British Journal of Pharmacology (2006) 148, 88–101 
www.nature.com/bjp

# Mutations of L293 in transmembrane two of the mouse 5-hydroxytryptamine$_{3A}$ receptor alter gating and alcohol modulatory actions

[1]Xiang-Qun Hu, [2]Volodya Hayrapetyan, [2]Jay J. Gadhiya, [2]Heather E. Rhubottom, [1]David M. Lovinger & *,[2]Tina K. Machu

[1]Laboratory for Integrative Neuroscience, National Institute of Alcohol Abuse and Alcoholism, Rockville, MD 20852, U.S.A. and [2]Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd., Fort Worth, TX 76107-2699, U.S.A.

**1** The goal of this study was to determine whether mutations of L293 at the 15′ position of TM2 in the 5-HT$_{3A}$ receptor alter macroscopic current kinetics, and if these changes could account for alterations in alcohol modulation. Receptor function was assessed in *Xenopus* oocytes under voltage-clamp and in HEK293 cells with whole-cell patch-clamp recording and rapid drug application.

**2** Examination of responses of L293C and L293S receptors to agonist alone revealed enhanced activation, deactivation, and desensitization rates relative to the wild-type receptor. The L293G mutation produced marked slowing of deactivation and desensitization rates. Increased potency of 5-HT and increased efficacy of the partial agonist, DA, was also observed in these mutant receptors.

**3** Ethanol and trichloroethanol (TCEt) enhancement of receptor function was reduced or eliminated in receptors containing L293 mutations to C, G, or S. The L293I mutant receptor retained ethanol and TCEt sensitivity. Ethanol and TCEt enhanced activation rate in the wild-type, but not the L293G and L293S receptors. No relationship was observed between any physicochemical property of the substituted amino acids and the change in alcohol potentiation of function.

**4** The changes in receptor-channel properties in the mutant receptors support the idea that the L293 residue has important roles in channel gating. Our findings indicate that loss of allosteric modulation by alcohols is not related in any simple way to changes in channel kinetic properties brought about by L293 mutants. We did not observe any evidence that L293 is part of an alcohol binding site.
*British Journal of Pharmacology* (2006) **148,** 88–101. doi:10.1038/sj.bjp.0706685;
published online 6 March 2006

**Keywords:** 5-HT$_3$; ethanol; alcohol; 5-HT; electrophysiology; receptor; mutant

**Abbreviations:** ANOVA, analysis of variance; EtOH, ethanol; HEK, human embryonic kidney; 5-HT, 5-hydroxytryptamine; LGIC, ligand-gated ion channel; MBS, modified Barth's solution; nACh, nicotinic acetylcholine; TCEt, 2,2,2-trichloroethanol; TM2, transmembrane 2

## Introduction

The 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor is a member of the 'cys-loop' family of ligand-gated ion channels (LGICs), of which the nicotinic acetylcholine (nACh) receptor is the prototype (Derkach *et al*., 1989). The 5-HT$_3$ receptor is present in the central and peripheral nervous systems, where it mediates fast synaptic transmission at postsynaptic sites and is thought to regulate neurotransmitter release presynaptically. Five subunits of the 5-HT$_3$ receptor have been cloned (Maricq *et al*., 1991; Davies *et al*., 1999; Dubin *et al*., 1999; Karnovsky *et al*., 2003; Niesler *et al*., 2003). To date, 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits are the only two of these subunits that have been demonstrated to have functional significance in the central or peripheral nervous systems. Recent studies suggest that the A homomer predominates in rodent brain (Morales & Wang, 2002).

The 5-HT$_3$ receptor has been implicated in acute alcohol intoxication, particularly in the subjective effects of ethanol (EtOH) (reviewed in McBride *et al*., 2004). In addition, 5-HT$_3$ receptor antagonists can reduce alcohol intake in laboratory animals (Knapp & Pohorecky, 1992) and alcoholic humans (Johnson *et al*., 1993). Alcohol potentiation of receptor function (Lovinger & White, 1991; Machu & Harris, 1994) likely contributes to the role of the receptor in intoxication and drinking behavior. EtOH and trichloroethanol (TCEt) enhance currents mediated by a maximal concentration of the 5-HT$_3$ partial agonist dopamine, suggesting that probability of opening is enhanced in the absence of any effect on agonist affinity (Lovinger *et al*., 2000). Rapid drug superfusion studies demonstrated that alcohols alter the kinetics of 5-HT$_3$ receptor-mediated currents in ways that stabilize and favor the open channel state (Zhou *et al*., 1998).

The search for alcohol and anesthetic binding sites in members of the superfamily of LGICs is an area of intense investigation. Amino acids at position 15′ of the second transmembrane domain 2 (TM2) and at position 16′ of the third transmembrane domain (TM3) of other LGICs, the $\gamma$-aminobutyric acid (GABA) type A and rho$_1$ receptors, as

*Author for correspondence; E-mail: tmachu@hsc.unt.edu

well as the glycine receptor, confer alcohol and anesthetic sensitivity (Belelli *et al*., 1997; Mihic *et al*., 1997). Changes in alcohol cutoff (Wick *et al*., 1998), as well as direction and degree of modulation by alcohols and anesthetics (Ye *et al*., 1998; Koltchine *et al*., 1999; Yamakura *et al*., 1999), accompany mutation-induced changes in molecular volume of these critical residues. An alcohol/anesthetic binding pocket is theorized to reside in a cavity between these amino acids in TM2 and TM3.

Alternatively, mutations at the 15′ position of TM2 of the LGIC superfamily of receptors could cause changes in receptor gating that result in altered sensitivity to alcohols and anesthetics. Indeed, Scheller & Forman (2002) have demonstrated that the α1(S270I) β2 γ2L GABA$_A$ receptor, containing a 15′ mutation in TM2 of the α1-subunit, has enhanced gating efficacy that is primarily due to reduced deactivation. Thus, the present study was undertaken to examine the hypothesis that mutations at the 15′ position of TM2 (L293) in the mouse 5-HT$_{3A}$ receptor result in gating changes that reduce or eliminate *n*-chain alcohol- and TCEt-induced enhancement of receptor function. Further, if gating changes and alterations in alcohol sensitivity are correlated, then the hypothesis that mutation-induced gating changes can be used to exclude amino acids as putative alcohol-binding domains could be examined. Mutations at L293 produced changes in allosteric modulation by alcohols, in agreement with Lopreato *et al*. (2003). In addition, channel kinetics were markedly altered in all mutants studied, suggesting that L293 may play an important role in gating dynamics, with no identifiable role as part of a putative alcohol-binding domain in the 5-HT$_{3A}$ receptor.

## Methods

### *Site-directed mutagenesis*

The cDNA encoding the mouse 5-HT$_{3A}$ receptor originally cloned from NCB-20 cells (gift from Dr D. Julius, San Francisco, CA, U.S.A.) was subcloned in pcDNA 3.1(−) (Invitrogen, Carlsbad, CA, U.S.A.). Numbering of the amino acids began with the initiating methionine. Mutant cDNAs were obtained with the unique site elimination method (Transformer Site Directed Mutagenesis Kit, Clontech, Palo Alto, CA, U.S.A.) or with QuikChange (Stratagene, La Jolla, CA, U.S.A.). Mutations were confirmed by dideoxynucleotide sequencing of double-stranded DNA at the Texas Tech University Biotechnology Core Facility, Lubbock, TX, U.S.A.

### *Expression of 5-HT$_{3A}$ receptors in* Xenopus laevis *oocytes*

All procedures for animal care and use were approved by the University of North Texas Health Science Center Animal Care and Use Committee. *Xenopus laevis* (*Xenopus* Express, Plant City, FL, U.S.A.) frogs were kept in tanks of dechlorinated tap water on a 12 h light/12 h dark cycle at 18°C and fed a diet of *Xenopus* Express sinking frog food once per week. Frogs were anesthetized by immersion in cold 0.12% 3-aminobenzoic acid ethyl ester for 20 min; sufficient level of anesthesia was assured by loss of nose flare, swallow reflex, and limb movement. After removal through a small incision in the frog's abdomen, ovarian lobes were placed in modified Barth's Solution (MBS) containing (in mM) NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES 10, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, and $CaCl_2$ 0.91 (pH 7.5).

Ovarian lobes were manually dissected into clumps of four to 10 oocytes and were then subjected to chemical separation and defolliculation. Clumps of oocytes were placed in medium containing 2 mg ml$^{-1}$ collagenase Type 2 (Sigma, St Louis, MO, U.S.A.) and (in mM) NaCl 83, KCl 2, $MgCl_2$ 1, and HEPES 10 (pH 7.5), and gently rocked for 2 h. Oocytes were then removed 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 (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and HEPES 5 (pH 7.5), plus 10 mg l$^{-1}$ streptomycin, 50 mg l$^{-1}$ gentamicin, 10,000 U l$^{-1}$ penicillin, 96 mg l$^{-1}$ sulfamethoxazole, 19 mg l$^{-1}$ trimethoprim, 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 EtOH, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. The cDNAs were then transcribed with T7 mMESSAGE mMACHINE (Ambion, Austin, TX, U.S.A.). An aliquot of cRNA was centrifuged at 15,000 × *g*, and the EtOH 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 day 2 to seven following injection.

### *Two electrode voltage-clamp electrophysiological recordings*

Oocytes were perfused (2 ml min$^{-1}$) in a 100 μl volume chamber with MBS *via* a roller pump (Cole-Parmer Instrument Co., Chicago, IL, U.S.A.). Two glass electrodes (1.2 mm outside diameter and 1–10 MΩ) filled with 3 M KCl were used to impale oocytes. A Warner Instruments Model OC-725B or OC-725C oocyte clamp (Hamden, CT, U.S.A.) was used to voltage-clamp oocytes to −70 mV. Clamping currents were plotted on a strip chart recorder (Cole Parmer Instrument Co., Chicago, IL, U.S.A.). Serotonin (Sigma, St Louis, MO, U.S.A.), EtOH (AAPER Alcohol and Chemical Co., Shelbyville, KY, U.S.A.) and other *n*-chain alcohols (Sigma), and TCEt (>99% pure; Sigma) were dissolved in MBS buffer. Serotonin was applied for 30 s or 1 min. *n*-Chain alcohols were perfused for 1 min prior to the application of 5-HT + *n*-chain alcohol for 30 s or 1 min; 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.

