current *I* is normalized with respect to the abovementioned mean ACh control response; *I*_{max} denotes the

maximal response current for the agonist, again relative to the ACh control response; and *n* is the Hill slope. It has been demonstrated that α7 nAChR total current (net charge over time) concentration-response curves are shifted to lower concentrations relative to transient peak response-concentration curves (Papke and Thinschmidt, 1998). Thus, we measured net charge as well as peak current responses for each oocyte response. Total current over a 20-second interval (including the 2-second drug application period) was measured, and the net charge response was obtained after subtraction of baseline current for the same 20-second period. Compound comparisons using the net charge method led to the same conclusions as for the peak current method (Table 1). The number of oocytes (*n*) tested at each concentration was ≥4 and is given in Fig. 2. Efficacy (*I*_{max}) estimates by peak current and net charge methods and potency (EC₅₀) estimates are presented in Tables 1 and 3 as arithmetic means ± one sample S.D. One-way ANOVA of log₁₀-transformed compound *I*_{max} and EC₅₀ data was used to compare all analogs with nicotine using Dunnett's multiple comparison post-test. Student's two-tailed *t* test was used to assess statistical significance between mean *I*_{max} and EC₅₀ estimates for the two enantiomers of a compound.

Radioligand Binding Experiments. Rat brain membrane radioligand binding experiments were carried out essentially as previously described (Kem et al., 2004). Frozen adult male Sprague-Dawley rat brains (Pel-Freeze Biologicals, Rogers, AZ), after thawing on ice and being sliced into smaller pieces, were homogenized with a 30-ml Wheaton glass homogenizing tube and pestle in ice-cold binding saline (120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 50 mM Tris-HCl buffer, pH 7.4). After the homogenate was centrifuged at 14,600g for 10 minutes, the resulting pellet was resuspended in fresh binding saline, homogenized, and centrifuged again, and the supernatant was discarded. A protein assay (BCA; Pierce, Rockford, IL) was then performed to obtain the protein concentration of the washed and pellet-resuspended membranes, which were stored at −82°C before use. Each tube in the binding experiment received 100 μg of rat brain membrane protein.

Washed membranes from cultured tsA201 cells expressing human α4β2 nAChRs were also used to assess the nAChR affinities of nicotine and most nicotine analogs. Although these cells are known to express both stoichiometries of this heteromeric receptor, nicotine binding curves in our study were well fitted, assuming a single population displaying the characteristics of the highest affinity (α4β2₃) form (Kuryatov et al., 2005). Cells at 80%–90% confluence were collected with a disposable cell scraper after removing the culturing media from the flask (75 cm²) and adding 6–10 ml of ice-cold Tris binding saline. The dislodged cells were collected in centrifuge tubes and spun down at 7700g for 8 minutes. The loose pellet was collected and homogenized as described above for rat brain membranes; after a protein assay, the washed membranes were stored at −85°C before use. For tsA201 cell membranes, 50 μg of protein per tube was used for radioligand binding experiments. SH-EP1 cells expressing human α7 nAChRs were similarly cultured, and their membranes (100 μg membrane protein per tube) were used in [¹²⁵I]α-BTX displacement assays (Zhao et al., 2003).

Radioligands selective for each nAChR subtype were obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA). Using conditions reported to optimally measure α4β2 nAChR affinity, 1.0 nM [³H]cytisine (34 Ci/mmol) displacement experiments were performed at 4°C as previously reported by Pabreza et al. (1991). Measurements of α7 nAChR affinity were done by displacement of [¹²⁵I]α-BTX (136 Ci/mmol) binding; the final test [¹²⁵I]α-BTX concentration was 0.32 nM. These experiments required incubation for 3 hours at 37°C to assure equilibration. Membranes were suspended in binding saline containing 2 mg/ml bovine serum albumin (Sigma-Aldrich) to reduce nonspecific binding. In each experiment, 48 disposable glass culture tubes that were each (total volume 0.50 ml) containing equal concentrations of cell membranes and radioligand but different concentrations of the compound of interest were always incubated together. For each radioligand, nonspecific binding was

measured in the presence of a final concentration of 1 mM nicotine hydrogen tartrate (Sigma-Aldrich). After incubation, radioligand bound to membranes in each tube was rapidly collected by vacuum filtration using a 48-position Brandel cell harvester (Gaithersburg, MD) and Whatman GF/C glass fiber filters that were presoaked in 0.5% polyethylenimine (Sigma-Aldrich) for 45 minutes to reduce nonspecific binding. The radiolabeled membranes were rapidly washed three times with 3 ml of ice-cold binding saline to remove the unbound radioligand. Filters containing [³H]cytisine bound membranes were collected in 20-ml scintillation tubes, suspended in 8 ml of 30% Scintisafe (Fisher Scientific, Atlanta, GA) scintillation fluid, and then counted in a Beckman LS-6500 liquid scintillation counter after standing overnight. Filters containing [¹²⁵I]α-BTX bound membranes were placed in 4-ml gamma vials and counted in a Beckman Instruments (Fullerton, CA) 5500B gamma counter.

Displacement assay binding data were analyzed using Prism software. The mean counts per minute for each compound concentration were obtained from four replicates; the data were fitted to a sigmoidal concentration-response curve for one-site binding, from which the Hill slope (*n*) and IC₅₀ were estimated as follows:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(X - \text{Log IC}_{50})n}).$$

Top (of the curve) is the maximal specific binding plateau of radioligand, Bottom (of the curve) is the minimum specific binding plateau observed at high concentrations of the displacing ligand, and *X* = Log (Compound). The *K_d* estimate for each radioligand and receptor, previously calculated from saturation binding experiments carried out using the same incubation conditions, was then used to calculate the equilibrium dissociation constant (*K_i*) value of the displacing ligand from the Cheng-Prusoff equation: *K_i* = IC₅₀ / [1 + (Radioligand)/*K_d*]. For [³H]cytisine, the binding *K_d* was 0.92 nM for rat brain membranes and 0.48 nM for human α4β2 receptors expressed in tsA201 cell membranes. For [¹²⁵I]α-BTX, the *K_d* was 0.67 nM for rat brain membranes and 0.97 nM for human α7 receptors expressed in SH-EP1 cell membranes. One-way ANOVA of log₁₀-transformed compound mean *K_i* values obtained from a number of individual experiments was used to compare analogs with nicotine using Dunnett's multiple comparison post-test. A Student's two-tailed *t* test was used to assess statistical significance between the mean *K_i* estimates for *cis*- and *trans*-enantiomers of a compound.

