

**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**

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**Abbreviations:** AChBP, acetylcholine binding protein;  $\alpha$ -BTX,  $\alpha$ -bungarotoxin; nAChR, nicotinic acetylcholine receptor

## 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. 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. While 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* AChBP 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**

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 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 has a number of potentially therapeutic effects. However, it is one of the most addictive of drugs when self-administered as a tobacco product. Nicotine is also widely used as a useful smoking cessation drug in non-smoked 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 biological investigations have revealed genes for 17 different subunits, so a large number of subunit combinations is theoretically possible and >15 pentameric subunit combinations have already been identified in mammalian tissues (Wu and Lukas, 2011). At least two  $\alpha$  subunits occur in each nAChR pentamer. Agonist binding sites are located within a groove between the N-terminal extracellular domains of an  $\alpha$  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.  $\alpha 7$  nAChRs are mainly homomeric complexes containing five  $\alpha 7$  monomers, though small concentrations of  $\alpha 7\beta 2$  nAChRs also occur in the brain (Moretti et al., 2014). All the other nAChRs contain two or three non- $\alpha$  subunits. Significant decreases in the concentrations of these two receptors have been found in the postmortem brains of Alzheimer's and Parkinson's disease patients (Burghaus et al., 2003). In many schizophrenics, 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- $\pi$ , 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; Hansen et al., 2005; Li et al., 2011). Crystal structures of the extracellular portions of the adult skeletal muscle  $\alpha 1$  subunit and the  $\alpha 10$  subunit binding  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) have been reported (Delissanti et al., 2007; Zouridakis et al., 2014). Recently, a crystal structure of the nearly whole  $\alpha 4_2\beta 2_3$  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 nicotines 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 4 of the 8 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.

[Fig. 1]



## 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  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral and MS spectrometric data of compounds whose syntheses were previously reported agreed with the published data. The chiral HPLC conditions for obtaining the individual enantiomers from the racemic 2'-, 3'- and 5'-methylnicotines are provided in the Supplement. 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.

### Functional experiments with *Xenopus* oocytes

Frogs were purchased, maintained and used under a UF IACUC 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 min. After the frog was completely unresponsive and immobile it was decapitated with a guillotine and its spinal cord pithed before excision of the ovary, which was placed into calcium-free Barth saline (88 mM NaCl, 1 mM KCl, 2.38 mM  $\text{NaHCO}_3$ , 0.82 mM  $\text{MgSO}_4$ , 15 mM HEPES, 0.012

mg/ml tetracycline hydrochloride (Sigma-Aldrich), pH  $7.3 \pm 0.1$ ), 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  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ , healthy stage 5 oocytes were transferred into dishes and incubated at  $17^{\circ}\text{C}$  overnight. Oocytes were routinely injected with 50 nl of a solution containing 20 ng  $\alpha 7$  mRNA or 50 nl of a mixture of  $\alpha 4$  and  $\beta 2$  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  $\alpha 4_2\beta 2_3$  nAChR, an equal weight (5ng) of RNA for the human  $\beta 2\text{AGS}\alpha 4$  concatamer was injected with 5 ng  $\beta 2$  RNA in each oocyte, since this ratio has been shown to minimize expression of alternative stoichiometries of this receptor (Kuryatov et al., 2005;2007). Injected oocytes were cultured in Barth's saline for 5-10 days at  $17^{\circ}\text{C}$  with daily changes in the saline prior to recordings.

Individual oocytes were placed into a 20  $\mu\text{l}$  oocyte perfusion chamber (Model OPC-1 connected to a ValveLink8.2 system, Automate 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  $\text{CaCl}_2$ , pH 7.3) containing 1  $\mu\text{M}$  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  $\alpha 7$  and  $-50$  mV for  $\alpha 4\beta 2$  nAChRs). The voltage-measuring microelectrode (filled with 3M KCl solution) resistance was

0.5-3.0 M $\Omega$  and the current-passing electrode (containing 250 mM CsCl and 100  $\mu$ M EGTA) resistance was 0.5-2.0 M $\Omega$ . 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 sec for  $\alpha 7$  and 5 sec for  $\alpha 4\beta 2$  receptors) to avoid a cumulative desensitization. A rapid flow rate (2.0 ml/sec) permitted complete replacement of the fluid bathing the oocyte at approximately 3X per second. Initially each oocyte received two control applications of a near maximal stimulatory concentration of ACh (1 mM ACh for  $\alpha 7$  and 100  $\mu$ M ACh for  $\alpha 4\beta 2$  receptors) to obtain a consistent response. ACh control applications alternated with test compound applications every 5 min. 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, USA) for analysis. The concentration-response curve for each compound was calculated by fitting the data using a 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 above-mentioned mean ACh control response and  $I_{\max}$  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  $\alpha 7$  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 s interval (including the 2 s drug application period) was measured and the net charge response was obtained after subtraction of baseline current for the same 20 s 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  $\geq 4$  and is given in Figure 2. Efficacy ( $I_{\max}$ ) estimates by peak current and net charge methods and potency ( $EC_{50}$ ) estimates are presented in Tables 1 and 3 as arithmetic means  $\pm$  one sample standard deviation. One way ANOVA of  $\log_{10}$  transformed compound  $I_{\max}$  and  $EC_{50}$  data was used to compare all analogs with nicotine, with Dunnett's multiple comparison post-test. Student's two-tailed t test was used to assess statistical significance between mean  $I_{\max}$  and  $EC_{50}$  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  $CaCl_2$ , 1 mM  $MgCl_2$ , 50 mM Tris-TrisHCl buffer, pH 7.4). After the homogenate was centrifuged at 14,600 x G for 10 min the resulting pellet was resuspended in fresh binding saline, homogenized, centrifuged again and the supernatant 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  $-82^{\circ}C$  before use. Each tube in the binding experiment received 100  $\mu g$  of rat brain membrane protein.