### *Data analysis for oocytes*

Peak current amplitudes were measured for all responses. 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 (25 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, U.S.A.) was used to calculate EC$_{50}$s, Hill coefficients, and two-way analysis of variance (ANOVA). Instat (San Diego, CA, U.S.A.) was used to perform *post hoc* tests.

### *Expression of 5-HT$_{3A}$ receptors in human embryonic kidney (HEK)293 cells*

Wild-type and mutant 5-HT$_{3A}$ cDNAs were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, U.S.A.). HEK293 cells grown in standard 35 mm diameter culture dishes were transfected with the wild-type or mutant 5-HT$_{3A}$ receptor cDNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Green fluorescent protein (pGreen Lantern, Invitrogen) was coexpressed with the 5-HT$_{3A}$ receptor subunits to permit selection of transfected cells under fluorescence optics.

### *Whole-cell patch-clamp electrophysiological recordings with rapid drug application*

Cells were resuspended in a phosphate-buffered saline solution containing 1 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) 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH and osmolality adjusted to 340 mOsm kg$^{-1}$ with sucrose). The solution constantly superfused cells at a rate of 2–3 ml min$^{-1}$. Patch pipettes had resistances of $\sim$5 M$\Omega$ when filled with (mM) 140 mM CsCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES (pH adjusted to 7.4 with CsOH and osmolality adjusted to 315 mOsm kg$^{-1}$ with sucrose). Only cells with a capacitance of 8–12 pF, isolated from neighboring cells, were used for experiments. Cells were voltage-clamped at $-60$ mV, unless otherwise indicated.

Alcohol and other pharmacological agents were dissolved directly into external solution, and applied to transfected HEK cells with a theta-tube that had been pulled to a tip diameter of $\sim$200 $\mu$m and whose lateral movement was controlled by a piezoelectric manipulator, as described previously (Zhou *et al*., 1998). Solution exchange rate for open pipette and whole-cell recording was estimated using the potential change induced by switching from the control solution to a 140 mM *N*-methyl-D-glucamine (NMDG) test solution at 0 mV in the absence of agonist, and the current rising phase was fit using an exponential function. The solution exchange time constants were $\sim$0.3 ms for an open pipette tip and $\sim$1.6 ms for whole-cell recording. 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 $\mu$m such that the cell was superfused by a solution containing agonist $\pm$ alcohol. Cessation of the computer-generated pulse reversed the pipette displacement, returning the cell to the standard external solution stream. In selected experiments, alcohols were applied alone for 30 s prior to application of agonist + alcohol.

Solution applications ranged in duration from 2 ms–10 s. Data were filtered at 2 kHz with a four-pole Bessel filter and digitized at 5–10 kHz. In the majority of experiments, data were acquired using an Axopatch 200B amplifier and pClamp 8.0 software (Axon Instruments, Foster City, CA, U.S.A.), and analyzed offline using pClamp 8.0 software. A few recordings were made using a Multiclamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Current amplitudes were measured relative to baseline current levels using cursors available in pClamp 8.0. Further data analysis and curve fitting were performed with Origin6.0 (Microcal Software, Northampton, MA, U.S.A.), pClamp8.0 (Axon), Statistica5.5 (StatSoft Inc., Tulsa, OK, U.S.A.) or GraphPad InStat3.0 (GraphPad Software Inc., San Diego, CA, U.S.A.) software. Concentration–response data were fit using the Hill equation, $I/I_{MAX} = 1/[1 + (EC_{50}/[\text{Agonist}])^{n_H}]$, where $I$ is the current amplitude activated by a given concentration of agonist ([Agonist]), $I_{MAX}$ is the maximum response of the cell, $n_H$ is the Hill coefficient and $EC_{50}$ is the concentration eliciting a half-maximal response.

Parameters of channel activation, deactivation and desensitization were estimated by fitting appropriate current components using exponential functions of the general form $\sum A_n e^{(-t/\tau n)} + A_s$, where $A_n$ is the relative amplitude of the respective component, $A_s$ is the steady-state current, $n$ is the optimal number of exponential components, $t$ is time and $\tau_n$ is the respective time constant. Curve fitting was achieved in Clampfit 9.0 using the Levenberg–Marquardt algorithm. Additional components were accepted only if they significantly improved the fit, as determined by an $F$-test performed by the analysis software.

Activation rates were derived from exponential fitting of the rising phase of agonist-activated current. Desensitization rates were derived from exponential fits to the current decay starting just after the current peak and extending to the end of agonist application. Deactivation rates were derived from exponential fits to the current decay after the removal of agonist following a 2- or 10-ms application of agonist. To facilitate direct comparison of monoexponentially and biexponentially fit data, a weighted summation of time constants ($\sum a_n \tau_n$) was used, where $a_n$ is the fractional contribution of the respective component, $\tau_n$ is the respective time constant, and $n$ is the optimal number of exponential components. The rate of current activation was also estimated by measuring the 10–30% slope of the initial inward current defined as the slope of a linear function fit to current between the time points at which current was 10 and 30% of the peak value (Zhou *et al*., 1998).

### *Statistical analysis of data obtained from 5-HT$_{3A}$ receptors expressed in HEK293 cells*

Graphpad Prism was used to calculate $EC_{50}$s, Hill coefficients, and two-way ANOVA. Instat was used to perform one-way ANOVA, Student's $t$-tests, and Bonferroni's multiple comparisons test. The criterion for statistical significance was set at $P < 0.05$ for all experiments. In the case of one- and two-way ANOVAs, the null hypothesis was that mutation did not produce an alteration relative to the wild-type receptor. For the second variable in the two-way ANOVAs, the null hypothesis was that no effect was produced across measurements, for example, no change in peak current amplitude with increasing concentrations of 5-HT.

## Results

### *Functional characteristics of wild-type and L293 mutant 5-HT$_{3A}$ receptors*

Amino acids at the 15′ positions of TM2 in the GABA and glycine receptors are critical for determining the direction of allosteric modulation by alcohols and anesthetics (Mihic *et al*., 1997). In the 5-HT$_{3A}$ receptor, L293 is at the 15′ position of TM2 (Figure 1a). We mutated L293 to amino acids with different physicochemical properties (Zimmerman *et al*., 1968; Zamyatnin, 1972; Hopp & Woods, 1981), Cys, Gly, Ile, Ser, Val, and Trp. All of the mutant receptors were functional, but the expression of L293V and L293W was low (below 100 pA with maximal 5-HT concentrations) in HEK293 cells, and therefore, they were not studied further. The L293I receptor also had relatively low expression (~150–500 pA with maximal 5-HT concentrations) in HEK293 cells; therefore, a limited number of studies were performed on the L293I mutant in this expression system.

Serotonin concentration–response curves were generated in oocytes expressing wild-type or mutant 5-HT$_{3A}$ receptors. Relative to the wild-type receptors, leftward shifts in the 5-HT concentration–response curves were observed in L293C, L293G, L293I, and L293S 5-HT$_{3A}$ receptors (Figure 1b). Two-way ANOVA demonstrated that mutation had a significant effect on the 5-HT concentration–response curves ($F_{(4, 302)} = 45.52$, $P < 0.0001$). In comparison with the wild-type

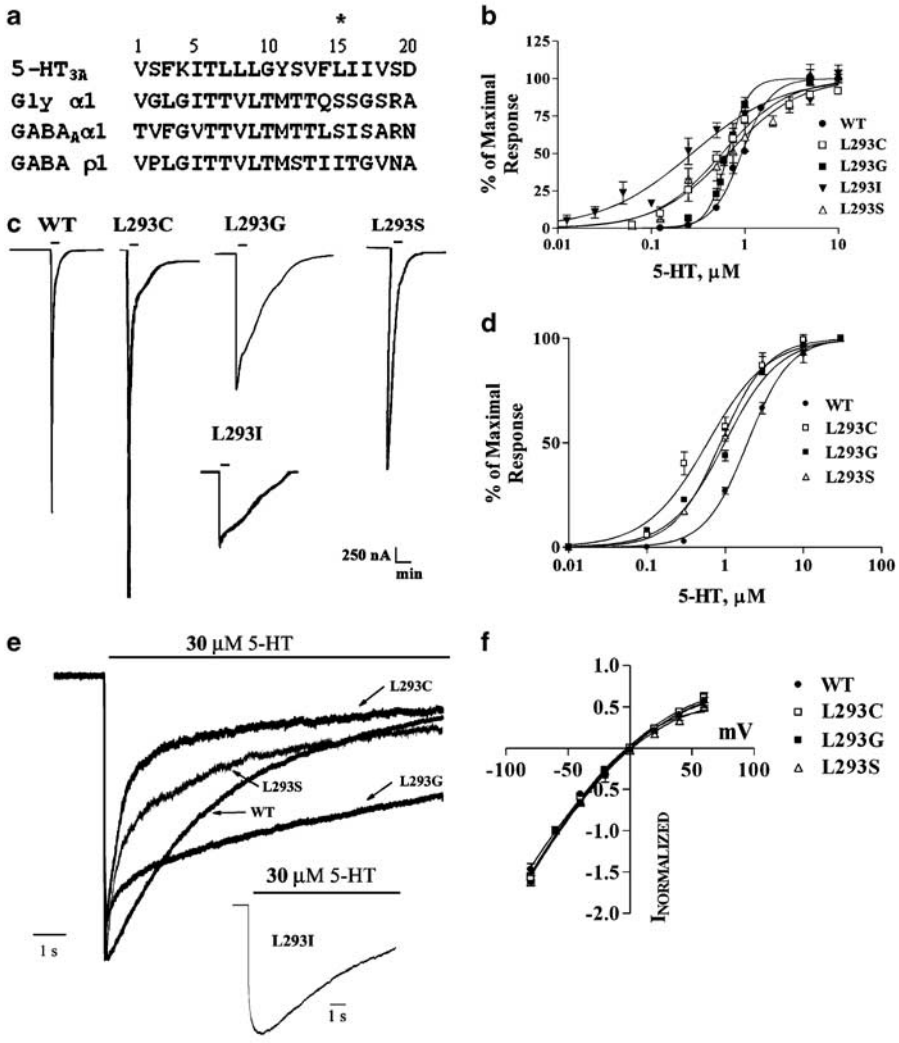


**Figure 1** (a) Alignment of the TM2 domains of the mouse 5-HT$_{3A}$, glycine α1, GABA$_A$ α1, and GABA ρ1 receptors. The asterisk indicates position 15′, the residue implicated in alcohol and anesthetic function. Serotonin concentration–response curves were generated in wild-type and L293 mutant 5-HT$_{3A}$ receptors. (b) Oocytes expressing wild-type or mutant receptors were perfused with 5-HT for 30 s ($n = 4$–9). Peak current amplitude data were normalized to the maximal 10 μM current response. (c) Representative current traces of wild-type and mutant 5-HT$_{3A}$ receptors expressed in oocytes in response to 10 μM 5-HT are shown. (d) in HEK293 cells expressing wild-type or mutant receptors, 5-HT concentration–response curves were generated ($n = 4$–14). Peak current amplitude data were normalized to the 30 μM 5-HT control response. (e) Representative current traces of wild-type and mutant 5-HT$_{3A}$ receptors in HEK293 cells in response to 30 μM 5-HT are shown for the indicated receptor constructs. (f) Current–voltage relationships for wild-type and L293 mutant 5-HT$_{3A}$ receptors are depicted, in which currents were evoked by 30 μM 5-HT ($n = 3$–4). Data are normalized to the currents obtained at −60 mV.