Computer Docking of Nicotine Analogs with nAChR Model Proteins. The protein crystal structures used for the *in silico* molecular docking were the *Lymnaea* AChBP, occupied by nicotine (Celie et al., 2004; Brookhaven Protein Data Bank accession code 1UW6), and a chimeric protein based on *Aplysia californica* AChBP containing binding site mutations to better resemble α7 nAChR, which was occupied by epibatidine (Li et al., 2011, Brookhaven Protein Data Bank accession code 3SQ6). The molecular docking of all nicotine analogs was preceded by a two-step optimization process aimed at obtaining consistent starting geometries. The random walk algorithm implemented in HyperChem version 8.0 (HyperCube Inc., Gainesville, FL) was used to sample the potential energy surface to identify the lowest-energy conformation of each molecule. In total, 1000 conformations per molecule were generated by varying randomly all dihedral angles and using an acceptance energy cutoff of 6 kcal/mol above the best. All conformations with an energy difference of less than 0.05 kcal/mol were considered duplicates; hence, only one was retained. Conformations with potentially "bad" (distance between any two atoms of less than 0.5 Å) van der Waals contacts were discarded. The lowest-energy conformation was further subjected to a optimization in GAMESS (Schmidt et al., 1993) using an STO-3G basis set to represent the wave function in the Hartree-Fock approximation. Molecular surface calculations for each nicotine analog were made using MSROLL (Dock 6.5 Manual; University of California San Francisco, San Francisco, CA). Each molecular surface was used as input for the sphere-generating program SPHGEN (Dock 6.5; University of California San Francisco). A cluster of 77 spheres that overlapped with the ligand (a nicotine or epibatidine molecule in the appropriate crystal structure) was

manually selected to be the point of interest in the subsequent docking calculations. SHOWBOX was used to construct a three-dimensional rectangle 6 Å in any direction from the sphere cluster. CHIMERA (Pettersen et al., 2004) was used to prepare each protein structure and convert the file type to the necessary Mol2 format. The box file was used as input for the GRID program, which calculated the necessary information concerning the steric and electrostatic environment within the area of the box in the 1UW6 and 3SQ6 protein structures.

The nicotine analogs used as inputs for docking were energy-minimized. DOCK6.5 (Allen et al., 2015) was then used to measure the predicted binding energies of the compounds within the binding pocket designated by the spheres. With ligand flexibility allowed, each compound was docked in a minimum of 20,000 orientations. Separate computations were done for docking to each of the following structures for the 1UW6 *Lymnaea* AChBP: 1) the water-free site, 2) the site containing persistent water A (Amiri et al., 2007) bound to the side chain oxygen on the complementary surface, 3) the site containing persistent water B binding to Tyr 192 (Amiri et al., 2007), and finally, 4) the site containing both of these waters. The fit of the lowest-energy docked structure of each docked nicotine compound relative to the 1UW6 nicotine-AChBP structure was estimated by calculating the root mean square deviation for the 11 ring atoms of nicotine (see Supplemental Table 2). The internet-available program Coote was used for these determinations. To allow comparison of the compound free energies of binding (calculated from the observed binding affinities of the compounds for the two human receptors) with the docking energies predicted for the two AChBPs, the predicted energies of the various analogs were first normalized with respect to the predicted nicotine docking energy. Then, the predicted docking energies of the compounds were normalized with respect to the experimental free energy changes (calculated from their *K_i* values) associated with their binding to SH-EP1 cell-expressed human α7 receptors (Table 2). The experimental and predicted free energies of binding (Δ*G*s) are presented in Table 5.

Results

Concentration-Response Relationships for ACh, Nicotine, and the Methylnicotines. We measured the functional properties of each methylnicotine—namely, the EC₅₀ (inversely related to potency) and *I*_{max} (maximal ACh-normalized current)—using *Xenopus* oocytes expressing human α7 or α4β2 nAChRs to allow comparison of the compound with nicotine and its enantiomer. The concentration-current response curves of these two nAChRs for ACh and nicotine are shown in Supplemental Fig. 1. In addition, the same curve for nicotine, but without the S.E. bars, is included as a dashed line in each concentration-response curve in Fig. 2 to allow visual comparison with the nicotine analog curve. Because the α7 receptor is much less sensitive to ACh than the α4β2 receptor, the standard calibrating agonist was 1000 μM ACh, but for the α4β2 nAChR, it was 100 μM ACh. The two calibrating ACh concentrations were chosen to produce near-maximal responses that would not cause cumulative desensitization during the experiment.

Our concentration-response data for α7 receptors (Fig. 2; Table 1) includes both the more readily recorded peak current responses as well as currents integrated over the entire time of agonist response (net charge method). Both types of data were obtained from the same response. When administered by standard methods of oocyte perfusion, including ours, the α7 peak current response for an administered concentration of agonist does not reflect the maximum degree of receptor activation possible with that agonist concentration, because receptor desensitization is more rapid than the solution change: the peak current occurs before the desired concentration is

Fig. 2. *Xenopus* concentration-response curves for nicotine and the methylnicotines on human $\alpha 7$ (left, locants A, C, E, G and I) and $\alpha 4\beta 2$ (right, locants B, D, F, H and J) nAChRs. Peak current responses were recorded by the two-electrode voltage-clamp method described in *Materials and Methods*. Net charge EC_{50} and I_{max} values were also estimated from the same oocytes, and data obtained from both methods are summarized in Tables 1 and 3. The error bars indicate ± 1 S.E.M. n = number of oocytes tested at a particular concentration. The complete concentration-response curves for nicotine and ACh, including numbers of oocytes tested at each concentration, are found in Supplemental Figs. 1 and 2. Et, ethyl; Me, methyl; N, nicotine; NorN, nor nicotine.

TABLE 1

Functional properties of nicotine analogs acting on human $\alpha 7$ receptors expressed in *Xenopus* oocytesPeak current and net charge estimates were obtained from each response. Mean estimates $\pm$ one sample S.D. of the maximum current response ($I_{\max}$) and potency (EC_{50}) are given.

Compound	EC_{50} (μ M)			$I_{\max}$ (% of ACh Response)			<i>n</i>
	Peak Current	Net Charge	PC/NC Ratio	Peak Current	Net Charge	PC/NC Ratio	
Nic (N)	65.3 $\pm$ 20	21.2 $\pm$ 7.4	3.1	91.5 $\pm$ 10	85.4 $\pm$ 20	1.1	7
1'-MeN	51.4 $\pm$ 11	16.7 $\pm$ 4.5	3.1	109 $\pm$ 11	90.3 $\pm$ 9.6	1.2	5
1'-EtNorN	85.2 $\pm$ 22	30.7 $\pm$ 12	2.8	54.1 $\pm$ 15***	55.9 $\pm$ 10	1.0	9
2'-MeN	9.65 $\pm$ 4.0***	3.15 $\pm$ 0.70***	3.1	82.4 $\pm$ 6.9	72.0 $\pm$ 7.2	1.1	5
3'- <i>Trans</i> -MeN	46.7 $\pm$ 14	13.8 $\pm$ 8.3	3.4	115 $\pm$ 25	64.6 $\pm$ 11	1.8	5
3'- <i>Cis</i> -MeN	171 $\pm$ 45*, ^{††}	68.1 $\pm$ 42*	2.5	92.5 $\pm$ 29	62.6 $\pm$ 8.5	1.5	5
4'- <i>Trans</i> -MeN	246 $\pm$ 58***	113 $\pm$ 32***	2.2	16.4 $\pm$ 4.6***	27.3 $\pm$ 4.6***	0.60	4
4'- <i>Cis</i> -MeN	277 $\pm$ 32***	186 $\pm$ 120**	1.5	1.81 $\pm$ 0.80***	8.08 $\pm$ 5.6***	0.20	4
5'- <i>Trans</i> -MeN	134 $\pm$ 53*	39.3 $\pm$ 15	3.4	67.4 $\pm$ 13	54.8 $\pm$ 6.6*	1.2	7
5'- <i>Cis</i> -MeN	1010 $\pm$ 860***, ^{††}	479 $\pm$ 110***, ^{††}	2.1	4.42 $\pm$ 2.0***, ^{††}	14.8 $\pm$ 7.2***, ^{††}	0.3	4

