N-terminal Domains in Mouse and Human 5-Hydroxytryptamine3A Receptors Confer Partial Agonist and Antagonist Properties to Benzylidene Analogs of Anabaseine

Ran Zhang, Natalie A. White, Ferenc S. Soti, William R. Kem, and Tina K. Machu

Primary Laboratory of Origin:
Dept. of Pharmacology and Neuroscience (RZ, NAW, TKM)
Dept. of Anesthesiology (TKM)
Texas Tech University Health Science Center, Lubbock, TX

Dept. of Pharmacology and Therapeutics (FSS, WRK)
University of Florida Health Sciences Center, Gainesville, FL
Running Title: Benzylidene-Anabaseines and 5-HT$_{3A}$ Receptors

Author of Correspondence (Note: current affiliation is different than the laboratory of origin):

Tina K. Machu, Ph.D.
Dept. of Pharmacology and Neuroscience
University of North Texas Health Science Center
3500 Camp Bowie Blvd.
Fort Worth, TX 76107-2699
817-735-2356
FAX: 817-735-0408
tmachu@hsc.unt.edu

Number of Text Pages:
Number of Tables: 1
Number of Figures: 5
Number of References: 34
Number of Words in Abstract: 241
Number of Words in Introduction: 620
Number of Words in Discussion: 1500

Abbreviations: 5-HT = 5-Hydroxytryptamine, serotonin; MWT = mouse wild-type 5-HT$_{3A}$ receptor; HWT = human wild-type 5-HT$_{3A}$ receptor; TM = transmembrane; MBS = modified Barth’s solution

DMXBA = GTS-21 = 3-(2, 4-dimethoxybenzylidene)-anabaseine
2-OHMBA = 3-(2-hydroxy, 4-methoxybenzylidene)-anabaseine
4-OHMBA = 3-(2-methoxy, 4-hydroxybenzylidene)-anabaseine
2, 4-DiOHBA = 3-(2, 4-dihydroxybenzylidene)-anabaseine
BA = anabaseine compounds containing a benzylidene substitution at the 3’ position

Recommended Section Assignment: Neuropharmacology
Abstract

The present study tested the hypothesis that mouse and human 5-Hydroxytryptamine$_{3A}$ (5-HT$_{3A}$) receptors may be differentially modulated by benzylidene analogs of anabaseine (BA) and that these analogs may be useful in assessing residues involved in receptor gating. Mouse and human wild-type and mouse and human chimeric 5-HT$_{3A}$ receptors expressed in *Xenopus* oocytes were evaluated with the two-electrode voltage clamp technique. Our previous studies demonstrated that 3-(2, 4-dimethoxybenzylidene)-anabaseine (DMXBA) is an antagonist at the MWT receptor, but that its metabolites 3-(2-hydroxy, 4-methoxybenzylidene)-anabaseine (2-OHMBA), 3-(2-methoxy, 4-hydroxybenzylidene)-anabaseine (4-OHMBA), and 3-(2, 4-dihydroxybenzylidene)-anabaseine (2, 4-DiOHBA) are partial agonists (Machu et al., 2001). In the HWT receptor, none of the BA compounds possessed partial agonist activity. BA compounds antagonized 1.5 $\mu$M 5-HT (EC$_{50}$) mediated responses in the HWT receptor with a rank order of potency (IC$_{50}$ in $\mu$M) of: 2-OHMBA (1.5 $\pm$ 0.1) $>$ DMXBA (3.1 $\pm$ 0.2) $>$ 4-OHMBA (7.4 $\pm$ 0.5) $>$ 2, 4-DiOHBA (12.8 $\pm$ 0.7). In mouse receptor chimeras containing N-terminal human receptor orthologs, 2-OHMBA inhibited 5-HT (EC$_{50}$) mediated currents with IC$_{50}$s of 2.0 $\pm$ 0.08 and 3.0 $\pm$ 0.13 $\mu$M, respectively. In human receptor chimeras containing N-terminal mouse receptor orthologs, 2-OHMBA displayed partial agonist activities with EC$_{50}$s of 1.3 $\pm$ 0.15 and 5.0 $\pm$ 0.4 $\mu$M; efficacies were 43 and 57%, respectively. Thus, amino acids present in the distal one-third of the N-terminus of mouse and human 5-HT$_{3A}$ receptors are necessary and sufficient to confer partial agonist or antagonist properties of 2-OHMBA.
Introduction

The 5-HT$_3$ receptor is a member of the cys-loop superfamily of ligand-gated ion channels, of which the nicotinic acetylcholine (nAch) receptor is the prototype (Maricq et al., 1991). The 5-HT$_3$ receptor mediates fast synaptic transmission in the central and peripheral nervous systems at postsynaptic sites. In addition, it is thought to regulate neurotransmitter release presynaptically (De Deurwaerdere et al., 1998; Turner et al., 2004). Five subunits of the 5-HT$_3$ receptor, A – E, have been cloned (Mariq et al., 1991; Davies et al., 1999; Dubin et al., 1999; Karnovsky et al., 2003; Niesler et al., 2003). However, only 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits have been demonstrated to have functional significance in the central or peripheral nervous systems. Although the 5-HT$_{3B}$ subunit must be co-expressed with the 5-HT$_{3A}$ subunit to be functional (Davies et al., 1999; Dubin et al., 1999), sole expression of the 5-HT$_{3A}$ subunit yields functional homomeric receptors. Recent studies suggest that the A homomer predominates in rodent brain (Morales et al., 2002).

