Published in final edited form as:

Neuropharmacology. 2008 June ; 54(8): 1153–1165.

The L293 Residue in Transmembrane Domain 2 of the 5-HT_{3A} Receptor is a Molecular Determinant of Allosteric Modulation by 5-Hydroxyindole

Xiang-Qun Hu and David M. Lovinger

Laboratory for Integrative Neuroscience, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892-8115

Abstract

Allosteric modulation of ligand-gated ion channels can play important roles in shaping synaptic transmission. The function of the 5-hydroxytryptamine (serotonin) type 3 (5-HT₃) receptor, a member of the Cys-loop ligand-gated ion channel superfamily, is modulated by a variety of compounds such as alcohols, anesthetics and 5-hydroxyindole (5-HI). In the present study, the molecular determinants of allosteric modulation by 5-HI were explored in N1E-115 neuroblastoma cells expressing the native 5-HT₃ receptor and HEK 293 cells transfected with the recombinant 5-HT_{3A} receptor using molecular biology and whole-cell patch-clamp techniques. 5-HI potentiated 5-HT-activated currents in both N1E-115 cells and HEK 293 cells, and significantly decreased current desensitization and deactivation. Substitution of Leu293 (L293, L15') in the second transmembrane domain (TM2) with cysteine (L293C) or serine (L293S) abolished 5-HI modulation. Other mutations in the TM2 domain, such as D298A and T284F, failed to alter 5-HI modulation. The L293S mutation enhanced dopamine efficacy and converted 5-HI into a partial agonist at the mutant receptor. These data suggest that 5-HI stabilizes the 5-HT_{3A} receptor in the open state by decreasing both desensitization and 5-HT unbinding/channel closing; and L293 is a common site for both channel gating and allosteric modulation by 5-HI. Our observations also indicate existence of a second 5-HI recognition site on the 5-HT_{3A} receptor which may overlap with the 5-HT binding site and is not involved in the positive modulation by 5-HI. These findings support the idea that there are two discrete sites for 5-HI allosteric modulation and direct activation in the 5-HT_{3A} receptor.

Keywords

5-HT_{3A} receptor; allosteric modulation; gating; desensitization; structure-function; electrophysiology

1. Introduction

Ligand-gated ion channels constitute a group of membrane-bound proteins that control the flux of ions across the cell membrane. The 5-hydroxytryptamine (serotonin) type 3 (5-HT₃),

Corresponding author: David M. Lovinger, Ph.D., Laboratory for Integrative Neuroscience, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, 5625 Fishers Lane, Room TS-13A, Bethesda, MD 20852, Phone: 301-443-2445, Fax: 301-443-3040, E-Mail: lovindav@mail.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

nicotinic acetylcholine (nACh), γ -aminobutyric acid type A (GABA_A), and glycine receptors belong to the Cys-loop ligand-gated ion channel superfamily, which mediate synaptic transmission and modulate neurotransmitter release (Ortells & Lunt, 1995; Reeves & Lummis, 2002). Members of the superfamily are pentamers formed by assembly of one (homomeric) or different (heteromeric) subunits. The subunits that form these receptors share similar topology, with each subunit possessing an extracellular N-terminal domain, four transmembrane domains (TMs) and loops connecting those TMs. The interfaces between subunits formed by the extracellular N-terminal domains comprise the agonist binding sites. The TM2 domains form the pore which is surrounded by an outer protein shell comprised of the other TMs.

