JPET Fast Forward. Published on May 2, 2003 as DOI:10.1124/jpet.103.050542

JPET #50542

A Mutation in Transmembrane Domain II of the 5-HT_{3A} Receptor Stabilizes Channel Opening and Alters Alcohol Modulatory Actions

J.S. Sessoms-Sikes[1], Margaret E. Hamilton[2], Li-Xin Liu[2], David M. Lovinger[3], and Tina K. Machu[2, 4]

Dept. of Pharmacology (MEH, L-XL, TKM)
Dept. of Anesthesiology (TKM)
Texas Tech University Health Sciences Center
3601 Fourth St.
Lubbock, TX 79430

Dept. of Molecular Physiology and Biophysics (JSS-S, DML)
Vanderbilt University School of Medicine
Nashville, TN 37232-0615

Running Title: Mutant 5-HT_{3A} Receptor: Kinetics and Alcohol Actions

Corresponding Author:
Tina K. Machu, Ph.D.
Texas Tech University Health Sciences Center
3601 Fourth St.
Lubbock, TX 79430
806-743-2425
FAX: 806-743-2744
tina.machu@ttuhsc.edu

Number of text pages: 25
Number of tables: 0
Number of figures: 6
Number of references: 34

Abstract Word Count: 196
Introduction Word Count: 749
Discussion Word Count: 1465

Nonstandard Abbreviations:
MBS=Modified Barth's Solution
TCEt=2,2,2,-Trichloroethanol
TM2=Transmembrane 2

Recommended Section Assignment: Neuropharmacology

Mutant 5-HT_{3A} receptors, in which changes were made at Ile294, position 16', of the second transmembrane domain, were assessed for alterations in macroscopic response kinetics and modulation by alcohols. Function of heterologously expressed receptors was measured in *Xenopus* oocytes in the two-electrode voltage clamp configuration and in HEK293 cells using whole cell patch-clamp electrophysiological recordings with rapid drug application. Compared to the wild-type receptor, a decrease in the 5-HT EC_{50} in the Ile294Thr mutant was observed, whereas an increase in the 5-HT EC_{50} in the Ile294Leu mutant was measured. Ile294Thr receptors showed marked reduction in the extent of desensitization. Ethanol and trichloroethanol (TCEt) enhanced 5-HT mediated currents in wild-type and Ile294Leu receptors, but inhibited or had little stimulatory effect in the Ile294Thr mutant. Kinetic analysis revealed that in the presence of TCEt, the slope of activation was unchanged in the Ile294Thr mutant and increased in the wild-type receptor. Alcohol cut-off was altered with wild-type = heptanol and Ile294Leu = hexanol. Kinetic changes in the Ile294Thr mutant which favor the open channel state, as well as reduction in the rate of channel activation in the presence of TCEt, likely underlie this mutant's altered response to n-chain alcohols.

JPET Fast Forward. Published on May 2, 2003 as DOI: 10.1124/jpet.103.050542
This article has not been copyedited and formatted. The final version may differ from this version.

The 5-Hydroxytryptamine$_3$ (5-HT$_3$) receptor is a member of the superfamily of ligand-gated ion channels (LGIC), of which the nicotinic acetylcholine (nAch) receptor is the prototype (Derkach et al., 1991; Peters et al., 1994). To date, two subunits of the 5-HT$_3$ receptor, 5-HT$_{3A}$ (Mariq et al., 1991, Belelli et al., 1995, Miyake et al., 1995, Lankiewicz et al., 1998) and 5-HT$_{3B}$ (Davies et al., 1999; Dubin et al., 1999) have been cloned. Recent reverse transcriptase and immunohistochemical studies demonstrated that the B subunit is largely expressed in the peripheral nervous system, suggesting that the predominant form in the brain is the A homomer (Morales et al., 2002). The most well-documented actions of 5-HT$_3$ receptors are to alter gastrointestinal motility and to regulate the vomiting reflex (Aarpo, 1991). Moreover, the 5-HT$_3$ receptor has been implicated in altering the voluntary intake of ethanol in humans (Johnson et al., 1993) and rodents (Knapp and Pohorecky, 1992; Hodge et al., 1993).

The 5-HT$_3$ receptor is modulated by pharmacologically relevant concentrations of n-chain alcohols and anesthetics. Alcohols and volatile anesthetics, in the presence of low 5-HT concentrations, potentiate native and heterologously expressed 5-HT$_3$ receptors (Lovinger and White, 1991; Machu and Harris, 1994). The mechanism for enhancement of receptor function by alcohols has been assessed in NCB-20 cells, which express the 5-HT$_3$ receptor endogenously. Ethanol and trichloroethanol (TCEt) enhanced peak currents evoked by a maximally effective concentration of dopamine, which is a weak partial agonist at the 5-HT$_3$ receptor (Lovinger et al., 2000). That alcohols would enhance receptor function under conditions of full agonist occupancy suggests that they increase probability of channel opening independent of any effect on agonist affinity. Whole cell patch-clamp electrophysiological recordings conducted with

rapid drug superfusion suggest that alcohols increase the activation rate, decrease the intrinsic desensitization rate, and decrease the deactivation rate of the 5-HT_3 receptor channel. These effects act in concert to favor and stabilize the open channel state (Zhou et al., 1998).

The binding domain in the 5-HT_3 receptor with which alcohols and/or anesthetics interact remains unknown. On the basis of bi-directional modulation of the nAch α7 receptor and 5-HT_{3A} receptors by ethanol, a chimera of the two was used to define a candidate region for ethanol sensitivity in these two receptors (Yu et al., 1996). The chimera was composed of the N-terminus of the nAch α7 receptor, and the balance 5-HT_{3A} receptor. Inhibition by ethanol was observed, consistent with the hypothesis that the N-terminus confers sensitivity. In contrast, amino acids at position 15' of the second transmembrane domain (TM2) and at position 16' of the third transmembrane domain (TM3) of other ligand-gated ion channels, the γ-aminobutyric acid (GABA) type A and rho_1 receptors, as well as the glycine receptor, determine the direction of alcohol and anesthetic modulation (Belelli et al., 1997; Mihic et al., 1997; Amin, 1999). Alcohol cut-off, which is the n-chain alcohol at which functional effects are lost altogether or are reduced to that of the n-1 alcohol, was altered in receptors containing mutations at these critical residues. Thus, mutant glycine receptors had the cut-off of GABA rho_1 receptors and *vice versa* (Wick et al., 1998). These results, plus correlation of molecular volume of these critical residues with direction or degree of modulation (Ye et al., 1998; Yamakura et al., 1999; Koltchine et al., 1999), has led to the speculation that an alcohol/anesthetic binding pocket may reside in a cavity between these amino acids in TM2 and TM3. On the other hand, the possibility that these drugs bind elsewhere in the receptor, with the mutations altering the transduction of the alcohol and anesthetic signal, cannot be ruled out.

The present study was undertaken to examine alcohol action on 5-HT_{3A} receptors containing mutations at Ile294 of TM2, a residue which has recently been shown to face the channel pore (Reeves et al., 2001; Panicker et al., 2002). Ile294 is at position 16' of TM2, neighboring the amino acid that is critical for n-chain alcohol and anesthetic modulation of the $GABA_A$, GABA rho_1, and glycine receptors at position 15' (Belelli et al., 1997; Mihic et al., 1997; Amin, 1999). We tested the hypothesis that mutation of Ileu294 in TM2 changes the response to n-chain alcohol sensitivity in the absence of any actions on an alcohol binding site. Ethanol and TCEt potentiation of receptor function is largely lost and change in n-chain alcohol cut-off occurs with mutation, suggesting that kinetics of channel gating have been altered rather than the dimension and/or physicochemical characteristics of an alcohol/anesthetic binding pocket.

Methods

Site-directed mutagenesis

The cDNA encoding the NCB-20 5-HT_{3A} receptor (gift from Dr. D. Julius, San Francisco, CA) was subcloned in pBluescript KS- (Stratagene, La Jolla, CA). Numbering of the amino acids began with the initiating methionine. Mutant cDNAs were obtained with the unique site elimination method (U.S.E. Mutagenesis kit, Pharmacia Biotech, Piscataway, NJ). In each mutagenesis reaction, selection pressure was created by deleting a unique restriction site in the plasmid DNA. Mutations were confirmed by dideoxynucleotide sequencing of double stranded DNA at the Texas Tech University Biotechnology Core Facility, Lubbock, TX.

Expression of 5-HT_{3A} receptors in *Xenopus* laevis oocytes

Ovarian lobes were obtained from *X. laevis* frogs and 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). Stage V and VI oocytes were dissected with fine surgical forceps in a hypertonic isolation medium containing (in mM) NaCl 108, KCl 2, EDTA 1, and HEPES 10 (pH 7.5) and placed in MBS. Dissected oocytes were incubated for 10 min in buffer containing 0.5 mg/ml collagenase Type IA and (in mM) NaCl 83, KCl 2, MgCl2 1, and HEPES 10 (pH 7.5) to remove the follicular cell layer. After several rinses with MBS, oocytes were placed in incubation medium composed of ND96, containing (in mM) NaCl 96, KCl 2, CaCl2 1.8, MgCl2 1, and HEPES 5 (pH 7.5) or MBS, plus 10 mg/l streptomycin, 50 mg/l gentamicin, 10,000 units/l penicillin, 0.5 mM theophylline, and 2 mM sodium pyruvate.

Wild-type and mutant 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 T3 mMESSAGE mMACHINE (Ambion, Austin, TX). An aliquot of cRNA was centrifuged at 15,000 x g, and the ethanol was removed. The pellet was resuspened in RNase free water, and 5-30 ng of cRNA were injected per oocyte. Oocytes were stored in incubation medium, and were recorded from days two through seven following injection.

Two electrode voltage-clamp 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 to -70 mV. Clamping currents were plotted on a strip chart recorder (Cole Parmer Instrument, Co., Chicago, IL). Serotonin (Sigma, St. Louis, MO), ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA) and other n-chain alcohols (Sigma), and 2,2,2,-Trichloroethanol (TCEt; >99% pure, Sigma) were dissolved in MBS buffer. Serotonin was applied for 30 s. N-chain alcohols were perfused for one min prior to the application of 5-HT + n-chain alcohol for 30 s; pre-application was used to ensure that the relatively low potency n-chain alcohols were equilibrated with their putative alcohol binding domains prior to exposure to 5-HT + n-chain alcohol. Equipotent concentrations of 5-HT (~ EC_1) were used for each receptor construct.

JPET Fast Forward. Published on May 2, 2003 as DOI: 10.1124/jpet.103.050542
This article has not been copyedited and formatted. The final version may differ from this version.

Data Analysis

The values in the 5-HT concentration response curves for wild-type and mutant 5-HT_{3A} receptors were expressed as a percentage of the respective maximal 5-HT (10 μM) responses. Unless otherwise noted, in all other experiments, data were expressed as percent change from the control, baseline response. Graphpad Prism (San Diego, CA) was used to calculate EC_{50}s, Hill coefficients, and two-way analysis of variance (ANOVA). Instat (San Diego, CA) was used to perform Student's t-tests.