Anabaseine, a naturally occurring toxin produced by nemertine worms, is structurally related to nicotine (Kem et al., 1997) and acts as an agonist at central and peripheral nAch receptors (de Fiebre et al., 1995; Kem et al., 1997). A novel derivative of anabaseine, 3-(2, 4-dimethoxybenzylidene)-anabaseine (DMXBA or GTS-21) has agonist activity at $\alpha 7$ but not other nAch receptors (de Fiebre et al., 1995; Meyer et al., 1998a). DMXBA is in clinical trials for the treatment of schizophrenia. DMXBA improves cognitive function in aging (Arendash et al., 1995) and brain-lesioned animals (Meyer et al., 1998b), and it also possesses neuroprotective properties (Meyer et al.,
These properties are believed to be due to the actions of DMXBA and its metabolites at the α7 nicotinic Ach receptor. After oral administration, DMXBA is extensively metabolized via O-dealkylation to 2-OHMBA, 4-OHMBA, and 2, 4-DiOHBA (Kem et al., 2004; Mahnir et al., 1998). All three metabolites of DMXBA have similar potencies as DMXBA and partial agonist efficacies equal to or greater than that of DMXBA (Kem et al., 2004).

The benzylidene anabaseine analogs have also been useful as molecular probes of the ligand binding domains of the α7 nicotinic receptor and 5-HT3A receptor. Papke and co-workers have demonstrated that DMXBA and its metabolites differ in partial agonist potency and efficacy in rat, monkey, and human α7 nicotinic Ach receptors (Papke et al., 2005; Stokes et al., 2004). In chimeric and point mutant α7 nicotinic Ach receptor electrophysiological studies, this group has shown that residues in Loops C, E, and F of the ligand binding domain that differ across species account for the differential pharmacology (Stokes et al., 2004). We have previously reported that DMXBA is an antagonist at the mouse 5-HT3A receptor, and its metabolites are partial agonists (Machu et al., 2001). Further, a –OH at the 2’ position is crucial for conferring partial agonist activity of ~ 50 – 60% of the response evoked by a maximal concentration of 5-HT.

In the present study, we examined the action of DMXBA and its metabolites on the HWT 5-HT3A receptor, which is ~ 84% identical in amino acid sequence to the MWT 5-HT3A receptor. All compounds inhibited the HWT 5-HT3A receptor, with 2-OHMBA being the most potent. Furthermore, 2-OHMBA is an apparent competitive antagonist of
the HWT 5-HT$_{3A}$ receptor. A human 5-HT$_{3A}$ receptor chimera, in which the distal one-third of the N-terminus is replaced with mouse residues, is gated by 2-OHMB, whereas a mouse 5-HT$_{3A}$ receptor chimera, in which the distal one-third of the N-terminus is replaced with human residues, is inhibited by 2-OHMB. These results suggest that the differences in 2-OHMB activity at MWT and HWT 5-HT$_{3A}$ receptors may be used to assess amino acids involved in initiation of gating of the receptor.
Methods

**Analogs of 3-Benzylideneanabaseine**

Syntheses of DMXBA and two O-demethylated analogs (Fig. 1A) were described in Kem et al., 2004. The dihydrochloride salts were dissolved in the appropriate physiological saline, and stock solutions were aliquoted and frozen. Because DMXBA is light-sensitive, the compounds were not exposed to strong light.

**Isolation of Xenopus laevis oocytes**

*X. laevis* frogs were kept in tanks of dechlorinated tap water on a 10 h light/14 h dark cycle at 19°C and fed a diet of AquaMax 500 grower (Purina Mills, St. Louis, MO) three times per week. Frogs were anesthetized by immersion in cold 0.12% 3-aminobenzoic acid ethyl ester for 20 min. After removal through a small incision in the frog’s abdomen, ovarian lobes were placed in modified Barth’s Solution (MBS) containing (in mM) NaCl 88, KCl 1, NaHCO₃ 2.4, HEPES 10, MgSO₄ 0.82, Ca(NO₃)₂ 0.33, and CaCl₂ 0.91 (pH 7.5).

Ovarian lobes were manually dissected into clumps of four to ten oocytes and were then subjected to chemical separation and defolliculation. Clumps of oocytes were placed in medium containing 2 mg/ml collagenase Type 2 and (in mM) NaCl 83, KCl 2, MgCl₂ 1, and HEPES 10 (pH 7.5) and gently rocked for two hours. Oocytes were then removed to fresh collagenase medium and rocked gently for an additional two hours. Lastly, oocytes were rinsed with MBS and stored in incubation media composed of ND96, containing (in mM) NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, and HEPES 5 (pH 7.5),
plus 10 mg/l streptomycin, 50 mg/l gentamicin, 10,000 units/l penicillin, 96 mg/l sulfamethoxazole, 19 mg/l trimethoprim, 0.5 mM theophylline, and 2 mM sodium pyruvate.

