EFFECTS OF 5-HYDROXYINDOLE ON THE AFFINITY OF AGONISTS FOR THE ALPHA7 NICOTINIC ACETYLCHOLINE RECEPTOR

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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The two most abundant neuronal nicotinic acetylcholine receptor (nAChR) subtypes are the α7 and the α4β2. The α7 subtype is characterized by high affinity binding to α-bungarotoxin and rapid desensitization. DMXBA (GTS-21) is a partial agonist for the α7 receptor that has been shown to enhance cognition. Positive allosteric modulators (PAMs) function by enhancing agonist activity without causing desensitization. 5-hydroxyindole (5-HI) is a positive allosteric modulator (PAM) of the α7 nAChR. I examined the effects of 5-HI on α7 receptor binding by: anabaseine-type agonists, tertiary amines, and quaternary ammonium agonists. Using radioligand competition binding assays we have shown that 5-HI enhances α7 receptor affinities for all three types of agonists. It was also found that 5-HI reduced the Hill slope for binding of all of the agonists except choline and succinylcholine. The anabaseine agonists and quaternary ammonium agonists (omitting choline) show a correlation between efficacy and IC_{50} ratios. Only tertiary amines displayed an apparent increase in Hill slope ratio with efficacy. Other known PAMs did not show DMXBA binding enhancements like 5-HI. Other indoles similar to 5-HI also did not change the IC_{50} or Hill slope values of DMXBA. We determined that 5-HI is not competing for the binding sites of the agonist epibatidine or the antagonist α-bungarotoxin on the α7 receptor.

CHAPTER 1
INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are members of a superfamily of ligand-gated ion channels. Other members of this family include $GABA_A$, $GABA_C$, glycine, and serotonin ($5HT_3$) receptors (Romanelli et al., 2007). Currently, 17 nAChR subunits have been cloned including five muscle-type subunits (α1, β1, δ, γ, and ε) and 12 neuronal subunits (α2- α10 and β2- β4). Unlike the muscle-type subunits, the neuronal subunits can exist in a wide array of combinations to form many different subtypes of receptors each with unique properties (Jensen et al., 2004).

Neuronal nAChRs are distributed throughout the central nervous system (CNS) and the peripheral nervous system where they participate in cholinergic synaptic transmission and also modulate the activity of non-cholinergic synapses (Gotti and Clementi, 2004). The two most abundant brain receptor subtypes are the heteromeric α4β2 and the homomeric α7 nAChRs, which respectively bind nicotine and the snake toxin α-bungarotoxin (α-BTX) with high affinity. These subtypes can be found on cortical and hippocampal neurons and have been of interest lately because of their proposed involvement in neuronal pathologies such as Alzheimer's disease (AD). AD is the most common form of dementia in the elderly, affecting nearly 10% of the population over age 65. It is characterized by plaques of β-amyloid peptides and neurofibrillary tangles in the brain. Decreased cholinergic transmission may manifest itself in the form of memory loss and cognitive impairments (Hogg et al., 2003). Brains of patients with AD show a loss of nAChRs in regions that correlate with the loss of cholinergic transmission. In the cerebral cortex, while levels of receptors containing the α4 subunit are greatly (~50%) reduced, α7 subunit receptor levels show much smaller decreases (Albuquerque et al., 2001). The α7 receptor is also highly permeable to calcium, an activator of many signal transduction

pathways in the cell. The cytoprotective properties seen by activating α7 nAChRs may be caused at least partly by its effects on calcium signaling (Dajas-Bailador et al., 2000). This makes the homomeric α7 receptor a potential target for developing drugs intended to increase cholinergic transmission and protect neuronal signaling pathways in neurodegenerative diseases (Kem, 2000).

GTS-21 (DMXBA) is a selective partial agonist for the α7 receptor that has been shown to enhance cognition and neuroprotection in animal studies and in clinical trials (Kem et al., 2004; Kem, 2006). Kitagawa et al. (2003) reported a phase I clinical trial on healthy young male volunteers that indicated improvements in attention. In 2006 Olincy et al. reported another phase I clinical trial on GTS-21. The drug was given to patients who suffered from schizophrenia. They found that GTS-21 had a positive effect on cognition.

Treating nAChRs with nicotinic agonists for therapeutic purposes may have some limitations. First, even nAChR subtype specific drugs might have unwanted side effects in other parts of the body where the nAChR is present (Clementi et al., 2000). Another problem is nAChR desensitization by agonists. This poses a particular issue for synaptic transmission mediated by α7 nAChRs because of their extremely rapid desensitization (Uteshev et al., 2002). An alternative therapeutic approach that would avoid the desensitizing effects of exogenous agonists would be the use of positive allosteric modulators (PAMs). For instance, diazepam is a PAM for certain $GABA_A$ receptors. PAMs bind to their receptor target and enhance the probability that endogenous agonists will bind to and open the channel, without opening the channel when acting alone (Hogg et al., 2005). PAMs could aid in the development of drugs for disorders such as AD because they reduce the problem of desensitization and other unwanted side effects associated with exogenous agonists.

A few compounds such as ivermectin (Krause et al., 1998), galantamine (Samochocki et al., 2003), PNU-120596 (Hurst et al., 2005), and 5-hydroxyindole (5-HI) (Zwart et al., 2002) have been found to be PAMs of α7 nAChRs. 5-HI, the aromatic moiety of 5-HT, was first shown to slow desensitization of 5-HT_3 receptors (Kooyman et al., 1993). In 2002, Zwart et al. showed that 5-HI also increased the potency and efficacy of ACh on α7 receptors as recorded electrophysiologically with *Xenopus* oocytes. 5-HI produced a maximal effect at concentrations around 2mM. Interestingly, in contrast with findings of Kooyman et al. for 5-HT_3 receptors, 5-HI did not affect the desensitization kinetics of ACh currents on α7 nAChRs (Zwart et al., 2002). Grantham et al. (2004) found that 5-HI enhanced the affinity for both selective and non-selective agonists for the α7 receptor, but not antagonists. They also reported that 5-HI does not compete directly for the ACh binding site with competitive antagonists on the α7 receptor.

We were curious to see if the degree of binding affinity enhancement by 5-HI on various α7 agonists correlated with their reported efficacies. In that case, 5-HI could be a useful tool in predicting whether new compounds are partial or full agonists at the α7 receptor by performing radioligand binding assays in the presence and absence of 5-HI. We chose to look at agonists of varying efficacies from three groups of agonists based on their chemical structures. The groups consisted of benzylidene-anabaseines, other tertiary amines and quaternary ammonium agonists (Figure 1-1). We also tested some of the other previously mentioned PAMs to determine if they produced similar effects to 5-HI on DMXBA binding. Finally, we investigated whether some indoles with structures similar to 5-HI can also produce a similar enhancement of receptor affinity for DMXBA.

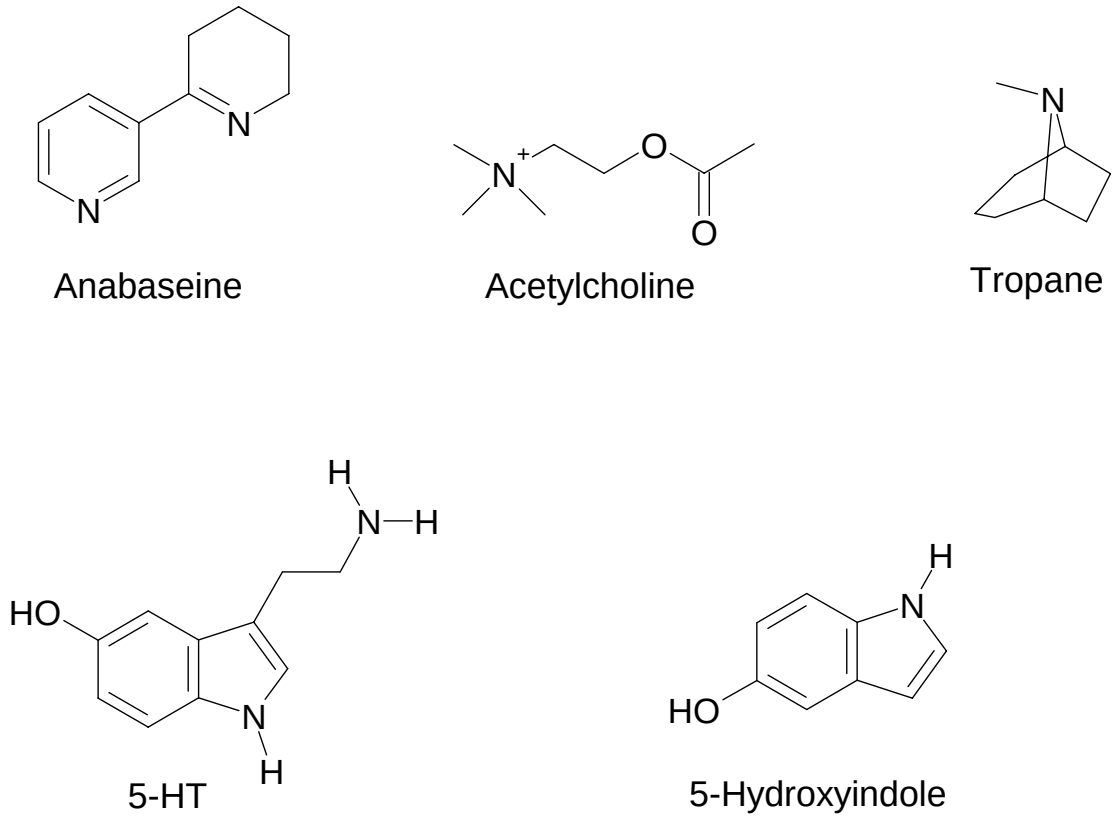


Figure 1-1. Examples of chemical structures from each of the three groups looked at along with 5-HT and 5-HI. Anabaseine is an example of an anabaseine compound. Acetylcholine is an example of a quaternary ammonium compound. Tropane is an example of a tertiary amine compound. 5-HT is an endogenous agonist for the 5-HT3 receptor. 5-HI is a metabolite of 5-HT that acts as a PAM at α7 nAChRs.