The 5-HT₃ receptor participates in a variety of physiological functions, such as cognitive processing, sensory transmission, regulation of autonomic function, integration of the vomiting reflex, pain processing and control of anxiety (Barnes & Sharp, 1999). Thus, agents that alter 5-HT₃ receptor function, including allosteric modulators, are of interest because altering receptor function could be a useful therapeutic strategy for diseases associated with the receptor. The 5-HT₃ receptor is not only the target of the natural agonist 5-HT but also a variety of allosteric modulators including the 5-HT analog 5-hydroxyindole (5-HI). Both 5-HT and 5-HI are metabolites of tryptophan (Mannaioni *et al.*, 2003). 5-HI has been shown to increase release of neurotransmitter in the cerebellum and hippocampus (Zwart *et al.*, 2002; Mannaioni *et al.*, 2003) and triggers convulsion in rats (Mannaioni *et al.*, 2003). 5-HI is a positive modulator of the 5-HT₃ receptor (Kooyman *et al.*, 1993; van Hooft *et al.*, 1997; Gunthorpe & Lummis, 1999) and $\alpha 7$ nicotinic acetylcholine receptor (Zwart *et al.*, 2002; Selina Mok & Kew, 2006). However, the molecular mechanisms by which 5-HI affects the 5-HT₃ receptor are still poorly understood. Locating sites involved in the binding and allosteric actions of 5-HI has proven elusive due to the complex effects on 5-HT_{3A} receptor-mediated responses and possible existence of more than one binding site. Previous studies suggested that 5-HI exhibits a combination of competitive and non-competitive actions at the 5-HT₃ receptor (Kooyman *et al.*, 1994), suggesting that there are at least two binding sites for 5-HI. One site likely overlaps with the orthosteric agonist binding site, whereas the second site may be in another region of the protein.

It has been established that amino acid residues in the transmembrane (TM) 2 domain and TM2-TM3 loop play critical roles in 5-HT₃ receptor gating (Filatov & White, 1995; Grosman *et al.*, 2000; Grosman & Auerbach, 2000) and allosteric modulation (Boileau & Czajkowski, 1999; Hu & Lovinger, 2005; Jones-Davis *et al.*, 2005) of the Cys-loop ligand-gated ion channels. To identify amino acid residue(s) that participate in 5-HI modulation, we examined the roles of selected residues from TM2 domain of the 5-HT_{3A} receptor by site-directed mutagenesis and whole-cell patch-clamp recording.

2. Materials and methods

2.1. Mutagenesis

Point mutation of the mouse 5-HT_{3A} receptor (gift from Dr. D. Julius, San Francisco, CA) was accomplished using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The successful incorporation of mutations was verified by sequencing the clones using an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The cDNAs were then subcloned into the vector pCDNA3.1 (Invitrogen) for expression in human embryonic kidney (HEK) 293 cells (passage ≤ 12).

2.2. Cell Culture and Transient Receptor Expression

N1E-115 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum

(FBS), and maintained in a humidified incubator at 37°C in 5% CO₂. HEK 293 cells (ATCC) were grown in minimum essential medium (MEM, Invitrogen) supplemented with 10% horse serum, and maintained in a humidified incubator at 37°C in 5% CO₂. HEK 293 cells were transiently transfected with the wild-type or mutant 5-HT_{3A} receptor cDNA using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Green fluorescent protein (pGreen Lantern, Invitrogen) was co-expressed with the 5-HT_{3A} receptor subunit to permit selection of transfected cells under fluorescence optics. Each 35 mm dish was transfected with 3 µg of cDNA encoding the wild-type or mutant receptors along with 1 µg green fluorescent protein cDNA.

2.3. Whole-Cell Patch-Clamp Recording

Whole-cell patch-clamp recordings were performed in cultured cortical neurons 1 week after plating, N1E-115 cells 1 day after cell passage, or HEK 293 cells 1–3 day after transfection. HEK 293 cells were re-plated on the day of the experiment to ensure that recordings were only made from single, isolated cells. Cells were continuously superfused with a solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with NaOH and osmolality adjusted to ~340 mosmol l⁻¹ with sucrose). Pipettes were pulled from borosilicate glass (TW-150F, World Precision Instruments, Sarasota, FL) using a two-stage puller (Flaming-Brown P-97; Sutter Instruments, Novato, CA) and had resistances of 2–5 µM when filled with pipette solution containing 140 mM CsCl, 2 mM MgCl₂, 10 mM EGTA, 10 mM HEPES (pH was adjusted to 7.2 with CsOH, and osmolality adjusted to ~315 mosmol l⁻¹ with sucrose). Membrane current was recorded in the whole-cell configuration using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at 20–22°C. Cells were held at -60 mV unless otherwise indicated. Data were acquired using pClamp9.0 software (Axon). Currents were filtered at 2 kHz and digitized at 5–10 kHz.