Construction of Chimeric Receptors

Mouse and human 5-HT\textsubscript{3A} receptor cDNAs, provided by Drs. D. Julius and A. Miyake, respectively, were subcloned into pBluescript KS\textsuperscript{−} and pCR-Script Amp SK\textsuperscript{(+)} (Stratagene, La Jolla, CA). Two unique restriction enzyme cleavage sites, Spe I, and Nar I, were introduced in both mouse and human 5-HT\textsubscript{3A} R cDNAs by site-directed mutagenesis (U.S.E. mutagenesis kit, Pharmacia Biotech Inc., Piscataway, N.J.) of the nucleotides encoding the conserved residues: Thr 181 and Arg 244 in the mouse receptor cDNA and Thr 176 and Arg 239 in the human receptor cDNA, respectively (Fig. 1B). Numbering of the amino acids in the two receptors began with the initiating methionine. Once the restriction sites were introduced, both receptor cDNAs were digested with the proper restriction enzymes and the appropriate fragments ligated. Chimeric cDNAs were confirmed by dideoxynucleotide sequencing at the Biotechnology Core Facility at Texas Tech University, Lubbock, TX.

Transcription of cDNA to cRNA

The wild-type and chimeric 5-HT\textsubscript{3A} receptor cDNAs were linearized with Not I, extracted with phenol-chloroform, precipitated with sodium acetate and ethanol, and resuspended in diethyl pyrocarbonate (DEPC) treated water. The cDNAs were then transcribed with the T3 mMESSAGE mMACHINE (Ambion, Austin, TX).
**Microinjection of oocytes with 5-HT₃ receptor cRNA**

An aliquot of cRNA was centrifuged at 15,000 x g, and the ethanol was removed with a tuberculin syringe. After air drying, the pellet was resuspended in a volume of DEPC water to yield a concentration of 5-50 ng of cRNA/50 nl. The cRNA was drawn up into a micropipette (10-20 µm tip size). In a volume of 50 nl, cRNA was injected into the animal/vegetal pole equator of each oocyte. Oocytes were stored in incubation medium in Corning cell well plates (Corning Glass Works, Corning, N.Y.) at room temperature. Incubation medium was changed daily. Oocytes were recorded from days two through seven following injection.

**Electrophysiological recordings**

Oocytes were perfused (2 ml/min) in a 100 µl volume chamber with MBS via a roller pump (Cole-Parmer Instrument, Co., Chicago, IL). Two glass electrodes (1.2 mm outside diameter and 1-10 megaohm resistance) filled with 3M KCl were used to impale oocytes. A Warner Instruments Model OC-725B or OC-725C oocyte clamp (Hamden, CT) was used to voltage clamp oocytes at –70 mV, and clamping currents were plotted on a strip chart recorder (Cole Parmer Instrument, Co., Chicago, IL).

To examine agonist or partial agonist effects, 5-HT or BA analogs were dissolved in MBS buffer and applied to oocytes for 30 sec. To examine antagonist effects, BA analogs were coapplied with 5-HT. Applications of 5-HT, BA analogs, or 5-HT plus BA analogs were performed every five min.
Data Analysis

The values in the 5-HT or BA analog concentration response curves (agonist effects) were expressed as a percentage of the respective maximal 5-HT (10, 25, or 200 µM) responses. Unless otherwise noted, in all other experiments (antagonist effects), data were expressed as a percent change from the control, baseline response. In all experiments, the control values were obtained by averaging the 5-HT mediated response before and after the response to 5-HT, BA analogs, or 5-HT plus BA analogs. In experiments where agonism was measured, the current measured from test drug stimulation was divided by the average response obtained with the maximal 5-HT concentration and multiplied by 100 to yield percent of maximal response. For antagonism, percent inhibition was calculated by subtracting the current obtained from the test drug plus 5-HT from the average current obtained with 5-HT alone; the difference was divided by the average 5-HT mediated current, and the quotient was multiplied by 100.

Graphpad Prism (San Diego, CA) was used to calculate EC$_{50}$s, IC$_{50}$s, and Hill coefficients. Within each data set, the average + SEM were calculated for each 5-HT or drug concentration, and curve-fitting was performed with these values. Thus, the error terms reported are fitted parameter errors. The equation used to calculate these parameters was: $I/I_{\text{control}} = 1/ [1 + [D / E(I)C_{50}] ^n]$, where $I$ is current, $I_{\text{control}}$ is the control current, $D$ is the drug concentration, EC$_{50}$ is the concentration of drug that produces 50% of the maximal response, IC$_{50}$ is the concentration of drug that produces 50% inhibition
of the response, and $n$ is the Hill coefficient. Linear regression analysis with Graphpad Prism was used to generate the Schild plot and to derive the $K_1$ value for 2-OHMB.

Two-way analysis of variance (ANOVA) was also performed with Graphpad Prism. The $F$ statistic for each two-way ANOVA is reported. The first subscript of the $F$-statistic is the degrees of freedom for the construct, drug, or interaction, and the second subscript represents the degrees of freedom of the error (residual).
Results

In a previous study, we demonstrated that DMXBA was an antagonist and demethylated analogs of DMXBA (Fig. 1A) were partial agonists at the mouse wild-type 5-HT$_{3A}$ receptor (MWT) (Machu et al., 2001). The rank order of potency for agonism was 5-HT > 2-OHMBA > 2, 4-DiOHBA > 4-OHMBA; both 2-OHMBA and 2, 4-DiOHBA had equivalent efficacies of ~ 63% of the maximal 5-HT evoked responses (summarized in Fig. 2A). In the present study, we examined the actions of DMXBA and its demethylated analogs on the human wild-type 5-HT$_{3A}$ receptor (HWT). No partial agonism was observed with any of these compounds (Fig. 2A). Antagonistic actions of the BA compounds (0.25 - 50 µM) were seen on 1.5 µM 5-HT mediated currents; 1.5 µM 5-HT is an EC$_{50}$ in the human receptor (see Fig. 3A). The rank order of potency (IC$_{50}$ in µM) and Hill coefficient were 2-OHMBA (1.5 ± 0.1, 1.5 ± 0.19) > DMXBA (3.1 ± 0.2, 1.3 ± 0.12) > 4-OHMBA (7.4 ± 0.5, 1.3 ± 0.11) > 2, 4-DiOHBA (12.8 ± 0.7, 1.2 ± 0.08). Given that 2-OHMBA had the greatest potency of the BA analogs at the mouse and human receptors and given that it also was a good partial agonist at the mouse receptor, it was used in all subsequent studies.