CHAPTER 2
MATERIALS AND METHODS

Chemicals

Dr. Ferenc Soti synthesized all benzylidene anabaseine compounds in the Kem lab. NMR was used to confirm the compound structure. All other chemicals were obtained from either Sigma Chemical Co. (St. Louis, MO), Tocris Bioscience (Ellisville, MO), or from Fisher Scientific (Fair Lawn, New Jersey).

Rat Brain Membranes

Whole Sprague-Dawley rat brains were received frozen and unstripped from males only from Pel-Freez Biologicals (Rogers, AR). They were prepared according to Marks and Collins (1982). The amount of protein was calculated using the BCA protein assay reagent kit from Pierce (Rockford, IL). The brain suspension was centrifuged at 11,000 rpm for 10 minutes. The pellet was collected and homogenized in binding saline (120 mM NaCl, 50 mM Tris-buffer, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$; pH=7.4) containing 2 mg/ml of bovine serum albumin (Sigma, St. Louis, MO). The homogenized brain was added to test tubes containing 200 µl of 2 mg/ml bovine serum albumin dissolved in binding saline, 50 µl of compound, and 50 µl of radioligand. For experiments with acetylcholine, the brain membrane suspension was incubated for 30 minutes at room temperature in a 200 µM concentration of DFP (Sigma, St. Louis, MO) to inhibit the enzyme acetylcholine esterase. Binding experiments used 200 µg of homogenized rat brain protein in a final volume of 500 µl.

Cell Culture

Cells from the SH-EP1 human epithelial cell line transfected to express the human α7 nAChR were obtained from Dr. Ronald J. Lukas (Barrow Neurological Institute, Phoenix, AZ). Cells were grown in Dulbecco's modified Eagle's medium from Irvine Scientific (Santa Ana,

CA). Each 500 ml bottle of medium was supplemented with 25 ml fetal bovine serum (MediaTech Inc., Herdon, VA), 50 ml heat inactivated horse serum (Gibco, Carlsbad, CA), 5 ml sodium pyruvate (100 mM, 11.0 mg/ml) (Irvine Scientific, Santa Ana, CA), 10 ml L-glutamine (200 mM, 29.2 mg/ml) (Irvine Scientific, Santa Ana, CA), 5 ml penicillin-streptomycin (10,000 units/ml) (Irvine Scientific, Santa Ana, CA), and 100 µl of 10 mg/ml amphotericin B (Sigma, St. Louis, MO). Hygromycin B (Calbiochem, La Jolla, CA) was added to the medium at a final concentration of 0.4 mg/ml. The cells were kept in a humidified environment with 5% CO_2 at 37° C. After reaching confluence, cells were collected in binding saline for binding experiments and prepared in the same manner as the rat brain. 70 µg of membrane protein was used for binding experiments in a final volume of 500 µl.

Radioligand Binding Experiments

[^{125}I]α-bungarotoxin (150 Ci/mmole) and [^{3}H]epibatidine (47 Ci/mmole) were obtained from PerkinElmer Life and Analytical Sciences (Billerica, MA) and GE Healthcare Bio-sciences Corp. (Piscataway, NJ). The final concentration of the radioligands in the experiment was 1 nM. 1 mM nicotine was used to calculate non-specific binding. In [^{125}I]α-bungarotoxin experiments the membranes were incubated for 2.5 hours at 37° C, while in [^{3}H]epibatidine binding experiments the membranes were incubated for 3 hours at room temperature. After incubation the samples were vacuum filtered on a Brandel cell harvester (Gaithersburg, MD) using Whatman GF/C glass filters that were presoaked for 45 minutes in 0.5% polyethylenimine. The samples were filtered three times with 3 ml of ice-cold binding saline with the same composition used for preparing the rat brain membranes. The filters containing [^{3}H]epibatidine were placed in 8 ml of 30% Scintisafe scintillation fluid (Fisher). Radioactivity was counted for 5 minutes using a Beckman 5500B biogamma counter for the [^{125}I]α-bungarotoxin, or a Beckman LS-6500

liquid scintillation counter for the [^{3}H]epibatidine. Each concentration of drug used for competition binding assays was performed in quadruplicate.

Binding Assay Data Analysis

Binding assay data were analyzed using GraphPad Prism software (San Diego, CA). The counts per minute were calculated based on the five minute counts to calculate radioactivity. Saturation assays were analyzed using a one site binding curve with the equation $Y=B_{max}*X/(K_d+X)$. The software then calculated the B_{max} for the tissue and K_d for the radioligand. Scatchard plots were derived from the saturation assays using the software in order to better visualize the data. Data was normalized by setting 100% equal to the specific binding value which was calculated by subtracting non-specific binding from total binding. The data was also normalized by setting zero equal to zero percent. When a curve had an obvious plateau at the top that did not equal 100%, the data was constrained by normalizing 100% to the average of the values of the points in the plateau. When a curve had an obvious plateau at the bottom that did not equal zero percent, the data was constrained by normalizing zero percent equal to the average of the values of the points in the plateau. Competition binding assays were analyzed using a sigmoidal dose response curve with a variable slope using the equation Y=Bottom+(Top-Bottom)/(1+10^((LogIC$_{50}$-X)*Hill slope. Hill slope and IC_{50} values were given by the software and obtained from the previous equation. K_i values were calculated using the Cheng-Prusoff equation ($K_i=IC_{50}/(1+(Ligand)/K_d)$). The K_d for [^{125}I]α-bungarotoxin binding to rat brain membranes was experimentally determined to be 0.32 nM. The K_d value for [^{3}H]epibatidine in SH-EP1 cells was experimentally determined to be 10 nM. Statistical values were calculated using GraphPad Prism software to perform unpaired, two-tailed T-tests.

CHAPTER 3
RESULTS

The effects of 5-HI on three chemically distinct groups of agonists for the α7 nAChRs were investigated. Competition binding assays performed with rat brain membranes were used to determine Hill slope and IC_{50} values for the agonists alone and in the presence of 5-HI at a concentration (2 mM) shown in pilot experiments to produce maximal effects.

IC_{50} Data

The 5-HI effect on the IC_{50} was examined for each of the three agonist groups. The IC_{50} values for the anabaseine agonists were consistently decreased by factors ranging from 2.43 ± 1.71 to 4.64 ± 0.670 (Table 3-1). The IC_{50} ratios for the tertiary amine compounds range from 3.43 ± 0.626 to 6.11 ± 0.359 (Table 3-2). The IC_{50} values of the quaternary ammonium compounds were decreased as well by factors ranging from 1.64 ± 0.178 to 4.96 ± 0.0379 (Table 3-3). Choline and succinylcholine show ratios that are lower then the other ratios in the quaternary ammonium compound group.

5-HI IC_{50} Effects

To test our hypothesis that the efficacy of the agonist might be predicted from binding enhancement by 5-HI, we examined whether efficacies and IC_{50} changes were related for each of the three compound groups. Table 3-4 lists the reported efficacies of the compounds we tested. Figure 3-1 shows a graph of the efficacies compared to the IC_{50} ratios for the anabaseine compounds. There is an apparent relationship between the efficacies and the IC_{50} ratios for these agonists. It appears that the IC_{50} ratios increase as the efficacy increases. The tertiary amine data (Figure 3-2) does not show a relationship between IC_{50} ratio and efficacy. Next, the efficacies of the five quaternary ammonium compounds were compared to their IC_{50} ratios. It was found that there was no apparent relationship between the two parameters unless the choline

point was omitted (Figure 3-3). The rationale for eliminating this compound from consideration in this figure will be discussed later.