Drugs were applied with a piezoelectric device (PZ-150M; EXFO Burleigh Products Group Inc., Victor, NY) through two-barrel theta glass tubing (TGC150, Warner Instruments, Hamden, CT) that had been pulled to a tip diameter of ~200 µm. The cell was placed in front of the stream of control solution. The piezoelectric device was driven by TTL pulses from the pClamp9.0 software. Voltage applied to the piezoelectric device produced a rapid lateral displacement (~50 µm) of the theta tubing to move the interface between control and drug solutions. Solution exchange rates for open pipette and whole-cell recording were 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 ~0.3 ms for an open pipette tip and ~1.6 ms for whole-cell recording.

2.4. Data Analysis

Data analysis and curve fitting were performed with Origin7.0 (Microcal Software, Northampton, MA), pClamp9.0 (Axon), or GraphPad InStat3.0 (GraphPad Software Inc., San Diego, CA) software. Concentration-response data for dopamine (DA) and 5-HT were fit using the Hill equation, $I/I_{\max \text{ 5-HT}} = 1/[1 + (EC_{50}/[\text{Agonist}])^{n_H}]$, where *I* is the current amplitude activated by a given concentration of agonist ([Agonist]), *I*_{max 5-HT} is the current amplitude produced by a maximally efficacious concentration of 5-HT (30 µM), *n*_H is the Hill coefficient and EC₅₀ is the concentration eliciting a half-maximal response.

Parameters of channel 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 *τ*_{*n*} is the respective time constant. Curve fitting was achieved in Clampfit9.0 using the Levenberg-Marquardt algorithm.

Additional components were accepted only if they significantly improved the fit, as determined by an F test performed using the analysis software.

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, whereas deactivation rates were derived from exponential fits to the current decay after the removal of agonist following a 2-ms application. To facilitate direct comparison of desensitization or deactivation with different components, a weighted summation of time constants ($\sum a_n \tau_n$) was used, where a_n is the fractional contribution of the respective component, τ_n is the respective time constant, and n is the optimal number of exponential components. The extent of desensitization was measured as the percentage of current loss relative to peak current using the equation $[(I_{\text{peak}} - I_{10\text{ s}}) / I_{\text{peak}}] \times 100$, where I_{peak} is the peak current amplitude, $I_{10\text{ s}}$ is the current amplitude at the end of 10 s application of 5-HT alone or 5-HT plus 5-HI.

In some experiments voltage ramps were applied to measure reversal potential. A ramp that changed the membrane potential between -80 and $+60$ mV with a slew rate of 0.5 mV/ms was applied during the peak of current activated by $3\text{ }\mu\text{M}$ 5-HT, 5 mM 5-HI, 5 mM 5-methoxyindole and 3-(2-hydroxyethyl)indole. Current activated by a voltage ramp in the absence of agonist was subtracted from the ramp-activated current in the presence of agonist prior to plotting and analyzing these data.

To facilitate comparison between different Cys-loop ligand-gated ion channels, a common TM2 domain numbering system is used (Miller, 1989). In this system, the amino acid residue at the putative cytoplasmic end of TM2 domain is assigned as position 1' (Fig. 3A).