Expression of 5-HT_{3A} receptors in HEK 293 cells

Wild-type and mutant 5-HT_{3A} cDNAs were subcloned into pcDNA3.1 (Invitrogen; Carlsbad, CA). HEK 293 cells grown in standard 35 mm diameter culture dishes were transfected by calcium phosphate precipitation as previously described (Lovinger and Zhou, 1994). Cells were maintained in a 95% O_2/ 5% CO_2 incubator, and recombinant receptor activity was measured beginning ~48 hours after transfection.

Whole Cell Patch-Clamp Electrophysiological Recordings with Rapid Drug Application

In all experiments, transfected cells were re-plated as single cells onto suspension dishes prior to initiating the recording. Cells were resuspended in a phosphate buffered saline solution containing 3 mM EDTA. The cell suspension was centrifuged, and cells were resuspended with light trituration in the external medium used for electrophysiological recording (see below).

Cells were then plated onto 35 mm diameter suspension dishes and were allowed to settle on the dish for at least 5 min before recording was initiated.

Whole-cell patch–clamp recordings were performed on transfected HEK293 cells bathed in external solution containing (in mM): 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 10 HEPES, 10 D-glucose (pH adjusted to 7.4 with NaOH and osmolality adjusted to 340 mOsm/kg with sucrose) as described previously (Zhou and Lovinger, 1996, Zhou et al, 1998) with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). The solution constantly superfused cells at a rate of 2-3 ml/min. Patch pipettes had resistances of 1-2 MΩ when filled with (mM): 140 CsCl, 10 Cs-EGTA and 10 HEPES (pH adjusted to 7.4 with CsOH and osmolality adjusted to 313-316 mosmol kg^{-1} with sucrose). Cells were voltage-clamped with the membrane potential held at -70 mV. After establishing a recording, the cell was lifted clear of the bottom of the suspension dish and was placed into a solution stream coming from the drug application micropipette. In selected experiments, alcohols were applied alone for 30 s prior to application of agonist + alcohol.

Alcohol and other pharmacological agents were dissolved directly into external solution, and applied to transfected HEK cells using two different techniques. In the majority of experiments, agonist was applied with a theta-tube whose lateral movement was controlled by a piezoelectric manipulator, as previously described (Zhou et al., 1998). The solution exchange time using this technique is 20 - 40 ms in the whole-cell recording mode. In a few experiments drug application was accomplished with a stepper-motor mounted on a micromanipulator (Warner Instruments Inc., Hamden, CT). In this system solution was applied from glass tubes

mounted on the manipulator, and solution exchange time was 22 ± 5 ms. At the beginning of each experiment the cell was placed in front of a solution stream of standard extracellular solution. Agonist application was initiated under computer control resulting in lateral displacement of the application pipette by 100-200 micrometers such that the cell was superfused by a solution containing agonist ± alcohol. Cessation of the computer-generated pulse reversed the pipette displacement, returning the cell to the standard external solution stream.

Solution applications ranged in duration from 1-30s. Data were filtered at 5 kHz with a 3 pole Bessel filter and digitized at 10 kHz. In the majority of experiments, data were acquired using an Axopatch 200 amplifier, and digitized using an ITC-16 analog/digital interface linked to a MacIntosh IIfx computer by means of IGOR software (Wavemetrics Inc., Lake Oswego, OR). In a few experiments data acquisition was accomplished using a TL-1-125 interface (Scientific Solutions, Mentor, OH) linked to a Pentium microcomputer running pClamp v5.5 software (Axon Instruments, Union City, CA.) Data were analyzed offline using either IGOR v3.0 or pClamp v8.0 software. Current amplitudes were determined using a cursor-based system to measure current at a particular time point, and these amplitudes were then subtracted from the baseline, pre-agonist current. Peak current was the maximal agonist-induced current, and was defined as the difference between the maximal current and the baseline pre-agonist holding (trough) current. Steady-state current was the stable current observed during prolonged (30 s) agonist application. Steady state currents were measured at 30 s. It must be noted that at 2 μM 5-HT (Fig. 2B, Fig. 5A), the Ile294Thr mutant receptor reached an apparent steady-state at 30 s. Very slight decrements in current were observed between 25 and 30 s of agonist exposure. Non-linear curve fitting and estimates of the time course of exponential decay were obtained using

IGOR software. The initial current slope was calculated for data points between 10 and 20% of the maximal current on the downward-sloping initial phase of current. Measurement of 10% of the peak response eliminates the contributions of non-linear components of current at the bottom of a presumed sigmoidal shaped response. Measurement at 20% of the peak current, rather than at higher percentages of peak current, ensures that slope can be measured accurately, with the least possible contamination from any other current component. All current amplitudes were normalized to whole cell capacitance and are reported as pA pF^{-1}. All averaged measurements are expressed as mean ± SEM values.

Results

Receptors containing mutations at Ile294 of the mouse 5-HT_{3A} receptor were initially compared with the wild-type receptor for their sensitivity to 5-HT (Fig. 1 A, B). In oocytes expressing the receptors, mutations at Ile294 resulted in shifts of the 5-HT concentration response curves (Fig. 1A), n=4. Two-way ANOVA demonstrated that mutation had a significant effect on the 5-HT concentration response curves ($F_{(2, 54)}$=3.88, p=0.03). The EC_{50} value (in μM) of the Ile294Thr 5-HT_{3A} receptor (0.65 $\pm$ 0.015) was significantly less than that of the wild-type receptor (0.93 $\pm$ 0.023), Student's t-test, $p < 0.0001$. The EC_{50} of the Ile294Leu mutant (1.15 $\pm$ 0.14) was significantly greater than that of the wild-type receptor, Student's t-test, p=0.0003. Hill coefficients were 3.2 $\pm$ 0.22 (wild-type), 4.1 $\pm$ 0.36 (Ile294Thr), and 3.1 $\pm$ 0.14 (Ile294Leu).

In HEK293 cells expressing wild-type and Ile294Thr 5-HT_{3A} receptors, 5-HT concentration response curves were generated, (Fig. 1B). Two-way ANOVA revealed that the curves were not significantly different ($F_{(1, 26)}$=3.36, p=0.08). EC_{50} values (in μM) and Hill coefficients were 3.82 $\pm$ 0.11 and 2.2 + 0.13 (wild-type) and 2.73 $\pm$ 0.18 and 1.7 $\pm$ 0.2 (Ile294Thr). It should be noted that the difference in 5-HT potency in oocytes compared to HEK 293 cells is due to the relatively slow agonist application in oocytes where current is measured after many of the receptors have undergone desensitization, in which case apparent agonist affinity and potency are increased. In addition, the observation in the oocyte expression system that the Hill coefficient of the Ile294Thr mutant was greater than that of the wild-type receptor may reflect an underestimation of the peak current and hence a steeper curve.

Currents generated in the presence of 5-HT in cells expressing wild-type or mutant receptors are presented in Fig. 2. In Fig. 2A, representative currents obtained from oocytes expressing wild-type, Ile294Thr, and Ile294Leu receptors are shown. Similar desensitization of the responses to 10 μM 5-HT were observed between wild-type and Ile294Leu receptors. In contrast, the most striking aspect of the currents generated in the Ile294Thr mutant expressed in oocytes is the slow decay toward baseline of current during the prolonged application of 10 μM 5-HT (Fig. 2A). Rapid current decay was observed in the wild-type receptor; however, recovery of currents in the Ile294Thr mutant was only ~ 33% at the end of the 30 s application of 5-HT (10 μM). Generally, a minimum of four min were required for the holding current to reach the baseline, pre-5-HT application levels. In Fig. 2B, current traces obtained in wild-type and Ile294Thr 5-HT_{3A} receptors expressed in HEK293 cells are shown. The most pronounced effect is the reduction in the amount of desensitization upon prolonged exposure to 10 μM 5-HT (30 s) in the Ile294Thr mutant. Furthermore, 5-HT-mediated current activated by a low concentration of agonist (2 μM) exhibited a sustained current in the Ile294Thr mutant in contrast to the rapid peak and desensitization of currents produced by the same agonist concentrations in the wild-type receptor (Fig. 2B). The insets in Fig. 2B are presented with an expanded time scale and highlight the differences in current onset and desensitization of the wild-type and mutant receptors.

With the rapid drug application protocol, we measured several of the parameters of whole-cell current in receptors expressed in HEK 293 cells that provide information about macroscopic kinetics and modulation of ion channel function by alcohols, as shown previously (Zhou et al., 1998). These include the initial slope of current during the activation phase, the 20-

80% rise-time, the Steady State/Peak (SS/Peak) current ratio, and the rate of current decay during prolonged agonist application. The Ile294Thr mutation did not produce a significant increase in initial current slope (101.2 + 35 pA pF^{-1} s^{-1}, n=5) relative to the wild-type receptor (89.3 + 62.7 pA pF^{-1} s^{-1}, n=3), (Student's t-test, * p=0.86). The 20-80% rise-time of the Ile294Thr mutant (1.7 + 0.46 s, n=5) was not significantly greater than that of the wild-type receptor (0.37 + 0.22 s, n=3), (Student's t-test, * p=0.09), given the large standard error in the measurements. However, a longer rise-time would indicate that during this phase of activation, the mutant is slower than the wild-type receptor. In the example shown in Fig. 2B in the inset at the extreme right of the figure, wild-type and Ile294Thr receptor current traces in response to 2 μM 5-HT are overlaid. The slower rise time in the mutant receptor is apparent. A greater than two-fold increase in SS/Peak current ratio was observed in the Ile294Thr mutant relative to the wild-type receptor upon application of 5-HT (10 μM) (Fig. 2C) (Student's t-test, * p=0.0006). This increase reflects a decrease in the extent of desensitization; i.e., steady state levels were achieved after receptors had desensitized ~ 60% upon prolonged exposure to 5-HT (10 μM). In contrast, in the wild-type receptor, currents achieved steady state after ~ 95% desensitization. Furthermore, the rate of desensitization (1/tau) was reduced in the presence of the Ile294Thr mutation as measured by the time constant for current decay (tau (τ)) following the peak response in the continued presence of agonist (Fig. 2D) (Student's t-test, * p=0.0006). The mutation decreases the rate and extent of desensitization of the $5\text{-}HT_{3A}$ receptor. One probable outcome of the changes in desensitization is the open state of the mutant receptor-channel would likely be stabilized relative to the wild-type receptor.

JPET Fast Forward. Published on May 2, 2003 as DOI: 10.1124/jpet.103.050542
This article has not been copyedited and formatted. The final version may differ from this version.

We next examined the modulatory actions of ethanol on wild-type and mutant 5-HT_{3A} receptors expressed in *Xenopus* oocytes and HEK293 cells (Fig. 3). The function of the wild-type and Ile294Leu 5-HT_{3A} receptors expressed in oocytes was similarly enhanced by ethanol over the range of concentrations tested (Fig. 3A). Stimulation increased from an average of 15% at ethanol (50 mM) to ~ 55% at ethanol (200 mM). In contrast, the Ile294Thr 5-HT_{3A} receptor was inhibited by ethanol, but the concentration-response curve for inhibition was relatively flat, ranging from ~ 15 to 27%. Two-way ANOVA demonstrated that mutation had a significant effect ($F_{(3, 54)}$= 228, $p < 0.0001$). The Ile294Thr 5-HT_{3A} receptor was significantly different from the wild-type receptor at all ethanol concentrations tested (Tukey's post-hoc test, * $p < 0.05$). In HEK293 cells (Fig. 3B), 5-HT-mediated currents were measured in the absence and presence of ethanol in the wild-type and Ile294Thr receptors. Stimulation of ~ 17 and 26% was observed in the wild-type receptor with 50 and 100 mM ethanol, respectively. No effects on receptor function were obtained in the Ile294Thr 5-HT_{3A} receptor with ethanol (10 - 100 mM). The percent change in response to ethanol were not significantly different between wild-type and Ile294Thr receptors, two-way ANOVA, ($F_{(1, 22)}$=3.47, p=0.08). Ethanol applied alone did not elicit any detectable current in the wild-type or Ile294Thr 5-HT_{3A} receptors expressed in ooctyes (n=5) or HEK293 cells (n=3-4) (data not shown).