Hill Slope Data

The data for the anabaseine compounds is found in Table 3-5. The average Hill slope of the anabaseine agonists was -2.94 (n=7). It was found that 5-HI consistently decreased the Hill slope of the anabaseine agonists by a factor that varied from 1.41 ± 0.188 (anabaseine) to 2.38 ± 0.787 (4-TFMeOBA) (Table 3-5). The data for the tertiary amine agonists is summarized in Table 3-6. The mean Hill slope for the tertiary amine compounds was 2.30 (n=7). The average Hill slope for the quaternary ammonium agonists was -2.98 (n=7). The Hill slopes of the quaternary ammonium agonists were decreased to a lesser degree by 5-HI, relative to the anabaseine compounds (Table 3-7). For both choline and succinylcholine there was not a significant effect of 5-HI on the Hill slopes.

5-HI Hill Slope Effects

The efficacies of the anabaseine compounds were then compared to the degree of enhancement of the Hill slopes (Figure 3-4). The efficacies of the anabaseine compounds were not obviously related to the Hill slope changes caused by 5-HI. Next, the tertiary amine compounds were examined for a relationship between efficacy and Hill slope ratios. It seems that there may be a relation between these two parameters. It appears that the Hill slope ratios increase as efficacy increases (Figure 3-5). The quaternary ammonium compounds did not show any apparent correlation between efficacy and change in Hill slope, unless the point for choline is omitted (Figure 3-6).

Effects of Other Allosteric Modulators on DMXBA Binding

To determine if other PAMs affected agonist binding in the same way as 5-HI, DMXBA was used as a standard agonist to perform competition assays on three other known PAMs:

ivermectin, galanthamine, and PNU-120596 (a Pfizer compound). Figure 3-7 shows the results of the binding assays, which show no obvious changes in Hill slope or IC_{50} value for DMXBA in the presence of these three PAMs.

Effects of Other Indoles Similar to 5-HI

To determine if other indoles which had similar core structures to 5-HI also behaved as PAMs, three other indoles were tested with DMXBA; 5-aminoindole, 5-methoxyindole, and indole-3-acetic acid. Figure 3-8 shows the results for these three indoles, using DMXBA as the displacing ligand. The three indoles did not influence the Hill slope or IC_{50} values for DMXBA.

Effect of 5-HI on [^{3}H]-Epibatidine Binding

Epibatidine is a potent agonist which has been found to bind to α7 receptors expressed in SH-EP1 cells (Peng et al., 2005). When 5-HI was added to DMXBA in the presence of radiolabled epibatidine, the binding curve was not shifted and the Hill slope did not change (Figure 3-9). This might be expected if 5-HI increased affinity for DMXBA and epibatidine equally so that no change in their competition would be expected. Figure 3-10 shows that 5-HI does not compete directly for the agonist binding site as epibatidine is not displaced with increasing concentrations of 5-HI. 5-HI also does not compete directly for the binding site of the antagonist α-bungarotoxin as shown in figure 3-11.

Table 3-1. IC_{50} values for anabaseine compounds without 5-HI, with 5-HI and IC_{50} ratios.

Anabaseine Compounds (n=3)	IC_{50} values (μM) without 5-HI	IC_{50} values (μM) with 2 mM 5-HI	IC_{50} Ratio (without/with)
Anabaseine	1.09 ± 0.0334	0.275 ± 0.0248	3.96 ± 0.241
DMXBA	0.496 ± 0.0288	0.123 ± 0.0204	4.03 ± 0.625
2,4 DHBA	0.00226 ± 0.000656	0.000487 ± 0.000176	4.64 ± 0.670
4-MeS-BA	1.20 ± 0.0682	0.313 ± 0.0448	3.83 ± 0.404
4-TFMeOBA	10.4 ± 1.57	3.13 ± 0.343	3.32 ± 0.373
OMPBA	3.21 ± 0.257	1.32 ± 0.820	2.43 ± 1.71
4-OH-GTS-21	0.404 ± 0.0270	0.0948 ± 0.0105	4.26 ± 0.362

IC_{50} values were determined using GraphPad Prism software. Each value is the mean ± SEM of three experiments. Concentrations were done in quadruplicate for each experiment.

Table 3-2. IC_{50} values for tertiary amines without 5-HI, with 5-HI and their IC_{50} ratios.

Tertiary Amine Compounds (n=3)	IC_{50} values (μM) without 5-HI	IC_{50} values (μM) with 2 mM 5-HI	IC_{50} Ratio (without/with)
Tropane	49.9 ± 18.4	8.17 ± 2.64	6.11 ± 0.359
Tropisetron	0.0666 ± 0.00735	0.0143 ± 0.00222	4.66 ± 0.977
Tropinone	130 ± 1.07	33.5 ± 1.81	3.88 ± 0.224
PNU-282987	0.0808 ± 0.00521	0.0160 ± 0.00308	5.05 ± 1.25
AR-17779	0.748 ± 0.0569	0.161 ± 0.0142	4.65 ± 0.154
Nicotine	2.52 ± 0.372	0.734 ± 0.152	3.43 ± 0.626
Cytisine	3.50 ± 0.433	0.750 ± 0.0274	4.67 ± 0.360

IC_{50} values were determined using GraphPad Prism software. Each value is the mean ± SEM of three experiments. Concentrations were done in quadruplicate for each experiment.

Table 3-3. IC_{50} values for quaternary ammonium agonists without 5-HI, with 5-HI and their IC_{50} ratios.

Quaternary Ammonium Compounds (n=3)	IC_{50} values (μM) without 5-HI	IC_{50} values (μM) with 2 mM 5-HI	IC_{50} Ratio (without/with)
ACh	13.2 ± 0.724	2.66 ± 0.151	4.96 ± 0.0379
Choline	260 ± 57.4	159 ± 53.3	1.64 ± 0.178
Succinylcholine	210 ± 15.5	126 ± 5.82	1.67 ± 0.102
Carbachol	48.7 ± 3.23	11.5 ± 2.09	4.23 ± 0.582
TMA	11.1 ± 1.89	2.91 ± 0.632	3.81 ± 0.348
ETMA	13.3 ± 2.04	3.54 ± 0.790	3.76 ± 0.852
MG-624	0.284 ± 0.0267	0.0834 ± 0.0158	3.41 ± 0.328

IC_{50} values were determined using GraphPad Prism software. Each value is the mean ± SEM of three experiments. Concentrations were done in quadruplicate for each experiment.

Table 3-4. Reported efficacies for the compounds used with 5-HI.

Compound	Efficacy (ACh=100%)	Receptor type	Reference
Benzylidene-anabaseines:			
Anabaseine	100%	Rat	(Kem et al., 2004)
DMXBA	35%	Rat	(Stokes et al., 2004)
2,4 DHBA	65%	Rat	(Hunter et al., 1994)
4-MeS-BA	7.5%	Rat	(Papke et al., 2004 a)
4-TFMeOBA	5%	Rat	(Papke et al., 2004 a)
OMPBA	0	Human	Kem lab unpub. data
4-OH-GTS-21	40%	Human	(Papke et al., 2005 a)
Tertiary Amines:			
Tropane	28%	Human	(Papke et al., 2005 b)
Tropisetron	30%	Human	(Papke et al., 2005 b)
Tropinone	64%	Human	(Papke et al., 2000 b)
PNU-282987	77%	Human	(Gronlien et al., 2007)
AR-17779	100%	Human	(Papket et al., 2005 a)
Nicotine	60%	Rat	(Papke et al., 2007)
Cytisine	90%	Human	(Papke et al., 2005 a)
Quaternary Ammoniums:			
ACh	100%	Human	(Papke et al., 2005 a)
Choline	100%	Rat	(Stokes et al., 2004)
Succinylcholine	25%	Rat	(Placzek et al., 2004)
Carbachol	60%	Human	Papke, personal comm.
TMA	N/A	N/A	N/A
ETMA	N/A	N/A	N/A
MG-624	50%	Human	Kem lab unpub. data

Efficacies are given as determined electrophysiologically by in *Xenopus* oocytes for the given agonists in relation to acetylcholine.

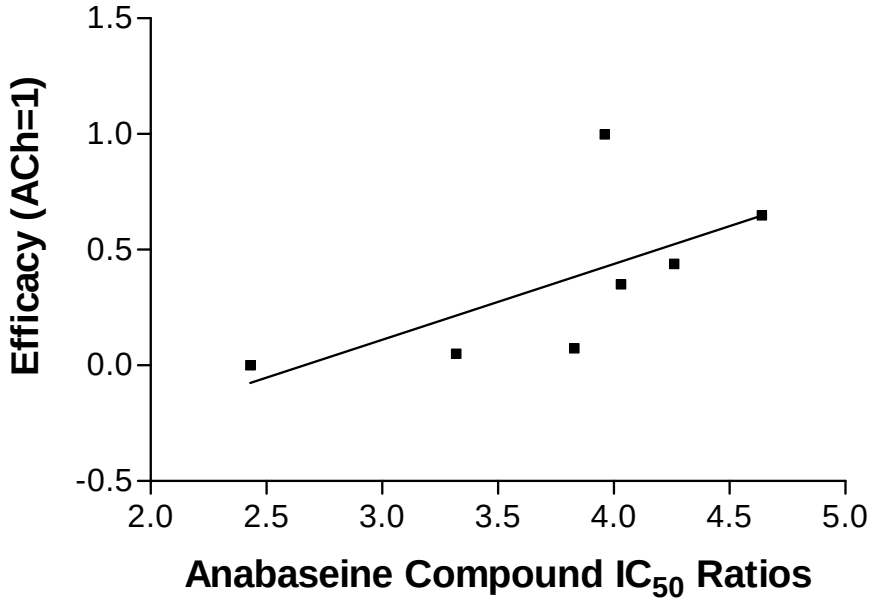


Figure 3-1. Graph of anabaseine compound efficacies vs. anabaseine compound IC_{50} ratios. The line was fitted using a linear regression analysis in GraphPad Prism software. The r^2 value for the linear regression fit is 0.4115.