receptor ($0.93 \pm 0.02\,\mu$M), $EC_{50}$ values in $\mu$M were significantly reduced in these mutant constructs: L293C ($0.57 \pm 0.04$), $P<0.001$; L293G ($0.66 \pm 0.01$), $P<0.01$; L293I ($0.29 \pm 0.04$), $P<0.001$; and L293S ($0.69 \pm 0.06$), $P<0.05$, Bonferroni's multiple comparisons test. The Hill slope of the wild-type receptor ($2.8 \pm 0.21$) was steeper than that of all mutants, except L293G: L293C ($1.3 \pm 0.1$), L293G ($3.8 \pm 0.3$), L293I ($0.95 \pm 0.1$), and L293S ($1.3 \pm 0.2$). Representative tracings at maximal 5-HT concentrations are shown for the wild-type and mutant 5-HT$_{3A}$ receptors in Figure 1c.

In HEK293 cells, 5-HT concentration–response curves were determined in wild-type, L293C, L293G, and L293S 5-HT$_{3A}$ receptors (Figure 1d). Similar to the results obtained in oocytes, 5-HT potency was enhanced in the mutant 5-HT$_{3A}$ receptors. A significant effect of mutation on the 5-HT concentration–response relationship was observed (two-way ANOVA, $F_{(3,238)} = 36.19$, $P<0.0001$). The $EC_{50}$ values for 5-HT (in $\mu$M) in the mutant receptors were significantly reduced compared to that of the wild-type receptor ($1.99 \pm 0.08$); L293C ($0.61 \pm 0.08$), $P<0.001$; L293G ($1.14 \pm 0.06$), $P<0.001$; and L293S ($0.92 \pm 0.08$), $P<0.001$, Bonferroni's multiple comparisons test. Hill coefficients for the receptors were: wild-type ($1.5 \pm 0.1$), L293C ($1.3 \pm 0.1$), L293G ($1.2 \pm 0.1$), and L293S ($1.4 \pm 0.1$). The likely explanation for the larger $EC_{50}$ values obtained in HEK293 cells compared to oocytes is that agonist application is relatively slow in oocytes. In oocytes, current is measured after many of the receptors have undergone desensitization, in which case apparent agonist affinity and potency are increased. Representative ion current tracings for the receptors expressed in HEK293 cells are shown in Figure 1e. Differences in current decay in traces obtained from oocytes and HEK293 cells likely reflect the longer recordings in oocytes (minutes) than in HEK293 cells (seconds). In L293 mutant receptors expressed in oocytes or HEK293 cells, resting currents in the absence of agonist were not increased relative to the wild-type receptor. Application of the 5-HT$_{3A}$ receptor competitive antagonist, MDL72222, to wild-type or L293 mutant receptors expressed in HEK293 cells did not produce a change in holding current (data not shown). Therefore, the mutations do not appear to produce spontaneous opening of a significant fraction of the available channels, as has been reported for $GABA_A$ receptors mutated in TM2 (Thompson *et al*., 1999; Findlay *et al*., 2001).

### *Lack of change in current/voltage relationship*

In the experiments shown in Figure 1f, the effects of L293 mutations on the current–voltage ($I/V$) relationship for the receptor channel were assessed. The $I/V$ relationships were obtained by measuring peak current amplitude evoked by $30\,\mu$M 5-HT at holding potentials ranging from $-80$ to $+60$ mV. Slight inward rectification of the $I/V$ curve was observed, and mutation did not affect the overall shape of the curve. Reversal potentials were calculated, and the values obtained were wild-type ($2.4 \pm 0.9$), L293C ($1.9 \pm 0.7$), L293G ($2.2 \pm 0.8$), and L293S ($3.5 \pm 0.7$). One-way ANOVA revealed no significant differences in reversal potential ($F_{(3,10)} = 0.79$, $P = 0.52$), suggesting that the L293 mutations do not affect relative ion permeability of the channel pore.

### *Mutation of L293 and changes in gating*

The increased 5-HT potency observed with mutation may reflect a change in gating of the 5-HT$_{3A}$ receptor. Previous studies have demonstrated that TM2 mutations in members of the superfamily of LGICs can produce changes in the coupling of agonist binding to channel opening, with alterations in affinity and efficacy of agonists (Forman & Zhou, 1999; Sessoms-Sikes *et al*., 2003). The first approach we took to explore this issue was to examine the efficacy of DA, a weak partial agonist at the mouse wild-type receptor (van Hooft & Vijverberg, 1996), Figure 2a. A saturating concentration of DA, 3 mM, was used. Relative efficacy is expressed as a percent of the response evoked by $30\,\mu$M 5-HT. All mutations increased DA efficacy, with a rank order of L293G ($79.1 \pm 1.9\%$) > L293C ($69.4 \pm 3.7\%$) > L293S ($61.5 \pm 1.9\%$) > wild type ($24.8 \pm 1.3\%$). One-way ANOVA demonstrated a significant effect of mutation on efficacy ($F_{(3,31)} = 60.39$, $P<0.0001$). All mutants exhibited a significantly greater DA efficacy than the wild-type receptor, $^*P<0.001$, Bonferonni's multiple comparisons test. Representative tracings of 5-HT- and DA-activated currents are presented in Figure 2b. In the wild-type receptor, DA- and 5-HT-activated currents differ in two ways, rate of current onset and presence of observable desensitization (i.e., current decay during agonist application). Receptors containing mutations at L293 demonstrate faster activation and, in some

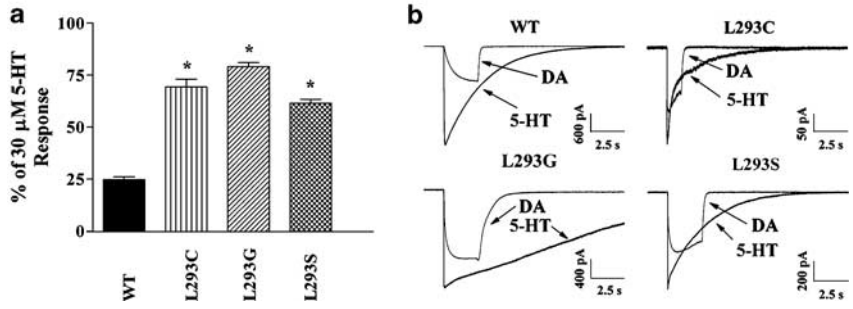


**Figure 2** Pharmacology of L293 mutant receptors is altered relative to the wild-type receptor. (a) HEK293 cells expressing wild-type or mutant 5-HT$_{3A}$ receptors were superfused with the 5-HT$_{3A}$ receptor partial agonist, DA (3 mM), for 2.5–5 s. Peak current data were normalized to the control baseline response obtained with $30\,\mu$M 5-HT ($n = 8$–15). (b) Representative current traces of wild-type and the indicated mutant receptors expressed in HEK293 cells and activated by 5-HT and DA are depicted.

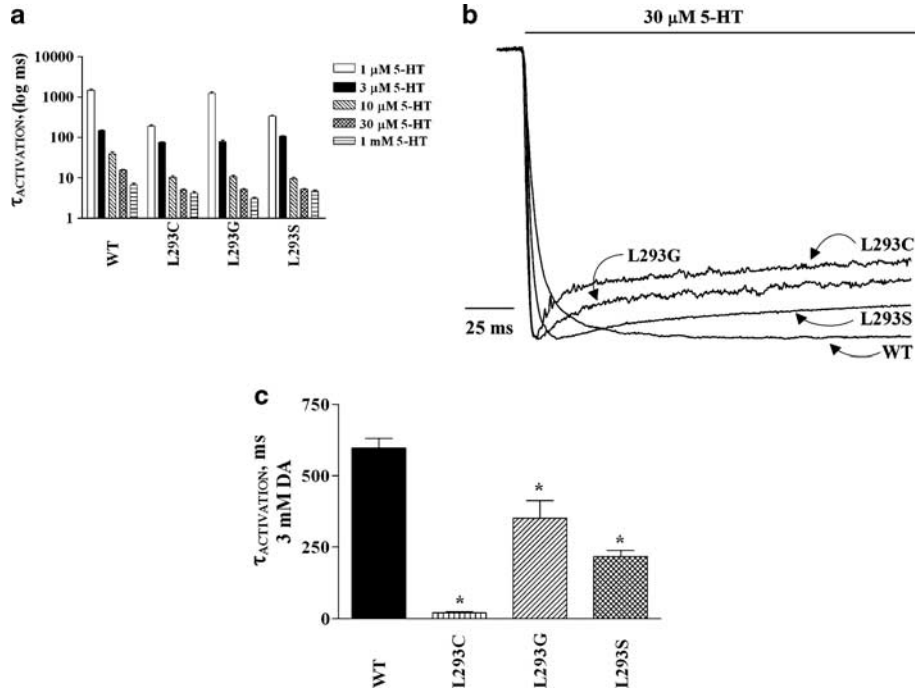


**Figure 3** Mutation of L293 reduces activation time constants. In (a), wild-type and mutant receptors expressed in HEK293 cells were exposed to 5-HT, 1 $\mu$M–1 mM, for 250 ms–10 s, $n=3$–52. (b) Representative tracings of wild-type, L293C, L293G, and L293S receptors in response to 30 $\mu$M 5-HT are presented. (c) Activation was measured in wild-type and L293 mutant receptors in response to the partial agonist, DA, 3 mM, applied for 2.5–5 s ($n=7$–13).

cases, also exhibited observable desensitization in response to DA.