To examine the nature of antagonism produced by 2-OHMBA, 5-HT concentration response curves were performed in the absence or presence of increasing concentrations of 2-OHMBA (Fig. 3A). In the 5-HT concentration response curve generated in the absence of 2-OHMBA, data were normalized to the 25 µM 5-HT baseline response. In the 5-HT concentration response curve generated in the presence of 2-OHMBA, data were normalized to the 200 µM 5-HT baseline response. Parallel shifts
in the 5-HT concentration response curves were observed as 2-OHMBA concentrations were increased from 2 to 50 µM. 5-HT EC50s increased from 1.5 + 0.01 µM (no 2-OHMBA) to 5.6 + 0.03 µM (2 µM 2-OHMBA), 12.8 + 0.4 µM (10 µM 2-OHMBA), and 84.5 + 0.6 µM (50 µM 2-OHMBA). Hill slopes were 3.4 + 0.26 (no 2-OHMBA), 1.6 + 0.15 (2 µM 2-OHMBA), 2.2 + 0.15 (10 µM 2-OHMBA), and 1.3 + 0.15 (50 µM 2-OHMBA). Increases in 5-HT EC50s with increasing concentrations of 2-OHMBA are suggestive of a competitive form of antagonism.

To more fully examine the apparent competitive antagonism by 2-OHMBA, a Schild plot was generated (Fig. 3B). The pA2, x-intercept, is the negative logarithm of the Ki. The pA2 was, 6.11 with a Ki of 0.78 µM. A slope of - 0.93 + 0.17 was obtained, which was significantly different than zero. In a separate set of experiments, we assessed the effects of pre-equilibration of 2-OHMBA on its inhibitory actions at the human wild-type receptor. Inhibition of 1.5 µM 5-HT mediated currents by 2-OHMBA (2 µM) was measured with and without a one min pre-incubation with 2-OHMBA (2 µM). Without pre-incubation, 48.95 + 4.67% inhibition of 5-HT mediated currents was observed. With pre-incubation, 69.95 + 4.9% inhibition of 5-HT mediated currents was observed (n= 4, p= 0.02, Student’s t-test), data not shown. These results lend support to our hypothesis that 2-OHMBA is a competitive antagonist, given that pre-equilibration of a competitive antagonist would be expected to enhance inhibition. In contrast, pre-equilibration of a channel blocking compound would be expected to have minimal effect, given that inhibition is use-dependent.
Given that 2-OHMB is a partial agonist at the mouse 5-HT3A receptor and an apparent competitive antagonist at the human 5-HT3A receptor, it was used as a tool to probe regions of the receptor that contribute to ligand binding. Previous work (Eisele et al., 1993; Bouzat et al., 2004) has demonstrated that the ligand binding sites of the 5-HT3A receptor are localized in the N-terminal domains. Thus, to verify that the two receptors’ differential sensitivity to 2-OHMB is conferred by the N-termini, we constructed and characterized two chimeras. The chimera, M244H, in which the N-terminus is mouse and the balance of the receptor is human, had a 5-HT EC50 of 1.2 ± 0.05 µM and a Hill slope of 2.1 ± 0.21, whereas the mouse wild-type receptor had a 5-HT EC50 of 0.9 ± 0.06 µM and a Hill slope of 3.1 ± 0.67. The chimera, H239M, in which the N-terminus is human and the balance of the receptor is mouse, had a 5-HT EC50 of 3.9 ± 0.03 µM, which is significantly greater than that of MWT and HWT receptors (Table 1), and a Hill slope of 2.6 ± 0.43 (data not shown).

To test the hypothesis that the identity of the N-terminus determines the pharmacological action of 2-OHMB, we tested the drug in both M244H and H239M (Fig. 4). Partial agonist activity of 2-OHMB was observed in M244H (Fig. 4A). The EC50 of 1.3 ± 0.15 µM for 2-OHMB in M244H was similar to that observed in MWT receptor (2.0 ± 0.24 µM), (Machu et al., 2001); Hill slopes were 2.4 ± 0.58 (M244H) and 2.1 ± 0.42 (MWT). Likewise, the maximal efficacy of 2-OHMB was apparently slightly less in M244H (43.0 ± 5.7 %) than MWT (63.6 ± 4.8 %). Two-way ANOVA revealed that the concentration response curves were not significantly different \( F_{(1,134)} = 3.39, p = 0.07 \), however. A significant effect of 2-OHMB concentration was observed
Representative tracings of maximal 5-HT and 2-OH MBA concentration evoked currents in M244H are shown in Fig. 4B. In H239M, 2-OH MBA had no partial agonist activity (data not shown). The drug inhibited 4 µM 5-HT (~EC50) mediated currents with an IC50 of 2.0 ± 0.08 µM and a Hill slope of 1.56 ± 0.10 (Fig. 4C). Antagonism produced in the chimera was slightly, but significantly less than that produced by 2-OH MBA in HWT (IC50 = 1.5 ± 0.1 µM), [F(1,82) = 46.57, p < 0.0001]; Table 1. Percent inhibition changed as a function of 2-OH MBA concentration [F(8,82) = 290.51, p < 0.0001], but no interaction was obtained between receptor construct and drug concentration [F(8,82) = 1.8, p = 0.09]. Representative currents produced by 5-HT in the absence and presence of 2-OH MBA in H239M are depicted in Fig. 4D.