n, the number of oocytes used.**P* < 0.05, ***P* < 0.01, ****P* < 0.001, comparison with nicotine. (These *P* values were obtained from one-way ANOVA of log-transformed data with Dunnett's multiple comparison post-test.)†*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001, comparison of *cis*- and *trans*-enantiomers; these *P* values were obtained from a two-tailed *t* test and, thus, are not corrected for multiple comparisons. Et, ethyl; Me, methyl; N, nicotine; NorN, nornicotine.

reached at the oocyte membrane (Papke and Thinschmidt, 1998). Nevertheless, the peak current response should allow us to quantitatively compare the $\alpha 7$ response of a methylated nicotine relative to that of its enantiomer or nicotine, since the diffusion limited rates of equilibration of these compounds with the receptor should be nearly the same. In support of this assumption, net charge responses obtained from the agonist pulses yielded the same conclusions as for the peak current responses, except that the net charge (NC) potency (EC_{50}) estimates were 2- to 3-fold smaller than the Peak Current measured (EC_{50}) estimates in Table 1).

Another consideration in the interpretation of the *Xenopus* oocyte functional data obtained with $\alpha 4\beta 2$ nAChRs is related to their subunit stoichiometry. Our functional measurements of the methylnicotine effects were nearly complete when the

coexpression of $\alpha 4\beta 2$ nAChRs with different stoichiometries and pharmacological properties was shown to be of general occurrence (Moroni et al., 2006). The $\alpha 4\beta 2\beta 2$ receptor with low sensitivity to AChandnicotine was reported to have a nicotine EC_{50} that is $\sim 50\times$ higher than for the high-sensitivity $\alpha 4\beta 4\beta 3$ receptor (Tavares et al., 2012). In our *Xenopus* oocyte experiments we routinely injected equal amounts of the two mRNAs. The nicotine concentration-response curve (Supplemental Fig. 1) that we obtained for $\alpha 4\beta 2$ nAChRs was well fitted with Prism, assuming a single population of $\alpha 4\beta 2$ nAChRs. When the $\alpha 4\beta 2$ mRNA ratio was either 4:1 or 1:4, we still obtained EC_{50} values for nicotine that were almost the same as when the mRNA ratio was 1:1 (results not shown). The $I_{\max}$ value of nicotine obtained with the 1:1 mRNA-injected oocytes was nearly identical with that of the 100 μ M ACh response,

TABLE 2

Nicotine analog binding to mammalian $\alpha 7$ nAChRs as measured by displacement of [¹²⁵I] α -BTX-specific bindingMean K_i estimates $\pm$ one sample S.D. are given

Compound	Rat Brain Receptor			Human Receptor (SH-EP1 Cell)		
	K_i (nM)	-Hill Slope	<i>n</i>	K_i (nM)	-Hill Slope	<i>n</i>
Nic (N)	808 $\pm$ 85	3.0	6	551 $\pm$ 140	1.1	5
1'-MeN	817 $\pm$ 74	2.5	3	917 $\pm$ 200***	1.5	4
1'-EtNorN	2620 $\pm$ 340***	2.3	4	2370 $\pm$ 32***	1.2	4
2'-MeN	181 $\pm$ 34***	2.9	7	144 $\pm$ 24***	1.2	4
3'- <i>Trans</i> -MeN	752 $\pm$ 120	2.4	4	756 $\pm$ 54*	1.2	4
3'- <i>Cis</i> -MeN	3120 $\pm$ 500***, ^{††}	3.1	4	4780 $\pm$ 12***, ^{††}	1.0	3
4'- <i>Trans</i> -MeN	3910 $\pm$ 30***	2.4	4	6850 $\pm$ 630***	1.5	5
4'- <i>Cis</i> -MeN	2930 $\pm$ 58***, ^{††}	2.4	4	3300 $\pm$ 16***, ^{††}	1.3	2
5'- <i>Trans</i> -MeN	2130 $\pm$ 76***	1.5	3	1970 $\pm$ 420***	1.1	4
5'- <i>Cis</i> -MeN	34,800 $\pm$ 2600***, ^{††}	2.0	4	22,500 $\pm$ 2600***, ^{††}	1.1	4

n = the number of experiments.**P* < 0.05, ***P* < 0.01, ****P* < 0.001, comparison with nicotine. (These *P* values were obtained from one-way ANOVA of log-transformed data with Dunnett's multiple comparison post-test.)†*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001, comparison of *cis*- and *trans*-enantiomers; these *P* values were obtained from a two-tailed *t* test and, thus, are not corrected for multiple comparisons. Et, ethyl; Me, methyl; N, nicotine; NorN, nornicotine.

TABLE 3

Functional properties of nicotine analogs acting on human $\alpha 4\beta 2$ receptors expressed in *Xenopus* oocytes

Mean estimates $\pm$ one sample S.D. are given.

Compound	EC ₅₀ (μ M)	I _{max} (% ACh Response)	n
Nic (N)	0.430 $\pm$ 0.33	93.8 $\pm$ 14	11
1'-Et-NorN	3.65 $\pm$ 2.1***	26.8 $\pm$ 10***	6
1'-MeN	1.38 $\pm$ 0.45***	22.5 $\pm$ 5.4***	5
2'-MeN	0.738 $\pm$ 0.089	83.4 $\pm$ 14	5
3'-Trans-MeN	0.74 $\pm$ 0.42	52.9 $\pm$ 14***	5
3'-Cis-MeN	3.83 $\pm$ 1.2***,tt	51.4 $\pm$ 5.4***	5
4'-Trans-MeN	0.254 $\pm$ 0.04	55.7 $\pm$ 27***	5
4'-Cis-MeN	0.285 $\pm$ 0.14	32.7 $\pm$ 14***	4
5'-Trans-MeN	>300***	<1***	4
5'-Cis-MeN	>300***	<1***	4

n = the number of oocytes used.