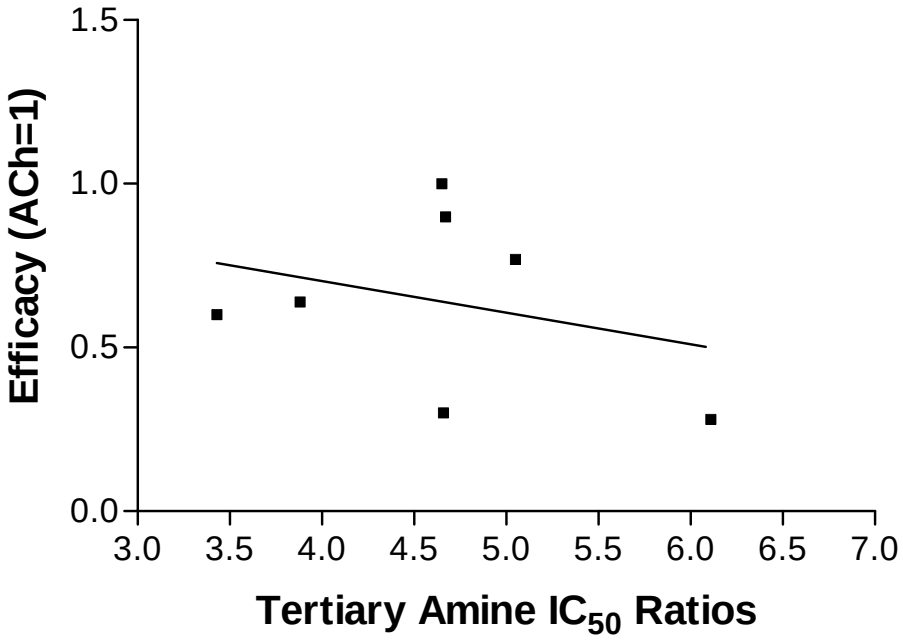


Figure 3-2. Graph of tertiary amine compound efficacies vs. tertiary amine IC_{50} ratios. The line was fitted using a linear regression analysis in GraphPad Prism software. The r^2 value for the linear regression fit is 0.8874.

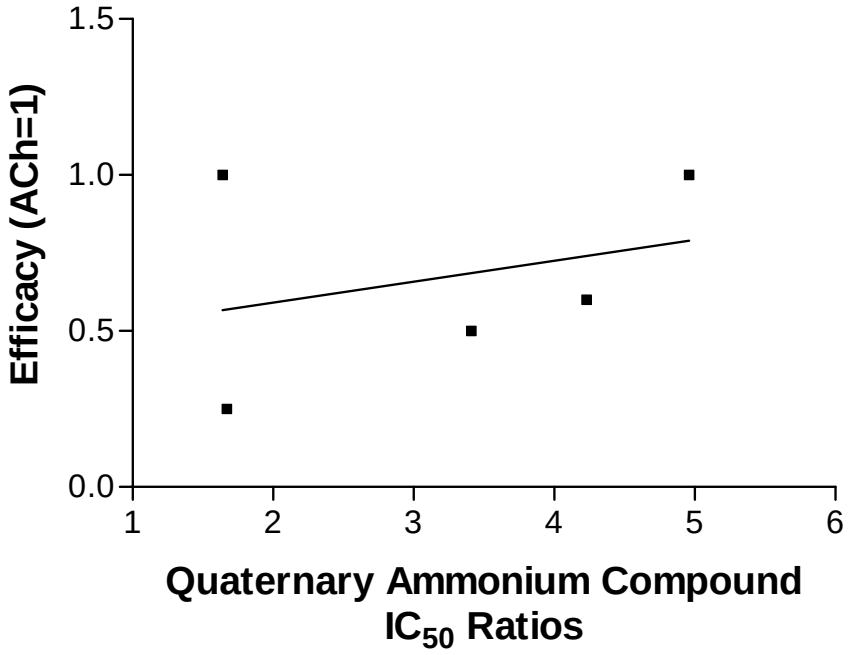


Figure 3-3. Graph of quaternary ammonium compound efficacies vs. quaternary ammonium IC_{50} ratios. The line was fitted using a linear regression analysis in GraphPad Prism software. The r^2 value for linear regression fit is 0.09396.

Table 3-5. Hill slope values for anabaseine compounds without 5-HI, with 5-HI and their Hill slope ratios.

Anabaseine Compounds (n=3)	Hill Slope Without 5-HI	Hill Slope With 2 mM 5-HI	Hill Slope Ratio (without/with)
Anabaseine	-2.50 ± 0.320	-1.77 ± 0.0218	1.41 ± 0.188
DMXBA	-3.34 ± 0.478	-1.59 ± 0.103	2.10 ± 0.294
2,4 DHBA	-2.57 ± 0.268	-1.59 ± 0.296	1.62 ± 0.137
4-MeS-BA	-3.00 ± 0.463	-1.74 ± 0.217	1.72 ± 0.249
4-TFMeOBA	-3.62 ± 1.09	-1.52 ± 0.233	2.38 ± 0.787
OMPBA	-3.31 ± 0.399	-2.10 ± 0.243	1.58 ± 0.379
4-OH-GTS-21	-2.23 ± 0.0464	-1.52 ± 0.200	1.47 ± 0.214

Hill slope values were determined using GraphPad Prism software. Each value is the mean ± SEM of three experiments. Concentrations were done in quadruplicate for each experiment.

Table 3-6. Hill slope values for tertiary amine compounds without 5-HI, with 5-HI and their Hill slope ratios.

Tertiary Amine Compounds (n=3)	Hill Slope Without 5-HI	Hill Slope With 2 mM 5-HI	Hill Slope Ratio (without/with)
Tropane	-1.86 ± 0.293	-1.62 ± 0.0686	1.15 ± 0.216
Tropisetron	-1.70 ± 0.0664	-1.60 ± 0.0964	1.06 ± 0.0384
Tropinone	-2.20 ± 0.2710	-1.70 ± 0.0345	1.29 ± 0.1690
PNU-282987	-2.27 ± 0.121	-1.56 ± 0.134	1.46 ± 0.108
AR-17779	-2.86 ± 0.728	-1.83 ± 0.223	1.56 ± 0.550
Nicotine	-2.69 ± 0.971	-1.77 ± 0.222	1.52 ± 0.353
Cytisine	-2.55 ± 0.898	-1.55 ± 0.0954	1.65 ± 0.131

Hill slope values were determined using GraphPad Prism software. Each value is the mean ± SEM of three experiments. Concentrations were done in quadruplicate for each experiment.

Table 3-7. Hill slope values for quaternary ammonium compounds without 5-HI, with 5-HI and their Hill slope ratios.

Quaternary Ammonium Compounds (n=3)	Hill Slope without 5-HI	Hill Slope with 2 mM 5-HI	Hill Slope Ratio (without/with)
ACh	-2.32 ± 0.174	-1.39 ± 0.0473	1.67 ± 0.176
Choline	-4.96 ± 0.868	-6.88 ± 2.90	0.721 ± 0.224
Succinylcholine	-3.40 ± 0.448	-3.66 ± 0.231	0.929 ± 0.183
Carbachol	-2.66 ± 0.327	-1.53 ± 0.202	1.74 ± 0.0910
TMA	-3.06 ± 0.663	-2.42 ± 0.579	1.26 ± 0.803
ETMA	-1.98 ± 0.746	-1.29 ± 0.158	1.53 ± 0.0991
MG-624	-2.49 ± 0.139	-1.95 ± 0.0464	1.28 ± 0.0667

Hill slope values were determined using GraphPad Prism software. Each value is the mean ± SEM of three experiments. Concentrations were done in quadruplicate for each experiment.

