N-Terminal Domains in Mouse and Human 5-Hydroxytryptamine_{3A} 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

Department of Pharmacology and Neuroscience (R.Z., N.A.W., T.K.M.) and Department of Anesthesiology (T.K.M.), Texas Tech University Health Sciences Center, Lubbock, Texas; and Department of Pharmacology and Therapeutics (F.S.S., W.R.K.), University of Florida Health Sciences Center, Gainesville, Florida

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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 (DMXA) is an antagonist at the mouse wild-type 5-HT_{3A} receptor, but that its metabolites 3-(2-hydroxy, 4-methoxybenzylidene)-anabaseine (4-OHBA), and 3-(2,4-dihydroxybenzylidene)-anabaseine (2,4-DiOHBA) are partial agonists (*J Pharmacol Exp Ther*, 299: 1112–1117, 2001). In the human wild-type (HWT) 5-HT_{3A} receptor, none of the BA compounds possessed partial agonist or antagonist properties of 2-OHMBA.

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 (Mariq 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 through 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 coexpressed 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 and Wang, 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 (DMXA or GTS-21), has agonist activity at α7 but not at other nACh receptors (de Fiebre et al., 1995; Meyer et al., 1998a). DMXA is in clinical trials for the treatment of schizophrenia. DMXA improves cog...
nitive function in aging (Arendash et al., 1995) and brain-lesioned animals (Meyer et al., 1998b), and it also possesses neuroprotective properties (Meyer et al., 1998b). These properties are believed to be due to the actions of DMXBA and its metabolites at the \( \alpha 7 \) nicotinic ACh receptor. After oral administration, DMXBA is extensively metabolized via O-dealkylation to 2-OHMBA, 4-OHMBA, and 2,4-DiOHBA (Mahnir et al., 1998; Kem et al., 2004). 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 \( \alpha 7 \) nicotinic receptor and 5-HT\(_{3A}\) receptor. Papke and co-workers have demonstrated that DMXBA and its metabolites differ in partial agonist potency and efficacy in rat, monkey, and human \( \alpha 7 \) nicotinic ACh receptors (Stokes et al., 2004; Papke et al., 2005). In chimeric and point mutant \( \alpha 7 \) 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-HT\(_{3A}\) receptor and that its metabolites are partial agonists (Machu et al., 2001). Furthermore, a \(-OH\) at the 2' position is crucial for conferring partial agonist activity of \( \sim 50-60\% \) 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-HT\(_{3A}\) receptor, which is \( \sim 84\% \) identical in amino acid sequence to the mouse wild-type (MWT) 5-HT\(_{3A}\) receptor. All compounds inhibited the HWT 5-HT\(_{3A}\) 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-OHMBA, 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-OHMBA. These results suggest that the differences in 2-OHMBA activity at MWT and HWT 5-HT\(_{3A}\) receptors may be used to assess amino acids involved in initiation of gating of the receptor.

### Materials and Methods

**Analogs of 3-Benzylideneanabaseine.** Syntheses of DMXBA and two O-demethylated analogs (Fig. 1A) were described by 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 ice-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 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\(_3\), 10 mM HEPES, 0.82 mM MgSO\(_4\), 0.33 mM Ca(NO\(_3\))\(_2\), and 0.91 mM CaCl\(_2\), 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 83 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\),...
and 10 mM HEPES, pH 7.5, and gently rocked for 2 h. Oocytes were then removed and added to fresh collagenase medium and rocked gently for an additional 2 h. Lastly, oocytes were rinsed with MBS and stored in incubation media composed of ND96 containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.5, plus 10 mg/l streptomycin, 50 mg/l gentamicin, 10,000 U/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-HT3A receptor cDNAs, provided by Drs. D. Julius and A. Miyake, respectively, were subcloned into BlueScript KS+ and pCR-Script Amp SK+ (+) (Stratagene, La Jolla, CA). Two unique restriction enzyme cleavage sites, SpeI and Nael, were introduced in both mouse and human 5-HT3A receptor cDNAs by site-directed mutagenesis (U.S.E. mutagenesis kit; Amersham Biosciences, Inc., Piscataway, NJ) of the nucleotides encoding the conserved residues, Thr181 and Arg244 in the mouse receptor cDNA and Thr176 and Arg239 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 were ligated. Chimeric cDNAs were confirmed byideoxynucleotide sequencing at the Biotechnology Core Facility at Texas Tech University (Lubbock, TX).