Data are presented as mean $\pm$ S.E.M. Statistical significance was determined with the Student's t test or one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

2.5. Kinetic Simulations

Kinetic simulations of current measured in HEK 293 cells were generated using Berkeley-Madonna V8.3 software (Macey and Oster, Berkeley, CA, USA) and the Runge-Kutta 4 integration algorithm. Channel states and most rate constant estimates were as in the allosteric model developed by Solt et al. (2007) except where noted. For simulation of continuous 5-HT application, 10 sec of simulated current was generated in the presence of $30\text{ }\mu\text{M}$ 5-HT with all channels initially set in the unliganded, closed state (R). For simulation of "deactivation", 20 sec of simulated current was generated with all channels initially set in the triple-liganded, open state (A_3O) and agonist concentration set to 0. The figure showing simulated current was generated in Prism software using exported simulated traces.

3. Results

3.1. Positive modulation of 5-HT₃ receptor-mediated current by 5-HI

To study the effects of 5-HI, we examined whole-cell currents activated by 5-HT in N1E-115 neuroblastoma cells expressing the native mouse 5-HT₃ receptor (Neijt *et al.*, 1988; Hope *et al.*, 1993), and subsequently in HEK 293 cells transiently expressing the recombinant mouse 5-HT_{3A} receptor. Inward current with a rapid onset was observed upon application of a maximally efficacious concentration of 5-HT ($30\text{ }\mu\text{M}$) for 10 s to either N1E-115 cells or transfected HEK 293 cells (Fig. 1A, left). Co-application of 5 mM 5-HI with 5-HT enhanced the current mediated by the 5-HT_{3A} receptor in both cell lines (Fig. 1A, right). The average peak current amplitude activated by 5-HT in the presence of 5-HI was $\sim 120\%$ of that in the absence of 5-HI (N1E-115: $125 \pm 6\%$; HEK293: $119 \pm 8\%$), which is comparable to previous findings in N1E-115 cells (Kooyman *et al.*, 1993).

The 5-HT₃ receptor-mediated current decayed toward baseline in both cell lines during prolonged agonist exposure (Fig. 1B). Desensitization time course was best fit using a bi-exponential function in N1E-115 cells, whereas it could be fit adequately with a mono-exponential function in HEK 293 cells transfected with the wild-type receptor. Desensitization (measured as weighted desensitization time constant) in N1E-115 cells was considerably faster than in the HEK 293 cells (Table 1, $p < 0.01$). Co-application of 5-HI decreased the rate of current decay in the presence of agonist in both cell lines. This finding is consistent with the decrease in desensitization rate previously observed during 5-HI application (Kooyman *et al.*, 1993; Gunthorpe & Lummis, 1999). 5-HI did not alter the number of components of desensitization in either cell line. However, the proportion of the fast component in N1E-115 cells was reduced by 5-HI from ~70% to ~20%. In addition, 5-HI significantly increased the time constants of both fast and slow components (Table 1). 5-HI also drastically increased desensitization time constant in HEK 293 cells expressing the wild-type receptor (Table 1). We also examined the impact of 5-HI co-application on the extent of desensitization of 5-HT₃ receptor-mediated current by normalizing the loss of current amplitude at the end of 10 s application of 5-HT over the peak current amplitude (see Materials and Methods). Although the extent of desensitization (measured as percent current decay) in the absence of 5-HI was greater in N1E-115 cells than in HEK 293 cells (N1E-115: $95.8 \pm 0.8\%$; HEK 293: $81.8 \pm 1.6\%$, $p < 0.01$), 5-HI significantly decreased this parameter in both cell lines (N1E-115: $25.8 \pm 0.8\%$, $p < 0.01$; HEK 293: $24.2 \pm 1.1\%$, $p < 0.01$) to a similar extent ($p = 0.3\%$).