In previous studies we have demonstrated that wild-type 5-HT_{3A} receptor function is more robustly enhanced by TCEt than by ethanol (Lovinger and Zhou, 1996; Zhou et al., 1998), and we have also performed extensive kinetic analysis of TCEt's actions at this receptor (Zhou et al., 1998). Therefore, it was of interest to examine the actions of TCEt on Ile294Thr receptors and to compare the receptor-channel macroscopic kinetic parameters of channel function in the

presence of TCEt in wild-type and Ile294Thr receptors. We first examined the effect of TCEt on wild-type and Ile294Thr 5-HT_{3A} receptors expressed in *Xenopus* oocytes; the Ile294Leu mutant was not studied because of the lack of change in modulation by ethanol due to this mutation (Fig. 4). A concentration dependent enhancement of 5-HT-evoked currents was seen in wild-type receptors, ranging from ~ 55 – 2646% with 0.25 – 10 mM TCEt. A biphasic response was observed in oocytes expressing the Ile294Thr mutant. Receptor function was inhibited by ~ 58 and 34% at 0.25 and 5 mM TCEt, respectively. No effects were observed at 1 and 2 mM TCEt, whereas stimulation occurred at 5 and 10 mM TCEt. Two-way ANOVA revealed that the curves were significantly different ($F_{(1,78)}$=106, $p < 0.0001$). TCEt applied alone did not elicit any detectable current in the wild-type or Ile294Thr 5-HT_{3A} receptors expressed in ooctyes (n=4-6) (data not shown).

In studies with rapid drug application, we focused on measuring kinetic parameters of wild-type and Ile294Thr 5-HT_{3A} receptors expressed in HEK293 cells in the absence and presence of 3 mM TCEt. No detectable currents were observed in wild-type or Ile294Thr 5-HT_{3A} receptors when TCEt was applied alone (n=3-4) (data not shown). Representative tracings of 5-HT evoked currents generated in wild-type and mutant receptors are shown (Fig. 5A). As demonstrated previously (Zhou et al., 1998), TCEt enhanced wild-type receptor function, panel a. On the other hand, Ile294Thr receptor currents were slightly inhibited by TCEt, with an apparent decrease in the slope of activation, panel b. The insets (panels a and b) in Fig. 5A are presented using an expanded time scale and highlight the onset of current in the wild-type and mutant receptors. In Fig. 5A, panels c and d, wild-type and Ile294Thr receptor current traces in response to 5-HT or 5-HT + TCEt are overlaid; these are the same traces as presented in panels a

and b, but are overlaid to illustrate the differences between the constructs. Activation is slower in the mutant (panel c, inset), but is even slower in the mutant in the presence of TCEt (panel d, inset). Both peak and isochronal (4.5 s) measurements were made in each receptor construct (Fig. 5B), and data were expressed as (($I_{TCEt}/I_{control}$) x 100), where I_{TCEt} is the current obtained in the presence of TCEt and $I_{control}$ is the current obtained in its absence. Both peak and isochronal measurements were performed to take into account the fact that peak currents occur at different time points and that desensitization is also altered in the two constructs. Clearly, in the wild-type receptor both peak (Student's paired t-test, * p=0.04) and isochronal currents (Student's paired t-test, * p=0.04) were increased, whereas in the mutant receptor an insignificant decrease was observed in peak (Student's paired t-test, p=0.37) and isochronal currents (Student's paired t-test, p=0.17). The wild-type and Ile294Thr receptor peak (Student's t-test, + p=0.03) and isochronal currents (Student's t-test, + p=0.01) were different from each other.

Measurements of initial slope and rise-time (20 – 80% of the response), which represent activation of the receptor, in HEK293 cells were performed in the presence of TCEt and expressed as a percentage of that obtained in its absence (Fig. 5C). Activation rate of the wild-type receptor was enhanced ~ 70 % in the presence of TCEt. The slope of 61.4 $\pm$ 18 pA pF^{-1} s^{-1} in the absence of TCEt was significantly different than the slope of 87.8 $\pm$ 20.6 pA pF^{-1} s^{-1} in the presence of TCEt (Student's paired t-test, * p=0.03). No effect was observed in the mutant receptor (Student's t-test, p=0.42). The slopes were significantly different between the wild-type and Ile294Thr receptors (Student's t-test, + p=0.004). Rise-time was unaffected by the presence of TCEt in either construct (wild-type: Student's paired t-test, p=0.26, mutant: Student's paired t-test, p=0.81). The rise-times of the wild-type and mutant receptors were not significantly

different from one another (Student's t-test, p=0.66). Finally, SS/Peak current ratios were compared in the absence and presence of TCEt in the wild-type and mutant receptors (Fig. 5D). As seen in Fig. 5A, wild-type receptors desensitize rapidly in the presence of 2 μM 5-HT and thus the SS/Peak current ratios are less than 0.1. Addition of TCEt increases both peak and steady state currents in the wild-type receptor, and the increase in the SS/Peak current ratio in the presence of TCEt is significant (Student's paired t-test, p=0.03). Minimal desensitization takes place in the Ile294Thr 5-HT_{3A} receptor (Fig. 5A), which is reflected by a SS/Peak current ratio that is greater than 0.8. The addition of TCEt does not further increase the SS/Peak current ratio in the mutant (Fig. 5D), (Student's paired t-test, p=0.13). The SS/Peak current ratios are different between wild-type and Ile294Thr 5-HT_{3A} receptors in the presence of 2 μM 5-HT (Student's t-test, + $p < 0.0001$) or 2 μM 5-HT + 3 mM TCEt (Student's t-test, + $p < 0.0001$).

Alcohol cut-offs were determined in wild-type and mutant 5-HT_{3A} receptors expressed in *Xenopus* oocytes. The alcohol cut-off for a protein is the n-chain alcohol at which functional effects are lost altogether or are reduced to that of the n-1 alcohol. Jenkins et al. (1996) previously showed that hexanol and lower chain alcohols stimulate 5-HT_{3A} receptor evoked currents but that octanol and higher chain alcohols produce inhibition. A series of n-chain alcohols were examined. In Fig. 6A, pentanol (0.25-4 mM) enhanced the function of both wild-type and Ile294Leu 5-HT_{3A} receptors, although the mutant receptor was less sensitive as determined by two-way ANOVA ($F_{(1,39)}$ =53, $p < 0.0001$). The actions of hexanol on wild-type and mutant receptors were examined. In B and C, hexanol (0.25 – 10 mM) enhanced the function of wild-type receptors, but inhibited or had no effect on Ile294Leu and Ile294Thr receptors. Where responses to hexanol (0.1-1 mM) were measured, two-way ANOVA yielded

significant effect of mutation ($F_{(6,52)}$ = 198, p < 0.0001), and two-way ANOVA also revealed a significant effect of mutation for hexanol at the 5 and 10 mM concentrations ($F_{(2, 25)}$ =54.96, p < 0.0001), (Tukey's post-hoc test, * p < 0.05, compared to wild-type). These results suggest that the alcohol cut-off for the Ile294Leu 5-HT_{3A} receptor is hexanol. Actions of heptanol (0.1-1 mM) and octanol (0.03125 – 1 mM) were evaluated in wild-type and Ile294Thr receptors (Fig. 6D). Minimal potentiation of 5-HT mediated currents (< 27%) was observed in wild-type receptors over the range of heptanol concentrations tested, suggesting that the alcohol cut-off for the wild-type receptor is heptanol. Robust inhibition of the Ile294Thr mutant was produced with heptanol, and the wild-type and Ile294Thr receptors' concentration response curves were significantly different, two-way ANOVA ($F_{(1,24)}$ = 112.5, p < 0.0001). The Ile294Thr mutant was modulated by octanol to a similar extent as the wild-type receptor, two-way ANOVA ($F_{(1,25)}$ = 2.45, p=0.1302). Inhibition that is observed with higher concentrations of octanol in both of the receptors has been previously reported for the wild-type receptor and is believed to represent a distinct alcohol site(s) from the potentiating site(s) (Jenkins et al., 1996). Given that the Ile294Thr 5-HT_{3A} receptor is inhibited by all n-chain alcohols, it is difficult to ascertain alcohol cut-off for the inhibition observed in the mutated receptor.

Discussion

In the present study, we examined the effects of two mutations at position 16' of TM2 of the 5-HT_{3A} receptor on kinetics of channel gating, allosteric modulation by n-chain alcohols, and n-chain alcohol cut-off. The two mutations, Ile294Leu and Ile294Thr, were conservative and non-conservative, respectively. Ile and Leu are very similar, with Ile having slightly greater hydrophobicity. Thr has less bulk and hydrophobicity, but greater polarity than Ile. Ile294 faces the channel pore and therefore is a poor candidate as a residue that forms an alcohol binding domain, given that n-chain alcohols enhance 5-HT_{3A} receptor function. We tested the hypothesis that mutation of Ile294 may alter the receptor's response to n-chain alcohols in the absence of any change in a putative alcohol binding domain. In the Ile294Thr mutant, the rate and extent of desensitization was reduced relative to that observed in the wild-type receptor. We have demonstrated previously that TCEt similarly enhances activation rate and decreases the intrinsic desensitization rate in the wild-type receptor (Zhou et al., 1998). Thus, we propose that the loss of enhancing actions of TCEt in the Ile294Thr receptor is not due to a change in an alcohol binding domain, but instead occurs as a consequence of kinetic changes that favor the open channel state and likely occlude alcohol effects.

Each of the members of the superfamily of ligand-gated ion channels has been mutated in the TM2 domain, but the difficulty in studying such receptors lies with the fact that the pore lining domains are critical in participating in the conformational changes that occur when the channels open. This gating process may be fundamentally disturbed in a variety of ways (reviewed in Colquhoun, 1998). Changes in desensitization are demonstrated in the present study, where the Ile294Thr 5-HT_{3A} receptor shows a marked reduction in extent of

desensitization, as well as rate of desensitization. Other mutation induced changes that have been reported are changes in affinity, shift from antagonism to agonism (Bertrand et al., 1992), loss of allosteric modulation (Findlay et al., 2001), and production of tonically open channels (Findlay et al., 2001). Collectively, these data suggest that mutations in TM2 alter the state dependence of these channels by shifting the probability of closed -> open-> desensitized transitions. Such changes would have a high likelihood of interfering with allosteric changes in channel gating such as those produced by alcohols.