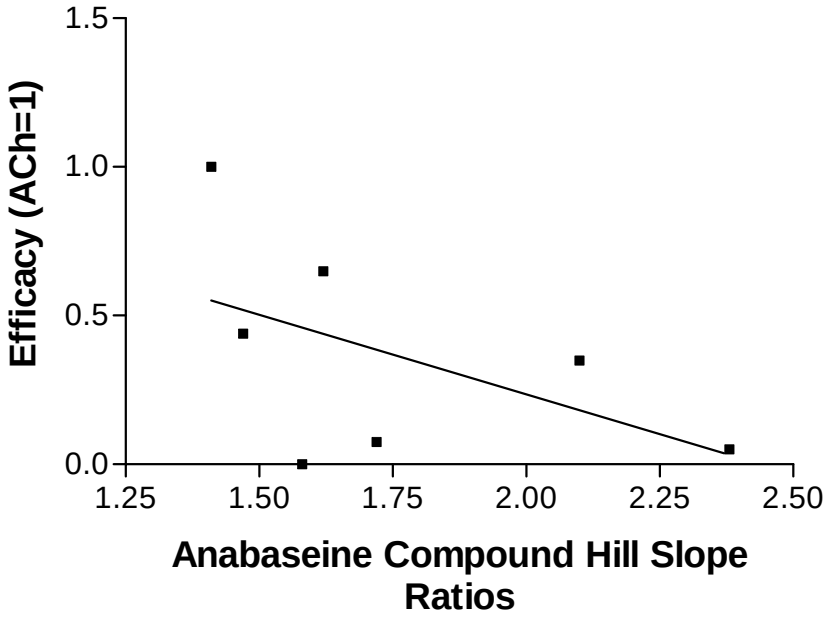


Figure 3-4. Graph of anabaseine compound efficacies vs. anabaseine compound Hill slope ratios. The line was fitted using a linear regression analysis in GraphPad Prism software. The r^2 value for linear regression fit is 0.2701.

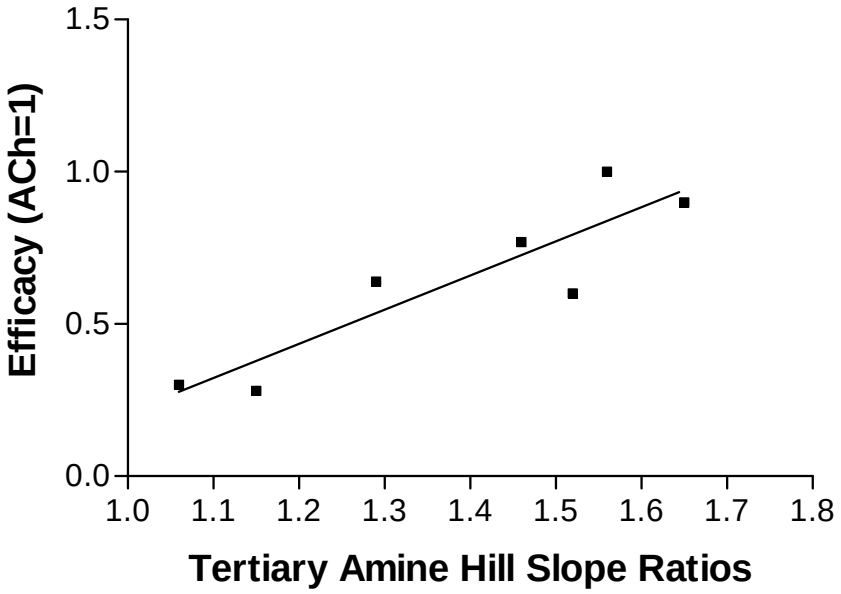


Figure 3-5. Graph of tertiary amine compound efficacies vs. tertiary amine compound Hill slope ratios. The line was fitted using a linear regression analysis in GraphPad Prism software. The r^2 value for linear regression fit is 0.8087.

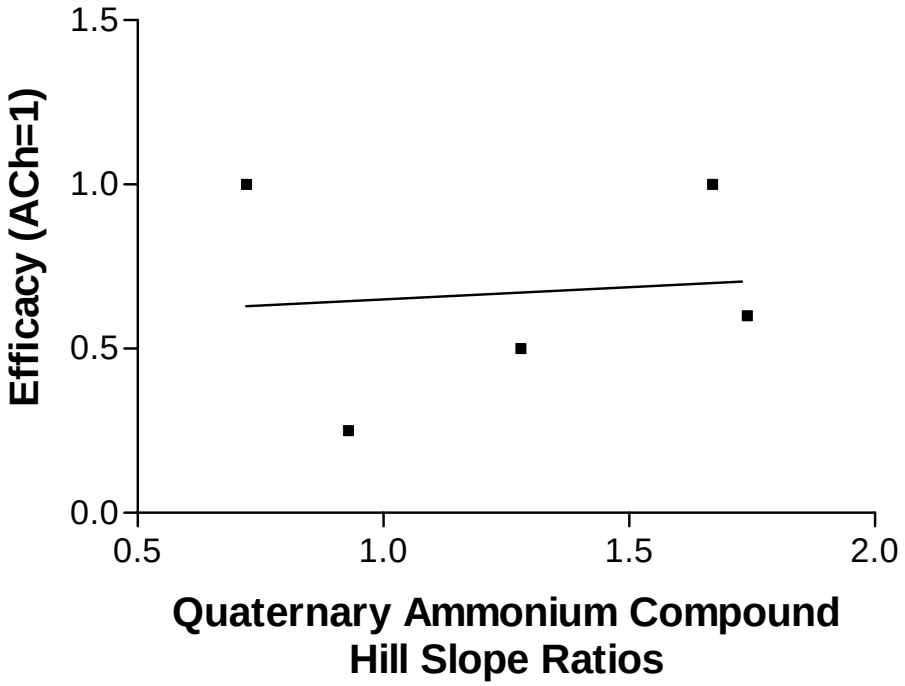
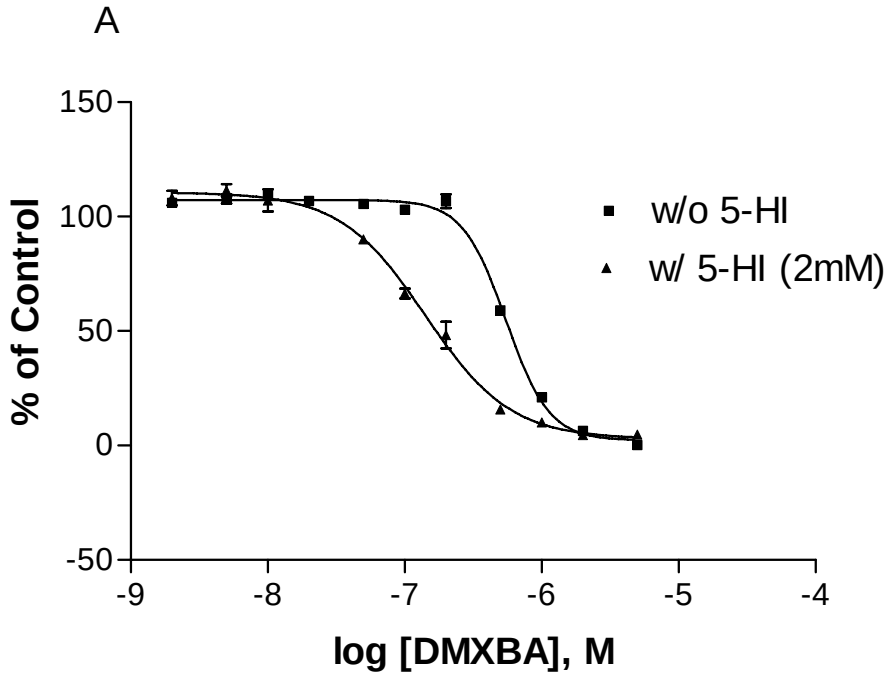
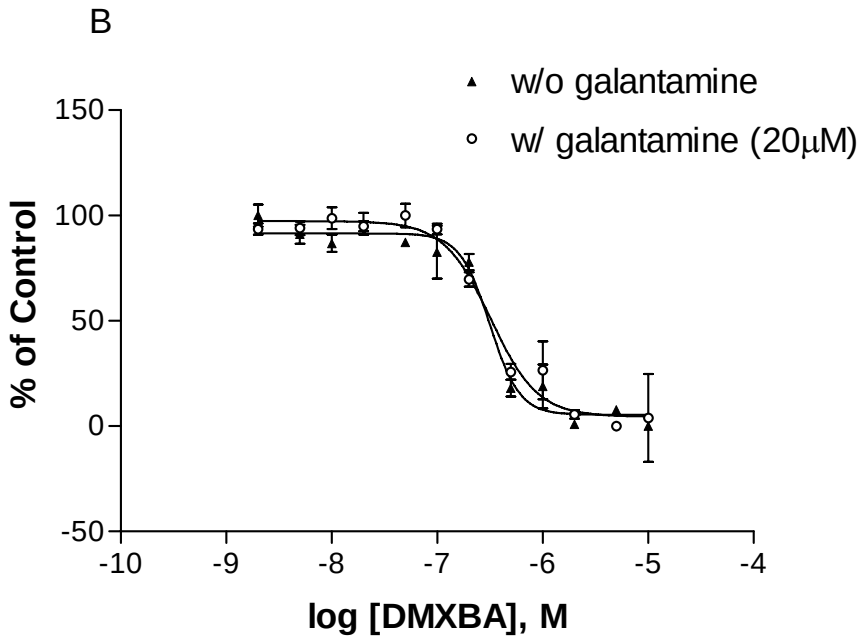


Figure 3-6. Graph of quaternary ammonium compound efficacies vs. quaternary ammonium compound Hill slope ratios. The line was fitted using a linear regression analysis in GraphPad Prism software. The r^2 value for linear regression fit is 0.01034.