*P < 0.05, **P < 0.01, ***P < 0.001, comparison with nicotine. (These P values were obtained from one-way ANOVA of log-transformed data with Dunnett's multiple comparison post-test.)

^tP < 0.05, ^{tt}P < 0.01, ^{ttt}P < 0.001, comparison of *cis*- and *trans*-enantiomers; these P values were obtained from a two-tailed t test and, thus, are not corrected for multiple comparisons. Et, ethyl; Me, methyl; N, nicotine; NorN, nor nicotine.

which was ~60% of the I_{max} for ACh. In published studies in which $\alpha 4$ and $\beta 2$ cDNAs were injected into the nucleus, the I_{max} for the high-affinity form was 30%–60% of the maximal ACh current response (Moroni et al., 2006). Thus, our I_{max} values for $\alpha 4\beta 2$ receptors are consistent with previously published values for I_{max} obtained with the high-affinity $\alpha 4\beta 2_3$ receptor. Our more recent experiments, in which we coinjected an $\alpha 4\beta 2$ concatemer mRNA with the $\beta 2$ mRNA, which should yield only $\alpha 4\beta 2_3$ -like functional receptors (Supplemental Fig. 2), indicate that the near-maximal concatemeric responses for several key analogs were very similar to the maximum responses in our routine *Xenopus* experiments (Supplemental Fig. 3), further supporting our contention that the latter primarily assessed the properties of the high-affinity stoichiometry $\alpha 4\beta 2_3$ subtype.

TABLE 4

Nicotine analog binding to mammalian $\alpha 4\beta 2$ nAChRs measured by displacement of [³H]cytisine-specific binding

Mean K_i estimates $\pm$ one sample S.D. are given.

Compound	Rat Brain Receptor			Human Receptor (tsA201)		
	K _i (nM)	–Hill Slope	n	K _i (nM)	–Hill Slope	n
Nic (N)	3.26 $\pm$ 0.78	0.85	6	3.53 $\pm$ 0.92	0.89	5
1'-MeN	147 $\pm$ 18***	0.84	4	158 $\pm$ 79**	0.87	8
1'-EtNorN	83.1 $\pm$ 20***	0.80	5	91.6 $\pm$ 22***	1.0	4
2'-MeN	3.13 $\pm$ 1.5	1.3	4	6.65 $\pm$ 1.7**	1.4	4
3'-Trans-MeN	73.7 $\pm$ 13***,ttt	0.94	4	161 $\pm$ 37***,ttt	0.92	6
3'-Cis-MeN	770 $\pm$ 71***	1.0	6	1380 $\pm$ 400***	1.2	4
4'-Trans-MeN	14.9 $\pm$ 5.1***	1.0	6	14.0 $\pm$ 6.9**	1.1	6
4'-Cis-MeN	24.4 $\pm$ 7.1***,t	1.1	6	32.6 $\pm$ 16***,t	1.8	3
5'-Trans-MeN	150 $\pm$ 26***	1.0	4	219 $\pm$ 76***	1.0	4
5'-Cis-MeN	10,600 $\pm$ 1620***,ttt	0.87	5	8330 $\pm$ 1030***,ttt	0.97	6

n = the number of experiments.

*P < 0.05, **P < 0.01, ***P < 0.001, comparison with nicotine. (These P values were obtained from one-way ANOVA of log-transformed data with Dunnett's multiple comparison post-test.)

^tP < 0.05, ^{tt}P < 0.01, ^{ttt}P < 0.001, comparison of *cis*- and *trans*-enantiomers; these P values were obtained from a two-tailed t test and, thus, are not corrected for multiple comparisons. Et, ethyl; Me, methyl; N, nicotine; NorN, nor nicotine.

The radioligand binding data measuring ligand affinity for $\alpha 4\beta 2$ AChRs are unlikely to be affected by the presence of the low-affinity $\alpha 4\beta 2_2$ receptors, since we measured displacement at such a low concentration (1 nM) of [³H]cytisine that few of these receptors would be occupied (Moroni et al., 2006; Tavares et al., 2012).

The variance estimates for the mean potencies (EC₅₀) and binding constants (K_i values) of the compounds tended to increase as their mean values increased, so we used a log₁₀ transformation of the estimates to better approximate a normal distribution of the individual estimates for a compound. This resulted in more-similar variance estimates among the compounds and allowed one-way ANOVA to be used to analyze comparisons of all compounds with nicotine.

Functional and radioligand binding data for each methyl-nicotine will now be presented and discussed in order of the five pyrrolidinium ring atoms 1' to 5' (Fig. 1), numbered according to the International Union for Pure and Applied Chemistry nomenclature. Data for $\alpha 7$ nAChRs will be presented initially, since interactions of these compounds with this receptor subtype have not been previously reported.

1'-Methylnicotine and 1'-Ethyl-nornicotine. Ample evidence exists that the monocationic form of nicotine and of most other ionizable nAChR agonists is the form that binds to nAChRs with highest affinity (Barlow and Hamilton, 1962; Jeng and Cohen, 1980; Kem et al., 2004). Since the published pK_a for ionization of the 1'-N of nicotine (8.05, Fujita et al., 1971) is 0.65 U above physiologic pH of 7.4, and the methyl-nicotine analogs were found to have similar pK_a values (Supplemental Table 1), our pharmacological data in Tables 1–6 largely reflect the properties of the monocationic species and are uncorrected for the small differences in ionization we observed for the 1'-ethyl-, 2'-methyl-, and 5'-methylnicotine analogs (see Supplemental Table 1 for estimation of the percentage of monocationic species for compounds expected to show the largest differences in ionization).

We first examined the consequences of adding an additional methyl substituent to the 1'-N of nicotine and found major differences between the two receptors. For $\alpha 7$ receptors, the

inferior to that of nicotine (Table 3). We also found that racemic 1'-propylnornicotine displayed an almost 1000-fold decrease in potency relative to nicotine at $\alpha 4\beta 2$ receptors, in agreement with a previous study (Glassco et al., 1994), as well as a greatly reduced potency at $\alpha 7$ receptors (results not shown). Thus, nicotine interaction with $\alpha 4\beta 2$ receptors is particularly sensitive to the presence of bulky quantitatively alkyl groups at the 1'-N position.

2'-Methylnicotines. *Trans*-2'-methylation produced unique changes relative to the other six carbon-methylated nicotines, which displayed reduced agonist properties relative to nicotine. 2'-Methylnicotine displayed agonist properties superior

The number within parentheses is the % increase in ΔG of the analog relative to the ΔG for nicotine; a plus value represents a reduced affinity of the analog for the receptor relative to nicotine.