Mutation at Ile294 produced a number of changes in gating and allosteric modulation in the 5-HT_{3A} receptor. The EC_{50} for the Ile294Thr 5-HT_{3A} receptor was significantly reduced relative to the wild-type receptor when oocytes were used as an expression system. Although not significantly lower, the EC_{50} for the Ile294Thr 5-HT_{3A} receptor was reduced in HEK293 cells. In contrast, the Ile294Leu mutant, when expressed in oocytes, had a greater EC_{50} than the wild-type receptor. The Ile294Thr and not the Ile294Leu mutant lost positive allosteric modulation by ethanol. These results are consistent with the findings of others that demonstrate that a TM2 mutation induced increase in agonist potency correlates with a decrease in alcohol or anesthetic modulatory actions (Yamakura et al., 1999). A mutant 5-HT_{3A} receptor with greater agonist potency than the wild-type receptor is more likely to open at any given 5-HT concentration. From a mechanistic standpoint, the resting conformation of such a mutant is more thermodynamically favored to transition from a closed to open state. Positive allosteric modulation is typically lost when probability of opening is increased, as exemplified by loss of ethanol modulatory actions at high concentration of 5-HT in the wild-type receptor (Machu and Harris, 1994).

The most striking change produced by the Ile294Thr mutation was the reduction in the rate and extent of desensitization compared to wild-type receptors. Changes in desensitization in the mutant receptor suggest that once activated by 5-HT, it is more likely than the wild-type receptor to remain in the open channel state. The most parsimonius explanation for the reduction in enhancing effect of ethanol and TCEt on the Ile294Thr 5-HT_{3A} receptor that we have observed is that the open channel state is already favored in the absence of alcohols and that the mutation actually mimics the actions of alcohols, albeit to a greater extent, on desensitization. Thus, the Ile294Thr mutation may actually mask the stimulatory effects of alcohols. Our data support this conclusion in that TCEt significantly increases steady-state to peak current ratios in the wild-type receptor, but not the Ile294Thr5-HT_{3A} receptor. Further supporting evidence comes from work in the $GABA_A$ receptor. Mutations in either TM2 or in the N-terminus have been described that produce tonically open channels, an extreme case of increased probability of channel opening wherein a certain fraction of receptors are constantly open. These receptors have very reduced or no sensitivity to allosteric modulation by alcohols and anesthetics (Thompson et al., 1999; Findlay et al., 2001; Zhang et al., 2002).

Two contrasting effects on activation were observed in the Ile294Thr 5-HT_{3A} receptor. Both the initial slope and the 20 – 80% rise-time were increased, although not significantly, in the Ile294Thr mutant relative to the wild-type receptor. An increase in activation rate would favor the open channel state in the mutant relative to the wild-type receptor. However, an increase in rise-time means that the time required for opening is longer in the mutant, suggesting that the wild-type receptor is more favored to open when this parameter is considered. It is

important to note that the 20-80% rise-time is a less precise measure of activation, since it may be contaminated with other components of the whole-cell current. We suggest that the desensitization of some of the mutant channels may be occurring while others are still opening. While it is the summation of all factors that contribute to the stabilization of the open state, we suggest that the effects of the mutation on rate and extent of desensitization are likely to be the most important. That is, once the Ile294Thr receptor opens, it stays open which prolongs the later stages of current onset, as is evidenced by the overlaid tracings in Fig. 5A, panel c.

TCEt had contrasting effects on 5-HT mediated currents in the wild-type and Ile294Thr 5-HT_{3A} receptors. In the wild-type receptor, TCEt increased the initial slope, but had no effect on 20 - 80% rise-time. In the mutant receptor, the initial slope of current is reduced, although nonsignificantly, by TCEt. Rise-time in the mutant receptor was unaffected by TCEt. Taken together, these data suggest that inhibition of activation of channel opening is third mechanism through which the potentiating effects of alcohol are lost in the mutant receptor. In fact, inhibition of activation may in part account for blockade of mutant receptor function (oocyte expression system) with TCEt at lower concentrations and of mutant receptor function (HEK293 cell expression system) at 3 mM TCEt. Thus, slower current activation, combined with reduced desensitization, likely contributes to loss of alcohol effects. The biphasic response observed over the TCEt concentrations tested (0.125- 10 mM), with potentiation occurring at 5 and 10 mM, may reflect TCEt binding at lower affinity sites whose allosteric modulation is less affected or unaffected by the mutation.

The kinetic data that we have shown provide us with a compelling argument that the lack of stimulatory action of alcohols on the Ile294Thr 5-HT_{3A} receptor is not due to an alteration of a binding pocket for alcohols and anesthetics. Another supporting piece of evidence is the finding that Ile294 faces the pore of the channel (Reeves et al., 2001; Panicker et al., 2002). Given the fact that alcohols enhance 5-HT_{3A} receptor function, it would be difficult to reconcile the notion of an alcohol binding domain that included amino acids in the pore. A pore lining pocket would be more consistent with an inhibitory, channel-blocking, action of alcohols. Furthermore, in the face of other evidence presented, our observation that the Ile294Leu mutant has a reduced cut-off relative to the wild-type receptor does not lend credence to the idea of a binding pocket at Ile294 and is likely a coincidental finding.

In summary, we have shown that the Ile294Thr 5-HT_{3A} receptor has a markedly altered kinetic profile in comparison with the wild-type receptor. Changes in the extent and rate of desensitization and increased activation rate, as well as slowing of activation in the presence of alcohols, can explain the loss of stimulatory effects of ethanol and TCEt. These results, coupled with the finding that Ile294 faces the pore of the channel, cast doubt that Ile294 is part of an alcohol binding domain. Our results suggest that loss of alcohol and anesthetic modulation of function and changes in alcohol cut-off should not be used as the sole criteria for identifying a putative alcohol binding domain. Kinetics of channel function should be evaluated in a series of mutants at an apparently critical residue before an alcohol binding site should be invoked.

References

Amin J (1999) A single hydrophobic residue confers barbiturate sensitivity to γ-aminobutyric acid type C receptor. *Mol Pharmacol* **55:**411-423.

Belelli D, Balcarek JM, Hope AG, Peters JA, Lambert JJ and Blackburn TP (1995) Cloning and functional expression of a human 5-Hydroxytryptamine type 3As receptor subunit. *Mol Pharmacol* **48:**1054-1062.

Belelli D, Lambert JJ, Peters JA, Wafford K and Whiting PJ (1997) The interaction of the general anesthetic etomidate with the γ-aminobutyric acid A receptor is influenced by a single amino acid. *Proc Natl Acad Sci USA* **94:**11031-11036.

Bertrand D, Devillers-Thiery A, Revah F, Galzi J-L, Hussy N, Mulle C, Bertrand S, Ballivet M and Changeux J-P (1992) Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain. *Proc Natl Acad Sci USA* **89:**1261-1265.

Colquhoun D (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**924-947.

Davies PA, Pistis M, Hanna MC, Peters JA, Lambert JJ, Hales TG and Kirkness EF (1999) The 5-HT_{3B} subunit is a major determinant of serotonin-receptor function. *Nature* **397:**359-363.

Derkach V, Surprenant A and North RA (1989) 5-HT_3 receptors are membrane ion channels. *Nature* **339:**706-709.

Dubin AE, Huvar R, D'Andreas MR, Pyati J, Zhu JY, Koy KC, Wilson SJ, Galindo JE, Glass CA, Lin L, Jackson MR, Lovenberg TW and Erlander MG (1999) The pharmacological and functional characteristics of the serotonin 5-HT_{3A} receptor are specifically modified by a 5-HT_{3B} receptor subunit. *J Biol Chem* **274:**30799-30810.

Findlay GS, Susumu U, Harrison NL and Harris RA (2001) Allosteric modulation in spontaneously active mutant γ-aminobutryic $acid_A$ receptors. *Neurosci Lett* **305:**77-80.

Hodge CW, Samson HH, Lewis RS and Erickson HL (1993) Specific decreases in ethanol but not water-reinforced responding produced by the 5-HT antagonist ICS 205-930. *Alcohol* **10:**191-196.

Jenkins A, Franks NP and Lieb WR (1996) Actions of general anesthetics on 5-HT3 receptors in N1E-115 neuroblastoma cells. *Br J Pharmacol* **117:**1507-1515.

Johnson BA, Campling GM, Griffiths P, and Cohen PJ (1993) Attenuation of some alcohol-induced mood changes and the desire to drink by 5-HT_3 receptor blockade: a preliminary study in healthy male volunteers. *Psychopharmacol* **112:**142-144.

Karlin A and Akabas MH (1995) Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* **15:**1231-1244.

Knapp DJ and Pohorecky LA (1992) Zacopride, a 5-HT_3 receptor antagonist, reduces voluntary ethanol consumption in rats. *Pharmacol Biochem and Behav* **41:**847-850.

Koltchine VV, Finn SE, Jenkins A, Nikolaeva N, Lin A and Harrison NL (1999) Agonist gating and isoflurane potentiation in the human gamma-aminobutyric acid type A receptor determined by the volume of a second transmembrane domain residue. *Mol Pharmacol* **56:**1087-1093.

Lankiewicz S, Lobitz N, Wetzel CHR, Rupprecht R, Gisselmann G and Hatt H (1998) Molecular cloning, functional expression, and pharmacological characterization of 5-hydroxytryptamine$_3$ receptor cDNA and its splice variants from guinea pig. *Mol Pharmacol* **53:**202-212.

Lovinger DM and White G (1991) Ethanol potentiation of 5-Hydroxytryptamine$_3$ receptor-mediated ion current in neuroblastoma cells and isolated adult mammalian neurons. *Mol Pharmacol* **40:**263-270.

Lovinger DM, Sung KW and Zhou Q (2000) Ethanol and trichloroethanol alter gating of 5-HT3 receptor-channels in NCB-20 neuroblastoma cells. *Neuropharmacol* **39:**561-570.

Lovinger DM and Zhou Q (1993) Trichloroethanol potentiation of 5-Hydroxytrypamine$_3$ receptor mediated ion current in nodose ganglion neurons from the adult rat. *J Pharmacol Exp Ther* **265:**771-776.

Machu TK and Harris RA (1994) Alcohols and anesthetics enhance the function of 5-Hydroxytryptamine$_3$ receptors expressed in Xenopus laevis oocytes. J Pharmacol Exp Ther **271:**898-905.

Maricq AV, Peterson AS, Brake AJ, Myers RM and Julius D (1991) Primary structure and functional expression of the 5-HT$_3$ receptor, a serotonin-gated ion channel. *Science* **154:**432-434.

Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA and Harrison NL (1997) Sites of alcohol and volatile anaesthetic action on GABA$_A$ and glycine receptors. *Nature* **389:**385-389.

Miyake A, Mochizuki S, Takemoto Y and Akuzawa S (1995) Molecular cloning of human 5-Hydroxytryptamine$_3$ receptor: heterogeneity in distribution and function among species. *Mol Pharmacol* **48:**407-416.

Morales M and Wang SD (2002) Differential composition of 5-hydroxytryptamine3 receptors synthesized in the rat CNS and peripheral nervous system. *J Neurosci* **22:**6732-6741.

Panicker S, Cruz H, Arrabit C and Slesinger PA (2002) Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. *J Neurosci* **22:**1629-1639.