3.2. Role of L293 in the modulatory action of 5-HI

The sequence alignment of the amino acid residues around TM2 across members of Cys-loop ligand-gated ion channels is displayed in Fig. 2A. Residue L293 is in the same 15' position as a residue in the GABA_A receptor that has important roles in channel gating (Findlay *et al.*, 2001; Scheller & Forman, 2002) and modulation by alcohols and general anesthetics (Mihic *et al.*, 1997; Wick *et al.*, 1998). This amino acid residue is thought to reside on the non-pore-facing side of TM2 domain (Panicker *et al.*, 2002; Reeves *et al.*, 2001). We recently demonstrated that alcohol enhancement of 5-HT_{3A} receptor-mediated current was abolished with L293C and L293S mutations (Hu *et al.*, 2006). One effect of alcohols is to decrease desensitization, and this alcohol action was also lost in receptors with mutations at L293. The actions of 5-HI on the 5-HT_{3A} receptor are similar to those observed for alcohols. It is possible that the allosteric interaction described for alcohol/anesthetics might involve mechanisms similar to those involved in modulation by 5-HI. We therefore examined the action of 5-HI in the L293C and L293S mutant receptors.

Interestingly, currents activated by 30 μ M 5-HT in the L293C and L293S receptor were slightly inhibited by co-application of 5 mM 5-HI (Fig. 2B). The average current amplitudes produced by co-application of 5-HT and 5-HI in the L293C and L293S receptors were $80.5 \pm 5.6\%$ and $73.0 \pm 3.4\%$, respectively, of the current amplitude activated by 5-HT alone. In both the L293C and L293S receptors there was a rebound current upon termination of co-application of 5-HT and 5-HI. Desensitization of 5-HT-activated current was faster in the L293C and L293S receptors than in the wild-type receptor (Fig. 2C, Table 1, ANOVA, $p < 0.01$). Unlike the wild-type receptor, a bi-exponential function was required to adequately fit the current decay in both the L293C and L293S receptors. The newly introduced fast component accounted for ~65% of the total current decay for both mutant receptors. Neither the time constant nor proportion of the fast and slow components of desensitization was significantly altered by 5-HI in the L293C and L293S receptors. 5-hydroxyindole did not alter the weighted desensitization time constant in either L293C ($p = 0.9$) or L293S ($p = 0.4$) receptors (Table 1). In addition, the extent of desensitization remained unchanged in the presence of 5-HI in both L293C ($91.7 \pm 1.1\% \rightarrow 91.3 \pm 0.9\%$, $p = 0.4$) and L293S ($88.8 \pm 1.4\% \rightarrow 89.4 \pm 1.1$, $p = 0.7$) receptors.

A recent study in the $\alpha 7$ nACh receptor suggested that 5-HI enhancement of ACh-activated current involves T266, the 6' residue of the TM2 domain (Placzek *et al.*, 2004). In addition, we have recently demonstrated that D298, an amino acid in the TM2-TM3 loop of the 5-HT_{3A} receptor participating in forming the outer mouth of the channel pore (although this residue may be part of the extended TM2 α -helix structure), plays a critical role in gating and desensitization as well as Ca²⁺ modulation (Hu & Lovinger, 2005). Therefore we tested whether the T284 (6' residue of the TM2 domain in the 5-HT_{3A} receptor) and D298 residues are involved in 5-HI action with the T284F and D298A mutations. The average current amplitudes in response to co-application of 5-HT and 5-HI in the T284F and D298A receptors were $123.1 \pm 1.5\%$ and $126.9 \pm 9.3\%$ of the current amplitude in the presence of 5-HT alone, respectively (Fig. 3D). Desensitization of T284F or D298A receptor-mediated current was faster than in the wild-type receptor (Fig. 3E, Table 1, ANOVA, $p < 0.01$). A bi-exponential function was required to adequately fit desensitization in these two mutant receptors (Table 1). The fast component of desensitization introduced by the mutations accounted for 70–80% of the total current amplitude. Co-application of 5-HI with 5-HT abolished the fast component of desensitization in the T284F receptor and reduced the fast component from ~80% to ~50% of the total current amplitude in the D298A receptor. The time constants for both fast and slow components of desensitization were significantly increased in the D298A receptor by 5-HI ($p < 0.01$; Table 1). The weighted desensitization time constants for both T284F and D298A receptor were also increased by 5-HI ($p < 0.01$; Table 1). In addition, the extent of desensitization was significantly reduced in both T284F ($99.0 \pm 0.4\% \rightarrow 78.8 \pm 1.2\%$, $p < 0.01$) and D298A ($99.5 \pm 0.3\% \rightarrow 91.5 \pm 0.4\%$, $p < 0.01$) receptors, although the effects were not as large as those observed in the wild-type receptor (ANOVA, $p < 0.01$).