Transcription of cDNA to cRNA. The wild-type and chimeric 5-HT3A receptor cDNAs were linearized with NotI, extracted with phenol-chloroform, precipitated with sodium acetate and ethanol, and resuspended in diethyl pyrocarbonate-treated water. The cDNAs were then transcribed with the T3 mMESSAGE mMACHINE (Ambion, Austin, TX). Two unique restriction enzyme cleavage sites, SpeI and Nael, were introduced in both mouse and human 5-HT3A receptor cDNAs by site-directed mutagenesis (U.S.E. mutagenesis kit; Amersham Biosciences, Inc., Piscataway, NJ) of the nucleotides encoding the conserved residues, Thr181 and Arg244 in the mouse receptor cDNA and Thr176 and Arg239 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 were ligated. Chimeric cDNAs were confirmed byideoxynucleotide sequencing at the Biotechnology Core Facility at Texas Tech University (Lubbock, TX).

Microinjection of Oocytes with 5-HT3 Receptor cRNA. An aliquot of cRNA was centrifuged at 15,000g, and the ethanol was removed with a tuberculin syringe. After air drying, the pellet was resuspended in a volume of diethyl pyrocarbonate-treated water. The cDNAs were then transcribed with the T3 mMESSAGE mMACHINE (Ambion, Austin, TX).

Electrophysiological Recordings. Oocytes were perfused (2 mM/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 3 M KCl were used to impale oocytes. A Warner Instruments Model OC-725B or OC-725C oocyte clamp (Hamden, CT) was used to volt- age clamp oocytes at −70 mV, and clamping currents were plotted on a strip chart recorder (Cole Parmer Instrument, Co.).

To examine agonist or partial agonist effects, 5-HT or BA analogs were dissolved in MBS buffer and applied to oocytes for 30 s. 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 5 min.

Data Analysis. The values in the 5-HT or BA agonist 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 of the other experiments (antagonist effects), data were expressed as a percentage 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 percentage of maximal response. For antagonism, percentage 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.

Results

In a previous study, we demonstrated that DMXBA was an antagonist and that demethylated analogs of DMXBA (Fig. 1A) were partial agonists at the MWT 5-HT3A receptor (Machu et al., 2001). The rank order of potency for agonism was 5-HT > 2-OHMB > 2,4-DiOHBA > 4-OHMB; both 2-OHMB 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 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 EC50 in the human receptor (see Fig. 3A). The rank order of potency (IC50 in μM) and Hill coefficient were 2-OHMB (1.5 ± 0.1, 1.5 ± 0.19) > DMXBA (3.1 ± 0.2, 1.3 ± 0.12) > 4-OHMB (7.4 ± 0.5, 1.3 ± 0.11) > 2,4-DiOHBA (12.8 ± 0.7, 1.2 ± 0.08). Given that 2-OHMB had the greatest agonistic effects 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 of the subsequent studies.