A
150
100
50
0
-50
% of Control
w/o 5-HI
w/ 5-HI (2mM)
-9
-8
-7
-6
-5
-4
log [DMXBA], M


B
w/o galantamine
w/ galantamine (20μM)
150
100
50
0
-50
% of Control
-10
-9
-8
-7
-6
-5
-4
log [DMXBA], M

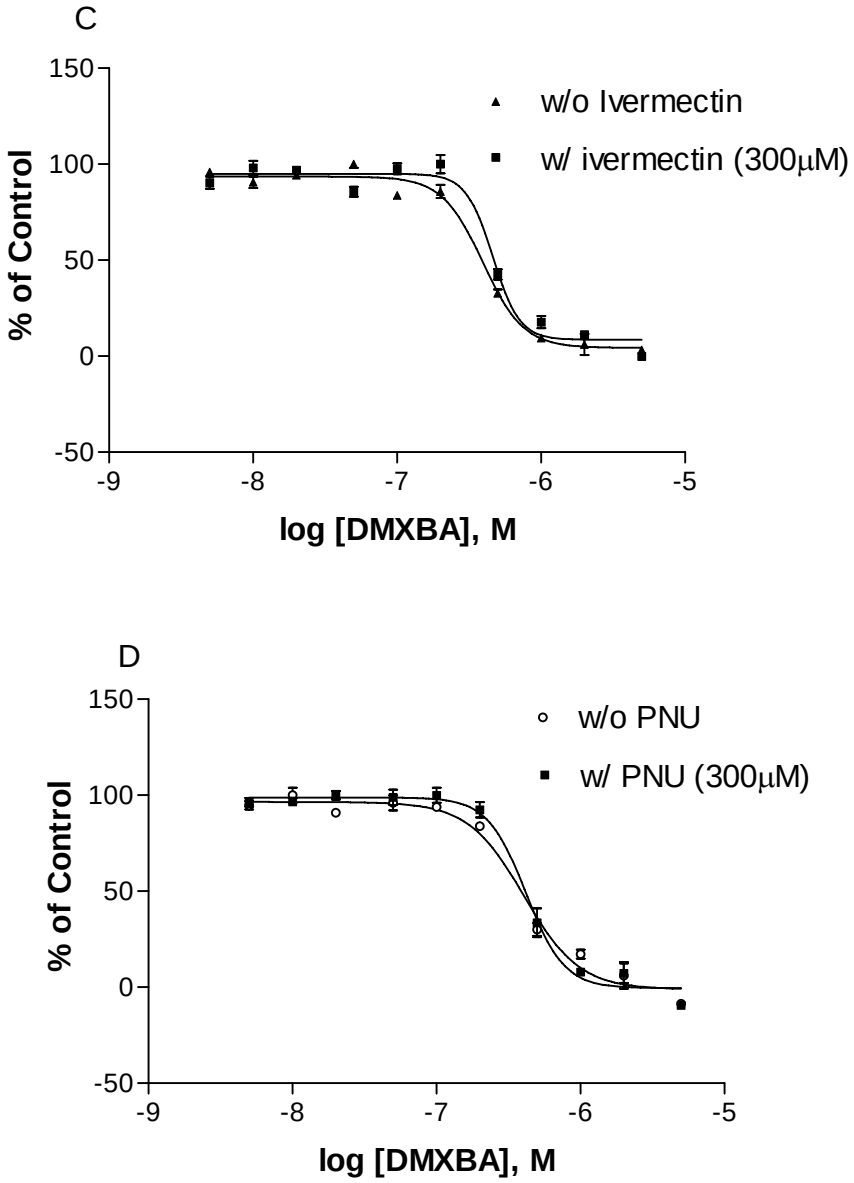
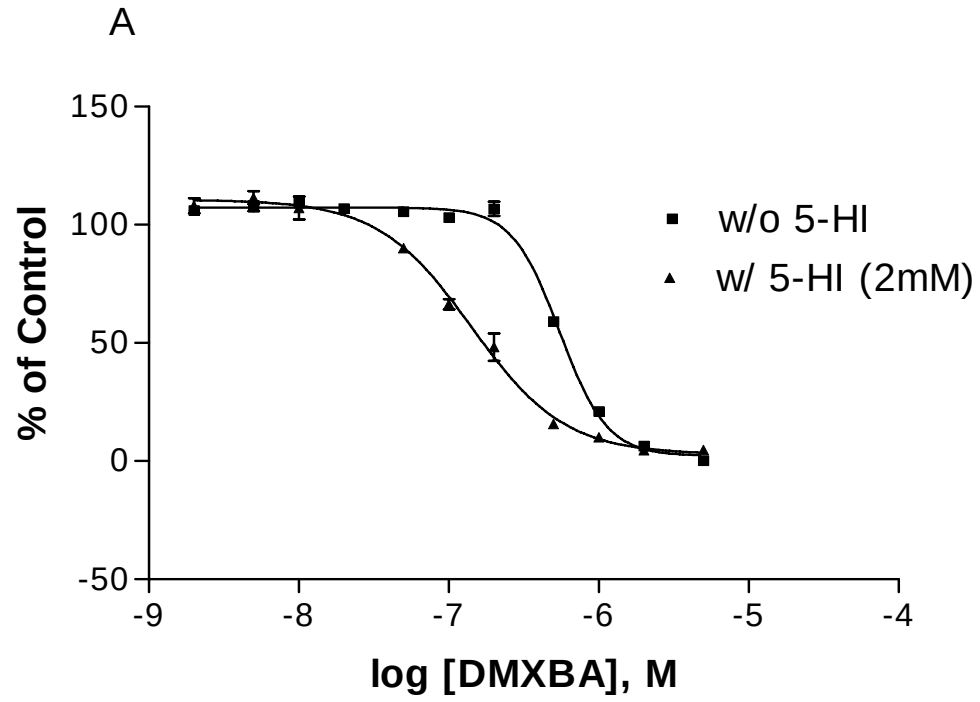
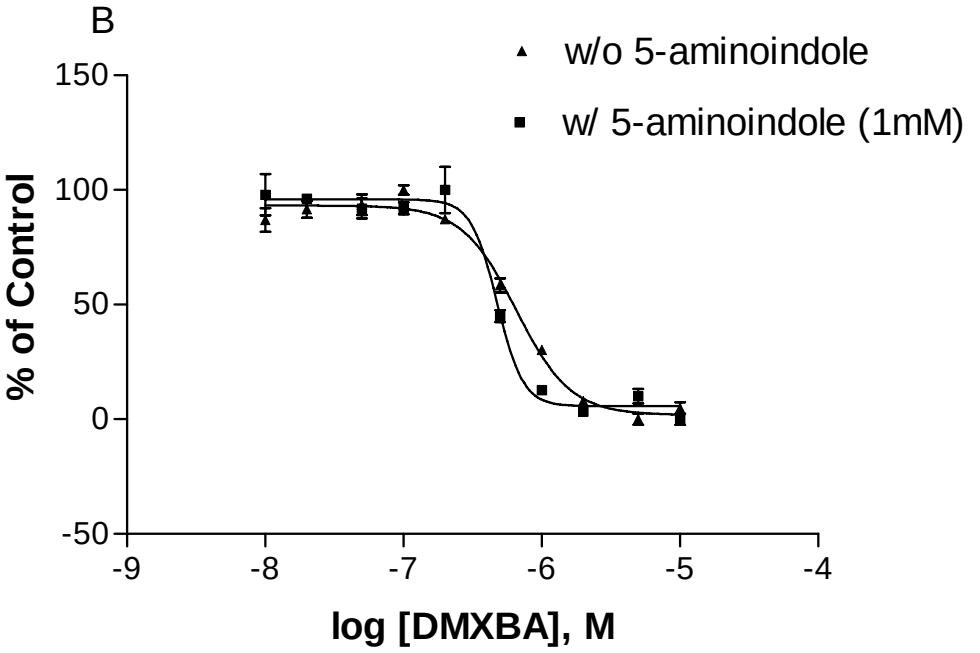


Figure 3-7. Graphs of competition binding assays performed with DMXBA in rat brain membranes using [^{125}I]-α-bungarotoxin. A) DMXBA with 5-HI. B) DMXBA with galantamine. C) DMXBA with ivermectin. D) DMXBA with PNU-120596.