We also examined the effect of 5-HI at lower concentrations on 5-HT_{3A} receptor-mediated current in both the wild-type and L293S receptors (Fig. 3A). As shown in Fig. 3B, 5-HI at 30 μ M decreased desensitization of 5-HT-activated current in the wild-type receptor ($p < 0.05$). The increase in desensitization time constant was 5-HI concentration-dependent in the wild-type receptor, reaching a maximum at ~300 μ M 5-HI. In addition, 5-HI also produced a concentration-dependent reduction in the extent of desensitization in the wild-type receptor (30 μ M: $70.7 \pm 1.6\%$; 100 μ M: $45.9 \pm 3.0\%$; 300 μ M: $39.4 \pm 2.2\%$; and 1 mM: $29.3 \pm 0.4\%$). However, the desensitization rate and relative contribution of the fast and slow components in the L293S receptor were not altered by either 100 μ M or 1 mM 5-HI (Fig. 3B). The weighted desensitization time constant remained unchanged when either concentration of 5-HI was co-applied with 5-HT in the L293S receptor (100 μ M: $p = 0.8$; 1 mM: $p = 0.7$). Furthermore, the extent of desensitization remained unchanged in the presence of 100 μ M ($86.6 \pm 2.2\%$, $p = 0.7$) and 1 mM ($88.2 \pm 2.9\%$, $p = 0.9$) 5-HI.

5-HI was without effect on the peak current amplitude in the wild-type receptor at 30 μ M ($99.8 \pm 1.3\%$ of control). Concentration-dependent enhancement of 5-HT-activated current was seen with 5-HI concentrations ≥ 100 μ M in the wild-type receptor ($108.6 \pm 1.7\%$, $116.4 \pm 2.0\%$ and $118.1 \pm 2.9\%$ of the control for 100 μ M, 300 μ M and 1 mM 5-HI, respectively). 5-HI at 100 μ M caused no change in the peak current amplitude in the L293S receptor ($97.3 \pm 2.0\%$ of control). However, 5-HI at 1 mM slightly inhibited 5-HT-activated current in the L293S receptor; and the current amplitude in the presence of 1 mM 5-HI was $90.1 \pm 1.7\%$ of control.

The impact of 5-HI on responses to a lower concentrations of 5-HT was also examined. Fig. 4 depicts the current activated by 1 μ M and 3 μ M 5-HT in the absence and presence of 5 mM 5-HI in the wild-type, L293C and L293S receptors. In the wild-type receptor 5-HT at 1 μ M activated a slowly rising current that showed minimal decay during 10 s of agonist application (Fig. 4A, left). In the presence 5-HI, the current activated by 5-HT developed gradually and never attained a true steady-state value during agonist application (Fig. 4A, right). The initial phase of current activation appeared similar to that observed with 5-HT alone, but current in