### *Receptor activation and L293 mutation*

To further investigate the effects of mutation on gating, we examined the kinetics of receptor activation, deactivation, and desensitization in response to 5-HT and DA. The kinetics of receptor activation were estimated by treating the initial phase of current as an exponentially developing process and fitting this initial current phase to obtain the time constant of activation, $\tau$. A smaller $\tau$ is indicative of a faster rate of activation of channel opening. As 5-HT concentrations increased, the $\tau$ values (in ms) for current activation decreased. In all but a few instances, the mutant receptors had smaller $\tau$s than the wild-type receptor (Figure 3a); two-way ANOVA demonstrated an effect of 5-HT ($F_{(4,299)}=663.1$, $P<0.0001$) and mutation ($F_{(3,299)}=125.4$, $P<0.0001$) on $\tau$. An interaction between 5-HT and mutation was also detected ($F_{(12,299)}=110.8$, $P<0.0001$). Because of low expression, activation rates were not measured in the L293I mutant receptor. However, from the current tracing of L293I presented in Figure 1e, activation appears slower than that observed in the other receptors. The peak current amplitude is reached slowly and may be contaminated by desensitization. Representative tracings of responses to 30 $\mu$M 5-HT are shown in Figure 3b. When $\tau$ was measured in response to saturating concentrations of the partial agonist, DA, the L293 mutant receptors all had smaller time constants than the wild-type receptor (Figure 3c), one-way ANOVA, $F_{(3,31)}=37.9$, $P<0.0001$, $^*P<0.05$, Bonferrroni's multiple comparisons test.

**Table 1** Activation rate, 10–30% slope (pA/ms)

| | *Control* | *EtOH* (100 mM) |
|---|---|---|
| Wild type | $-0.18\pm0.08$ | $-0.33\pm0.11$[a] |
| L293G | $-0.06\pm0.01$ | $-0.06\pm0.01$ |
| L293S | $-0.27\pm0.04$ | $-0.28\pm0.03$ |
| | *Control* | *TCEt* (1 mM) |
| Wild type | $-0.16\pm0.04$ | $-1.03\pm0.27$[a] |
| L293G | $-0.08\pm0.01$ | $-0.08\pm0.01$ |
| L293S | $-0.28\pm0.04$ | $-0.27\pm0.05$ |

[a]Slope in the presence of alcohol is significantly different from that in its absence, paired *t*-test, $P<0.01$ ($n=8$–11).

In each individual construct, activation by saturating concentrations of 5-HT was faster than that with 3 mM DA, an expected outcome given the lower efficacy of DA.

Activation rate of wild-type and mutant 5-HT$_{3A}$ receptors was also assessed using a measure of the initial slope during the onset of 5-HT-activated current. The rate of current onset was determined from data points during the rising phase of current between 10 and 30% of peak current amplitude (Table 1). This alternate measure of channel activation rate is unlikely to be contaminated by desensitization (Zhou *et al*., 1998). One-way ANOVA revealed that mutation had a significant effect on slope ($F_{(2,24)}=5.5$, $P=0.01$), but neither L293G nor L293S was significantly different from the wild-type receptor; however, the two mutants differed from one another (Tukey–Kramer multiple comparisons test).

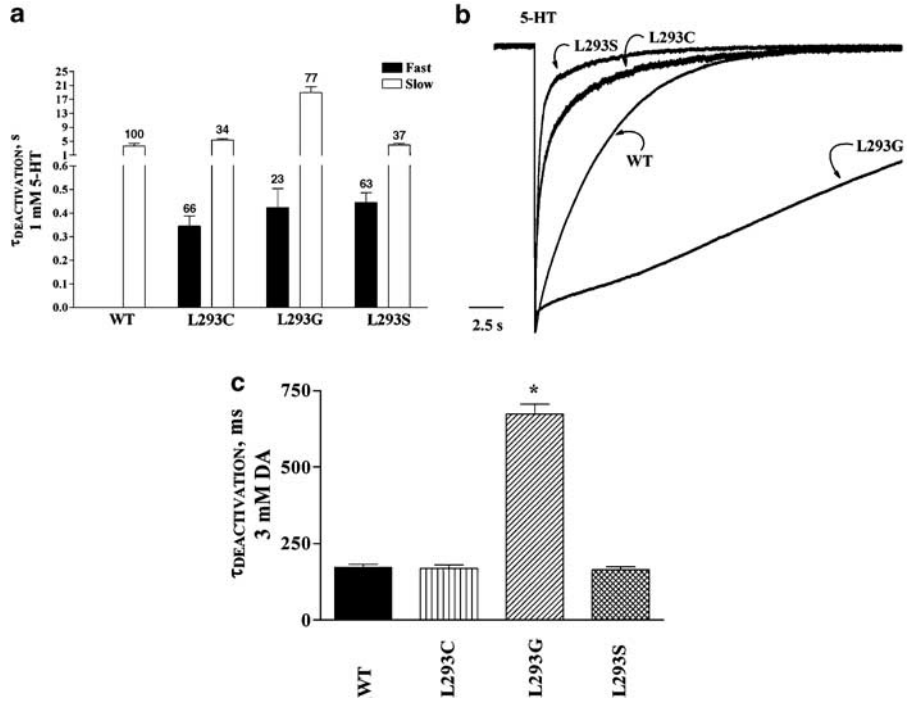


**Figure 4** Deactivation time constants of L293 mutant receptors are changed relative to the wild-type receptor. (a) HEK293 cells expressing wild-type or mutant receptors were superfused with 1 mM 5-HT for 2 ms. Monoexponential (wild-type) and biexponential (mutants) fits of current decay were obtained, with fast (black bar) and slow (clear bar) components ($n = 6$–14). Percent of fast and slow components of the total current amplitudes are indicated above each bar. In (b), representative current traces for deactivation for wild-type, L293C, L293G, and L293S receptors are shown. (c) HEK293 cells were exposed to 3 mM DA for 0.5–5 s ($n = 5$–16). Monoexponential (fast) decay of current was observed for all receptor constructs.

### *Changes in deactivation kinetics with L293 mutation*

Deactivation, a function of agonist unbinding and channel closing, was measured with both 5-HT and DA in wild-type and L293 mutant 5-HT$_{3A}$ receptors (Figure 4a–c). Application of 5-HT and DA was for 2 and 10 ms, respectively, time points that minimize contamination with desensitization. When receptors were activated with 1 mM 5-HT, monoexponential decay of current with an average time constant of $3.6 \pm 0.28$ s was observed in the wild-type receptor, whereas biexponential decay was observed in the mutant receptors (Figure 4a). Fast decay time constants of the mutant receptors were comparable at $\sim$0.3–0.4 s. In contrast, slow decay ranged from $\sim$3.6 to 18 s. From visual inspection of the ion current traces (Figure 4b), it is clear that all mutants display a component of decay that is faster than the wild-type receptor. In addition, all mutants display the slow decay component. The L293G mutant does, however, express less of the fast component ($\sim$23% of total) in comparison to the other mutants ($\sim$63–66%). The percentages of the fast and slow components are indicated above the bars for each receptor construct in Figure 4a. The slow decay in L293G is much slower than that observed in the wild-type receptor.

When receptors were activated by DA, deactivation was monoexponential in all receptors studied. All receptors, except L293G, demonstrated relatively fast deactivation time constants after the removal of 3 mM DA, as expected for a low-affinity agonist (Figure 4c). One-way ANOVA demonstrated a significant effect of mutation on deactivation ($F_{(3,42)} = 216.6$, $P < 0.0001$). L293G had a significantly greater deactivation time constant than the wild-type receptor, $^*P < 0.001$, Bonferonni's multiple comparisons test. Collectively, with respect to deactivation, the open channel state is favored in wild-type and L293G mutant receptors to a greater extent than in the other L293 mutant receptors.

### *Desensitization and L293 mutation*

Desensitization, the decay of current in response to a prolonged application of 30 $\mu$M 5-HT, was measured in wild-type and L293 mutant 5-HT$_{3A}$ receptors. Peak and steady-state currents were measured, with the peak current being the maximum current obtained during agonist application. Steady-state current was the current observed at the end of a 10 s agonist application. It should be noted that this duration was not sufficient to achieve a true 'steady-state' current in the L293G mutant. Inspection of representative tracings (Figure 1e) reveals that the mutant receptors display both fast and slow desensitization components, whereas the wild-type receptor shows only a slow component. The time constants for desensitization are presented in Figure 5a. In the mutant receptors, the $\tau$ values for the fast component of desensitization ranged from 0.45–0.49 s. The slow component $\tau$ values in the wild-type and mutant receptors ranged from 3.5–4.8 s. The slow component of the L293G mutant receptor could not be well fit and thus was not determined. The percent contribution of the fast and slow components of desensitization is indicated above the bars in Figure 5a. While L293C and L293S mutants had fast components that constitute 67 and 65% of the total current, respectively, the fast component of the L293G mutant receptor represented only 22% of the current response. Ratios of steady state to peak currents are shown in Figure 5b and

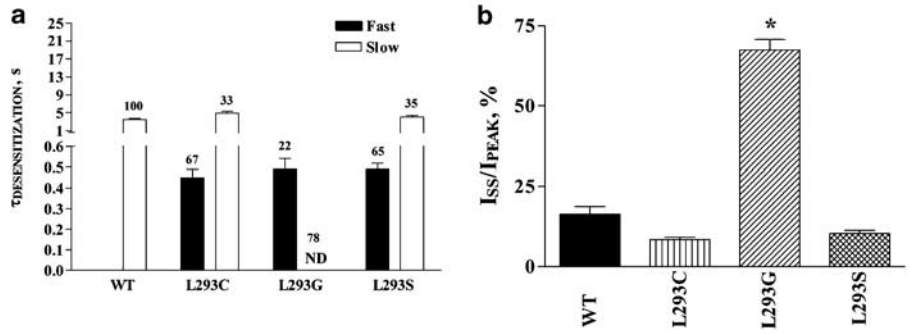


**Figure 5** Desensitization is altered in all L293 mutant receptors. Wild-type and mutant receptors expressed in HEK293 cells were exposed to 30 $\mu$M 5-HT for 10 s. (a) Monoexponential (wild-type) and biexponential (mutants) current decays were observed, with fast (black bar) and slow (clear bar) components ($n = 7$–13), although the slow component of L293G could not be fit (ND = not determined). Percent of fast and slow components of the total current amplitudes are indicated above each bar. Steady state to peak current ratios derived from data obtained in (a) are presented in (b). Extent of desensitization is represented, with less in L293G relative to the wild-type receptor.