^a%ΔG change relative to ΔG for nicotine.
^bPredicted nicotine −ΔG = 34.1 kcal/mol; normalized to 8.88.
^cPredicted nicotine −ΔG = 37.5 kcal/mol; normalized to 8.88.

TABLE 6

Radioligand binding affinities of six additional methylnicotine analogs for rat brain $\alpha 4\beta 2$ and $\alpha 7$ receptorsMean K_i estimates $\pm$ one sample S.D. are given (n = the number of experiments). The P values were obtained from a two-tailed t test comparing the two enantiomers and, thus, are not corrected for multiple comparisons. The four Me(R)N compounds are methylated methyl(R)-nicotines.

Compound	$\alpha 4\beta 2$ Receptor			$\alpha 7$ Receptor		
	K_i (nM)	–Hill Slope	n	K_i (nM)	–Hill Slope	n
A84543 ^a	3.44 $\pm$ 0.40			340 $\pm$ 50		
5'- <i>Trans</i> -MeA84543	18.5 $\pm$ 17 ^{tt}	1.0	4	603 ^{ttt} $\pm$ 170	1.9	5
5'- <i>Cis</i> -MeA84543	201 $\pm$ 26	1.0	3	2140 $\pm$ 830	1.8	5
5'- <i>Trans</i> -Me(R)N	480 $\pm$ 140 ^t	1.1	3	3920 $\pm$ 2300	2.9	2
5'- <i>Cis</i> -Me(R)N	204 $\pm$ 89	1.0	5	426	2.8	1
3'- <i>Trans</i> -Me(R)N	553 $\pm$ 140 ^{ttt}	1.1	4	6720 $\pm$ 710 ^{tt}	3.6	3
3'- <i>Cis</i> -Me(R)N	4340 $\pm$ 1400	1.1	4	22,000 $\pm$ 3600	1.7	3

^t P < 0.05, ^{tt} P < 0.01, ^{ttt} P < 0.001, comparison of *cis*- and *trans*-enantiomers (These P values were obtained from a two-tailed t test).^aA84543 K_i $\pm$ S.E.M. binding data of Abreo et al. (1996), Ogunjirin et al. (2015) reported K_i values of 0.15 and 1 nM for A84543 and nicotine, respectively, at rat brain $\alpha 4\beta 2$ receptors.

to nicotine at $\alpha 7$ receptors and was at least as active as nicotine at $\alpha 4\beta 2$ receptors. $\alpha 7$ nAChR potency and equilibrium binding affinity (Table 1) were enhanced about 7-fold for the human receptor in comparison with nicotine. At the $\alpha 4\beta 2$ receptor, 2'-methylnicotine potency and $I_{\max}$ were not statistically different from nicotine (Table 3). The small increase in binding affinity at the $\alpha 4\beta 2$ receptor was only statistically significant for the human receptor (Table 4). Our data for 2'-methylnicotine binding to rat brain $\alpha 4\beta 2$ receptors was consistent with the data of Wang et al. (1998) for racemic 2'-methylnicotine, taking into consideration that our 2'-methyl-(S)-nicotine has a much higher (>40-fold) binding affinity than 2'-methyl-(R)-nicotine (Kem, unpublished results).

3'-Methylnicotines. *Cis*-3'-methylnicotine displayed less potency (~3-fold at $\alpha 7$ and ~10-fold at $\alpha 4\beta 2$ receptors) relative to nicotine (Fig. 2; Tables 1 and 3). There was also a statistically significant (~2-fold) decrease in the $\alpha 4\beta 2$ receptor $I_{\max}$. *Cis*-3'-methylnicotine displayed less affinity for $\alpha 7$ (approximately 4-fold for rat and 8-fold for human) and $\alpha 4\beta 2$ (approximately 200-fold less for rat and 400-fold less for human) receptors relative to nicotine (Tables 2 and 4). Although the affinity of *trans*-3'-methylnicotine for the $\alpha 7$ receptor was similar to that of nicotine, its affinity for $\alpha 4\beta 2$ receptors was ≥ 30 -fold less than for nicotine.

4'-Methylnicotines. At both receptors (especially the $\alpha 7$), the efficacies of both 4'-methylnicotines were greatly reduced relative to nicotine (Fig. 2; Tables 1 and 3). Whereas the potencies of the two enantiomers were inferior to that of nicotine at $\alpha 7$ receptors, on the human $\alpha 4\beta 2$ receptor the potencies were not significantly different from that of nicotine. Relative binding affinities of the two enantiomers at the two receptors differed: at both $\alpha 7$ receptors, the *cis*-enantiomer displayed the highest affinity, but at rat and human $\alpha 4\beta 2$ receptors, the *trans*-enantiomer displayed the highest affinity.

5'-Methylnicotines. Analysis of the pharmacological properties of *cis*-5'-methylnicotine revealed its limited ability to interact with $\alpha 4\beta 2$ receptors. Agonist potency was reduced >700-fold, and $I_{\max}$ was reduced >100-fold (Table 3). The binding K_i values for rat and human $\alpha 4\beta 2$ receptors were also increased >2000-fold and >700-fold, respectively (Table 4). Previous radioligand binding studies also indicated that methylation at this position had detrimental effects on nicotine binding affinity for rat brain high-affinity ($\alpha 4\beta 2$) receptors and that the binding K_i of

trans-5'-methylnicotine was less affected than that of the *cis*-form (Lin et al., 1994; Wang et al., 1998).

Although *cis*-5'-methylnicotine also displayed the greatest reduction in interaction with $\alpha 7$ receptors, *trans*-5'-methylnicotine was a relatively potent agonist, with potency decreasing only 2- to 3-fold and $I_{\max}$ decreasing ~1.5-fold relative to nicotine (Table 1). Binding affinities for the human and rat $\alpha 7$ nAChRs were only decreased ~3-fold relative to nicotine (Table 2).

Relationships between Receptor Affinity and Receptor Potency. Compound $EC_{50} = K_d/(E + 1)$, where K_d is the equilibrium dissociation constant for initial binding to the inactive state, and E is the equilibrium constant associated with receptor activation (Auerbach, 2016). If K_i estimates are related to the K_d , then our EC_{50} estimates might show some relationship to the K_i values, at least for compounds displaying low E values. We plotted K_i versus EC_{50} estimates for each alkylnicotine to determine whether they might be related (Fig. 3, A and C). We used EC_{50} estimates obtained with the net charge method of analysis for EC_{50} and $I_{\max}$ characterization of the functional properties of the human $\alpha 7$ receptor in this figure. Both the $\alpha 7$ and $\alpha 4\beta 2$ plots showed a relatively strong correlation between these two variables (Fig. 3A, $\alpha 7$ correlation coefficient $r^2 = 0.90$; Fig. 3C, $\alpha 4\beta 2$ $r^2 = 0.61$). Since the $\alpha 7$ net charge-determined EC_{50} values (Table 1) were ~3-fold less than the peak current EC_{50} estimates, using our standard 2-second agonist exposure (and ~10-fold less for a 20-second exposure; Kem, unpublished results), the actual $\alpha 7$ receptor EC_{50} for each compound may be as much as 10-fold less than the peak current EC_{50} reported in Table 1 and used in Fig. 3A of this figure.