A
150
100
50
0
-50
% of Control
-9
-8
-7
-6
-5
-4
log [DMXBA], M
w/o 5-HI
w/ 5-HI (2mM)


B
150
100
50
0
-50
% of Control
-9
-8
-7
-6
-5
-4
log [DMXBA], M
w/o 5-aminoindole
w/ 5-aminoindole (1mM)

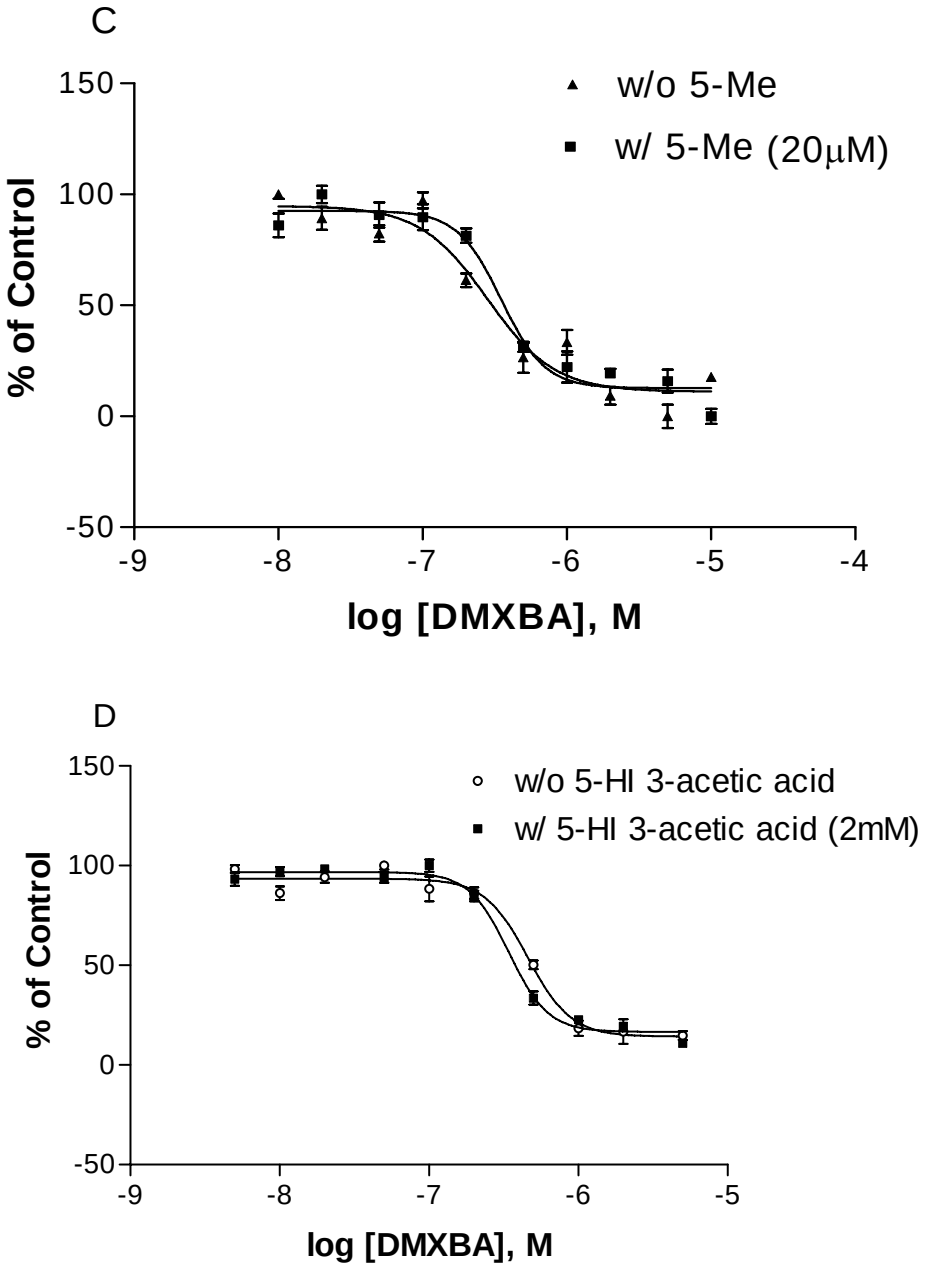


Figure 3-8. Graphs of competition binding assays performed in rat brain membranes using [^{125}I] α-bungarotoxin. A) DMXBA with 5-HI. B) DMXBA with 5-aminoindole. C) DMXBA with 5-methoxyindole. D) DMXBA with 5-hydroxyindole acetic acid.

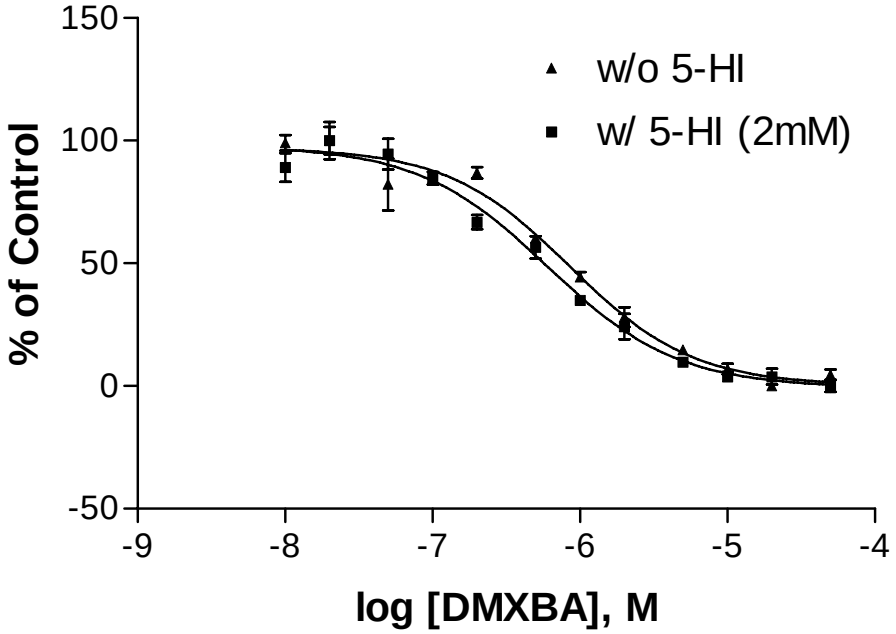


Figure 3-9. Graph of competition binding assay of DMXBA vs. [^{3}H]-epibatidine in SH-EP1 cells expressin human α7 nAChRs.

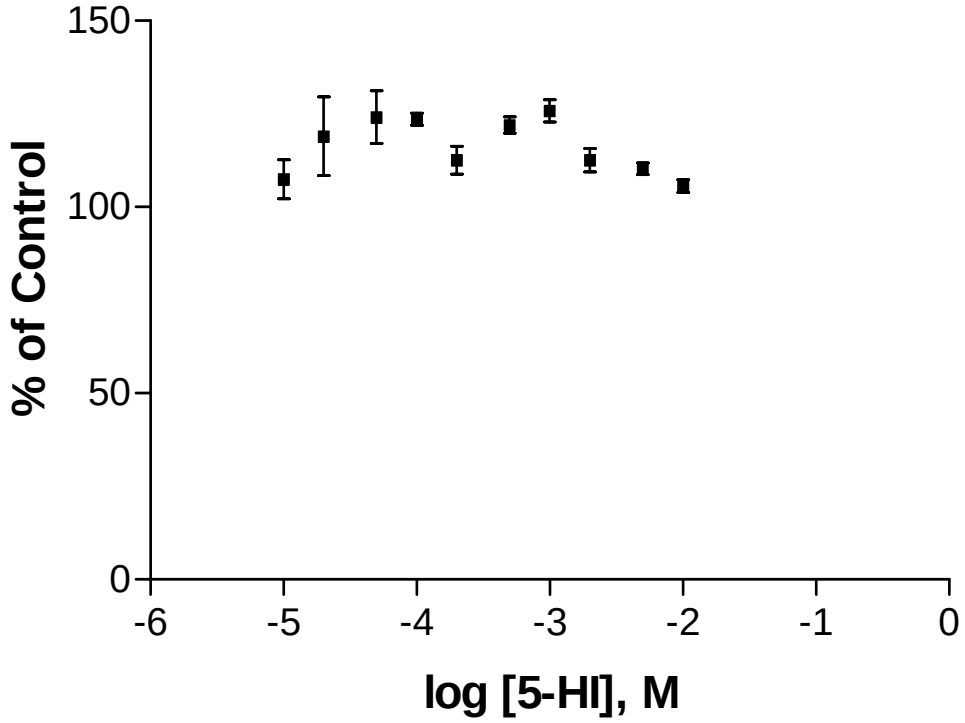


Figure 3-10. Graph of competition binding assay between 5-HI vs. [^{3}H]-epibatidine in SH-EP1 cells expressing human α7 nAChRs.