Peters JA, Lambert JJ and Malone HM (1994) Electrophysiological studies of 5-HT_3 receptors, in 5-Hydroxytryptamine$_3$ Receptor Antagonists (King FD, Jones BJ and Sanger GJ eds), pp115, CRC Press, Boca Raton.

Reeves DC, Goren EN, Akabas MH and Lummis SC (2001) Structural and electrostatic properties of the 5-HT3 receptor pore revealed by substituted cysteine accessibility mutagenesis. *J Biol Chem* **276:**42035-42042.

Thompson S-A, Smith MZ, Wingrove PB, Whiting PJ and Wafford KA (1999) Mutation at the putative $GABA_A$ ion-channel gate reveals changes in allosteric modulation. *Br J Pharmacol* **127:**1349-1358.

Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, Ye Q, Harrison NL and Harris RA (1998) Mutations of γ-amino acid and glycine receptors change alcohol cut-off: evidence for an alcohol receptor? *Proc Natl Acad Sci USA* **95:**6504-6509.

Yamakura T, Mihic SJ and Harris RA (1999) Amino acid volume and hydropathy of a transmembrane site determine glycine and anesthetic sensitivity of glycine receptors. *J Biol Chem* **274:**23006-23012.

Ye Q, Kolthcine VV, Mihic SJ, Mascia MP, Wick MJ, Finn SE, Harrison NL and Harris RA (1998) Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position α267. *J Biol Chem* **273:**3314-3319.

Yu D, Zhang L, Eisele′ J-L, Bertrand D, Changeux J-P and Weight FF (1996) Ethanol inhibition of nicotinic acetylcholine type α7 receptors involves the amino-terminal domain of the receptor. *Mol Pharmacol* **50:**1010-1016.

Zhang L, Hosoi M, Fukuzawa M, Sun H, Rawlings RR and Weight FF (2002) Distinct molecular basis for differential sensitivity of the serotonin type 3A receptor to ethanol in the absence and presence of agonist. *J Biol Chem* **277:**46256-46264.

Zhou Q, Verdoorn TA and Lovinger DM (1998) Alcohols potentiate the function of 5-HT_3 receptor-channels on NCB-20 neuroblastoma cells by favouring and stabilizing the open channel state. *J Physiol* **507.2**:335-352.

Footnotes

Financial support was provided by grants from the Alcoholic Beverage Medical Research Foundation (TKM) and NIAAA, AA12643 (TKM) and NIAAA, AA08986 (DML).

Reprint Requests:
Tina K. Machu, Ph.D.
Texas Tech University Health Sciences Center
3601 Fourth St.
Lubbock, TX 79430
806-743-2425
FAX: 806-743-2744
tina.machu@ttuhsc.edu

[1]Dept. of Pharmacology
Duke University Medical Center
Research Drive
Durham, NC 27710

[2]Dept. of Pharmacology
Texas Tech University Health Sciences Center
3601 Fourth St.
Lubbock, TX 79430

[3]Laboratory of Integrative Neuroscience
National Institute of Alcohol Abuse and Alcoholism
Room 158H Park 5 Building
12420 Parklawn Drive
Rockville, MD 20852

[4]Dept. of Anesthesiology
Texas Tech University Health Sciences Center
3601 Fourth St.
Lubbock, TX 79430

FIGURE LEGENDS

Figure 1. Serotonin concentration response curves were generated in wild-type, Ile294Leu, and Ile294Thr $5HT_{3A}$ receptors. (A) Oocytes expressing wild-type or mutant receptors were perfused with 5-HT for 30 s, n=4-5. Peak current data were normalized to the maximal 10 μM current response. The EC_{50} values (in μM) were 0.93 $\pm$ 0.023 (wild-type), 1.15 $\pm$ 0.14 (Ile294Leu), and 0.65 $\pm$ 0.015 (Ile294Thr). Hill coefficients were 3.2 $\pm$ 0.22 (wild-type), 4.1 $\pm$ 0.36 (Ile294Thr), and 3.1 $\pm$ 0.14 (Ile294Leu). (B) In HEK293 cells expressing wild-type and Ile294Thr 5-HT_{3A} receptors, 5-HT concentration response curves were generated, n=3-5. Peak current data were normalized to the maximal 30 μM current response. EC_{50} values (in μM) and Hill coefficients were 3.82 $\pm$ 0.11 and 2.2 $\pm$ 0.13 (wild-type) and 2.73 $\pm$ 0.18 and 1.7 $\pm$ 0.2 (Ile294Thr).

Figure 2. Kinetic properties of $5HT_{3A}$ receptors are altered in the presence of the Ile294Thr mutation in TM2. (A) Representative current traces from wild-type, Ile294Leu, and Ile294Thr 5-HT_{3A} receptors expressed in *Xenopus* oocytes in response to 10 μM 5-HT are shown. (B) Representative currents traces from wild-type and Ile294Thr $5HT_{3A}$ receptors expressed in HEK293 are depicted. Responses were activated by 2 μM 5-HT or 10 μM 5-HT delivered by rapid application. Asterisks indicate the traces evoked by 10 μM 5-HT. The duration of drug application is denoted by the bars above the respective traces. The first two insets provide an expanded time scale for comparisons of 2 μM 5-HT and 10 μM 5-HT evoked currents. The third inset is a comparison of the 2 μM 5-HT mediated currents in the wild-type and Ile294Thr receptors. (C) Ratios of steady-state (Iss) to peak (Ipeak) current values (pA) in response to 10μM 5-HT were obtained for wild-type (black, n=13) and Ile294Thr (white, n=16) samples. In all graphs, bars represent the mean ± SEM for the indicated numbers of samples. (D) The rate of

current decay in the presence of a prolonged application of 10 μM 5-HT was significantly slowed in the Ile294Thr mutant receptors (n = 4) compared with wild-type 5-HT_{3A} receptors (n = 7).

Figure 3. The Ile294Thr mutation in the $5HT_{3A}$ receptor alters ethanol sensitivity. (A) Oocytes expressing Ile294Leu, Ile294Thr, and wild-type 5-HT_{3A} receptors were perfused with ethanol (50-200 mM) in MBS for one min prior to the addition of 5-HT plus ethanol (50-200 mM) in MBS for 30 s (n=3-8). Concentrations of 5-HT (~ EC_1) used were 175, 250, and 100 nM for wild-type, Ile294Thr, and Ile294Leu 5-HT_{3A} receptors, respectively. Peak current data are presented as a % change from the baseline, control value. (B) HEK293 cells expressing either wild-type or Ile294Thr 5-HT_{3A} receptors were perfused with 0.5 μM 5-HT in the absence or presence of ethanol (10, 50, or 100 mM) for 30 s, (n=3–5) via a rapid drug delivery system. Peak current data are presented as a % of the baseline, control value.

Figure 4. Actions of TCEt were examined in wild-type and Ile294Thr 5-HT_{3A} receptors. *Xenopus* oocytes expressing either construct were perfused with 5-HT (175 nM = wild-type, 250 nM = Ile294Thr) in the absence or presence of TCEt (0.25 – 10 mM) for 30 s (n=4-6). Peak current data are presented as a % change from the control, baseline response obtained in the absence of TCEt. TCEt enhanced 5-HT mediated currents in wild-type receptors, but had a biphasic response in the Ile294Thr mutant receptor.

Figure 5. The potentiating effects of TCEt are diminished in the Ile294Thr 5-HT_{3A} receptor channels. (A) Representative current traces from wild-type (panel a) and Ile294Thr 5-HT_{3A}

(panel b) receptors expressed in HEK293 cells and activated by 2μM 5-HT or 2μM 5-HT + 3mM TCEt (denoted with asterisks). In panel c, wild-type (from panel a) and Ile294Thr mutant (from panel b) current traces in the presence of 2 μM 5-HT are overlaid. In panel d, wild-type (from panel a) and Ile294Thr mutant (from panel b) current traces in the presence of 2 μM 5-HT + 3mM TCEt are overlaid. In all four panels, the insets are provided in an expanded time scale to illustrate the activation phase of the current. The duration of drug application is denoted by the bars above the respective traces. (B) TCEt's potentiation of currents from wild-type (black, n=5) and Ile294Thr (white, n=5) receptors activated by 5-HT. Peak currents were determined by cursor measurement within a 30 s application of 5-HT plus TCEt, and isochronal measurements were determined where wild-type currents peaked at 4.5 s. Values are expressed as the percent change for each receptor in the presence of 2 μM 5-HT + 3mM TCEt relative to 2 μM 5-HT treatment alone. (C) The effects of TCEt on the activation kinetics of wild-type (black) and Ile294Thr (white) $5HT_3$ receptors in response to 2μM 5-HT treatment. Values for the initial slope (10% -20% response) and the rise-time (20% -80% response) in the presence of 3 mM TCEt are expressed as a percentage of control (2μM 5-HT alone) for each cell. Bars represent the mean ± SEM of n=7 (wild-type) and n=5 (Ile294Thr) paired samples. (D) Ratios of steady-state (Iss) to peak (Ipeak) current values (pA) in response to 2μM 5-HT alone (black), or 2μM 5-HT co-applied with 3mM TCEt (gray) were obtained with n=7 (wild-type) and n=4 (Ile294Thr). In all graphs, bars represent the mean ± SEM of the indicated sample size.

Figure 6. Cut-off of n-chain alcohol sensitivity was determined in wild-type, Ile294Leu, and Ile294Thr $5\text{-}HT_{3A}$ receptors. (A) Pentanol (0.25- 4 mM) concentration response curves were generated in oocytes expressing wild-type or Ile294Leu $5\text{-}HT_{3A}$ receptors (n=3-8). Ile294Leu

mutant receptors responses were enhanced by pentanol, albeit less than wild-type receptor responses, suggesting that the mutant receptor's alcohol cut-off is hexanol. (B) Oocytes were perfused with hexanol (0.125-1 mM) in MBS for one min prior to the addition of 5-HT plus hexanol (0.125-1 mM) in MBS for 30 s (n=4-7). (C) Low affinity hexanol (5 and 10 mM) actions on wild-type, Ile294Thr, and Ile294Leu 5-HT_{3A} receptors were examined. Oocytes were perfused with hexanol (5 or 10 mM) in MBS for one min prior to the addition of 5-HT plus hexanol in MBS for 30 s (n=3-6). (D) Heptanol (0.1- 1 mM) or octanol (0.03125- 1 mM) concentration response curves were generated in oocytes expressing wild-type or Ile294Thr 5-HT_{3A} receptors (n=3-5). In A-D, concentrations of 5-HT used were 175, 250, and 200 nM for wild-type, Ile294Thr, and Ile294Leu 5-HT_{3A} receptors, respectively. Values are expressed as percent changes from baseline responses.