Washed membranes from cultured TsA201 cells expressing human  $\alpha 4\beta 2$  nAChRs were also utilized 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 ( $\alpha 4_2\beta 2_3$ ) 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 cm<sup>2</sup>) and adding 6-10 ml of ice-cold Tris binding saline. The dislodged cells were collected in centrifuge tubes and spun down at 7,700 x G for 8 min. 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 protein per tube were used for radioligand binding experiments. SH-EP1 cells expressing human  $\alpha 7$  nAChRs were similarly cultured and their membranes (100 µg membrane protein per tube) were used in <sup>125</sup>I- $\alpha$ -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  $\alpha 4\beta 2$  nAChR affinity, 1.0 nM <sup>3</sup>H-cytisine (34 Ci/mmol) displacement experiments were performed at 4°C as previously reported by Pabreza et al. (1991). Measurements of  $\alpha 7$  nAChR affinity were done by displacement of <sup>125</sup>I- $\alpha$ -BTX (136 Ci/mmol) binding; the final test <sup>125</sup>I- $\alpha$ -BTX concentration was 0.32 nM. These experiments required incubation for three hours at 37°C to assure equilibration. Membranes were suspended in binding saline containing 2 mg/ml bovine serum albumin (Sigma-Aldrich) to reduce non-specific binding. In each experiment, 48 disposable glass culture tubes 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 pre-soaked 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  $^3\text{H}$ -cytisine bound membranes were collected in 20 ml scintillation tubes and suspended in 8 ml 30% Scintisafe (Fisher Scientific, Atlanta, GA) scintillation fluid, then counted in a Beckman LS-6500 liquid scintillation counter after standing overnight. Filters containing  $^{125}\text{I}$ - $\alpha$ -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 4 replicates; the data was fitted to a sigmoidal concentration response curve for one site binding, from which the Hill slope ( $n$ ) and  $\text{IC}_{50}$  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  $X=\text{Log (Compound)}$ , 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:  $K_i = \text{IC}_{50} / [1 + (\text{Radioligand}) / K_d]$ . For [ $^3\text{H}$ ]-cytisine, the binding  $K_d$  was 0.92 nM for rat brain membranes (RBM) and 0.48 nM for human  $\alpha 4\beta 2$  receptors expressed in TsA201 cell membranes. For  $^{125}\text{I}$ - $\alpha$ -Btx, the  $K_d$  was 0.67 nM for RBM and 0.97

nM for human  $\alpha 7$  receptors expressed in SH-EP1 cell membranes. One way ANOVA of  $\log_{10}$  transformed compound mean  $K_i$ s obtained from a number of individual experiments was used to compare analogs with nicotine, with 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* Acetylcholine Binding Protein (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  $\alpha 7$  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 v8.0 (HyperCube Inc., Gainesville, FL) was used to sample the potential energy surface to identify the lowest energy conformation of each molecule. One thousand conformations per molecule were generated by varying randomly all dihedral angles and using an acceptance energy cut-off of 6 kcal/mol above the best. All conformations with an energy difference of less than 0.05 kcal/mol were considered duplicates and 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 QM optimization in GAMESS (Schmidt et al., 1993) using an STO-3G basis set to represent the wavefunction in the Hartree-Fock approximation. Molecular surface calculations

for each nicotine analog were made using MSROLL (Dock 6.5 Manual, UCSF, San Francisco, CA). Each molecular surface was used as input for the sphere generating program SPHGEN (Dock 6.5, UCSF). 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 3-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 LWA (Amiri et al., 2007) bound to the side chain oxygen on the complimentary surface, (3) the site containing LWB binding to Tyr 192 (Amiri et al., 2007) and finally, and (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 Table S2). 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  $K_i$ s) associated with their binding to SH-EP1 cell expressed human  $\alpha 7$  receptors (Table 2). The experimental and predicted free energies ( $\Delta G$ s) are presented in Table 5.

[Figure 2]

## RESULTS

### Concentration-response relationships for ACh, nicotine and the methylnicotines

We measured the functional properties of each methylnicotine, namely, the  $EC_{50}$  (inversely related to potency) and  $I_{max}$  (maximal ACh-normalized current), using *Xenopus* oocytes expressing human  $\alpha 7$  or  $\alpha 4\beta 2$  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 Figure S1. In addition, the same curve for nicotine, but without the standard error bars, is included as a dashed line in each concentration-response curve in Figure 2 to allow visual comparison with the nicotine analog curve. Because the  $\alpha 7$  receptor is much less sensitive to ACh than the  $\alpha 4\beta 2$  receptor, the standard calibrating agonist was 1,000  $\mu M$  ACh, but for the  $\alpha 4\beta 2$  nAChR it was 100  $\mu M$  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  $\alpha 7$  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  $\alpha 7$  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 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 potency ( $EC_{50}$ ) estimates were 2-3-fold smaller (See PC/NC ratios in Table 1).