Within the N-termini of the mouse and human wild-type receptors, the major differences in amino acid composition are localized to the distal one-third domains, which are adjacent to the transmembrane one segments. Some 16 differences are present, as depicted in the alignment presented in Fig. 5A. To test the hypothesis that the distal one-third of the N-termini are responsible for the pharmacological action of 2-OH MBA, two additional chimeras were generated and tested. The chimera, H176M244H, contains the human receptor backbone with the distal one-third of the N-terminus replaced by the mouse receptor. Conversely, the mirror image chimera, M181H239M, contains the mouse receptor backbone with the distal one-third of the N-terminus replaced by the human receptor. H176M244H and M181H239M had similar 5-HT EC50 values of 1.7 ± 0.3 µM and 1.2 ± 0.03 µM, respectively. The H176M244H chimera’s EC50 was
significantly greater than MWT (Table 1). Hill slopes were 1.82 ± 0.95 (H176M244H) and 3.2 ± 0.25 (M181H239M), data not shown.

The actions of 2-OHMB A were examined in H176M244H and M181H239M (Fig. 5). Partial agonist activity was observed in H176M244H, with an EC$_{50}$ of 5.0 ± 0.4 µM and a Hill slope of 2.1 ± 0.32; maximal efficacy was 57.4 ± 9.6 % (Fig. 5B). The 2-OHMB A concentration response curve was slightly right shifted in H176M244H relative to MWT; two-way ANOVA demonstrated that the two curves were significantly different [F$_{(1,73)}$ = 15.08, p = 0.0002]; the EC$_{50}$ for the chimera was significantly greater (Table 1). A significant effect of 2-OHMB A was observed [F$_{(8,73)}$ = 23.04, p < 0.0001], but no interaction was obtained between receptor construct and drug concentration [F$_{(8,73)}$ = 1.35, p = 0.23]. Representative tracings of 5-HT and 2-OHMB A evoked currents in H176M244H are depicted in Fig. 5C. In M181H239M, no partial agonist activity of 2-OHMB A was observed (data not shown). As predicted, 2-OHMB A inhibited 1.2 µM 5-HT (EC$_{50}$) mediated currents (Fig. 5D), with an IC$_{50}$ of 3.0 ± 0.13 µM and a Hill slope of 1.28 ± 0.07. However, the drug was slightly less potent at M181H239M than at HWT. Inhibitory concentration response curves were significantly different with two-way ANOVA [F$_{(1,113)}$ = 177.2, p < 0.0001]; the IC$_{50}$ for M181H239M was significantly greater. Drug concentration also affected inhibition [F$_{(11,113)}$ = 193.7, p < 0.0001]. A significant interaction was observed between receptor construct and drug [F$_{(11,113)}$ = 15.08, p = 0.0003]. In Fig. 5E, typical tracings of 5-HT evoked currents, in the presence and absence of 2-OHMB A, in M181H239M are shown. Collectively, these results suggest that the identity of the distal one-third of the N-terminus is both necessary and
sufficient to determine whether 2-OHMBBA is a partial agonist or an antagonist at the 5-HT$_{3A}$ receptor.
Discussion

In the present study, we have demonstrated that benzylidene analogs of anabaseine have no partial agonist activity at the HWT 5-HT$_{3A}$ receptor, in contrast to their effects at the MWT receptor. Instead, they inhibit 5-HT mediated currents in the HWT receptor, with 2-OHMBA functioning as an apparent competitive antagonist. Our finding that the identity of the N-terminal domain, and in particular the identity of the distal one-third of the N-terminal domain, determines whether 2-OHMBA is a partial agonist or an antagonist, suggests that among the 16 differences between the MWT and HWT receptors in this region are residues that are both necessary and sufficient to confer drug action.

Collectively, the results presented suggest that 2-OHMBA is a competitive antagonist at the HWT 5-HT$_{3A}$ receptor. The slope of the Shild plot of the 2-OHMBA competition curves is -0.93, which is strongly suggestive of a competitive form of antagonism. However, the Hill slopes in the presence of 2-OHMBA are lower than that in its absence. In the literature, Hill slopes for 5-HT in the HWT range from 1.4 – 3.1 (Barann et al., 2002; Hapfelmeir et al., 2003; Hope et al., 1999), and our values fall close to that range. In the absence of an antagonist, trough and peak currents are observed across a narrow range of 5-HT concentrations, typically 0.3 – 6 µM. Slight deviations in EC values across sets of experiments can significantly impact the Hill coefficient, in the absence and presence of antagonists. In addition, 2-OHMBA likely has a second, non-competitive site of action, which may be in the channel pore. In MWT and HWT
receptors, which have identical amino acid compositions in transmembrane two, which lines the channel pore of the receptors, a “tail current” is sometimes observed on wash-out of BA compounds that exceed 50 µM concentrations (Machu et al., 2001 and unpublished observations). This tail current may indicate relief from open channel block. It is unlikely that channel blockade plays any significant component in the Schild analysis presented in Fig. 3, given that the maximum 2-OH MBA concentration used was 50 µM. Finally, the chimera H176M244H, in which the distal one-third of the human receptor is replaced with mouse orthologs, is sufficient to change 2-OH MBA from an antagonist to a partial agonist with efficacy approaching that observed in the MWT receptor. It is possible that 2-OH MBA may have a tightly coupled negative allosteric modulatory site in the N-terminus of the human receptor. If so, the substitution of N-terminal mouse orthologs would have to simultaneously eliminate or significantly reduce binding of 2-OH MBA to this site and alter the agonist binding site to permit drug recognition and channel opening. The most parsimonious hypothesis is that 2-OH MBA occupies the agonist recognition site in both the MWT and HWT receptors, but cannot initiate gating in the HWT receptor.