Fig. 1A

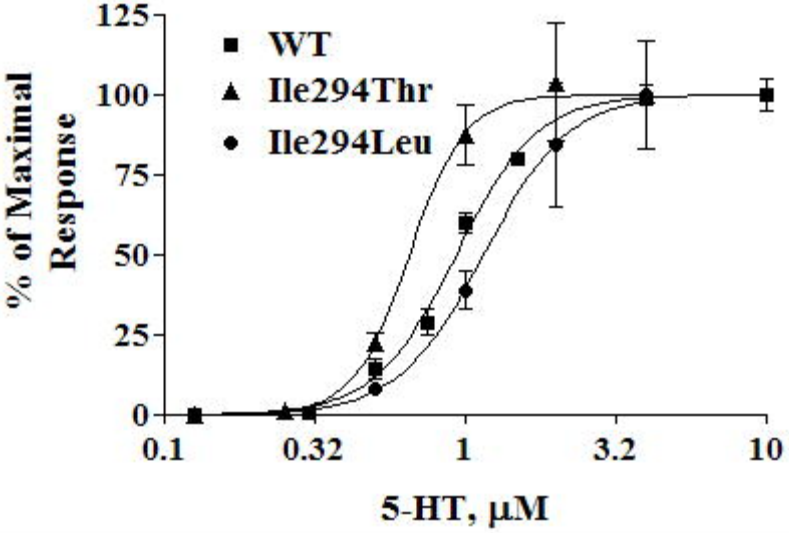

125
100
75
50
25
0
% of Maximal Response
WT
Ile294Thr
Ile294Leu
0.1
0.32
1
3.2
10
5-HT, μM

Fig. 1B

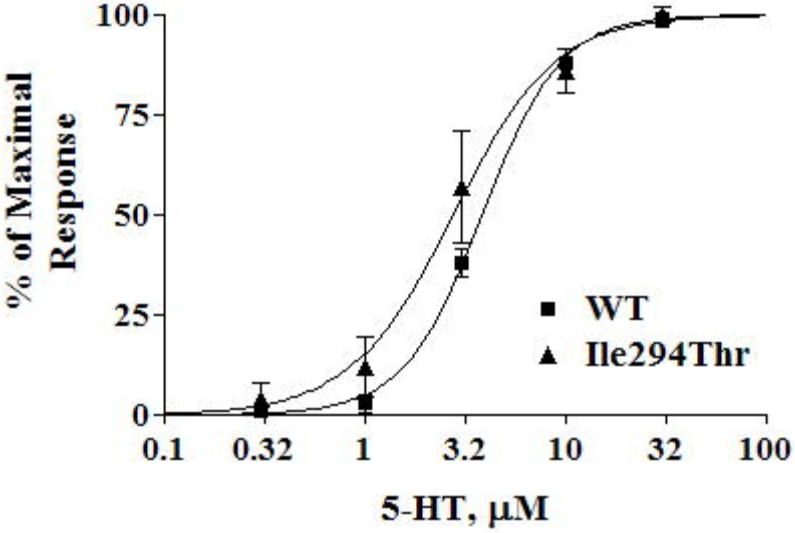

100
75
50
25
0
% of Maximal Response
WT
Ile294Thr
0.1
0.32
1
3.2
10
32
100
5-HT, μM

Fig. 2A

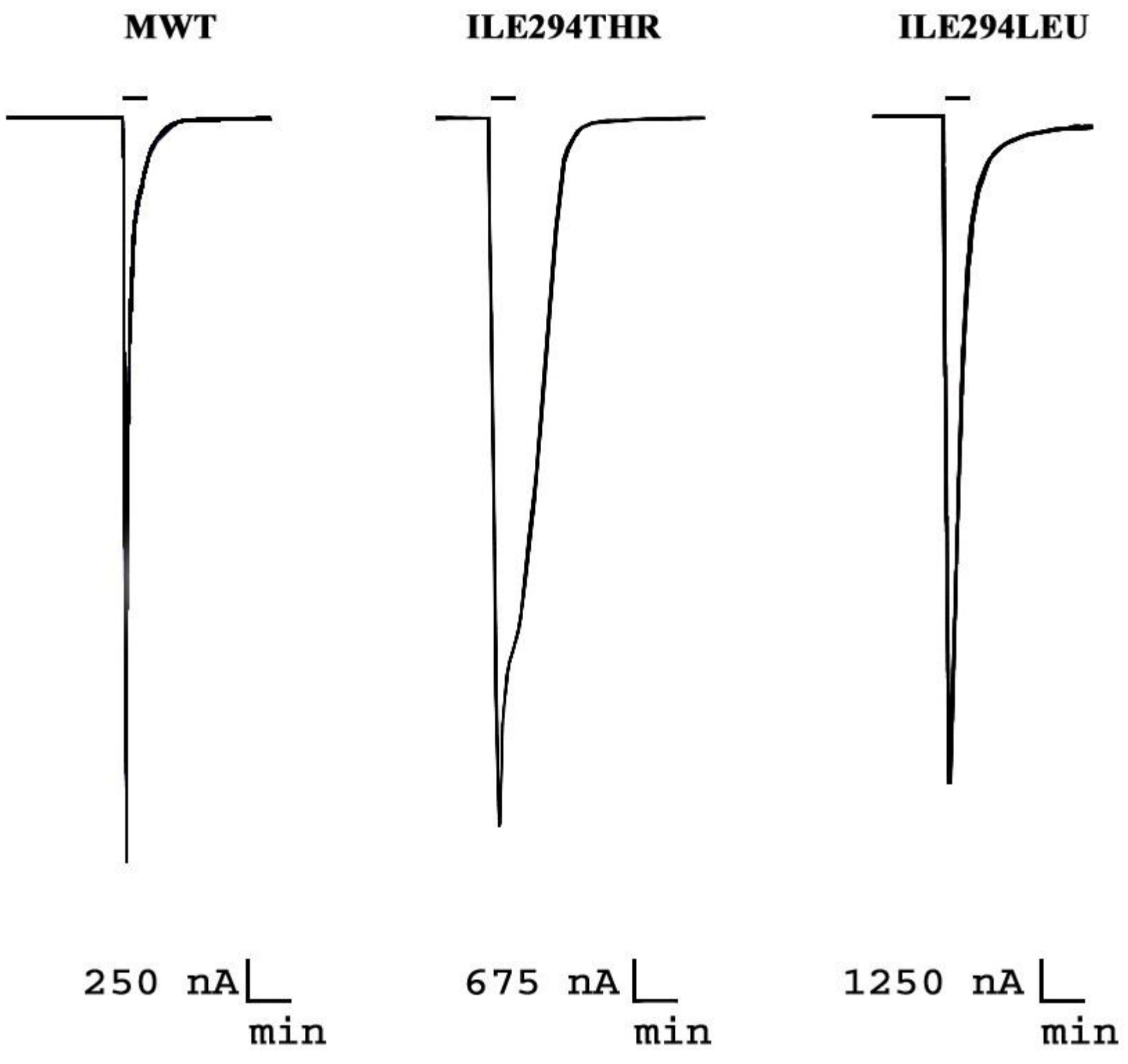
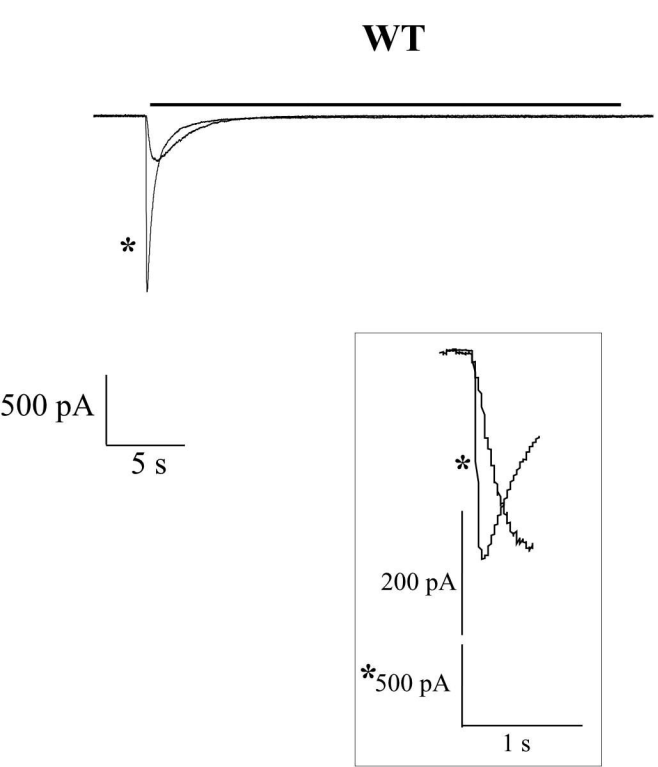
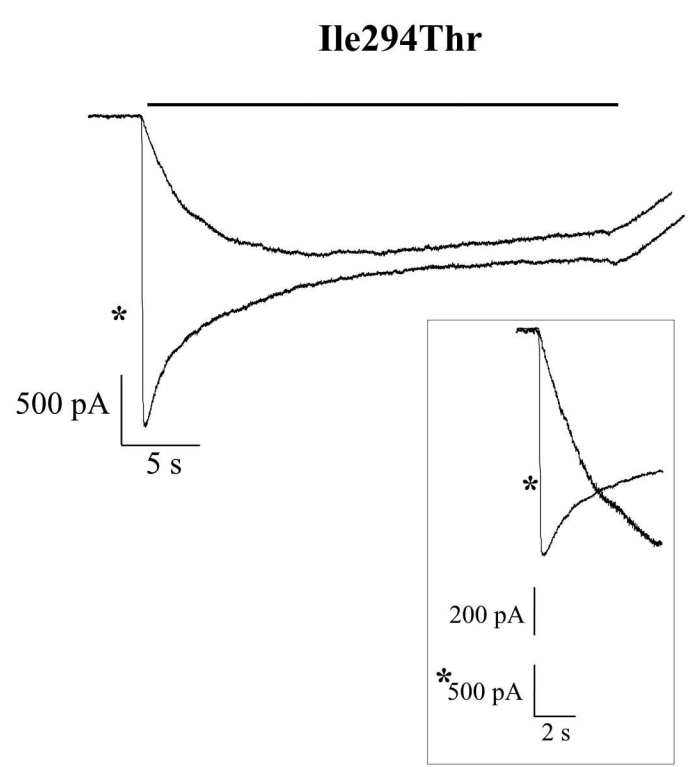