[Tables 1&2]

Another consideration in the interpretation of the *Xenopus* oocyte functional data obtained with  $\alpha 4\beta 2$  nAChRs regards their subunit stoichiometry. Our functional measurements of the methylnicotine effects were nearly complete when the co-expression of  $\alpha 4\beta 2$  nAChRs with different stoichiometries and pharmacological properties was shown to be of general occurrence (Moroni et al., 2006). The  $\alpha 4_3\beta 2_2$  low ACh-nicotine sensitivity receptor was reported to have a nicotine  $EC_{50}$  that is ~50X higher than for the high sensitivity  $\alpha 4_2\beta 4_3$  receptor (Tavares et al., 2012). In our *Xenopus* oocyte experiments we routinely injected equal amounts of the two mRNAs. The nicotine concentration-response curve (Figure S1) that we obtained for  $\alpha 4\beta 2$  nAChRs was fitted well 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  $\mu$ M ACh response, which was ~60% of the  $I_{\max}$  for ACh. In published studies where  $\alpha 4$  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  $\alpha 4_2\beta 2_3$  receptor. Our more recent experiments co-injecting an  $\alpha 4\beta 2$  concatamer mRNA with the  $\beta 2$  mRNA, which should yield only  $\alpha 4_2\beta 2_3$ -like functional receptors (Fig S2), indicate that the near maximal concatameric responses for several key analogs were very similar to the maximum responses in our routine *Xenopus* experiments (Fig. S3), further supporting our contention that the latter primarily assessed the properties of the high affinity stoichiometry  $\alpha 4_2\beta 2_3$  subtype.

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  $\alpha 4_3\beta 2_2$  receptors, since we measured displacement at such a low concentration (1 nM) of [ $^3$ H]-cytisine that few of these receptors would be occupied (Tavares et al., 2012; Moroni et al., 2006).

The variance estimates for the mean potencies ( $EC_{50}$ ) and binding constants ( $K_i$ s) of the compounds tended to increase as their mean values increased, so we used a  $\log_{10}$  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 methylnicotine will now be presented and discussed in order of the five pyrrolidinium ring atoms 1' to 5' (Figure 1) numbered according to the International Union for Pure and Applied Chemistry nomenclature. Data for receptor  $\alpha 7$

nAChRs will be presented initially since interactions of these compounds with this receptor subtype have not been previously reported.

*[Tables 3 and 4]*

### **1'-Methylnicotine and 1'-Ethylnornicotine**

Ample evidence exists that the monocationic form of nicotine and of most other ionizable nAChR agonists is the form which binds to nAChRs with highest affinity (Barlow and Hamilton, 1962; Jeng and Cohen, 1980; Kem et al., 2004). Since the published pK<sub>a</sub> for ionization of the 1'-N of nicotine (8.05, Fujita et al., 1971) is 0.65 units above physiological pH of 7.4 and the methylnicotine analogs were found to have similar pK<sub>a</sub>s (Table S1), 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 Table S1 for estimation of the % 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 agonist properties (Figure 2, Table 1) and binding affinities (Table 2) of the quaternary nitrogen analog, 1'-methylnicotinium iodide, were similar to those of nicotine, but they were diminished at  $\alpha 4\beta 2$  receptors (Figure 2, Tables 3 and 4). Thus, methyl quaternization of the pyrrolidinium nitrogen is well tolerated by  $\alpha 7$  but not  $\alpha 4\beta 2$  nAChRs.

Replacing the 1'-N-methyl group of nicotine with a larger alkyl group, as in 1'-ethylnornicotine, also differentially affected agonistic properties at these two receptors. The ethyl substituent was relatively well tolerated by the  $\alpha 7$  receptor: only the I<sub>max</sub> was statistically

different from that of nicotine (Table 1). However, at  $\alpha 4\beta 2$  nAChRs 1'-ethyl-nornicotine potency and efficacy was inferior to that of nicotine (Table 3). We also found that racemic 1'-propyl-nornicotine displayed almost a thousand-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, who displayed reduced agonist properties relative to nicotine. 2'-methylnicotine displayed agonist properties superior 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 7fold 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) higher 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 (Figure 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). While 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  $\geq 30$  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 (Figure 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  $I_{\max}$  >100 fold (Table 3). The binding  $K_i$ 's for rat and human  $\alpha 4\beta 2$  receptors also were increased >2,000 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'-methyl-nicotine was less affected than that of the cis-form (Lin et al., 1994; Wang et al., 1998).

While cis-5'-methylnicotine also displayed the greatest reduction in interaction with  $\alpha 7$  receptors, trans-5'-methylnicotine was a relatively potent agonist, potency decreasing only 2-3 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).

*[Figure 3]*

### **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 et al., 2016). If  $K_i$  estimates are related to the  $K_d$ , then our  $EC_{50}$  estimates might show some relationship to the  $K_i$ s, 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 (Figures 3A 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 (Part A,  $\alpha 7$  correlation coefficient  $r^2 = 0.90$ ; Part C,  $\alpha 4\beta 2$   $r^2 = 0.61$ ). Since the  $\alpha 7$  net charge determined  $EC_{50}$ s (Table 1) were ~3 fold less than the peak current  $EC_{50}$  estimates, using our standard 2 sec 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 Part A of this Figure.