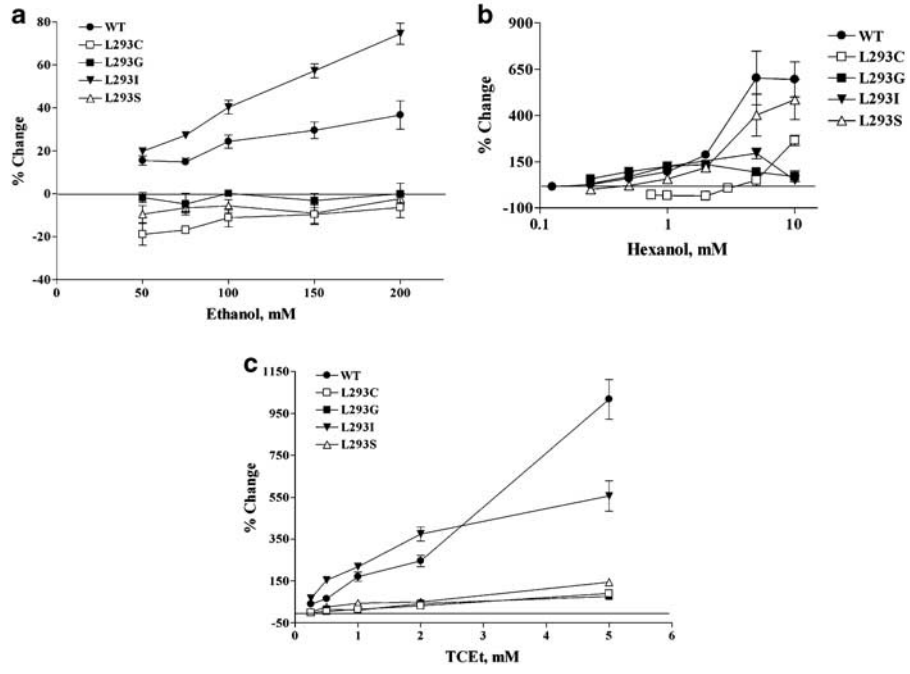


**Figure 6** Modulatory actions of alcohols in wild-type and mutant receptors expressed in *Xenopus* oocytes. (a) Wild-type and mutant receptors were exposed to EtOH for 1 min prior to exposure to 5-HT plus EtOH for 1 min. Concentrations of 5-HT ($\sim EC_1$) were 175, 62.5, 150, 25, and 62.5 nM for wild-type, L293C, L293G, L293I, and L293S 5-HT$_{3A}$ receptors ($n = 4$–8). (b) Actions of hexanol on wild-type and mutant 5-HT$_{3A}$ receptors expressed in *Xenopus* oocytes. Wild-type and L293 mutant receptors were exposed to hexanol for 1 min prior to exposure to 5-HT plus hexanol for 1 min. $EC_1$ concentrations of 5-HT (described in a) were used ($n = 3$–6). Peak current data are presented as percent change from the control baseline value. (c) Wild-type and mutant receptors were exposed to 5-HT plus TCEt for 30 s. $EC_1$ concentrations of 5-HT (described in a) were used ($n = 4$–8). Peak current data are presented as percent change from the control baseline value.

reflect the extent of receptor desensitization during the 10 s period of 5-HT application. The L293G mutant desensitized to a lesser degree than the wild-type receptor (one-way ANOVA, $F_{(3,49)} = 217.4$, $P < 0.0001$; Bonferonni's multiple comparison test, $^*P < 0.001$). Collectively, with respect to the time course and extent of desensitization, the open channel state is favored over the closed or desensitized states in the wild-type and L293G mutant receptors to a greater degree than in the other L293 mutant receptors.

### *Effects of L293 mutation on alcohol modulation of receptor function*

Initially, we used the *Xenopus* oocyte expression system to perform alcohol concentration–response studies (Figure 6). The oocyte expression system was of particular utility in assessing the actions of alcohols on the function of L293I relative to that of other receptor constructs, given that L293I has such poor expression in HEK293 cells. Figure 6 shows

modulation of 5-HT-mediated currents by EtOH in *Xenopus* oocytes expressing the receptors. As shown in Figure 6a, EtOH (50–200 mM) enhanced wild-type receptor-mediated currents, with potentiation ranging in magnitude from ~15% at 50 mM to ~37% at 200 mM. L293I mutant receptor function was also enhanced by EtOH, to an even greater degree than that of wild-type receptors. In contrast, EtOH (50–200 mM) had slight inhibitory effects on C, G, and S mutant receptor function. Two-way ANOVA demonstrated that both mutation ($F_{(4, 101)} = 174.3$, $P < 0.0001$) and EtOH concentration ($F_{(4, 101)} = 19.2$, $P < 0.0001$) effects were significant. A significant interaction was observed between EtOH concentration and mutation ($F_{(16, 101)} = 4.8$, $P < 0.0001$). Stimulation of wild-type receptor function and inhibition of mutant receptor function by EtOH were rapidly reversible.

The modulatory actions of hexanol were assessed in wild-type and L293 mutant 5-HT$_{3A}$ receptors expressed in *Xenopus* oocytes to determine if differential responses to short- and long-chain alkanols were observed in the mutant receptors (Figure 6b). In the mouse wild-type receptor, hexanol is the longest *n*-chain alcohol that enhances receptor function (Jenkins *et al*., 1996). In the wild-type receptor, enhancement of 5-HT-evoked currents was observed with hexanol (0.25–10 mM) in a concentration-dependent manner. Hexanol enhanced the function of L293G and L293I receptors to an extent similar to the wild-type receptor, except at 5 and 10 mM ($P < 0.05$, Bonferroni's multiple comparisons test), where enhancement was reduced in the mutants. Hexanol enhanced the function of the L293S receptor, but at concentrations of 2 mM and lower, potentiation was less than that observed in the wild-type receptor ($P < 0.05$, Bonferroni's multiple comparisons test). The function of the L293C receptor was inhibited by low hexanol concentrations (0.75–2 mM) and enhanced by higher concentrations (5–10 mM), but to a lesser extent than that observed in the wild-type receptor ($P < 0.05$, Bonferroni's multiple comparisons test). Two-way ANOVA demonstrated an effect of mutation ($F_{(4, 117)} = 22.4$, $P < 0.0001$) and hexanol ($F_{(5, 117)} = 33.4$, $P < 0.0001$). An interaction between the two factors was also observed ($F_{(20, 117)} = 7.9$, $P < 0.0001$).

Enhancement of 5-HT-mediated currents by TCEt was robust in wild-type and L293I mutant receptors, with enhancement of ~40–1000% with 0.25–5 mM TCEt (Figure 6c). The potency and efficacy of TCEt was markedly reduced in the C, G, and S mutant receptors. In L293C and L293G 5-HT$_{3A}$ receptors, potentiation of receptor function of ~10–90% was observed at 1–5 mM TCEt. Similarly, enhancement of L293S receptor function ranged from ~43 to 144% at 1–5 mM TCEt. Mutation significantly affected enhancement of 5-HT-evoked currents (two-way ANOVA, $F_{(4, 104)} = 146$, $P < 0.0001$). A significant effect of TCEt concentration was also observed ($F_{(4, 104)} = 149$, $P < 0.0001$). An interaction between mutation and TCEt concentration was obtained as well, $F_{(16, 104)} = 39.9$, $P < 0.0001$.

The modulatory actions of EtOH and TCEt were also examined in wild-type and L293G, L293I, and L293S receptors expressed in HEK293 cells. The L293C mutant was eliminated from this part of the study because of low current amplitudes at the 5-HT concentrations used. Representative tracings of 5-HT-evoked currents in the absence and presence of EtOH (100 mM) are shown (Figure 7a). EtOH effects on peak 5-HT-activated currents were examined at $EC_{25}$ concentrations of 5-HT. EtOH enhanced wild-type receptor function, but had no effect on that of the L293G and L293S mutant receptors (Figure 7b). EtOH enhancement of wild-type receptor-mediated currents was rapidly reversible. One-way ANOVA was significant, $F_{(2, 21)} = 15.3$, $P < 0.0001$, Bonferonni's multiple comparisons test, $^*P < 0.001$, compared to the wild-type receptor.

Effects of TCEt on peak current amplitude were also measured in HEK293 cells. In Figure 7c, traces are presented for wild-type and mutant receptor responses to 5-HT in the absence and presence of TCEt. Averaged data for wild-type and mutant receptor responses to TCEt (1 mM) are depicted in Figure 7d. Similar to EtOH, TCEt had no effect on L293G receptor function, and it inhibited the L293S mutant ~15%. One-way ANOVA was significant, $F_{(2, 21)} = 532.9$, $P < 0.0001$, Bonferonni's multiple comparisons test, $^*P < 0.001$, compared to the wild-type receptor. TCEt (0.5–25 mM) has no inhibitory effects on peak currents in wild-type 5-HT$_3$ receptors at the agonist concentrations used for receptor activation. TCEt enhancement of 5-HT-mediated currents in native receptors declines as 5-HT concentrations are increased (Zhou *et al*., 1998). In native 5-HT$_3$ receptors in no-dose ganglion neurons, a leftward shift in the 5-HT concentration–response curve was observed in the presence of 5 mM TCEt (Lovinger & Zhou, 1993). Of the mutant 5-HT$_{3A}$ receptors, L293S, at an $EC_{25}$ concentration of 5-HT, was inhibited to the greatest extent by TCEt. Therefore, TCEt effects were examined over a range of 5-HT concentrations to ensure that this mutation abolishes the enhancement of agonist potency normally produced by this alcohol in the wild-type receptor. TCEt caused an apparent decrease in 5-HT potency in the L293S receptor. The 5-HT concentration–response curve was shifted rightward in the presence of TCEt (3 mM), Figure 7e. Two-way ANOVA demonstrated a significant effect of 5-HT ($F_{(3, 16)} = 215.3$, $P < 0.0001$) and TCEt ($F_{(1, 16)} = 6.8$, $P = 0.02$). No interaction was observed between 5-HT and TCEt ($F_{(3, 16)} = 1.2$, $P = 0.35$).