It was recently reported that there is a linear relationship between $\log I_{\max}$ and $\log EC_{50}$ for agonists at the mouse neuromuscular nAChR—that high efficacy is directly correlated with high potency (Auerbach, 2016). In spite of the differences in how efficacy was measured (single channel recording in the Auerbach study and oocyte net charge response here), a similar correlation was observed with our $\alpha 7$ data (Fig. 3B, $r^2 = 0.67$). In Fig. 3B, the $I_{\max}$ estimates for the most efficacious compounds (2'-MeNic, 1'-MeNic, and Nic) are very similar, although their EC_{50} values differ considerably, which would not be predicted from the Auerbach equation; it seems likely that desensitization may limit the $I_{\max}$ values measured for these highly potent agonists under our experimental conditions. In contrast to the $\alpha 7$ plot in 3B,

Fig. 4. Concentration-response curves for 5'-*trans*-methylA84543 stimulation of human $\alpha 4\beta 2$ and $\alpha 7$ nAChRs expressed in *Xenopus* oocytes. The error bars indicate $\pm$ one S.E.M. (The 5'-*cis*-methylA84543 enantiomer was much less active: at 30 μ M, the peak $\alpha 7$ response was approximately 10% of the 1 mM ACh response, and the $\alpha 4\beta 2$ response was $\sim$ 0% of the 100 μ M ACh response.)

the $\alpha 4\beta 2$ plot showed much more scatter when plotted in an identical fashion (Fig. 3D, $r^2 = 0.24$).

Modeling Methylnicotine Interaction with the Molluscan ACh Binding Proteins. Since high-resolution crystal structures of the two nAChRs are still unavailable, we attempted to predict the binding poses and relative docking free energies of the various methylnicotines using the Brookhaven Protein Data Bank *Lymnaea* AChBP-nicotine 1UW6 structure (Celie et al., 2004) and the mutated *A. californica* AChBP-epibatidine 3SQ6 structure, in which the binding site was mutated to be similar to the $\alpha 7$ site (Li et al., 2011). In the 1UW6 structure, the *cis*-side of the nicotine pyrrolidinium ring comes in closest proximity with the AChBP Trp143 indole side chain, and the 1'-N proton is also very near (2.6 Å) the Trp143 peptide carbonyl group and is assumed to form an H-bond with it (Celie et al., 2004). In contrast, the 2'-carbon of nicotine is not in close contact with any nonwater atoms in the AChBP binding site. Actually, the 2'-methyl is oriented toward the disulfide bond between the vicinyl half-cystines in the C-loop. The structures of the various methylnicotines (as monocations) were energy-minimized and docked to both AChBPs after removing the crystallized ligands from their respective ACh binding sites. The relative energies for docking the various methylnicotines (Table 5) to both AChBP structures predicted many of the differences in binding affinity that were observed, particularly when two of the most persistently bound water molecules in the *Lymnaea* AChBP-nicotine complex (Amiri et al., 2007) were retained within the binding site during docking (see Supplemental Table 2 for fit estimates). For the 3'- and 5'-methylnicotines, the docking score of the *cis*-methyl enantiomer predicted a less favorable free energy of binding relative to the *trans*-enantiomer, and *cis*-5'-methyl-nicotine was predicted to have the least-favorable free energy of binding of all the methyl analogs to both AChBPs (Table 5). The nicotine compound predictions for the *Aplysia* AChBP- $\alpha 7$ chimera binding site, containing no water molecules, were similar to those obtained for the *Lymnaea* AChBP.

Comparison of Free Energy Changes Associated with Compound Binding to Human nAChRs with Predicted Free Energy Changes for Binding to AChBPs. The ΔG s (Table 5) of the various analogs relative to the ΔG for nicotine binding at the same receptor site, expressed within the parentheses as a percent change, are most salient. The relative ΔG changes associated with methylation were much greater for $\alpha 4\beta 2$ receptors than for $\alpha 7$ receptors. The major exception was 2'-methylnicotine, which actually showed a greater free energy decrease (lower K_i) relative to nicotine at the $\alpha 7$ receptor. This was only predicted for the *Aplysia* AChBP- $\alpha 7$ chimera, whose orthosteric binding site most resembles the $\alpha 7$ binding site. Another important observation was that the free energy of binding difference (-1.9 kcal/mol) for nicotine, between the human $\alpha 4\beta 2$ receptor ($\Delta G = -10.8$ kcal/mol) and the $\alpha 7$ receptor ($\Delta G = -8.9$ kcal/mol), almost disappeared in free energy comparisons for most other analogs other than the 1'-*N*-methylnicotinium and 1'-*N*-ethylnornicotine. This important observation will be discussed later.

Influence of Methyl Substituent Configuration on Receptor Interaction in Other Methylated Nicotine Analogs. Abreo et al. (1996) synthesized and tested the nicotine analog A84543, in which an oxymethylene group forms a bridge between the 3-pyridyl ring and the 2'-(S) position of the pyrrolidinium ring so that the pyridyl ring retains the same chirality as in nicotine. This compound displayed very high affinity and potency at $\alpha 4\beta 2$ receptors, exceeding that of nicotine, indicating that this receptor not only tolerates a greater distance between the two nicotine ring N atoms but also binds this compound more readily than nicotine. To determine whether A84543 would be similarly affected by 5'-methylation, we synthesized (see Supplemental Data) and tested the two 5'-methylA84543 diastereomers. The rat brain $\alpha 4\beta 2$ receptor affinity (Table 6) of *trans*-5'-methylA84543 was 7-fold higher than the *cis*-5'-methylA84543. The rat brain high-affinity receptor K_i for A84543 has been reported to be 0.15 ± 0.01 (Abreo et al., 1996) and 3.44 ± 0.40 nM (Ogunjirin et al., 2015), and the $\alpha 7$ receptor K_i was reported to be 340 ± 50 nM (Ogunjirin et al., 2015). Although the difference we observed between the two methylated A84543 enantiomers was approximately $10\times$ less than it was for the 5'-methylnicotines, it is clear that the preferential binding of the *trans*-5'-methylnicotine also applies to *trans*-5'-methylA85443. Like A84543, both methyl enantiomers interacted with $\alpha 7$ receptors with much lower affinity relative to $\alpha 4\beta 2$ receptors, with the *cis*-enantiomer showing the least affinity and potency (Table 6). The efficacies of *trans*-5'-methylA84543 (Fig. 4) at human $\alpha 7$ and $\alpha 4\beta 2$ receptors were similar to those of *trans*-5'-methylnicotine (Tables 1 and 3).