Fig. 2B





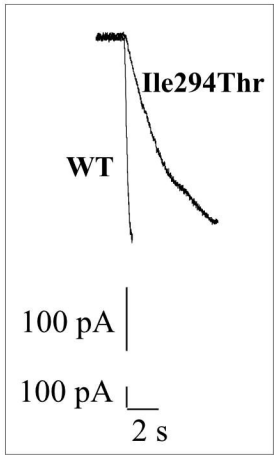


Fig. 2C

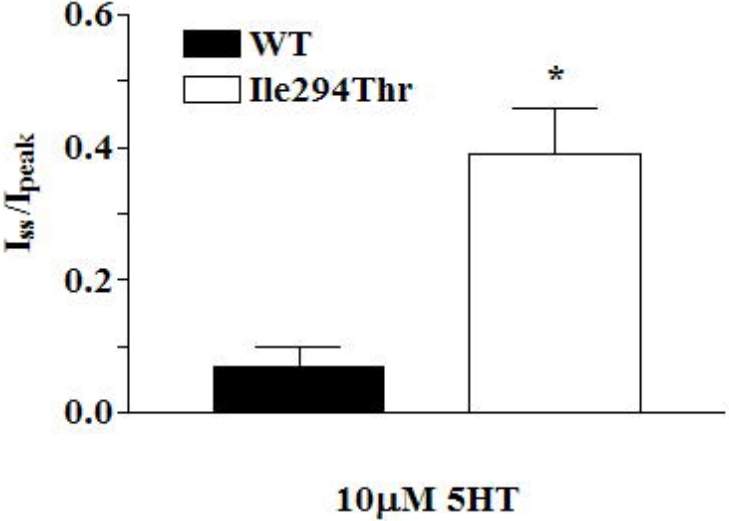


Fig. 2D

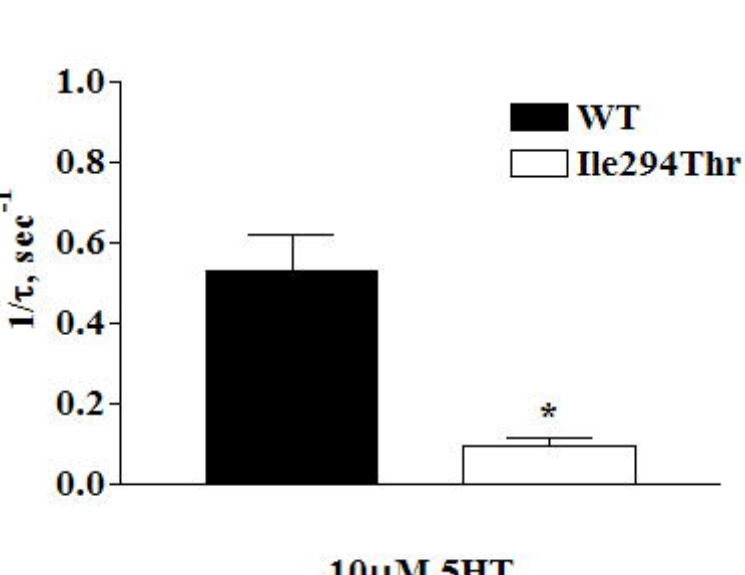


Fig. 3A

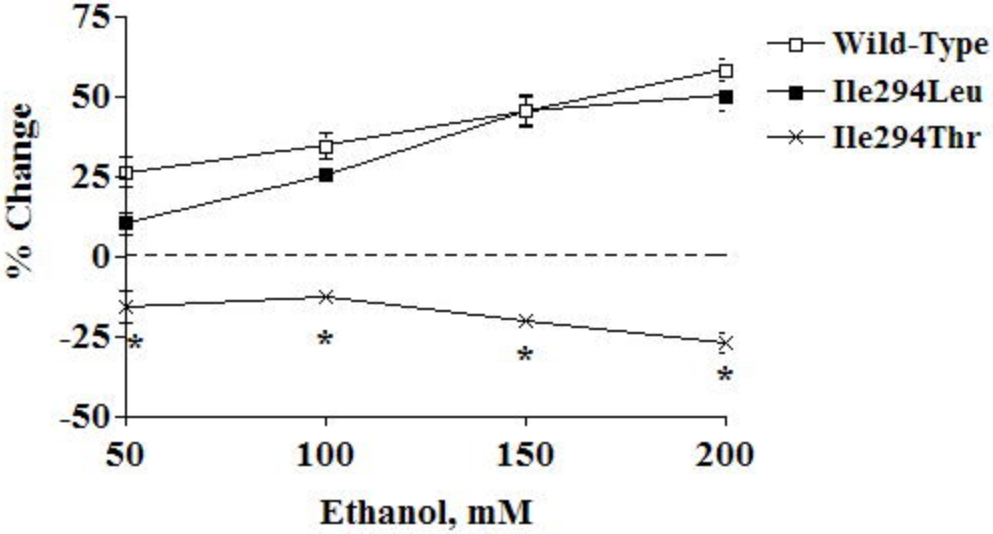

75
50
25
0
-25
-50
% Change
50
100
150
200
Ethanol, mM
Wild-Type
Ile294Leu
Ile294Thr
*
*
*
*