### *Residue 293 mutations alter alcohol effects on activation rate*

Activation rate was assessed in the absence and presence of EtOH or TCEt with the initial slope measure described previously. Table 1 shows that activation rates were increased in the presence of EtOH (100 mM) in the wild-type receptor, in agreement with an increase in 5-HT-mediated currents induced by EtOH. No change was observed in the L293G and L293S receptors, mutant receptors in which EtOH also did not alter peak current amplitude. Two-way ANOVA demonstrated an effect of mutation ($F_{(2, 48)} = 9.9$, $P = 0.0002$), but not EtOH ($F_{(1, 48)} = 1.5$, $P = 0.22$) on activation rate. An interaction between mutation and EtOH was also detected ($F_{(2, 48)} = 1.2$, $P = 0.3$). The effect of TCEt on activation rates was measured and is presented in Table 1. Similar to EtOH, TCEt enhanced activation rates of wild-type receptors, but not L293G and L293S receptors. However, in the wild-type receptor, the increase in activation rate by TCEt was several fold greater than that observed with EtOH, correlating well with the greater efficacy of TCEt. Two-way ANOVA revealed an effect of mutation ($F_{(2, 54)} = 15.4$, $P < 0.0001$) and TCEt ($F_{(1, 54)} = 14$, $P = 0.0004$). An interaction between the two factors was also observed ($F_{(2, 54)} = 14.5$, $P < 0.0001$).

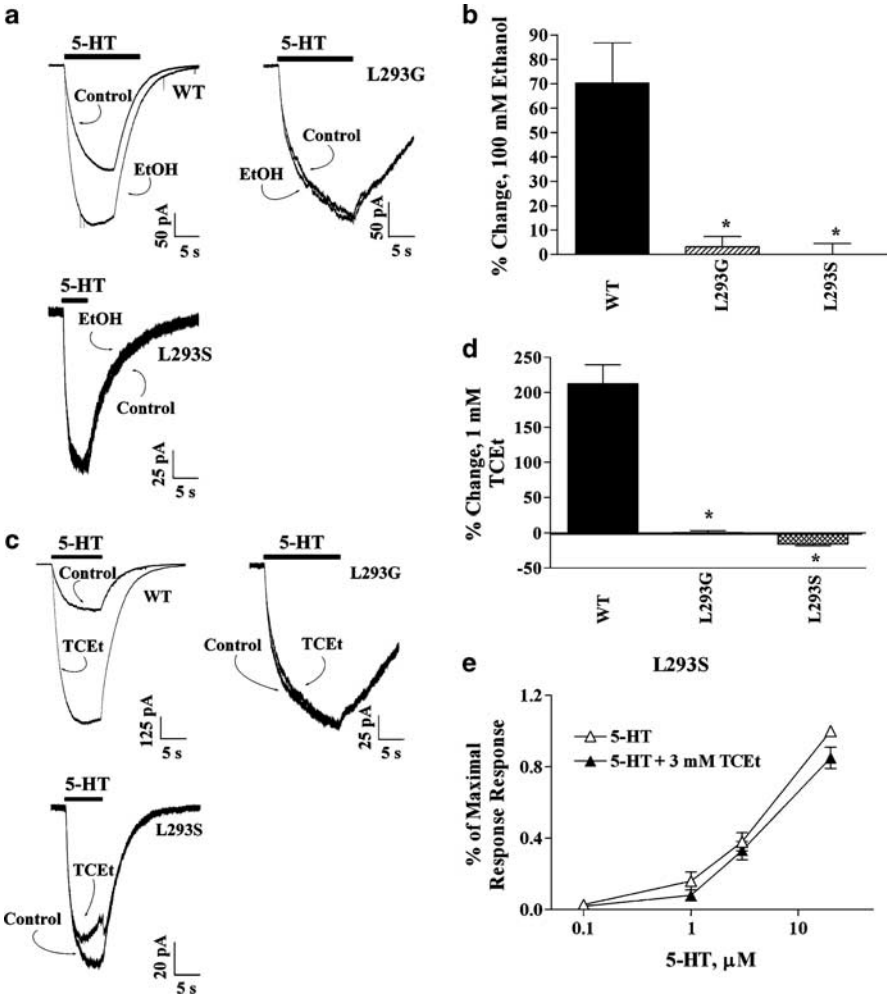


**Figure 7** Actions of EtOH and TCEt on wild-type and mutant receptors expressed in HEK293 cells. (a) Representative tracings of wild-type and mutant receptors superfused with 5-HT in the absence and presence of EtOH (100 mM) for 5–15 s are shown. 5-HT concentrations ($\sim EC_{25}$) were 1, 0.5, and 0.5 $\mu$M for wild-type, L293G, and L293S receptors. In (b), percent change of the 5-HT-induced current in the presence of EtOH (100 mM) is presented ($n=8$). (c) Representative traces of wild-type and mutant receptors superfused with 5-HT in the absence and presence of TCEt (1 mM) for 5–15 s are depicted. $EC_{25}$ concentrations of 5-HT (described in a) were used. (d) The percent change produced by TCEt (1 mM) in 5-HT-mediated currents is shown ($n=8$–11). (e) 5-HT concentration–response curves were performed in the presence and absence of TCEt (3 mM). Responses are reported as percent of the 20 $\mu$M 5-HT maximal response ($n=3$).

### *Residue 293 mutations alter TCEt's effects on desensitization*

Our previous work has demonstrated that TCEt slows desensitization in the wild-type receptor (Zhou *et al*., 1998). In Table 2, we report that TCEt (1 mM) decreased the slow phase of desensitization in the wild-type receptor by $\sim$4-fold. In contrast, TCEt significantly enhanced the fast phase of desensitization in the L293S mutant. As can be observed in the tracings in Figure 8a, TCEt slowed the rate of current decay during prolonged agonist application in wild-type receptors, but enhanced the decay in L293S receptors. Given the observation in the oocyte expression system that the L293I receptor's function was enhanced by TCEt, it was of interest to determine whether TCEt slowed desensitization in this mutant. In Figure 8b, TCEt (1 mM and 10 mM) effectively eliminated desensitization of current responses evoked by a saturating concentration of 5-HT (25 $\mu$M); current decay could not be well fit, and therefore taus for desensitization are not presented for this mutant. In addition, 1 mM TCEt enhanced peak current amplitudes by 36.7$\pm$3.3%, whereas 10 mM TCEt changed peak current amplitudes by 2.5$\pm$2.5% above control currents.

**Table 2** Tau, desensitization (s)

| | *Fast phase* | | *Slow phase* | |
|---|---|---|---|---|
| | *Control* | *TCEt* (1 mM) | *Control* | *TCEt* (1 mM) |
| MWT | Not observed | | 3.3$\pm$0.4 | 12.1$\pm$1.5[a] |
| L293S | 0.38$\pm$0.04 | 0.16$\pm$0.01[a] | 3.2$\pm$0.2 | 2.7$\pm$0.09 |

[a]Time constant in the presence of TCEt is significantly different from that in its absence, paired *t*-test, $P<0.001$ ($n=4$–18).

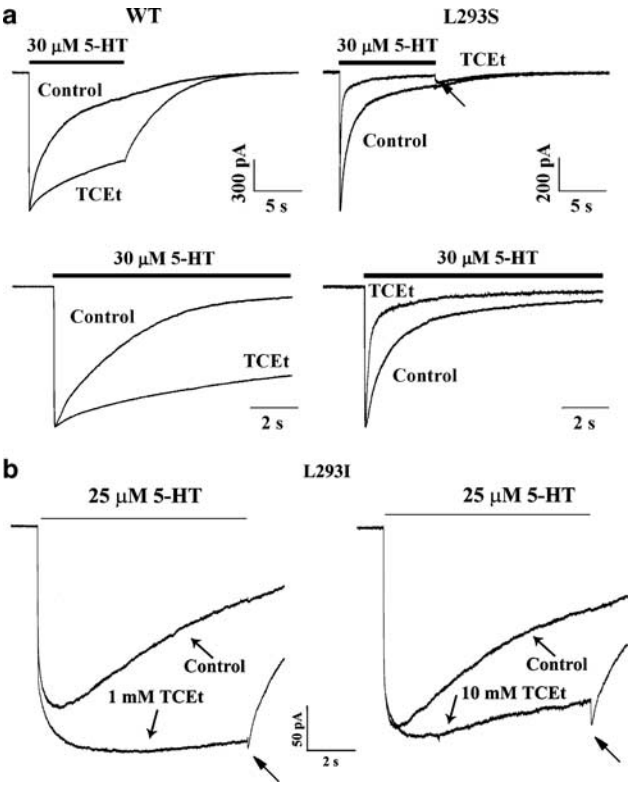


**Figure 8** Representative tracings demonstrating desensitization in wild-type, L293S, and L293I 5-HT$_{3A}$ receptors are presented. (a) Wild-type receptors (left) and L293 mutant receptors (right) were exposed to 30 $\mu$M 5-HT for 10 s, in the absence or presence of TCEt (1 mM). Both rate and extent of desensitization were markedly reduced by TCEt in the wild-type receptor. In contrast, decay enhanced in the L293S mutant receptor. In L293S, the percent contribution of the fast phase of desensitization was increased from $60.5 \pm 4.4\%$ in the absence of TCEt to $74.8 \pm 2.0$ in the presence of TCEt. Note the surge current at the termination of 5-HT plus TCEt application (arrow), which may be an indication of relief from open channel block. In (b), representative tracings of L293I exposed to 25 $\mu$M 5-HT, in the absence and presence of TCEt (1 mM, left panel and 10 mM, right panel). Desensitization is nearly eliminated in the presence of TCEt, and surge currents (arrows) are present upon cessation of drug application.