It was recently reported that there is a linear relationship between  $\text{Log } I_{\max}$  and  $\text{Log } EC_{50}$  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 et al. study and oocyte net charge response here, a similar correlation was observed with our  $\alpha 7$  data (Figure 3B,  $r^2=0.67$ ). In Figure 3B the  $I_{\max}$  estimates for the most efficacious compounds (2'MeNic, 1'MeNic and Nic) are very similar, though their  $EC_{50}$ s differ considerably, which would not be predicted from the Auerbach equation; it seems likely that desensitization may limit the  $I_{\max}$ s measured for these highly potent agonists under our experimental conditions. In contrast to the  $\alpha 7$  plot in 3B, the  $\alpha 4\beta 2$  plot showed much more scatter when plotted in an identical fashion (Figure 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 *Aplysia californica* AChBP-epibatidine 3SQ6 structure where 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 non-water atoms in the AChBP binding site. Actually, the 2'-methyl is oriented towards 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 methyl-nicotines (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 Table S2 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'-methylnicotine 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  $\Delta G$ s (Table 5) of the various analogs relative to the  $\Delta G$  for nicotine binding at the same receptor site, expressed within the parentheses as a percent change, are most salient. The relative  $\Delta G$  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.

[Table 5]

## **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 binds this compound more readily than nicotine. To determine whether A84543 would be similarly affected by 5'-methylation, we synthesized (See Supplement) 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'-methyl-A84543. The rat brain high affinity receptor  $K_i$  for A84543 has been reported to be  $0.15 \pm 0.01$  (Abreo et al., 1996) and  $3.44 \pm 0.40$  nM (Ogunjirin et al., 2015) and the  $\alpha 7$  receptor  $K_i$  was reported to be  $340 \pm 50$  nM (Ogunjirin et al., 2015). While the difference we observed between the two methylated A84543 enantiomers was approximately 10X less than it was for the 5'-methyl-nicotines, it is clear that the preferential binding of the trans-5'-methylnicotine also applies to trans-5'-methyl A85443. 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'-methyl-A84543 (Figure 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 tran-5'methyl-(S)nicotine.

*[Figure 4, Table 6]*

## 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 physiological 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  $pK_{a1}$  was 8.00 and its ionization was 80% at pH 7.4 (Table S1). The  $pK_{a1}$  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  $pK_{a1}$ .

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-ethylnornicotine, 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 its interaction with the  $\alpha 7$  nAChR subtype and may not be essential (Puskar et al., 2011; van Arnam et al., 2013). The lesser effects on  $\alpha 7$  interaction of these two 1'-N modifications suggests that a small increase in bulk of the pyrrolidinium group does not adversely affect cation- $\pi$  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 group and that may favor formation of an H bond between the 1'NH<sup>+</sup> 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, 2011; 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. While 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 Arnum 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 pyrrolidinyll N than on the three C atoms (1', 2' and 5') attached to the nicotine 1'N (Elmore and Dougherty, 2002). Regardless of mechanism, the significant diminution in 5'cis-methyl nicotine binding to both receptors indicates that the 5'- carbon occupies a very critical position that can exert much control of nicotine's interaction with these receptors. The superior activity of 5'trans-methylA84543 relative to 5'cis-methylA84543 (Table 6 and Figure 4) is consistent with the notion that the binding orientation this compound, though it contains an oxymethylene bridge between the pyridyl and pyrrolidinyll rings, is very similar to that of nicotine. Our data regarding the relative affinities of the 5'methyl(R)nicotine enantiomers indicates 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 nicotines on both receptors, as well as for cis-4'-methylnicotine binding to the rat  $\alpha 4\beta 2$  (but not the  $\alpha 7$  receptor). While 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 (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<sup>+</sup> hydrogen-bonding to Trp B and/or

the cation- $\pi$  bonding forces between nicotine and this receptor being directed towards 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, 2002). The four carbon atoms of the pyrrolidinium ring are in a relatively planar “envelope” conformation in crystalline and aqueous solution conditions. While 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 calculated, 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 nicotines on both receptors. Although the functional properties differed, the measured  $-\Delta G$  (Kcal/mol) values for 1'-methylnicotinium, both 3'-methylnicotines and both 5'-methylnicotines were very similar for the two receptors. It is tempting 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**

While 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. CNS 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., 2017). 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 suggests 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 promising



approach, as long as adverse effects exerted through autonomic or neuromuscular nAChRs can be avoided (Sun et al., 2019; Potasiewicz 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 DMXBA (GTS-21), 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 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 methylnicotines 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 methylnicotines and how they differ from nicotine. Crystal structures, especially of 2'methyl- and 5'trans-methylnicotines bound to AChBPs,  $\alpha 4\beta 2$  and  $\alpha 7$  receptors, would also be informative.