To examine the nature of antagonism produced by 2-OHMB, 5-HT concentration response curves were performed in the absence or presence of increasing concentrations of 2-OHMB (Fig. 3A). In the 5-HT concentration response curve generated in the absence of 2-OHMB, data were normalized to the 25 μM 5-HT baseline response. In the 5-HT concentration response curve generated in the presence of 2-OHMB, data were normalized to the 200 μM 5-HT baseline response. Parallel shifts in the 5-HT concentration response curves were observed as 2-OHMB concentrations were increased from 2 to 50 μM. 5-HT EC50 values increased from 1.5 ± 0.01 μM (no 2-OHMB) to 5.6 ± 0.03 μM (2 μM 2-OHMB), 12.8 ± 0.4 μM (10 μM 2-OHMB), and 84.5 ± 0.6 μM (50 μM 2-OHMB). Hill slopes were 3.4 ± 0.26 (no 2-OHMB), 1.6 ± 0.15 (2 μM 2-OHMB), 2.2 ± 0.15 (10 μM 2-OHMB), and 1.3 ± 0.15 (50 μM 2-OHMB). Increases in 5-HT EC50 values 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 $K_I$. The pA₂ was 6.11 with a $K_I$ of 0.78 μM. A slope of $-0.93 \pm 0.17$ was obtained, which was significantly different from 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 1-min preincubation with 2-OHMBA (2 μM). Without preincubation, 48.95 ± 4.67% inhibition of 5-HT-mediated currents was observed. With preincubation, 69.95 ± 4.9% inhibition of 5-HT-mediated currents was observed ($n = 4, p = 0.02, \text{Student's} \ t \ \text{test}; \ \text{data not shown}$). 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-OHMBA 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-HT$_{3A}$ receptor are localized in the N-terminal domains. Thus, to verify that differential sensitivity of the two receptors to 2-OHMBA 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 EC$_{50}$ 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 EC$_{50}$ 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 EC$_{50}$ of 3.9 ± 0.03 µM, which is significantly greater than that of MWT and HWT 5-HT$_{3A}$ 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-OHMBA, we tested the drug in both M244H and H239M (Fig. 4). Partial agonist activity of 2-OHMBA was observed in M244H (Fig. 4A). The EC$_{50}$ of 1.3 ± 0.15 µM for 2-OHMBA in M244H was similar to that observed in MWT 5-HT$_{3A}$ 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-OHMBA was apparently slightly less in M244H (43.0 ± 5.7%) than MWT (63.6 ± 4.8%). However, two-way ANOVA revealed that the concentration-response curves were not significantly different ($F_{1,134} = 3.39, p = 0.07$). A significant effect of 2-OHMBA concentration was observed [$F_{1,134,134} = 42.13, p < 0.0001$]. Representative tracings of maximal 5-HT and 2-OHMBA concentration-evoked currents in M244H are shown in Fig. 4B. In H239M, 2-OHMBA had no partial agonist activity (data not shown). The drug inhibited 4 µM 5-HT-mediated (−EC$_{50}$) currents with an IC$_{50}$ 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-OHMBA in HWT (IC$_{50}$ = 1.5 ± 0.1 µM) [$F_{1,182} = 46.57, p < 0.0001$] (Table 1). Percentage inhibition changed as a function of 2-OHMBA concentration [$F_{0.82, 290.51} = 290.51, p < 0.0001$], but no interaction was obtained between receptor construct and drug concentration [$F_{1,182} = 1.8, p = 0.09$]. Representative currents produced by 5-HT in the absence and presence of 2-OHMBA 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 1 (TM1) segments. Approximately 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 terminus are responsible for the pharmacological action of 2-OHMBA, 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 EC$_{50}$ values of 1.7 ± 0.3 and 1.2 ± 0.03 µM, respectively. The EC$_{50}$ of H176M244H chimera was significantly greater than that of MWT (Table 1). Hill slopes were 1.82 ± 0.96 (H176M244H) and 3.2 ± 0.25 (M181H239M) (data not shown).

The actions of 2-OHMBA 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-OHMBA concentration-response curve was slightly right-shifted in H176M244H relative to MWT; two-way ANOVA demonstrated that the two curves were significantly different [$F_{1,173} = 15.08, p = 0.0002$]; the IC$_{50}$ for the chimera was significantly greater (Table 1). A significant effect of 2-OHMBA 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-OHMBA-evoked currents in H176M244H are depicted in Fig. 5C. In M181H239M, no partial agonist activity of 2-OHMBA was observed (data not shown). As predicted, 2-OHMBA inhibited 1.2 µM 5-HT-mediated (EC$_{50}$) 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 [$F_{1,113} = 193.7, p < 0.0001$]. A significant interaction was observed between receptor construct and drug [$F_{1,113} = 15.08, p = 0.0003$]. In Fig. 5E, typical tracings of 5-HT-evoked currents, in the presence and absence of 2-OHMBA, 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-OHMBA 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 5-HT$_{3A}$ receptor. Instead, they inhibit 5-HT-mediated currents in the HWT 5-HT$_{3A}$ 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...

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**TABLE 1**

Wild-type and chimeric 5-HT$_{3A}$ receptor potencies and efficacies

| Two-way ANOVA revealed a significant effect of receptor construct on 5-HT concentration-response curves [$F_{0.82, 290.51} = 290.51, p < 0.0001$].|
|---|---|---|
| 5-HT | 2-OHMBA | 2-OHMBA |
| | EC$_{50}$ | EC$_{50}$/E$_{max}$ | IC$_{50}$ |
| | µM | nM | µM |
| MWT | 0.9 ± 0.06 | 2.0 ± 0.24, 63% | NA |
| M244H | 1.2 ± 0.02 | 1.3 ± 0.15, 43% | NA |
| H176M244H | 1.7 ± 0.3* | 5.0 ± 0.4, 57% | NA |
| HWT | 1.5 ± 0.01 | NA | 1.5 ± 0.1 |
| H239M | 3.9 ± 0.03d, NA | 2.0 ± 0.08e | 3.0 ± 0.13d |
| M181H239M | 1.2 ± 0.03 | NA | 3.0 ± 0.13d |

*NA, not applicable.
* $p < 0.01$ compared with MWT.
* $p < 0.001$ compared with MWT.
* $p < 0.05$ compared with HWT.
* $p < 0.001$ compared with HWT, Bonferroni’s multiple comparison test.
between the MWT and HWT 5-HT₃A receptors in this region are residues that are both necessary and sufficient to confer drug action.