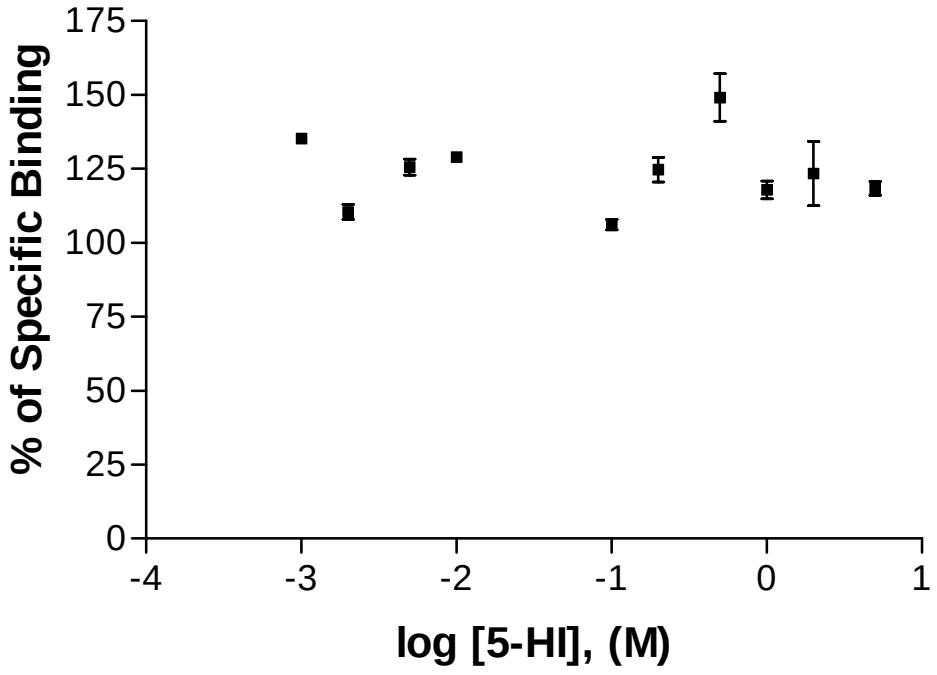


Figure 3-11. Graph of competition binding assay between 5-HI vs. [^{125}I] α-bungarotoxin in rat brain membranes.

CHAPTER 4
DISCUSSION

We have shown that 5-HI enhances the apparent affinity of a variety of agonists for the α7 nAChR. In addition, 5-HI reduces the Hill slopes for agonist displacement of [^{125}I] α-bungarotoxin to the α7 nAChR. Although our data are from rat brain membranes we have also done preliminary experiments with SH-EP1 cells expressing the human α7 receptor that indicates that 5-HI works in the same manner on the human receptor. Our original hypothesis was that the IC_{50} enhancements would vary with efficacy. Our data has not been able to prove this hypothesis. We have found that performing competition binding assays with an agonist in the presence of 5-HI is not a reliable method to determine the efficacy of the agonist. Only the anabaseine type compounds, out of the three groups of agonists tested, show that such a relationship between efficacy and IC_{50} enhancement may exist (Figure 3-1). The tertiary amine group shows no apparent correlation between IC_{50} ratios and efficacy (Figure 3-2). The efficacies are not known for all of the quaternary ammonium agonists. Therefore, TMA and ETMA have been omitted from the graphs for the quaternary ammonium compounds. Figure 3-3 does not reveal an apparent relation between the IC_{50} ratios and efficacy of the quaternary ammonium compounds, unless the point for choline is omitted. It should be considered that the reported efficacies used to determine relationships with IC_{50} and Hill slope ratios are a combination of rat and human receptor efficacies.

When we discovered that 5-HI was also changing the Hill slope values for the agonists, we decided to look for a relationship between the change in Hill slopes and the efficacies. For the anabaseine agonists and the quaternary ammonium agonists, we found that a relationship was not so obvious, unless the point for choline is omitted (Figures 3-4 and 3-6). The tertiary amines do show an apparent correlation between Hill slope ratios and efficacy (Figure 3-5). It may be that

for the tertiary amines, the Hill slope is a more sensitive measurement of differences between the compounds within the group. However, we recognize that the Hill slope is a complex variable to measure and therefore our data does not have a simplistic interpretation.

Our results for the quaternary ammonium compounds show two compounds that do not fit the pattern of the rest of the compounds in this category. Choline and succinylcholine both have Hill slope ratios that do not vary significantly from 1.0. Apparently 5-HI does not produce a change in Hill slope for these two compounds, but it does still produce an enhancement of IC_{50} values. The Hill slope value for choline when 5-HI is present has a very large standard of error, so this experiment will need to be repeated in order to ascertain that this data is consistent. Interestingly, the IC_{50} ratios of choline and succinylcholine also show differences from the other quaternary ammonium compounds. Their ratios are very small compared to not only the other quaternary ammonium compounds, but also compared to all of the agonists tested. It is possible that the chemical structures (Figure 4-1) of choline and succinylcholine allow them to compete with 5-HI for its binding site. Both molecules have polar hydrogen bonding groups that might compete for a common binding site in the 5-HI binding domain. It is possible that there are other quaternary ammonium agonists that we have not looked at that behave similarily to choline and succinylcholine.

Preliminary findings for OMPBA indicate that this anabaseine compound may be an antagonist for the α7 receptor (Kem lab, unpublished results). If this is the case, then we would not suspect 5-HI to change its Hill Slope and IC_{50} values. However, our data shows that 5-HI does change the Hill Slope and IC_{50} value for OMPBA. Further electrophysiological studies will need to be done on this compound to determine if it is indeed a competitive antagonist, a channel blocker or a desensitizing agent.

The quaternary ammonium compound MG-624 was previously determined to be an antagonist for the chick α7 nAChR (Gotti et al., 2000). Since 5-HI enhanced both the Hill slope and IC_{50} of this compound we were prompted to test its efficacy electrophysiologically. We found that MG-624 produced currents slightly greater then 50% of the ACh maximum current on human α7 receptors in *Xenopus* oocytes and therefore must be considered a partial agonist on this receptor (Kem lab, unpublished results).

The site of 5-HI binding and its mechanisms of action to potentiate nicotinic transmission are still unknown. Electrophysiological studies show that, unlike ivermectin, 5-HI does not need to be pre-applied to the receptor in order to potentiate the response to agonists (Zwart et al., 2002). We have shown that 5-HI is not competing for the binding site of agonists such as epibatidine (Figure 3-7) or antagonists such as α-bungarotoxin (Figure 3-8). 5-HI thus binds to an allosteric site on the receptor that apparently increases the probability of its activation when agonist is bound. Computer docking experiments could be helpful in locating the binding site for 5-HI. Another useful approach might be to use the acetylcholine-binding protein isolated from molluscan glial cells that binds to ACh (Talley et al., 2006). The ligand binding domain of the protein is similar to that of the α7 nAChR ligand binding domain. A crystal structure of 5-HI bound to the acetylcholine-binding protein might reveal where 5-HI binds on the α7 receptor. However, it is possible that 5-HI binds to sites on the receptor that are not present on the acetylcholine-binding protein.

It is interesting that 5-HI, as well as most other nicotinic receptor PAMs, has only been shown to act on α7 nAChRs and not heteromeric nicotinic receptor subtypes. 5-HI also potentiates the actions of agonists on the homomeric $5\text{-}HT_3$ receptor, which is closely related in sequence to the α7 nAChR (Van Hooft et al., 1997; Papke et al., 2004). This suggests that 5-HI

(and other α7-selective PAMs) may need to bind to more than the two or three binding sites offered by α subunits in the heteromeric nAChRs to exert its PAM effect. Further binding and functional investigations of 5-HI with the α7 nAChR are needed to understand the mechanisms of action of this allosteric modulator.

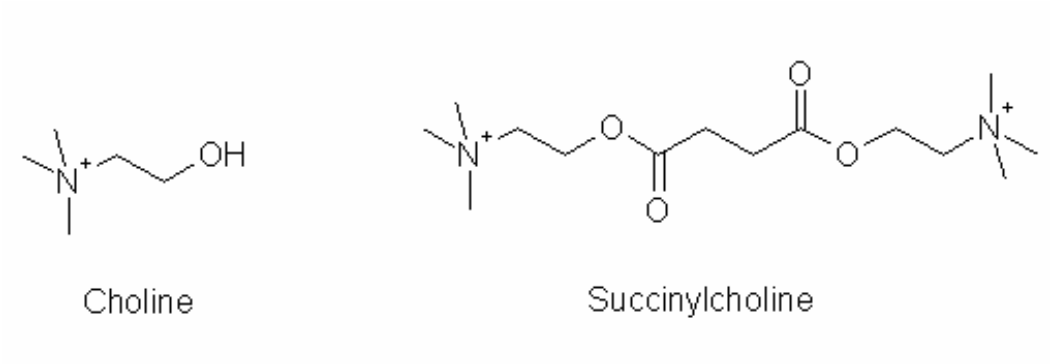


Figure 4-1. Chemical structures of choline and succinylcholine.

APPENDIX A

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BIOGRAPHICAL SKETCH

Kelly Nicole MacDougall was born in 1981 in Duluth, Minnesota to Deborah Yvonne Wiesen and David Bruce Wiesen. She attended The College of Saint Scholastica in Duluth, Minnesota where she completed her undergraduate degree in biology. Upon graduation she entered the Inderdisciplinary Program in Biomedical Sciences at the University of Florida College of Medicine. She joined the laboratory of Dr. William R. Kem in the department of Pharmacology and Therapeutics to expand her knowledge of drug discovery and cognitive disorders.

Kelly married Justin Allen MacDougall in 1999. Together they have two daughters, Madison and Brooklyn. After graduation Kelly plans to attend the University of Florida College of Pharmacy to pursue a Doctor of Pharmacy degree.