DMXBA and its metabolites all inhibited the HWT receptor, with IC50s ranging from 1.5 – 12.8 µM. Interestingly, the presence of either –OH or –OCH3 at the 2’ or 4’ positions of the benzylidene ring yielded antagonism of HWT receptor function, suggesting that neither functional group has a drug molecule site specific assignment required for binding of the drug to the HWT receptor per se. However, the identity of the moiety at the 2’ and 4’ positions does appear to play a role in drug potency. The –OH at
the 2’ position appears to be necessary for maximal potency given that DMXBA, which has a –OCH3 at the 2’ position, has a two-fold lower potency. Switching the moieties, with a 2’ –OCH3 and a 4’ –OH reduced the potency even more. The identity of the moiety at the 4’ position appeared to be the more critical of the two, given that 2, 4-DiOHBA had an 8.5 fold lower potency than 2-OHMBMA. In contrast, in the MWT receptor, a –OH at the 2’ or 4’ position is critical for partial agonist activity (Machu et al., 2001). However, placement of the –OCH3 at the 2’ position and –OH at the 4’ position resulted in weak partial agonistic actions (with eight-fold lower potency) of the BA compound at the mouse MWT receptor. Collectively, these results suggest a much greater precision of drug molecule interaction with the agonist binding domain to initiate gating in MWT receptors than to inhibit 5-HT binding in HWT receptors.

The function of 2-OHMBMA as a partial agonist or an apparent competitive antagonist is dictated by the identity of the distal one-third of the N-terminus, which contains Loops C and F of the ligand binding domain. Loops C and F are among six Loops (A-F) identified in the cys loop family of ligand-gated ion channels that participate in ligand recognition (see Sine, 2002, for a review). There are two recognition sites for ligands, and each recognition site is at the interface of two subunits. Loops A, B, and C are on the principal face, and Loops D, E, and F are on the complementary face. Among the 16 differences between mouse and human receptors in the distal one-third of the N-terminus, seven are near or within Loop C, and nine are near or within Loop F (Fig. 5A). Among Loops A, B, D, and E, only three differences between mouse and human receptors are present. Interspecies 5-HT3A receptor chimeras implicate Loop C in the
differential potency of m-chlorophenylbiguanide in human and rat receptors (Mochizuki et al., 1999) and in the presence and absence of phenylbiguanide agonist activity in human and guinea pig receptors (Lankiewicz et al., 1998), respectively. Furthermore, mouse-human 5-HT\(_{3A}\) receptor chimeras have been used to demonstrate that Loop C is partly responsible for conferring curare potency (Hope et al., 1999). Taken together, these results suggest that Loops C and/or F may contribute to the partial agonist and apparent competitive antagonists actions of 2-OHMB A in mouse and human 5-HT\(_{3A}\) receptors, respectively.

Site-directed mutagenesis studies in the 5-HT\(_{3A}\) receptor strongly support the roles of numerous residues in Loops A-F in ligand binding. A number of studies have investigated the role of Loop C. The interaction of multiple residues has been suggested to confer differences in curare potency between mouse and human receptors (Hope et al., 1999). Mutations at Phe226, Ile228, Asp229, Ile230, and Tyr234 reduced affinity of \([^3H]\)-granisetron, and in some cases, eliminated binding (Suryanarayanan et al., 2005; Thompson et al., 2005). Mutations of Glu224 and Glu235 altered binding of \([^3H]\)mCPBG and \([3H]\)GR65630 (Schreiter et al., 2003). Further, Ala substitutions at F226, I228, and Y234 altered the relative efficacies of the partial agonist 2-methyl 5-HT and/or 5-HT, suggesting a role of these residues in gating (Suryanarayanan et al., 2005). Likewise, mutations of Tyr234 to unnatural amino acids point to the aromatic ring as playing a role in both binding and gating (Beene et al., 2004). In Loop F, Thompson et al. (2005) reported that mutations at Trp195, Ser203, and S206 alter \([^3H]\)-granisetron binding. Collectively, these results support the idea that Loop C and/or Loop F residues
may participate in the differential actions of 2-OHMBBA at the mouse and human 5-HT\textsubscript{3A} receptor.