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**Table 1. Functional properties of nicotine analogs acting on human  $\alpha 7$  receptors expressed in *Xenopus* oocytes.** Peak current (PC) and net charge (NC) estimates were obtained from each response. Mean estimates  $\pm$  one sample standard deviation of the maximum current response ( $I_{\max}$ ) and potency ( $EC_{50}$ ) are given. (n = the number of oocytes used)

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

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

<sup>†</sup> $p < 0.05$ , <sup>††</sup> $p < 0.01$ , <sup>†††</sup> $p < 0.001$  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.

**Table 2. Nicotine analog binding to mammalian  $\alpha 7$  nAChRs as measured by displacement of [ $^{125}$ I]- $\alpha$ -BTX specific binding.** Mean  $K_i$  estimates  $\pm$  one sample standard deviation are given.

(n = the number of experiments)

Compound	Rat Brain Receptor			Human Receptor (SH-EP1 Cell)		
	$K_i$ (nM)	-Hill Slope	n	$K_i$ (nM)	-Hill Slope	n
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	2,620 $\pm$ 340***	2.3	4	2,370 $\pm$ 32***	1.2	4
2'-MeN	181 $\pm$ 34***	2.9	7	144 $\pm$ 24***	1.2	4
3'Trans-MeN	752 $\pm$ 120	2.4	4	756 $\pm$ 54*	1.2	4
3'Cis-MeN	3,120 $\pm$ 500*** <sup>ttt</sup>	3.1	4	4,780 $\pm$ 12*** <sup>ttt</sup>	1.0	3
4'Trans-MeN	3,910 $\pm$ 30***	2.4	4	6,850 $\pm$ 630***	1.5	5
4'Cis-MeN	2,930 $\pm$ 58*** <sup>ttt</sup>	2.4	4	3,300 $\pm$ 16*** <sup>ttt</sup>	1.3	2
5'Trans-MeN	2,130 $\pm$ 76***	1.5	3	1,970 $\pm$ 420***	1.1	4
5'Cis-MeN	34,800 $\pm$ 2,600*** <sup>ttt</sup>	2.0	4	22,500 $\pm$ 2,600*** <sup>ttt</sup>	1.1	4

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

<sup>t</sup> $p < 0.05$ , <sup>tt</sup> $p < 0.01$ , <sup>ttt</sup> $p < 0.001$  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.

**Table 3. Functional properties of nicotine analogs acting on human  $\alpha 4\beta 2$  receptors**

**expressed in *Xenopus* oocytes.** Mean estimates  $\pm$  one sample standard deviation are given. (n = the number of oocytes used).

Compound	EC <sub>50</sub> ( $\mu$ M)	I <sub>max</sub> (% ACh Response)	n
Nic (N)	0.430 $\pm$ 0.33	93.8 $\pm$ 14	11
1'-Et-NorN	3.65 $\pm$ 2.1 <sup>***</sup>	26.8 $\pm$ 10 <sup>***</sup>	6
1'-MeN	1.38 $\pm$ 0.45 <sup>***</sup>	22.5 $\pm$ 5.4 <sup>***</sup>	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 <sup>***</sup>	5
3'Cis-MeN	3.83 $\pm$ 1.2 <sup>*** ttt</sup>	51.4 $\pm$ 5.4 <sup>***</sup>	5
4"Trans-MeN	0.254 $\pm$ 0.04	55.7 $\pm$ 27 <sup>***</sup>	5
4'Cis-MeN	0.285 $\pm$ 0.14	32.7 $\pm$ 14 <sup>***</sup>	4
5"Trans-MeN	>300 <sup>***</sup>	<1 <sup>***</sup>	4
5'Cis-MeN	>300 <sup>***</sup>	<1 <sup>***</sup>	4

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

<sup>t</sup> $p < 0.05$ , <sup>tt</sup> $p < 0.01$ , <sup>ttt</sup> $p < 0.001$  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.

**Table 4. Nicotine analog binding to mammalian  $\alpha 4\beta 2$  nAChRs measured by displacement of [ $^3\text{H}$ ]-cytisine specific binding.** Mean  $K_i$  estimates  $\pm$  one sample standard deviation are given. (n = the number of experiments)

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*** <sup>††</sup>	0.94	4	161 $\pm$ 37*** <sup>††</sup>	0.92	6
3'Cis-MeN	770 $\pm$ 71 ***	1.0	6	1,380 $\pm$ 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*** <sup>†</sup>	1.1	6	32.6 $\pm$ 16** <sup>†</sup>	1.8	3
5'Trans-MeN	150 $\pm$ 26***	1.0	4	219 $\pm$ 76 ***	1.0	4
5'Cis-MeN	10600 $\pm$ 1620** <sup>†††</sup>	0.87	5	8330 $\pm$ 1030*** <sup>††</sup>	0.97	6

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

<sup>†</sup> $p < 0.05$ , <sup>††</sup> $p < 0.01$ , <sup>†††</sup> $p < 0.001$  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.