the presence of 5-HI continued to increase at time points where current plateaued with agonist alone (Fig. 4A, inset). The average current amplitude at the end of a 10 s agonist application in the presence of 5-HI was $329.6 \pm 34.5\%$ of current amplitude in response to agonist alone. However, in both the L293C and L293S receptors the initial rising phase of the current activated by $1 \mu\text{M}$ 5-HT was faster than that observed in the wild-type receptor; and the current amplitude was inhibited by 5-HI (Fig. 4B). The average peak current amplitude in the presence of 5-HI was $68.5 \pm 3.4\%$ and $71.0 \pm 2.9\%$ of control (agonist alone) for the L293C and L293S receptors, respectively. 5-HT at $3 \mu\text{M}$ activated a relative fast rising current in the wild-type receptor (Fig. 4C, left). The response activated by 5-HT was enhanced by co-application of 5-HI ($134.7 \pm 10.3\%$ of the control; Fig. 4C, right). The current decayed after reaching peak in the absence of 5-HI and plateaued in the presence of 5-HI (Fig. 4C, inset). 5-HI inhibited $3 \mu\text{M}$ 5-HT-activated current in the L293S receptor ($77.1 \pm 5.6\%$ of the control; Fig. 4D). A rebound current appeared at the end of co-application of 5-HT and 5-HI in the L293C and L293S receptors.

To determine whether 5-HI alters deactivation of 5-HT-activated current, a short pulse (2 ms) of 1 mM 5-HT was followed by washout with either control solution or 5 mM 5-HI-containing solution. The current activated by a brief application of 5-HT decayed gradually back to baseline levels in both the wild-type and L293S receptors (Fig. 5A). However, deactivation in the L293S receptor appeared to be faster than in the wild-type receptor. As shown in Fig. 5A, 5-HI slowed deactivation in the wild-type receptor but not in the L293S receptor. The current decay after removal of 5-HT in the absence or presence of 5-HI followed a mono-exponential time-course in the wild-type receptor, whereas it was best described by a bi-exponential function in the L293S receptor. 5-HI significantly increased the weighted deactivation time constant in the wild-type receptor ($p < 0.01$), whereas it did not alter this value in the L293S receptor ($p = 0.5$, Fig. 5B).

3.3. Conversion of 5-HI into an agonist by the L293S mutation

Dopamine is a partial agonist at the 5-HT₃ receptor (van Hooft & Vijverberg, 1996), and application of a maximally efficacious concentration of dopamine (3 mM) activated a small current in the wild-type receptor expressed in HEK 293 cells. On the other hand, 5 mM 5-HI did not induce any detectable current when applied in the absence of 5-HT in the wild-type receptor (Fig. 6A, middle). In the L293S receptor 3 mM dopamine activated a current of larger amplitude relative to $30 \mu\text{M}$ 5-HT than that observed in the wild-type receptor. Surprisingly, 5 mM 5-HI showed agonist activity when applied in the absence of 5-HT to the L293S receptor, activating a relatively small amplitude current (Fig. 6A, right). The currents activated by various concentrations of 5-HI are presented in Fig. 6B. A rebound current occurred upon removal of high concentrations ($\geq 5 \text{ mM}$) of 5-HI. Concentration-response curves for agonists were constructed by normalizing the amplitude of current activated by a range of dopamine and 5-HI concentrations to that produced by a maximally-effective 5-HT concentration ($I/I_{30 \mu\text{M} \text{ 5-HT}}$) (Fig. 6C). The amplitude of current activated by dopamine was concentration-dependent in both the wild-type and L293S receptors. The concentration-response curve for dopamine was shifted to the left and maximal dopamine efficacy was increased in the L293S receptor. The L293S receptor exhibited a 3.5-fold increase in dopamine potency (EC_{50}) (L293S: $0.04 \pm 0.01 \text{ mM}$; wild-type: $0.14 \pm 0.05 \text{ mM}$; $p < 0.01$). The L293S mutation also decreased the Hill slope of the concentration-response curve for dopamine (wild-type: 2.10 ± 0.16 ; L293S: 1.17 ± 0.25 ; $p < 0.05$). The maximal response to dopamine was also greatly increased by the L293S mutation, from ~ 30 to $\sim 65\%$ of the current activated by $30 \mu\text{M}$ 5-HT ($p < 0.01$). In the wild-type receptor 5-HI alone failed to activate a response at concentrations up to 10 mM . However, a detectable current was observed at $300 \mu\text{M}$ 5-HI in the L293S receptor (Figs. 6B and C). The amplitude of current activated by 5-HI was also concentration-dependent in the L293S receptor and the maximal response to 5-HI was $\sim 25\%$ of the current activated by $30 \mu\text{M}$ 5-HT (Fig. 6C). Fitting the pooled data with the logistic equation revealed an EC_{50}

value of 1.05 ± 0.02 mM and a Hill slope of 2.31 ± 0.19 for 5-HI activation of the L293S receptor.