The recent crystallization and structural determination of the acetylcholine binding protein (AchBP) (Brejc et al., 2001) has been used to generate homology-models of the N-terminal domains of the 5-HT\textsubscript{3A} receptor (Reeves et al., 2003; Maksay et al., 2003). These models have been very useful in elucidating the possible roles of amino acids in Loops A-F in stabilizing the architecture of the ligand binding site and in spatially orienting agonists and antagonists in the ligand binding site. Maksay and co-workers (2003) have compared mouse and human 5-HT\textsubscript{3A} receptor models, and suggest that Loop C orthologs, mouse D229/ human E224 and mouse I230/ human S225, by virtue of differences in side chain length and size, respectively, not only change intramolecular interactions, but also alter the spatial orientation of curare. Whether these residues are involved in differences in 2-OHMBBA binding in mouse and human receptors will be of interest to determine. Loop F has not been clearly resolved in the AchBP (Brejc et al., 2001) and has not been modeled in the 5-HT\textsubscript{3A} receptor.

Downstream gating events have been linked to residues in the N-terminal domains of the 5-HT\textsubscript{3A} receptor. Movement of Trp 183 in Loop B is thought to initiate motions of \(\beta2 - \beta3\) and the Cys loops of the extracellular domain, which straddle the transmembrane (TM) 2 – TM3 linker region (Bouzat et al., 2004; Reeves et al., 2005). Movement of the Cys loop may provide the molecular switch to initiate isomerization of proline in the TM2 – TM3 linker, which causes the ion channel to open (Lummis et al.,
2005). However, the roles of other residues in Loops A, C, D, E, and F in initiating the cascade of molecular motions that results in channel gating are poorly understood. We suggest that the roles of individual amino acids in Loops C and/or F in initiation of gating may be probed through the substitution of human orthologs in the mouse 5-HT₃A receptor, which converts the actions of 2-OH MBA from partial agonism to antagonism in the mutant receptor.
References


Papke RL, McCormack TJ, Jack BA, Wang D, Bugaj-Gaweda B, Schiff HC, Buhr JD, Waber AJ and Stokes C (2005) Rhesus monkey alpha7 nicotinic acetylcholine receptors:


Suryanarayanan A, Joshi PR, Bikadi Z, Muthalagi M, Kulkarni TR, Gaines C and Schulte MK (2005) The Loop C region of the murine 5-HT$_{3A}$ receptor contributes to the
differential actions of 5-Hydroxytryptamine and \( m \)-Chlorophenylbiguanide. *Biochem* 44:9140-9149.


Footnotes:

This work was supported by NS 43438 to TKM.

Reprint Requests:

Tina K. Machu, Ph.D.
Dept. of Pharmacology and Neuroscience
University of North Texas Health Science Center
3500 Camp Bowie Blvd.
Fort Worth, TX  76107-2699
817-735-2356
FAX: 817-735-0408
tmachu@hsc.unt.edu
Figure Legends

Figure 1 A) The benzylidene-anabaseine structure is shown. Substitutions with hydroxy (OH) or methoxy (OCH₃) were made at the R₁ and R₂ positions, and these analogs were assessed for activity in the MWT or HWT. The R₁ group represents the 2’ position and the R₂ group represents the 4’ position. B) A schematic diagram of the relative locations of the two shift-points used to make N-terminal chimeras of mouse and human 5-HT₃ARs is shown. The chimeric receptor cDNAs were constructed with mutagenically installed restriction enzymes cleavage sites, Spe I and Nar I, corresponding to amino acid residues, Thr181, and Arg244 in the mouse receptor Thr176 and Arg 239 in the human receptor, respectively. The vertical lines demarcate the end of the N-termini.

Figure 2 The actions of benzylidene-anabaseine analogs were tested in MWT and HWT. A) As previously described (Machu et al.; 2001), the rank order of partial agonist efficacy at the MWT is 5-HT > 2-OHMB = 2, 4-OHBA > 4-OHMB = 4-OHMBA. DMXBA had no partial agonist activity at the MWT. None of the analogs tested had partial agonist activity at the HWT. In the MWT, concentrations of compounds used were: 2-OHMB (10 µM), DMXBA (25 µM), 4-OHMB (50 µM), and 5-HT (10 µM), n= 4 - 13. In the HWT, concentrations of compounds used were: all BA analogs (25 µM) and 5-HT (25 µM), n= 4 - 8. B) The antagonist actions of BA analogs were tested in the HWT. Serotonin was applied in the absence and presence of BA analogs (0.25 – 50 µM)
for 30 s (n = 4 - 9). The 1.5 μM 5-HT evoked currents were antagonized by the analogs with a rank order of potency of 2-OHMBA > DMXBA > 4-DiOHMBA > 2, 4-DiOHBA.

Figure 3 The nature of antagonism of 2-OHMBA at the HWT was examined. A) Serotonin concentration response curves were performed in the absence and presence of increasing concentrations of 2-OHMBA (2 – 50 μM), n = 3 - 9. Serotonin (0.25 – 1000 μM) in the absence or presence of 2-OHMBA was applied for 30 s, and responses were normalized to that obtained with 25 μM 5-HT (5-HT curve generated in the absence of 2-OHMBA) or 200 μM 5-HT (5-HT curve generated in the presence of 2-OHMBA), both of which produce maximal responses. B) A Schild plot was generated, and an apparent competitive antagonism by 2-OHMBA was indicated.