In oocytes and HEK293 cells, none of the alcohols produced any direct activation of the receptors in the absence of 5-HT, as evidenced by the observation that application of EtOH, hexanol, or TCEt alone did not alter baseline current levels (data not shown). Direct activation of GABA$_A$ receptors by alcohols and anesthetics is associated with loss of allosteric enhancement (Thompson *et al*., 1999; Findlay *et al*., 2001). Therefore, direct activation by alcohols cannot be a mechanism through which alcohol-induced enhancement of function is occluded or lost in the L293 mutant receptors tested.

## Discussion

The 15′ residue of TM2, L293, was assessed for its roles in gating and alcohol modulatory effects in the 5-HT$_{3A}$ receptor. This residue is thought to reside on the protein-facing side of TM2 (Reeves *et al*., 2001; Panicker *et al*., 2002) and corresponds to amino acids in GABA and glycine receptors that have been implicated in forming a hydrophobic-binding domain for alcohols and anesthetics (Belelli *et al*., 1997; Mihic *et al*., 1997). Thus, we examined effects of mutation at this 5-HT$_{3A}$ receptor residue on gating and alcohol actions. Mutations at L293 produced changes not only in 5-HT potency and DA efficacy but also in channel kinetics. Our results are consistent with numerous previous reports (Thompson *et al*., 1999; Findlay *et al*., 2001; Scheller & Forman, 2002; Sessoms-Sikes *et al*., 2003), which have indicated that alterations in TM2 amino acids strongly affect channel gating. Therefore, it was of interest for us to determine whether mutation-induced changes in channel kinetics predict alcohol sensitivity and whether changes in kinetics might be useful in assessing L293 as a part of a putative alcohol-binding domain in the 5-HT$_{3A}$ receptor.

Changes in kinetic parameters of channel function were observed in all L293 mutant receptors. In general, relative to the wild-type receptor, activation of channel opening in the mutants was enhanced across a series of increasing concentrations of 5-HT and saturating concentrations of DA. All but L293G and L293I desensitized much more rapidly than the wild-type receptor. Deactivation rates were very similar to desensitization rates, suggesting that desensitization contaminates the deactivation measurements, as has been reported by another group (Mott *et al*., 2001). In all mutants, increases in activation rate and increases in DA partial agonist efficacy suggest an increase in probability of opening. However, all constructs with the exception of the L293G mutant close more rapidly during agonist application (desensitization) or following agonist removal (deactivation) in comparison to the wild-type receptor. These effects would tend to counteract the increase in probability of opening. Thus, stability of the open state relative to other states in the mutant receptors is a complex function of agonist concentration, relative agonist efficacy and duration of agonist exposure. It should be noted that the most conservative mutation, L293I, produced significant changes in desensitization, suggesting that small changes in amino-acid branching at the 15′ position produce marked changes in receptor conformation associated with gating.

It must be pointed out that measurement of kinetics of whole-cell current does not provide direct evidence of probability of channel opening. Single-channel analysis of the mutant receptors would be the most straightforward way to determine whether probability of opening has been altered. However, single-channel conductance of 5-HT$_{3A}$ receptors is $\sim$0.4 pS (Kelley *et al*., 2003), and thus single-channel currents are exceedingly difficult to measure. In contrast, the 5-HT$_{3A/B}$ receptor has greater single-channel conductance ($\sim$13 pS; Kelley *et al*., 2003), but is insensitive to EtOH and much less sensitive to TCEt than the homomeric receptor (Hayrapetyan *et al*., 2005). Mutation of three residues in the large cytoplasmic loop of the 5-HT$_{3A}$ receptor to that contained in the 5-HT$_{3B}$ receptor confers conductance of $\sim$13 pS to the A homomer. However, it is unknown whether the mutations in the higher conducting 5-HT$_{3A}$ receptor would alter alcohol sensitivity, and that would have to be determined before alcohol effects on TM2 mutants could be studied on such a background. Thus, at present, we are limited to inferring changes in open probability from changes in whole-cell current kinetics and partial agonist efficacy.

In all L293 mutant receptors, except L293I, alcohol enhancement of 5-HT-mediated currents was markedly reduced or eliminated. No mutation-induced change in kinetic parameter predicted L293 mutant sensitivity to EtOH or TCEt. The alcohol-sensitive L293I receptor exhibited slower desensitization relative to the wild-type 5-HT$_{3A}$ receptor, as did the alcohol-insensitive L293G receptor. Deactivation was faster in the alcohol-insensitive L293C and L293S mutant receptors and slower in the alcohol-insensitive L293G mutant receptor relative to the wild-type receptor. Activation was not measured in the alcohol-sensitive L293I mutant receptor, but all alcohol-insensitive mutant receptors, in general, had shorter time constants of activation (faster rate) than the wild-type receptor. However, the initial current slope, an alternate measure of activation of L293G and L293S, was not significantly different from that of the wild-type receptor, suggesting that activation rates may not be good predictors of alcohol sensitivity. All L293 mutant receptors showed leftward shifts in 5-HT concentration–response curves and increases in DA efficacy, suggesting an increase in probability of channel opening. EtOH enhancement of wild-type 5-HT$_{3A}$ receptor function is not observed during application of high 5-HT concentrations (Lovinger & White, 1991; Machu & Harris, 1994), when probability of channel opening is high. While increased probability of opening could contribute to loss of alcohol potentiation in the mutant receptors, the observation that L293I mutant receptor function was enhanced by alcohols does not support this hypothesis.

Although whole-cell current kinetics do not predict whether a mutant receptor will be sensitive to alcohols, they may be useful in assessing possible mechanisms of allosteric modulation. Our previous work suggests that EtOH and TCEt reductions in the desensitization rate of wild-type 5-HT$_{3A}$ receptors likely contribute to potentiation of channel function (Zhou *et al*., 1998), and we have confirmed these findings with TCEt in the current study. Desensitization of the alcohol-sensitive L293I mutant receptor is similarly reduced by TCEt. The L293S receptor is insensitive to EtOH and is inhibited by TCEt, and two possible mechanisms could account for the inhibition by TCEt. First, we have demonstrated that desensitization is enhanced by TCEt in this mutant. In addition, the serine mutation could enhance a channel-blocking effect of TCEt. The surge current that is observed in the tracings of 5-HT plus TCEt that occurs upon cessation of drug application (Figures 7c and 8) may be relief from open channel block (Zhou *et al*., 1998). Alternatively, it may reflect resensitization of channels occurring more quickly than agonist unbinding. Either mechanism could result in slight inhibition of peak current amplitude by TCEt. Finally, both 1 and 10 mM TCEt produced surge currents upon drug cessation in the L293I mutant, with 10 mM TCEt producing a larger surge current. That observation, combined with the finding that 1 mM, but not 10 mM, TCEt enhanced peak current amplitudes, suggests that a channel-blocking effect may contribute to the lack of potentiating response at this higher TCEt concentration.

There is little correlation between physicochemical characteristics of amino-acid substitutions at L293 and alcohol modulation of the 5-HT$_{3A}$ receptor. The amino acids tested C, G, and S, which either eliminate or markedly reduce enhancement by alcohols, vary in molecular volume (Zamyatnin, 1972), hydrophilicity (Hopp & Woods, 1981), hydropathy (Kyte & Doolittle, 1982), and polarity (Zimmerman *et al*., 1968). The isoleucine substitution is the only one that retains sensitivity. Despite their similarities, the L293I receptor, in the absence of alcohols, has markedly different kinetic properties than the wild-type receptor. The L293 mutants possess activation, deactivation, and desensitization kinetics. These results suggest that leucine at the 293 position 'constrains' the wild-type receptor in a thermodynamically stable, closed conformational state, but it is not clear what properties of this amino-acid side chain underlie this constraint.

The relationship between loss of alcohol modulation with mutations at L293 and the role of this residue as a putative alcohol-binding pocket is unclear. A binding site model would predict changes in alcohol/anesthetic actions based on side-chain bulk, and no such effect was observed in the present study. We would anticipate that with substitution of smaller amino acids, alcohol stimulatory effects would be enhanced because more drug molecules could theoretically bind within the 'pocket'. Furthermore, we would predict that an 'optimized' pocket volume for alcohols would exist and that the pocket size would vary for EtOH and TCEt, alcohols of considerably different molecular volume. Given that the observations made in the present study did not indicate the existence of such a relationship between amino-acid side-chain volume and alcohol potentiation, examination of alcohol cutoff is not useful for this residue. Further, if EtOH and hexanol occupy the same binding site and the binding site is too small for EtOH, then hexanol, as the larger molecule, should not bind and therefore should not modulate function. This was not the case as EtOH slightly inhibited and hexanol enhanced both L293G and L293S function. Isoleucine has the same molecular volume as leucine, yet the L293I mutant had reduced sensitivity to hexanol relative to the wild-type receptor. Collectively, the lack of a relationship between the loss of enhancing effect of alcohols and any physicochemical property of substituted amino acid at L293 and the lack of consistency between the modulation by EtOH and that produced by hexanol in the mutant receptors suggest that changes in alcohol modulation are more likely to be the result of more generalized changes in channel conformation that give rise to changes in gating properties.