Collectively, the results presented suggest that 2-OHMB is a competitive antagonist at the HWT 5-HT₃A receptor. The slope of the Schild plot of the 2-OHMB competition curves is -0.93, which is strongly suggestive of a competitive form of antagonism. However, the Hill slopes in the presence of 2-OHMB are lower than that in its absence. In the literature, Hill slopes for 5-HT in the HWT range from 1.4 to 3.1 (Hope et al., 1999; Barann et al., 2002; Hapfelmeier et al., 2003), 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 to 6 μM. Slight deviations in EC values across sets of experiments can significantly affect the Hill coefficient in the absence and presence of antagonists. In addition, 2-OHMB probably has a second noncompetitive site of action, which may be in the channel pore. In MWT and HWT 5-HT₃A receptors that have identical amino acid compositions in

![Fig. 4](image-url)

Fig. 4. The actions of 2-OHMB 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 of the human receptor (M244H) were perfused with 5-HT (10 μM) or 2-OHMB (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 of the mouse receptor (H239M) were perfused with 5-HT (EC₅₀) in the absence and presence of 2-OHMB (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 percentage inhibition of the respective control 5-HT-mediated response. D, representative tracings in H239M demonstrate that 2-OHMB antagonizes 5-HT-mediated currents.
TM2, which lines the channel pore of the receptors, a “tail current” is sometimes observed upon washout of BA compounds that exceed 50 μM concentrations (Machu et al., 2001; T. K. Machu, T. F. Frye, and C. L. Shanklin, 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 maximal 2-OHMBA 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-OHMBA from an antagonist to a partial agonist with efficacy approaching that observed in the MWT 5-HT3A receptor. It is possible that 2-OHMBA 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-OHMBA to this site and alter the agonist binding site to permit drug recognition and channel opening. The most parsimonious hypothesis is that 2-OHMBA occupies the agonist recognition site in both the MWT and HWT 5-HT3A receptors but cannot initiate gating in the HWT 5-HT3A receptor.

DMXBA and its metabolites all inhibited the HWT 5-HT3A receptor, with IC50 values ranging from 1.5 to 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 5-HT3A receptor function, suggesting that neither functional group has a drug molecule site-specific assignment required for binding of the drug to the HWT 5-HT3A 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 2-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-diOHBDA had an 8.5-fold lower potency than 2-OHMBA. In contrast, in the MWT 5-HT3A 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 8-fold lower potency) of the BA compound at the mouse MWT 5-HT3A receptor. Collectively, these results suggest a much greater precision of drug molecule interaction with the agonist binding domain to initiate gating in MWT 5-HT3A receptors than to inhibit 5-HT binding in HWT 5-HT3A receptors.

The function of 2-OHMBA 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 (for review see Sine, 2002). 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-HT3A 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-OHMBa in mouse and human 5-HT3A receptors, respectively.

Site-directed mutagenesis studies in the 5-HT3A receptor strongly support the roles of numerous residues in loops A through 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 the binding of [3H]m-chlorophenylbiguanide and [3H]GR65630 (Schreiter et al., 2003). Furthermore, Ala substitutions at Phe226, Ile228, and Tyr234 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 Ser206 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-OHMBa at the mouse and human 5-HT3A receptor.

The recent crystallization and structural determination of the acetylcholine-binding protein (Brejc et al., 2001) has been used to generate homology models of the N-terminal domains of the 5-HT3A receptor (Maksay et al., 2003; Reeves et al., 2003). These models have been very useful in elucidating the possible roles of amino acids in loops A through F in stabilizing the architecture of the ligand binding site and in spatially orienting agonists and antagonists in the ligand binding site. Maksay et al. (2003) have compared mouse and human 5-HT3A receptor models and suggest that loop C orthologs, mouse Asp229/human Glu224 and mouse Ile230/human Ser225, 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-OHMBa binding in mouse and human receptors will be of interest to determine. Loop F has not been clearly resolved in the acetylcholine-binding protein (Brejc et al., 2001) and has not been modeled in the 5-HT3A receptor.

Downstream gating events have been linked to residues in the N-terminal domains of the 5-HT3A receptor. Movement of Trp183 in loop B is thought to initiate motions of β3 and the Cys loops of the extracellular domain that straddle the TM2 to 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 to 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-OHMA from partial agonism to antagonism in the mutant receptor.

References


Dr. Tina K. Machu, Dept. of Pharmacology and Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd., Ft. Worth, TX 76107-2699. E-mail: tmachu@hsc.unt.edu


Address correspondence to: Dr. Tina K. Machu, Dept. of Pharmacology and Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd., Ft. Worth, TX 76107-2699. E-mail: tmachu@hsc.unt.edu

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