Figure 4 The actions of 2-OHMBA were assessed in mouse-human and human-mouse chimeric 5-HT₃A receptors. A) The MWT and chimeric receptor containing the mouse 5-HT₃A receptor N-terminus and the balance the human receptor (M244H) were perfused with 5-HT (10 μM) or 2-OHMBA (0.25 – 10 μM) for 30 s. Partial agonist responses were normalized to that produced by 5-HT (10 μM), n = 4 - 10. B) Representative tracings of agonist responses in M244H are presented. C) The HWT and chimeric receptor containing the human 5-HT₃A receptor N-terminus and the balance the mouse receptor (H239M) were perfused with 5-HT (EC₅₀) in the absence and presence of 2-OHMBA (0.25 – 25 μM), n = 4 - 8. The EC₅₀ values of 5-HT were 1.5 μM for HWT and
4 µM for H239M. Data are expressed as a percent inhibition of the respective control 5-HT mediated response. D) Representative tracings in H239M demonstrate that 2-OHMBa antagonizes 5-HT mediated currents.

Figure 5  Chimeric 5-HT3A receptors containing substitutions in the distal one-third of the N-termini were assessed for 2-OHMBa activity. Alignment of the distal one-third of the N-termini of mouse and human 5-HT3A receptors reveals 16 amino acid differences (bold). The single underline designates Loop F, and the double underline designates Loop C. B) The human chimeric receptor with the distal one-third of the N-terminus replaced with mouse orthologs (H176M244H) and the MWT were perfused with 5-HT (10 µM) or 2-OHMBa (0.25 – 25 µM) for 30 sec. Partial agonist responses were normalized to that produced by 5-HT (10 µM), n = 4 - 6. C) Representative tracings of H176M244H agonist responses are presented. D) The mouse chimeric receptor with the distal one-third of the N-terminus replaced with human orthologs (M181H239M) and the HWT were perfused with 5-HT (EC50) in the presence and absence of 2-OHMBa (0.5 – 25 µM) for 30 s, n =5 - 8. The 5-HT EC50 values for HWT and M181H239M were 1.5 µM and 1.2 µM, respectively. Data are expressed as a percent inhibition of the respective control 5-HT mediated response. E) Representative tracings of M81H239M responses to 5-HT in the absence and presence of 2-OHMBa.
Table 1  Wild-type and Chimeric 5-HT$_{3A}$ Receptor Potencies and Efficacies

<table>
<thead>
<tr>
<th></th>
<th>5-HT EC$_{50}$, µM</th>
<th>2-OHMB A EC$_{50}$, µM/ Emax</th>
<th>2-OHMB A IC$_{50}$, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWT</td>
<td>0.9 ± 0.06</td>
<td>2.0 ± 0.24, 63%</td>
<td>NA</td>
</tr>
<tr>
<td>M244H</td>
<td>1.2 ± 0.02</td>
<td>1.3 ± 0.15, 43%</td>
<td>NA</td>
</tr>
<tr>
<td>H176M244H</td>
<td>1.7 ± 0.3$^a$</td>
<td>5.0 ± 0.4$^b$, 57%</td>
<td>NA</td>
</tr>
<tr>
<td>HWT</td>
<td>1.5 ± 0.01</td>
<td>NA</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>H239M</td>
<td>3.9 ± 0.03$^{b,d}$</td>
<td>NA</td>
<td>2.0 ± 0.08$^c$</td>
</tr>
<tr>
<td>M181H239M</td>
<td>1.2 ± 0.03</td>
<td>NA</td>
<td>3.0 ± 0.13$^d$</td>
</tr>
</tbody>
</table>

Two-way ANOVA revealed a significant effect of receptor construct on 5-HT concentration response curves ($F$(5, 349) = 92.5, $p < 0.0001$). $^a$ $p < 0.01$ compared to MWT; $^b$ $p < 0.001$ compared to MWT; $^c$ $p < 0.05$ compared to HWT; $^d$ $p < 0.001$ compared to HWT. Bonferroni’s multiple comparison test. NA = not applicable.
**Fig. 2**

A.  

![Graph showing % of Maximal Response for MWT and HWT](image)

B.  

![Graph showing % Inhibition with various drugs](image)

- **2-OHMBA** IC$_{50}$ = 1.5 μM
- **DMXBA** IC$_{50}$ = 3.1 μM
- **4-OHMBA** IC$_{50}$ = 7.4 μM
- **2, 4-DiOHBA** IC$_{50}$ = 12.8 μM
Fig. 3

A. 

B. 

Slope = -0.93 ± 0.17  
$K_i = 0.78 \, \mu M$
Fig. 5

A.  

M 5-HT3A 181 TSWLHTIQDINITLWR**SPEE**  
H 5-HT3A 176 TSWLHTIQDINISLWR**LPEK**  
M 5-HT3A 201 **VRSDKSIFINOGEWELLEVE**  
H 5-HT3A 196 **VKSDRSVFMNQGEWELGVL**  
M-5-HT3A 221 **QFKFESIDISNSYAEKMFY**  
H-5-HT3A 216 **FYREFSMESNSYNAEMKMRY**  
M-5-HT3A 241 **VIIRRR**  
H-5-HT3A 236 **VIIRRR**

B.  

% of Maximal Response  

% of Maximal Response vs. 2-OHMB, μM  

- MWT  
- EC_{50} = 2.0 μM  
- H176M244H  
- EC_{50} = 5.0 μM

C.  

H176M244H  

10 μM 2-OHMB  

10 μM 5-HT  

10 μM 5-HT  

1250 nA  

1 min.

D.  

% Inhibition  

% Inhibition vs. 2-OHMB, μM  

- HWT  
- IC_{50} = 1.5 μM  
- M181H239M  
- IC_{50} = 3.0 μM

E.  

M181H239M  

1 μM 5-HT  

6 μM 2-OHMB  

1 μM 5-HT  

1 μM 5-HT  

1250 nA  

1 min.