**Table 5. Nicotine analog free energies of binding to the two human nAChRs compared with free energies of binding predicted from AChBP crystal structure docking. The number within parentheses is the % increase in  $\Delta G$  of the analog relative to the  $\Delta G$  for nicotine; a plus value represents a reduced affinity of the analog for the receptor relative to nicotine.**

Compound	Human Receptor		AChBP	
	$\alpha 7$ (SH-EP1 Cell)	$\alpha 4\beta 2$ (tsA201 Cell)	Aplysia $\alpha 7$	Lymnaea
	- $\Delta G$ (Kcal/mol)		Predicted - $\Delta G$ (Kcal/mol)	
Nic (N)	8.88 (0%) <sup>1</sup>	10.77 (0%) <sup>1</sup>	8.882	8.883
1'-MeN	8.58 (+3%)	8.66 (+20%)	---	---
1'-EtNorN	7.99 (+10%)	8.96 (+17%)	---	---
2'-MeN	9.72 (-9%)	10.42 (+3%)	9.21	6.09
3'Trans-MeN	8.70 (+2%)	8.66 (+20%)	7.98	7.56
3'Cis-MeN	7.56 (+15%)	7.47 (+31%)	1.22	4.5
4'Trans-MeN	7.34 (+14%)	10.00 (+7%)	8.04	6.36
4'Cis-MeN	7.79 (+12)	9.54 (+11%)	6.3	5.01
5'Trans-MeN	8.11 (+9%)	8.48 (+21%)	8.37	7.16
5'Cis-MeN	6.60 (+26%)	6.47 (+40%)	8.33	3.66

<sup>1</sup> % $\Delta G$  change relative to  $\Delta G$  for nicotine

<sup>2</sup> Predicted nicotine - $\Delta G$ = 34.1 Kcal/mol; normalized to 8.88

<sup>3</sup> Predicted nicotine - $\Delta G$ = 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  $\alpha 7$  receptors.** Mean  $K_i$  estimates  $\pm$  one sample standard deviation 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.

Compound	$\alpha 4\beta 2$ Receptor			$\alpha 7$ Receptor		
	$K_i$ (nM)	-Hill Slope	n	$K_i$ (nM)	-Hill Slope	n
A84543 <sup>1</sup>	3.44 $\pm$ 0.40			340 $\pm$ 50		
5'Trans-MeA84543	18.5 $\pm$ 17 <sup>tt</sup>	1.0	4	603 <sup>ttt</sup> $\pm$ 170	1.9	5
5'Cis-MeA84543	201 $\pm$ 26	1.0	3	2,140 $\pm$ 830	1.8	5
5'Trans-Me(R)N	480 $\pm$ 140 <sup>t</sup>	1.1	3	3920 $\pm$ 2300	2.9	2
5'Cis-Me(R)N	204 $\pm$ 89	1.0	5	426	2.8	1
3'Trans-Me(R)N	553 $\pm$ 140 <sup>ttt</sup>	1.1	4	6720 $\pm$ 710 <sup>tt</sup>	3.6	3
3'Cis-Me(R)N	4340 $\pm$ 1400	1.1	4	22000 $\pm$ 3600	1.7	3

<sup>t</sup> $p < 0.05$ , <sup>tt</sup> $p < 0.01$ , <sup>ttt</sup> $p < 0.001$  Comparison of cis- and trans-enantiomers (These p values were obtained from a two-tailed t test.)

<sup>1</sup>A84543  $K_i \pm$ SEM binding data of Ogunjirin et al. (2015); Abreo et al (1996) reported  $K_i$ 's of 0.15 nM and 1 nM for A84543 and nicotine, respectively, at rat brain  $\alpha 4\beta 2$  receptors

## FIGURE LEGENDS

### Figure 1. Structure and IUPAC 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.

**Figure 2. *Xenopus* concentration-response curves for nicotine and the methylnicotines on human  $\alpha 7$  (Left) and  $\alpha 4\beta 2$  (Right) 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  $\pm 1$  SEM. 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 Supplement Figures 1S and 2S.

**Figure 3. Correlations between *Xenopus* oocyte functional and cultured cell receptor binding data.**  $I_{max}$  and  $EC_{50}$  estimates were determined from human  $\alpha 7$  receptors expressed in *Xenopus* oocytes using the net charge method, while  $K_i$ s were determined by [ $^{125}$ I]-BTX displacement using SH-EP1 cell membranes expressing human  $\alpha 7$  receptors (A,B) ,  $EC_{50}$ s from human  $\alpha 4\beta 2$  receptors expressed in oocytes and  $K_i$ s from tsA201 cell membranes (C,D) (A). Nicotine analog correlation between binding  $K_i$  from SH-EP1 cells and  $EC_{50}$  from  $\alpha 7$  receptor *Xenopus* oocytes; (B) Nicotine analog correlation between  $\alpha 7$  receptor net charge  $EC_{50}$  and  $I_{max}$  for each compound; (C) Nicotine analog correlation between and  $\alpha 4\beta 2$  receptor  $K_i$ s (tsA201

cells) and  $EC_{50}$ s (oocytes); and **(D)** Nicotine analog correlation between human  $\alpha 4\beta 2$  receptor  $EC_{50}$  and  $I_{max}$ .

**Figure 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 SEM. (The 5'cis-methylA84543 enantiomer was much less active: at 30  $\mu$ M the peak  $\alpha 7$  response was approximately 10% of the 1 mM ACh response and the  $\alpha 4\beta 2$  response was ~0 % of the 100  $\mu$ M ACh response).