Since we had prepared racemic 3'- and 5'-methylnicotines and then separated the diastereomeric (R)- and (S)-methylnicotines, this afforded the opportunity of determining whether the relative or absolute configurations of these two methyl substituents are critical for optimal interaction with the receptors. Our binding data shows that at the 3' position, the same relative configuration (*trans*) allows the highest affinity, irrespective of the pyridyl ring substituent configuration. However, it is the absolute configuration of the 5'-methyl substituent that determines relative activity, since the substituent in *cis*-5'-methyl

(R)-nicotine has the same absolute configuration as the methyl in *trans*-5'-methyl-(S)-nicotine.

Discussion

Dominant Role of the Cationic N-Alkyl Moiety. Ionization of the most basic N atom is essential for efficient binding of nicotine and most nicotinoids to various AChRs and AChBPs. The pyrrolidinyl N in nicotine has been reported to possess a pK_a of 8.05 and a pyridyl ring N pK_a of 3.85 (Fujita et al., 1971). Thus, at physiologic pH, there will be a mixture of the monocationic and unionized pyrrolidinyl forms, and their relative solution concentrations will depend upon the pH. Under the ionic strength conditions of our radioligand binding measurements, the nicotine pK_a was 8.00, and its ionization was 80% at pH 7.4 (Supplemental Table 1). The pK_a of the pyrrolidinium N was enhanced the most (to 8.44) by replacing the 1'-N-methyl with an ethyl group. Methylating the 2'- or 5'-positions produced very small increases in ionization at pH 7.4. Methylation at the 3' or 4' position would not be predicted to have a significant inductive effect on the pK_a .

Adding an additional methyl to the 1'-N of nicotine greatly diminished interaction with the $\alpha 4\beta 2$ receptor without much effect on interaction with the $\alpha 7$ receptor. Substituting the larger ethyl moiety for the 1'-N-methyl group, as in 1'-N-ethylnicotine, was deleterious for interaction with both receptors, especially the $\alpha 4\beta 2$ receptor. Formation of the 1'-N-Trp B carbonyl hydrogen bond seems to be important for nicotine binding to $\alpha 4\beta 2$ receptors (Xiu et al., 2009; Puskar et al., 2011). The high affinity of nicotine for the $\alpha 4\beta 2$ receptor may, at least partially, be due to this hydrogen bond, which seems less important for interaction with the $\alpha 7$ nAChR subtype and may not be essential (Puskar et al., 2011; Van Arnem et al., 2013). The lesser effects on $\alpha 7$ interaction of these two 1'-N modifications suggest that a small increase in bulk of the pyrrolidinium group does not adversely affect cation- π binding. The highly detrimental influence of increasing the bulk of substituents at the 1'-N for $\alpha 4\beta 2$ receptor interaction may be due to these bulky substituents interfering with formation of this hydrogen bond.

2'-Methylation Uniquely Increases Interaction with the $\alpha 7$ Receptor without Adversely Affecting Interaction with the $\alpha 4\beta 2$ nAChR. Unexpectedly, methylation at the 2'-carbon, which also connects the two rings, significantly enhances binding and agonist potency at the $\alpha 7$ nAChR without significantly affecting interaction with the $\alpha 4\beta 2$ nAChR. The 2'-methyl may form a hydrophobic interaction with some groups that may favor formation of an H-bond between the 1'-NH⁺ and the receptor, which otherwise seems to be absent. If binding site waters are present in the vicinity of the 1'-NH of nicotine, they might be displaced by a methyl substituent like the 2'-methyl (Barratt et al., 2006; Leung et al., 2012). Water molecules are present in the agonist-bound AChBP as well as in the agonist-free state of the AChBP binding site (Celie et al., 2004; Amiri et al., 2007), and it has been found (Forli and Olson, 2012; this study) that better docking predictions can be obtained when persistent water molecules observed in the nicotine-AChBP crystal binding site are included in the computer docking. The actual basis for the potentiating effect of the 2'-methyl substituent can only be determined by additional experimental and computational studies.

3'-Cis-Methylation Reduces Agonist Activity More than 3'-Trans-Methylation. Since the pyrrolidinium 3'-carbon is

next to the inter-ring bonding 2'-carbon, it was predicted that its methylation would alter the energy profile for inter-ring torsion and might even prevent the compound from attaining an optimal conformation for agonistic binding and to the receptor. Although both 3'-methylnicotines were less active than nicotine, we found that that *trans*-3'-methylnicotine was significantly more active than *cis*-3'-methylnicotine at both receptors. The thermodynamically preferred inter-ring angles for *cis*-3'- and *trans*-3'-methylnicotine and for nicotine are predicted to be very similar, but the energy barrier for rotation around the C2'-C3 bond is much higher (60 kcal/mol) for *cis*-3'-methylnicotine compared with 14 kcal/mol for *trans*-3'-methylnicotine (Seeman, 1984). One possibility is that the two rings must be able to move with respect to each other in the binding or receptor activation processes, and the presence of the 3'-methyl substituent to varying degrees inhibits these movements. The data in Table 6, indicating that the relative configuration of the 3'-methyl group is more important for activity and binding than its absolute configuration, is consistent with this interpretation.

5'-Cis-Methylation Causes a Drastic Loss of Activity at Both nAChRs. The 5'-position of the pyrrolidinium ring is adjacent to the 1'-N-hydrogen, which H-bonds to the peptide carbonyl of Trp 143 at the *Lymnaea* AChBP and by inference at Trp B of the $\alpha 4\beta 2$ nAChR (Xiu et al., 2009; Blum et al., 2010). The H-bond does not seem to be present or very strong at $\alpha 7$ receptors, at least for ACh, epibatidine, and varenicline (van Arnem et al., 2013). Since the 5'-cis-hydrogen of nicotine abuts the Trp 143 side chain in AChBP, we suggest that *cis*-methylation at this site significantly lowers the free energy change associated with nicotine's H-bonding to the equivalent Trp B (residue 149) in the $\alpha 4\beta 2$ receptor. Theoretical calculations of the positive charge density of the various carbon atoms in the pyrrolidinium ring of nicotine indicate that the positive charge is less centered on the pyrrolidinyl N than on the three C atoms (1', 2', and 5') attached to the nicotine 1'-N (Elmore and Dougherty, 2000). Regardless of mechanism, the significant diminution in 5'-*cis*-methylnicotine binding to both receptors indicates that the 5'-carbon occupies a very critical position that can exert much control over nicotine's interaction with these receptors. The superior activity of 5'-*trans*-methylA84543 relative to 5'-*cis*-methylA84543 (Fig. 4; Table 6) is consistent with the notion that the binding orientation of this compound, although it contains an oxymethylene bridge between the pyridyl and pyrrolidinyl rings, is very similar to that of nicotine. Our data regarding the relative affinities of the 5'-methyl-(R)-nicotine enantiomers indicate that it is the absolute configuration of the 5'-methyl group that determines the relative affinities of these enantiomers for both receptors.