Fig. 3B

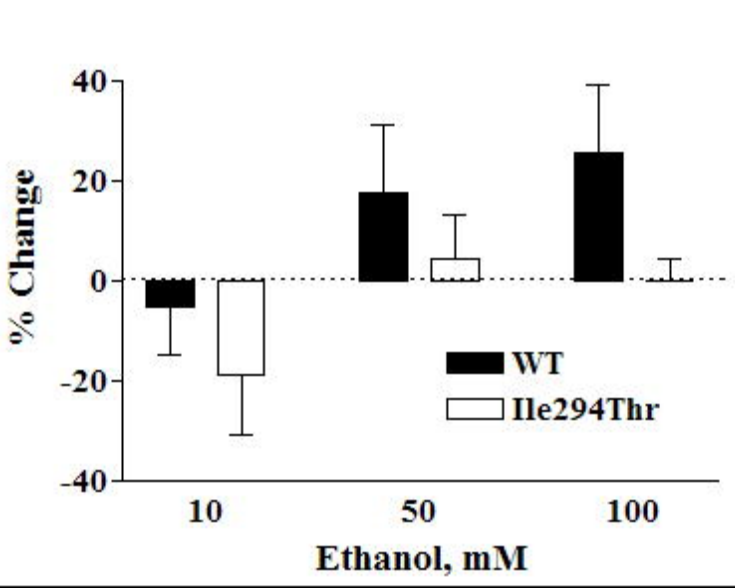


Fig. 4

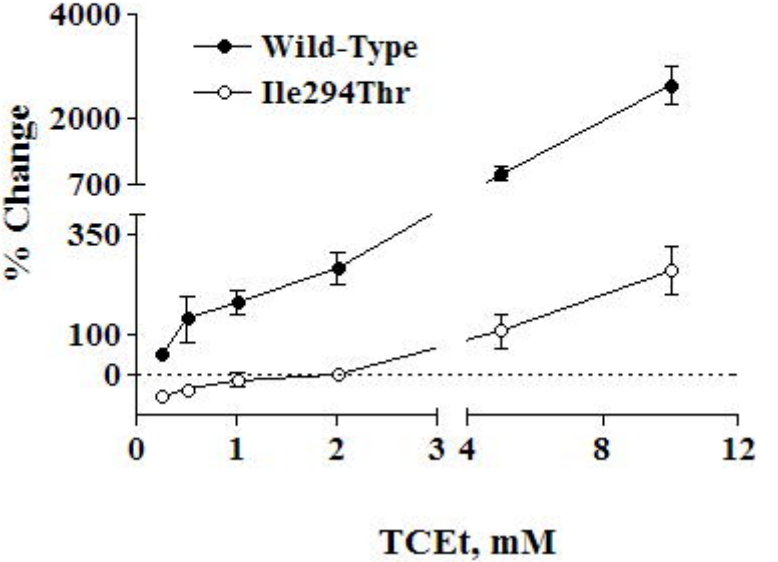

4000
2000
700
350
100
0
% Change
Wild-Type
Ile294Thr
0
1
2
3
4
8
12
TCEt, mM

Fig. 5A

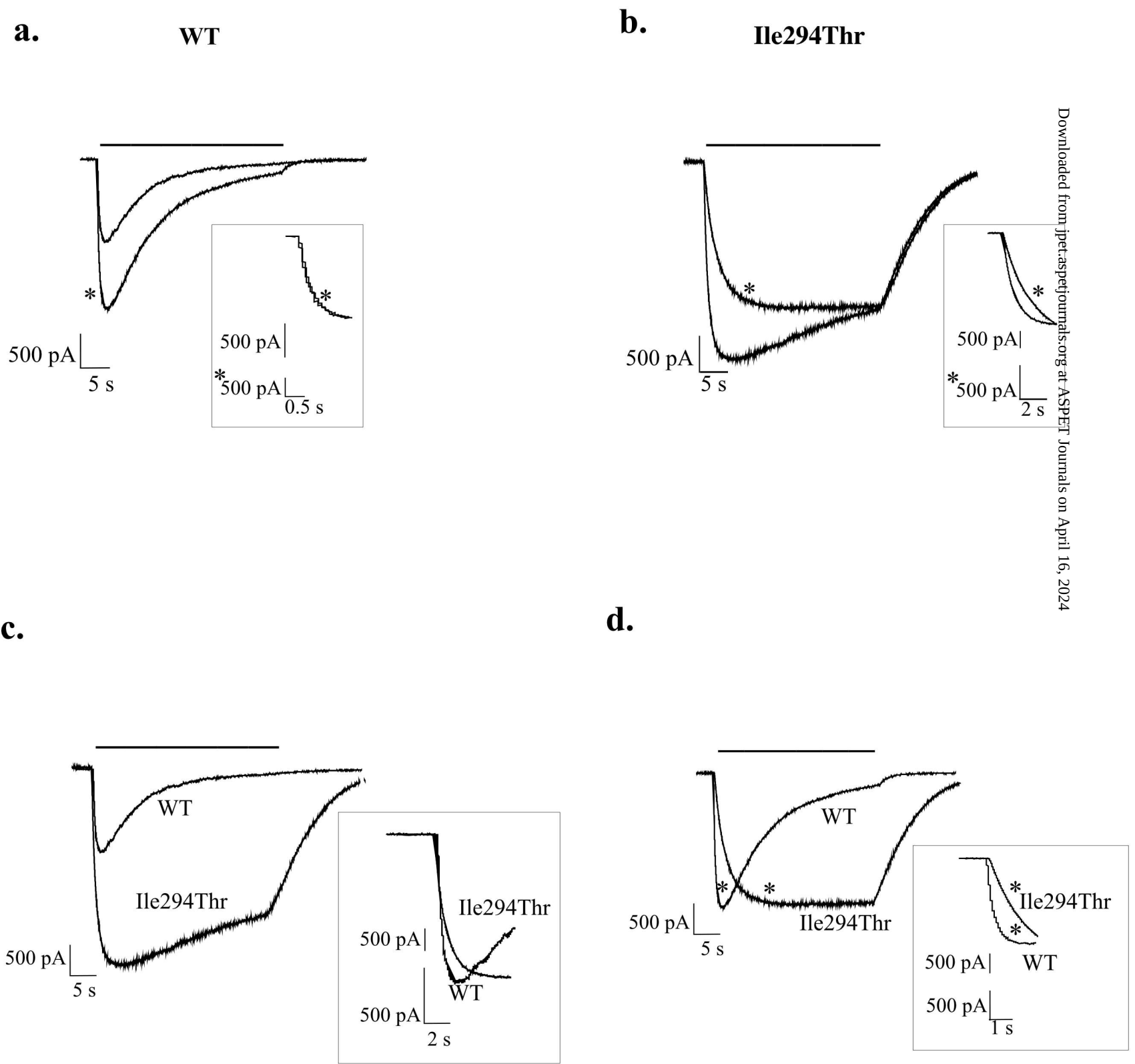


Fig. 5B

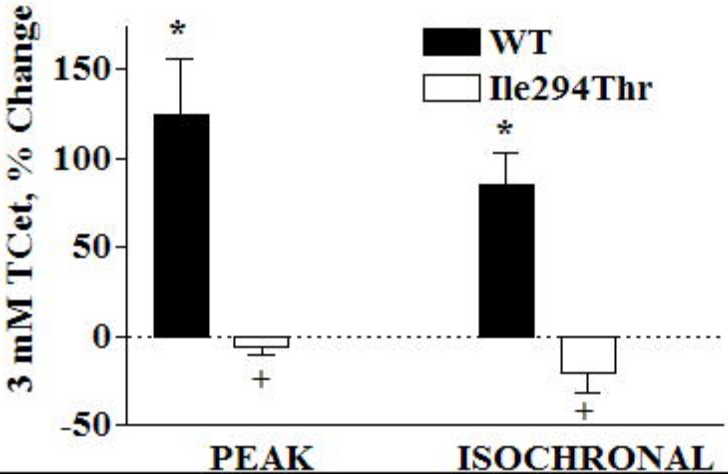


Fig. 5C

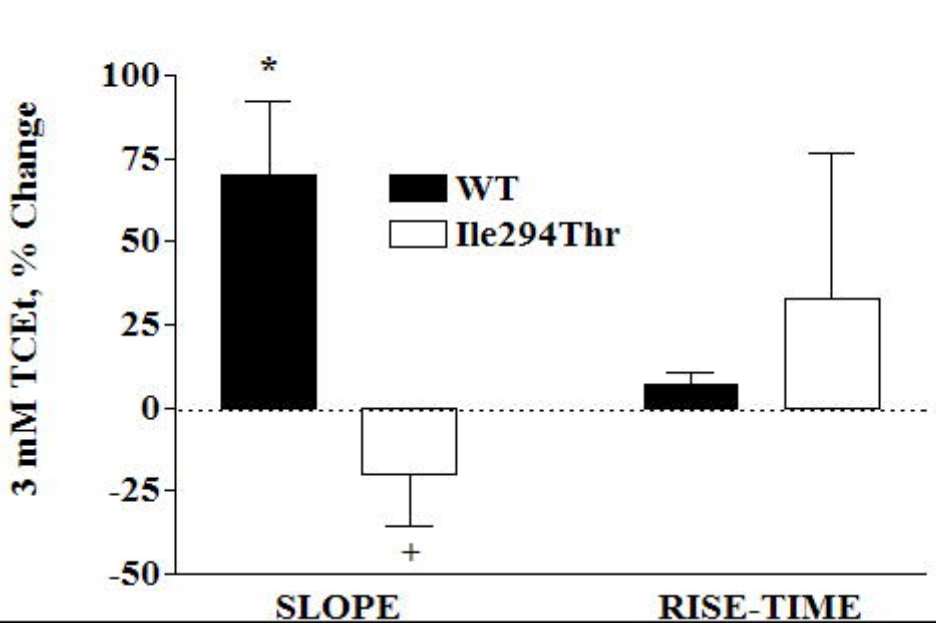

3 mM TCEt, % Change
100
75
50
25
0
-25
-50
*
+
WT
Ile294Thr
SLOPE
RISE-TIME

Fig. 5D

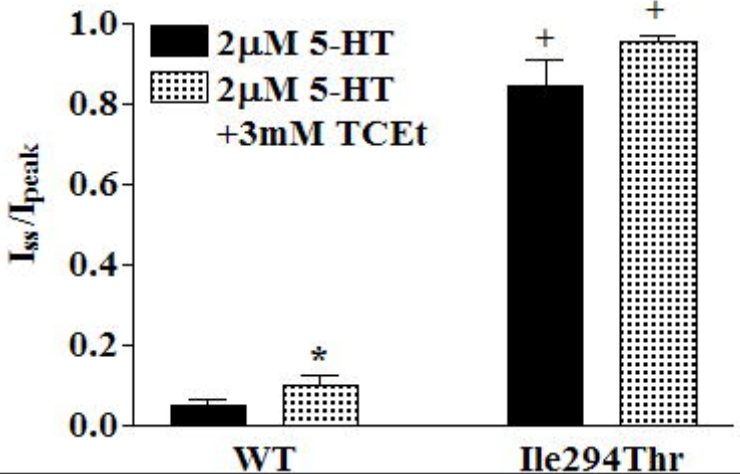

1.0
0.8
0.6
0.4
0.2
0.0
I_{ss}/I_{peak}
2μM 5-HT
2μM 5-HT
+3mM TCEt
*
+
+
WT
Ile294Thr

Fig. 6A

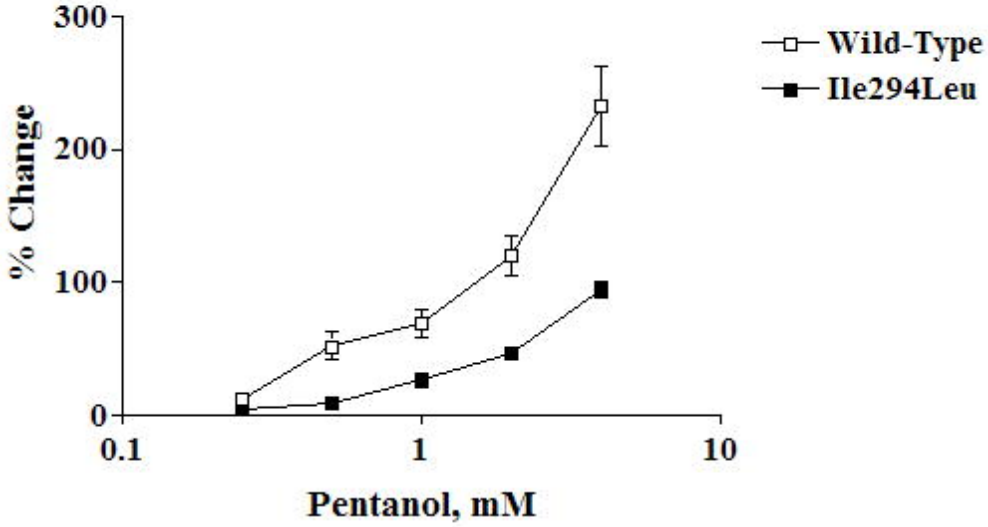
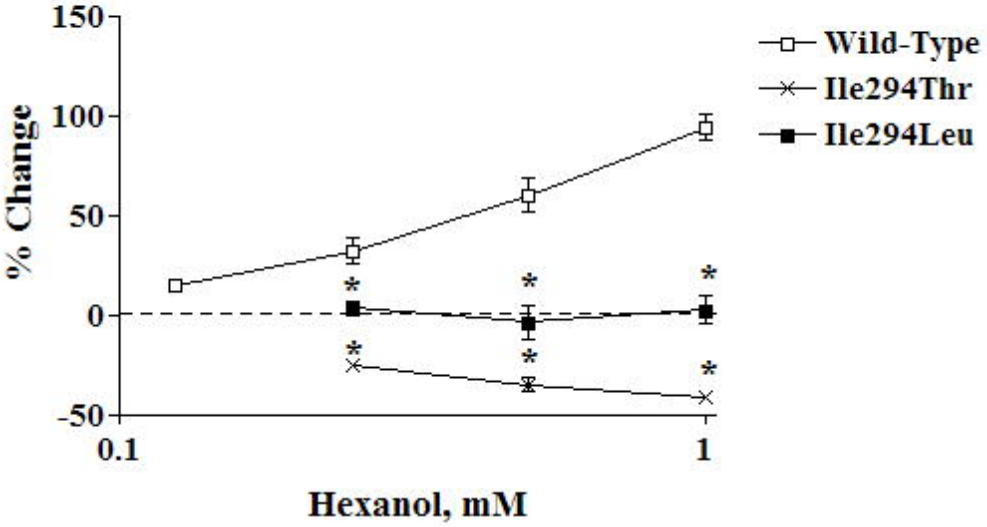


Fig. 6B


150
100
50
0
-50
% Change
0.1
1
Hexanol, mM
Wild-Type
Ile294Thr
Ile294Leu
*
*
*
*
*
*

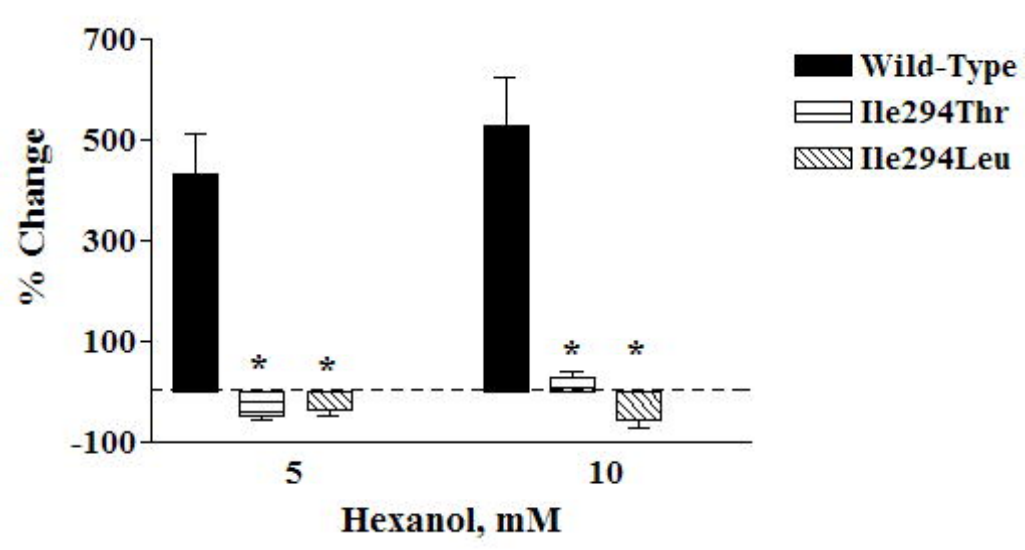
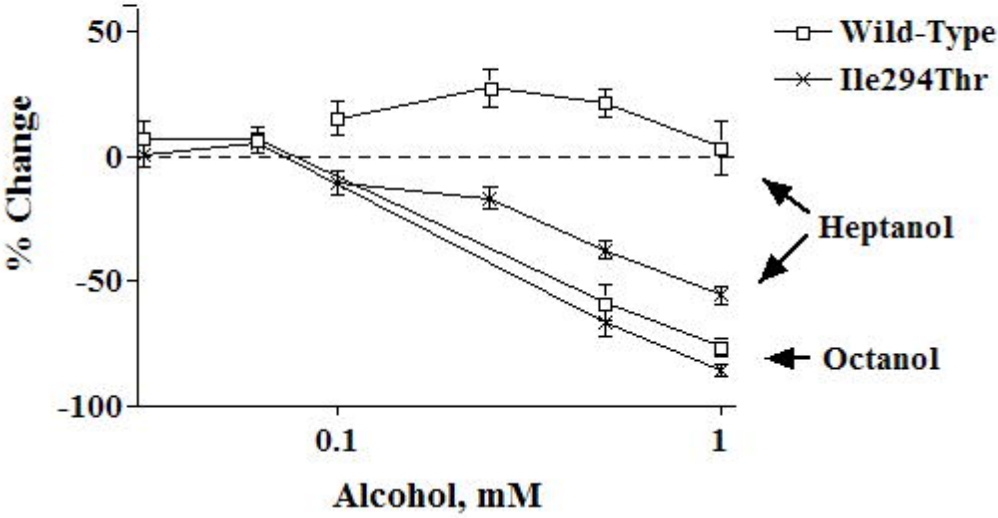

700
500
300
100
-100
% Change
*
*
*
*
5
10
Hexanol, mM
Wild-Type
Ile294Thr
Ile294Leu

Fig. 6D


50
0
-50
-100
% Change
0.1
1
Alcohol, mM
Wild-Type
Ile294Thr
Heptanol
Octanol