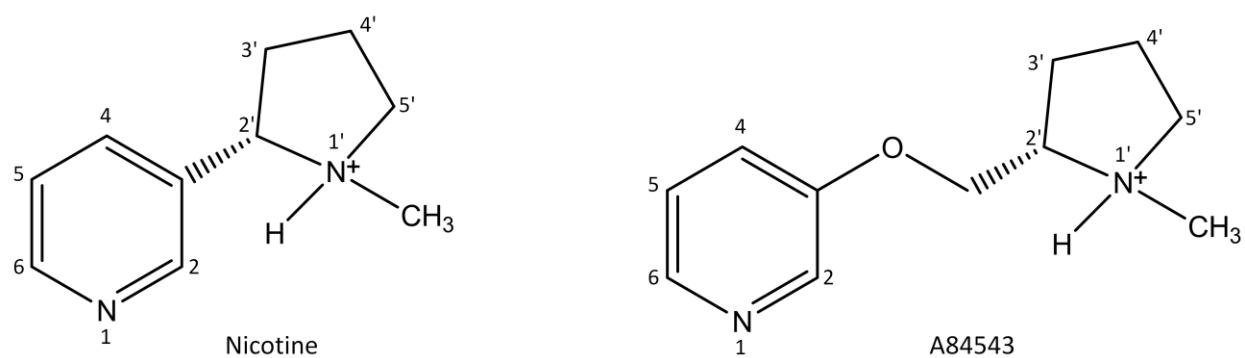
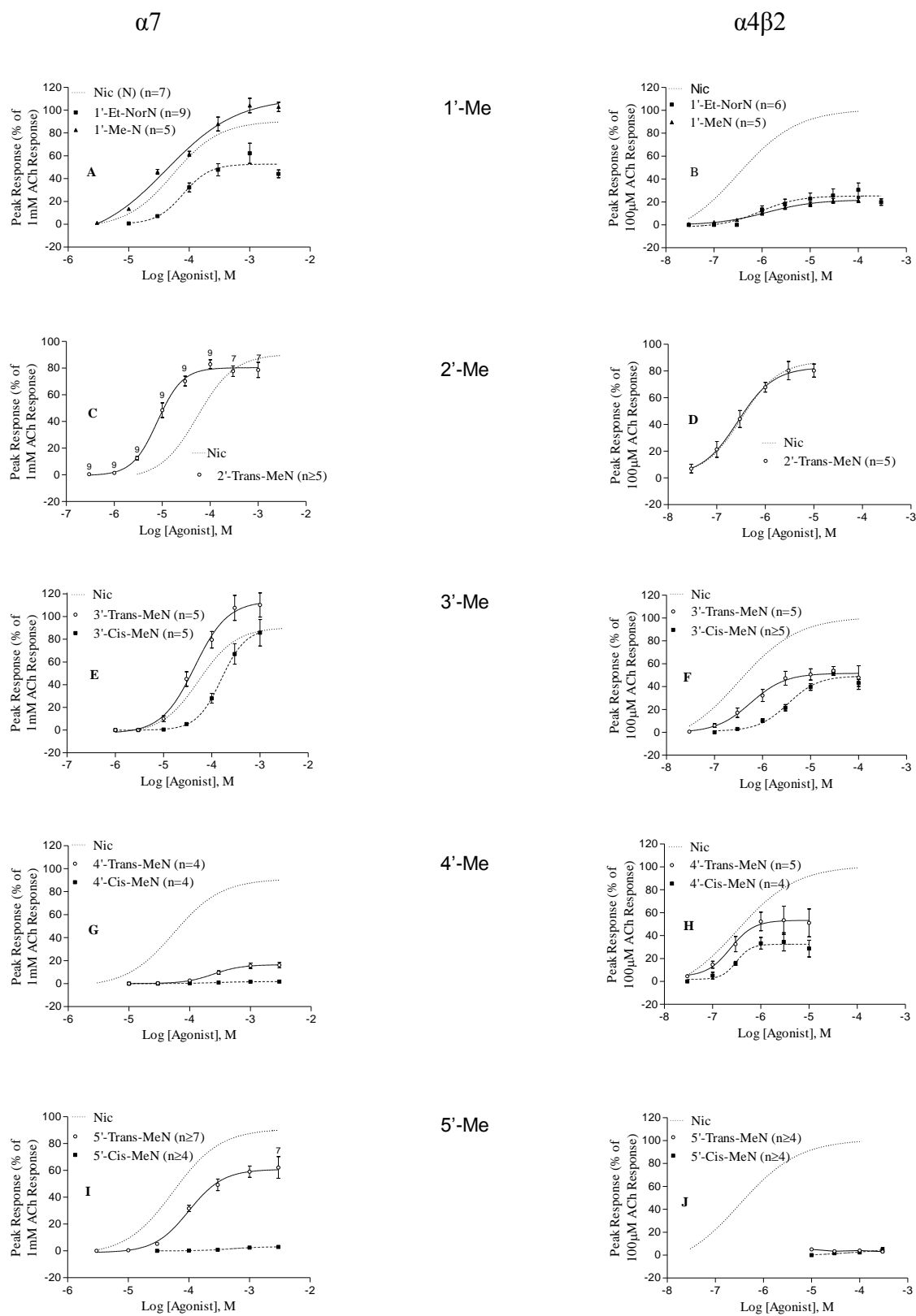
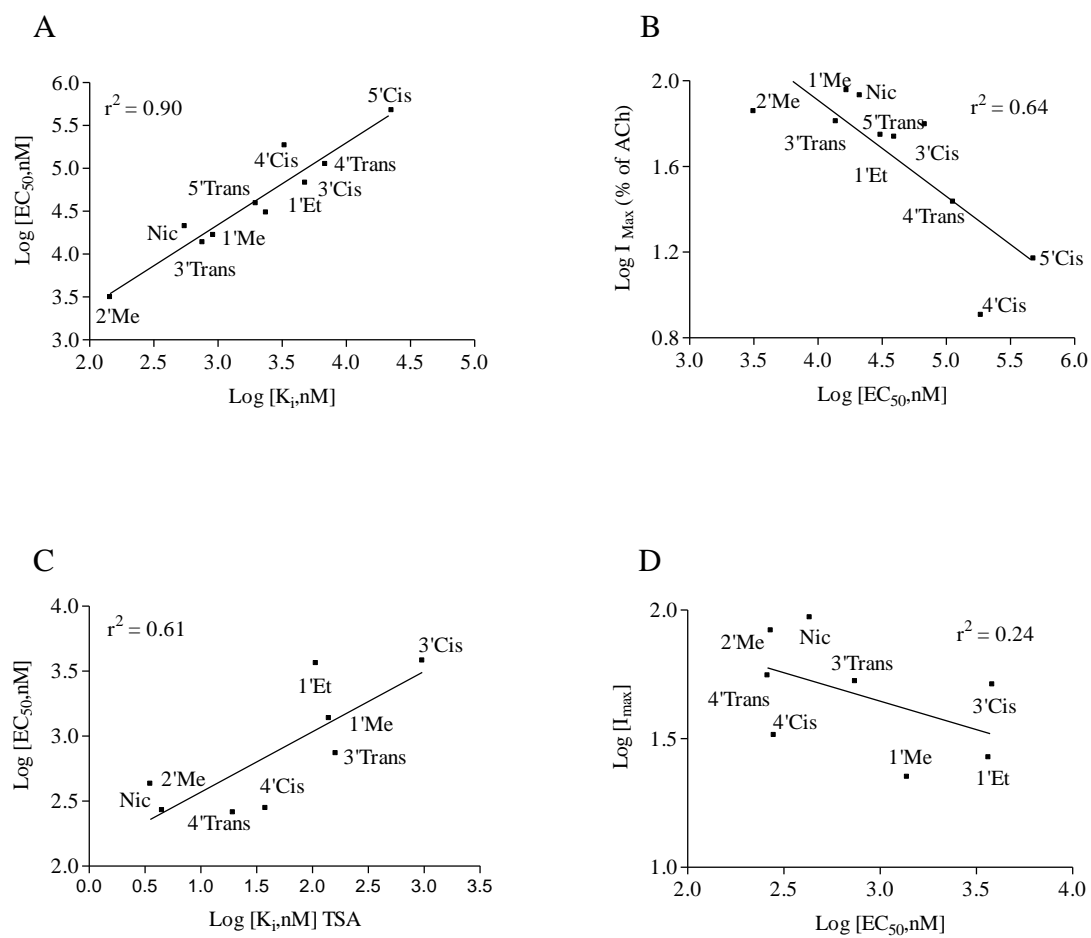
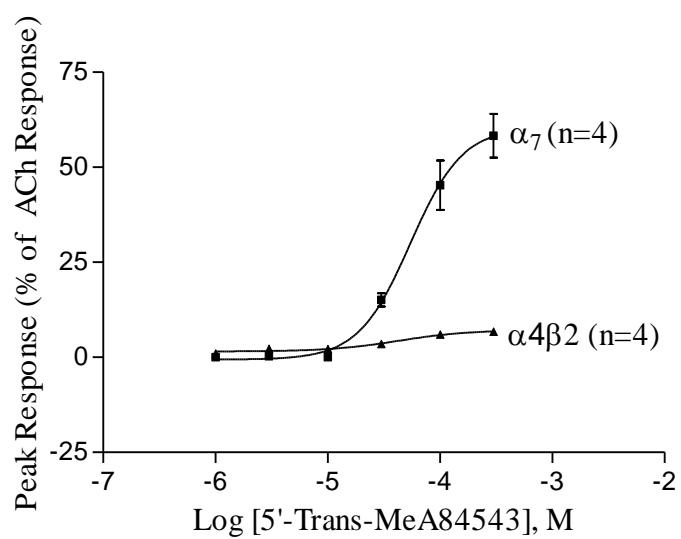
**Fig. 1**

Fig. 2



**Fig.3**



**Fig. 4**

**Authorship Contributions**

*Participated in research design:* Xing, Andrud, Slavo, Jahn, Corsino, Lindstrom, and Kem

*Conducted experiments:* Xing, Andrud, Cho, Habibi, Ziang, Slavo, Jahn, Corsino and Kem

*Contributed new reagents or analytic tools:* Lukas, Lindstrom and Slavo

*Performed data analysis:* Xing, Andrud, Slavo, Jahn, Corsino, Cho, Habibi, Ziang, Lindstrom, and Kem

*Wrote or contributed to the writing of the manuscript:* Kem, Xing, Jahn, and Slavo, Ziang