The effects of 5'-methylation were greatest on nicotine's interaction with the $\alpha 4\beta 2$ nAChR receptor. Based on the earlier radioligand binding studies with some of these nicotine analogs on $\alpha 4\beta 2$ receptors, we anticipated that all methylations would diminish nicotine's agonistic properties. Our radioligand binding data demonstrated a preferential reduction in binding of the 3'-*cis*- and 5'-*cis*-methylated nictines on both receptors, as well as for *cis*-4'-methylnicotine binding to the rat $\alpha 4\beta 2$ (but not the $\alpha 7$ receptor). Although the molecular basis for this behavior is not yet known, it suggests that interaction of the *cis*-side of the pyrrolidinium ring with a part of the receptor is of paramount importance. It seems likely, based on the close proximity of the *cis*-side of the pyrrolidinium ring to Trp 143 in AChBPs and Trp B in the $\alpha 4\beta 2$ X-ray

structure (Morales-Perez et al., 2016), that *cis*-methylation generally interferes with nicotine's interaction with this component of the aromatic "box." The lesser influence of *cis*-methylation on interaction with the $\alpha 7$ receptor could be due to the lesser importance of 1'-NH⁺ hydrogen bonding to Trp B and/or the cation- π bonding forces between nicotine and this receptor being directed toward an adjacent Tyr in the aromatic box (Dougherty et al., 2011).

The Docking of Nicotine and Its Methylated Analogs to AChBPs. The solution conformation of nicotine has been the subject of extensive experimental and theoretical investigations over the last few decades. NMR and theoretical studies have provided strong evidence that the preferred conformation of nicotine in aqueous solution is one in which the two rings of nicotine are twisted with respect to each other (Chynoweth et al., 1973; Pitner et al., 1978; Elmore and Dougherty, 2000). The four carbon atoms of the pyrrolidinium ring are in a relatively planar "envelope" conformation in crystalline and aqueous solution conditions. Although the 1'-N-methyl group of nicotine is capable of inversion, its *trans*-orientation with respect to the pyridyl ring substituent is of slightly lower free energy in solution. The relative positions of the two rings of nicotine in the *Lymnaea* AChBP crystal structure are very similar to that observed for nicotine in solution or in a vacuum, except that the N-methyl is *cis*- with respect to the pyridyl ring (Celie et al., 2004).

Comparison of the experimental free energies of binding of the various analogs (Table 5) revealed some interesting similarities in the binding energies of the 1'-3'-, 4'-, and 5'-methylated nictines on both receptors. Although the functional properties differed, the measured $-\Delta G$ (kcal/mol) values for 1'-methylnicotinium and both 3'-methylnicotine and both 5'-methylnicotine were very similar for the two receptors. It is tempting to interpret the much-reduced binding of these analogs to the $\alpha 4\beta 2$ receptor, such that they bind with similar affinity to the $\alpha 7$ receptor, as being due to their inability to form the 1'-NH hydrogen bond with the Trp B peptide carbonyl in this receptor.

Concluding Remarks. Although similarities in methylation effects are also of general interest for understanding the interaction of nicotine with AChRs, if selective enhancement or diminution of activity at one of the nAChR subtypes is desired in the design of new nicotinic drugs, then the differences become of paramount interest. Central nervous system drug design has largely focused on development of drug candidates selective for either $\alpha 4\beta 2$ or $\alpha 7$ receptors. Drug design using nicotine as the lead compound has focused on improving selectivity for the high-affinity heteromeric receptor subtypes, but our study demonstrates that the nicotine scaffold is capable of being modified so as to increase its interactions with $\alpha 7$ nAChRs. Enhancing cognitive function by separately stimulating a particular brain nAChR, $\alpha 4\beta 2$ or $\alpha 7$, has been a therapeutic goal for at least two decades, but a maintained occupation of either $\alpha 4\beta 2$ or $\alpha 7$ receptors seems to produce suboptimal stimulation of cognitive processes in humans (Kem et al., 2018). The two major nAChR subtypes are widely expressed in the brain, often in different parts of the same neuron or within different neurons in a common circuit. Our data for 2'-methylnicotine suggest that it may be possible to concurrently stimulate both receptors at an appropriate brain concentration. A dual receptor stimulation strategy might provide a more powerful cognitive effect than

when only one of the receptor subtypes is stimulated. There are some recent studies that suggest that this may be a promising approach, as long as adverse effects exerted through autonomic or neuromuscular nAChRs can be avoided (Potasiewicz et al., 2019; Sun et al., 2019).

By measuring binding affinities of the various nicotine analogs on both rat and human forms of each receptor, we have shown that these compounds display almost identical interactions with receptors of the two species. Some agonists, such as 3-(2,4-dimethoxybenzylidene)-anabaseine, display differences in their interactions with rat and human $\alpha 7$ nAChRs (Kem et al., 2004). The nearly identical binding behavior of the nicotine analogs in receptors of both species should facilitate behavioral predictions for humans based on rat models.

Some of the nicotine analogs considered in this paper have already been tested in rats and were found to reduce nicotine self-administration (Rowland et al., 2008). Of particular interest is the ability of *trans*-5'-methylnicotine to inhibit self-administration of nicotine, in spite of its greatly diminished affinity for $\alpha 4\beta 2$ receptors. We recently learned that this compound is a relatively potent partial agonist at $\alpha 6\beta 2^*$ nAChRs (Kem et al., unpublished results). Thus, some of the methylnicotine may interact differently with other nAChR subtypes than would be predicted from our current studies with the two major brain nAChRs.

Our study has revealed important differences as well as similarities in the orthosteric binding sites of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs that may be useful in designing new drug candidates based on the nicotine scaffold. "Dougherty"-type analyses (Cashin et al., 2005; Davis and Dougherty, 2015), investigating the effects of introducing electron-withdrawing substituents on Trp B and other members of the binding site "aromatic box," are likely to provide additional insights into the interactions of some of these methylnicotine and how they differ from nicotine. Crystal structures, especially of 2'-methylnicotine and 5'-*trans*-methylnicotine bound to AChBPs, $\alpha 4\beta 2$ and $\alpha 7$ receptors, would also be informative.

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Authorship Contributions

Participated in research design: Xing, Andrud, Jahn, Corsino, Slavov, Lindstrom, Kem.

Conducted experiments: Xing, Andrud, Cho, Jahn, Lu, Habibi, Corsino, Slavov, Kem.

Contributed new reagents or analytic tools: Slavov, Lindstrom, Lukas.

Performed data analysis: Xing, Andrud, Jahn, Lu, Cho, Habibi, Corsino, Slavov, Lindstrom, Kem.

Wrote or contributed to the writing of the manuscript: Xing, Jahn, Lu, Slavov, Kem.

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