The observation that L293 mutations at the 15′ position of TM2 in the 5-HT$_{3A}$ receptor disrupt the normal gating process, which likely in turn causes changes in allosteric modulation by alcohols, does not automatically rule out the possibility that L293 is part of an alcohol-binding pocket. The fact that L293 mutations destabilize the normal conformation of the receptor may render it very difficult to assess its role in alcohol binding with the tools that have been used in the past. Thiol reagents, which are proposed to act as alcohol 'surrogates', have been used to irreversibly modify cysteine mutants at the 15′ position of TM2 in the GABA$_A$ and glycine receptors (Mascia *et al*., 2000). Modification ablates alcohol and anesthetic action, which is offered as strong evidence that the 15′ residue in these receptors is part of an alcohol-binding pocket. However, the possibility that cysteine modification eliminates alcohol action through a gating mechanism has not been rigorously tested to date. It must be pointed out that recent studies suggest that the mutation S270I at the 15′ position of the $\alpha$1-subunit of the GABA$_A$ receptor enhances open probability by reducing channel closing rate (Scheller & Forman, 2002). In our studies, the L293C 5-HT$_{3A}$

receptor is slightly inhibited by EtOH and its enhancement by TCEt is markedly reduced, which complicates its use as a putative alcohol surrogate in a similar thiol modification study. In summary, our studies do not absolutely refute a possible role of L293 in the 5-HT$_{3A}$ receptor as a residue in an alcohol-binding pocket. However, they offer an alternate explanation, namely, that changes in channel conformation favoring the open channel state, as evidenced by changes in kinetic properties, may underlie mutation induced alterations in allosteric modulation by alcohols.

This work was supported by AA12643 (T.K.M.) and the NIAAA Division of Intramural Clinical and Basic Research (D.M.L.).

## References


BELELLI, D., LAMBERT, J.J., PETERS, J.A., WAFFORD, K. & WHITING, P.J. (1997). The interaction of the general anesthetic etomidate with the $\gamma$-aminobutyric acid A receptor is influenced by a single amino acid. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 11031–11036.

DAVIES, P.A., PISTIS, M., HANNA, M.C., PETERS, J.A., LAMBERT, J.J., HALES, T.G. & KIRKNESS, E.F. (1999). The 5-HT$_{3B}$ subunit is a major determinant of serotonin-receptor function. *Nature,* **397,** 359–363.

DERKACH, V., SURPRENANT, A. & NORTH, R.A. (1989). 5-HT$_3$ receptors are membrane ion channels. *Nature,* **339,** 706–709.

DUBIN, A.E., HUVAR, R., D'ANDREAS, M.R., PYATI, J., ZHU, J.Y., KOY, K.C., WILSON, S.J., GALINDO, J.E., GLASS, C.A., LIN, L., JACKSON, M.R., LOVENBERG, T.W. & ERLANDER, M.G. (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, G.S., SUSUMU, U., HARRISON, N.L. & HARRIS, R.A. (2001). Allosteric modulation in spontaneously active mutant $\gamma$-aminobutryic acid$_A$ receptors. *Neurosci. Lett.,* **305,** 77–80.

FORMAN, S.A. & ZHOU, Q. (1999). Novel modulation of a nicotinic receptor channel mutant reveals that the open state is stabilized by ethanol. *Mol. Pharmacol.,* **55,** 102–108.

HAYRAPETYAN, V., JENSCHKE, M., DILLON, G.H. & MACHU, T.K. (2005). Co-expression of the 5-HT$_{3B}$ subunit with the 5-HT$_{3A}$ receptor reduces alcohol sensitivity. *Mol. Brain Res.,* **142,** 146–150.

HOPP, T.P. & WOODS, K.R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. U.S.A.,* **78,** 3814–3828.

JENKINS, A., FRANKS, N.P. & LIEB, W.R. (1996). Actions of general anesthetics on 5-HT$_3$ receptors in N1E-115 neuroblastoma cells. *Br. J. Pharmacol.,* **117,** 1507–1515.

JOHNSON, B.A., CAMPLING, G.M., GRIFFITHS, P. & COHEN, P.J. (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. *Psychopharmacology,* **112,** 142–144.

KARNOVSKY, A.M., GOTOW, L.F., MCKINLEY, D.D., PIECHAN, J.L., RUBLE, C.L., MILLS, C.J., SCHELLIN, K.A.B., SLIGHTOM, J.L., FITZGERALD, L.R., BENJAMIN, C.W. & ROBERDS, S.L. (2003). A cluster of novel serotonin receptor 3-like genes on human chromosome 3. *Gene,* **319,** 137–148.

KELLEY, S.P., DUNLOP, J.I., KIRKNESS, E.F., LAMBERT, J.J. & PETERS, J.A. (2003). A cytoplasmic region determines single-channel conductance in 5-HT$_3$ receptors. *Nature,* **424,** 321–324.

KNAPP, D.J. & POHORECKY, L.A. (1992). Zacopride, a 5-HT$_3$ receptor antagonist, reduces voluntary ethanol consumption in rats. *Pharmacol. Biochem. Behav.,* **41,** 847–850.

KOLTCHINE, V.V., FINN, S.E., JENKINS, A., NIKOLAEVA, N., LIN, A. & HARRISON, N.L. (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.

KYTE, J. & DOOLITTLE, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.,* **157,** 105–132.

LOPREATO, G.F., BANERJEE, P. & MIHIC, S.J. (2003). Amino acids in transmembrane domain two influence anesthetic enhancement of serotonin-3A receptor function. *Mol. Brain Res.,* **118,** 45–51.

LOVINGER, D.M. & ZHOU, Q. (1993). Trichloroethanol potentiation of 5-hydroxytryptamine3 receptor-mediated ion current in nodose ganglion neurons from the adult rat. *J. Pharmacol. Exp. Ther.,* **265,** 771–776.

LOVINGER, D.M., SUNG, K.W. & ZHOU, Q. (2000). Ethanol and trichloroethanol alter gating of 5-HT3 receptor-channels in NCB-20 neuroblastoma cells. *Neuropharmacology,* **39,** 561–570.

LOVINGER, D.M. & 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.

MACHU, T.K. & HARRIS, R.A. (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, A.V., PETERSON, A.S., BRAKE, A.J., MYERS, R.M. & JULIUS, D. (1991). Primary structure and functional expression of the 5-HT$_3$ receptor, a serotonin-gated ion channel. *Science,* **154,** 432–434.

MASCIA, M.P., TRUDELL, J.R. & HARRIS, R.A. (2000). Specific binding sites for alcohols and anesthetics on ligand-gated ion channels. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 9305–9310.

MCBRIDE, W.J., LOVINGER, D.M., MACHU, T., THIELEN, R.J., RODD, Z.A., MURPHY, J.M., ROACHE, J.D. & JOHNSON, B.A. (2004). Serotonin-3 receptors in the actions of alcohol, alcohol reinforcement, and alcoholism. *Alcoholism: Clin. Exp. Res.,* **28,** 257–267.

MIHIC, S.J., YE, Q., WICK, M.J., KOLTCHINE, V.V., KRASOWSKI, M.D., FINN, S.E., MASCIA, M.P., VALENZUELA, C.F., HANSON, K.K., GREENBLATT, E.P., HARRIS, R.A. & HARRISON, N.L. (1997). Sites of alcohol and volatile anaesthetic action on GABA$_A$ and glycine receptors. *Nature,* **389,** 385–389.

MORALES, M. & WANG, S.D. (2002). Differential composition of 5-hydroxytryptamine3 receptors synthesized in the rat CNS and peripheral nervous system. *J. Neurosci.,* **22,** 6732–6741.

MOTT, D.D., ERREGER, K., BANKE, T.G. & TRAYNELIS, S.F. (2001). Open probability of homomeric murine 5-HT$_{3A}$ serotonin receptors depends on subunit occupancy. *J. Physiol.,* **535.2,** 427–443.

NIESLER, B., FRANK, B., KAPELLER, J. & RAPPOLD, G.A. (2003). Cloning, physical mapping and expression analysis of the human 5-HT3 serotonin receptor-like genes HTR3C, HTR3D and HTR3E. *Gene,* **319,** 137–148.

PANICKER, S., CRUZ, H., ARRABIT, C. & SLESINGER, P.A. (2002). Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. *J. Neurosci.,* **22,** 1629–1639.

REEVES, D.C., GOREN, E.N., AKABAS, M.H. & LUMMIS, S.C. (2001). Structural and electrostatic properties of the 5-HT$_3$ receptor pore revealed by substituted cysteine accessibility mutagenesis. *J. Biol. Chem.,* **276,** 42035–42042.

SCHELLER, M. & FORMAN, S.A. (2002). Coupled and uncoupled gating and desensitization effects by pore domain mutations in GABA(A) receptors. *J. Neurosci.,* **22,** 8411–8421.

SESSOMS-SIKES, J.S., HAMILTON, M.E., LIU, L.-X., LOVINGER, D.M. & MACHU, T.K. (2003). A mutation in transmembrane domain II of the 5-hydroxytryptamine$_{3a}$ receptor stabilizes channel opening and alters alcohol modulatory actions. *J. Pharmacol. Exp. Ther.,* **306,** 595–604.

THOMPSON, S.-A., SMITH, M.Z., WINGROVE, P.B., WHITING, P.J. & WAFFORD, K.A. (1999). Mutation at the putative GABA$_A$ ion-channel gate reveals changes in allosteric modulation. *Br. J. Pharmacol.,* **127,** 1349–1358.

VAN HOOFT, J.A. & VIJVERBERG, H.P. (1996). Selection of distinct conformational states of the 5-HT$_3$ receptor by full and partial agonists. *Br. J. Pharmacol.,* **117,** 839–846.

WICK, M.J., MIHIC, S.J., UENO, S., MASCIA, M.P., TRUDELL, J.R., BROZOWSKI, S.J., YE, Q., HARRISON, N.L. & HARRIS, R.A. (1998). Mutations of $\gamma$-amino acid and glycine receptors change alcohol cut-off: evidence for an alcohol receptor? *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 6504–6509.

YAMAKURA, T., MIHIC, S.J. & HARRIS, R.A. (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, V.V., MIHIC, S.J., MASCIA, M.P., WICK, M.J., FINN, S.E., HARRISON, N.L. & HARRIS, R.A. (1998). Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position $\alpha$267. *J. Biol. Chem.,* **273,** 3314–3319.

ZAMYATNIN, A.A. (1972). Protein volume in solution. *Prog. Biophys. Mol. Biol.,* **24,** 107–123.

ZHOU, Q., VERDOORN, T.A. & LOVINGER, D.M. (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.

ZIMMERMAN, J.M., ELIEZER, N. & SIMHA, R. (1968). The characterization of amino acids in proteins by statistical methods. *J. Theoret. Biol.,* **21,** 170–201.

*(Received October 31, 2005*
*Revised December 21, 2005*
*Accepted January 11, 2006*
*Published online 6 March 2006)*