3.4. Properties of 5-HI-activated currents

Both 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) and the 5-HT₃ receptor competitive antagonist MDL 72222 have been used to detect spontaneous channel activity in the 5-HT_{3A} mutant receptors (Zhang *et al.*, 2002; Bhattacharya *et al.*, 2004). The application of 100 μ M TMB-8 or 300 nM MDL 72222 failed to alter the holding current in cells expressing the L293S receptor (Fig. 7A), suggesting that the L293S mutation did not produce spontaneous channel activity. The current activated by 5 mM 5-HI alone in the L293S receptor was completely abolished by 300 nM MDL 72222 (Fig. 7B). Fig. 7C shows the current-voltage (I-V) relationship obtained with a voltage-ramp from -80 to $+60$ mV at the peak of current activated by 3 μ M 5-HT in the wild-type receptor and by 5 mM 5-HI in the L293S receptor. The shape of the I-V relationship was similar for both 5-HI and 5-HT; and the current reversed at 2.8 ± 0.9 mV for 5-HI in the L293S receptor and 3.2 ± 1.0 mV for 5-HT in the wild-type receptor ($p = 0.8$). We next tested whether the membrane potential influences the rebound current generated upon the termination of 5-HI application in the L293S receptor. The rebound current was preserved at holding potentials ranging from -80 to $+40$ mV (Fig. 7D).

In the present study, 5-HI was able to function as an agonist in the L293S receptor. Analogs of 5-HI, such as tryptamine, are weak partial agonists at the wild-type 5-HT₃ receptor (van Hooft & Vijverberg, 1996). These findings suggest a possible interaction of 5-HI analogs with the 5-HT recognition site on the 5-HT_{3A} receptor. Therefore we wished to find out whether other closely-related analogs of 5-HI (Fig. 8A, left) could activate the wild-type 5-HT_{3A} receptor. We examined the agonist activities of 5-methoxyindole (5-MI) and 3-(2-hydroxyethyl)indole (3-2-HEI) in HEK 293 cells transfected with the wild-type receptor. Both 5-MI and 3-2-HEI were unable to elicit a current in untransfected HEK 293 cells (data not shown). However, we observed that application of either compound alone at 5 mM directly activated inward current in HEK 293 cells transfected with the wild-type receptor (Fig. 8A, right). The onset of current was relatively slow in response to an application of either compound when compared to 5-HT-activated current. A rebound current upon agonist removal was observed following cessation of application of 5-MI, but not 3-2-HEI.

Averaged data indicate that 5-MI and 3-2-HEI were weak agonists at the wild-type receptor; the maximal responses to 5 mM 5-MI and 3-2-HEI were $\sim 30\%$ and $\sim 20\%$ of the current activated by 30 μ M 5-HT, respectively (Fig. 8B). The I-V relationship obtained with a voltage-ramp from -80 to $+60$ mV at the peak of current activated by the agonists, as shown in Fig. 8C, revealed similar rectification and reversal potential for current activated by 5-MI (3.0 ± 0.5 mV) and 3-2-HEI (2.8 ± 0.4 mV), and these current characteristics were comparable to that for 5-HT in the wild-type receptor.

A sequential application protocol was used to examine the effect of pre-incubation of 5-HI on 5-HT-activated current in the wild-type and L293 receptors (Fig. 9). Responses to 30 μ M 5-HT applications (250 ms and 10 s) in the absence of 5-HI immediately after pre-incubation with 5 mM 5-HI for 20 s were slightly reduced in the wild-type receptor (Fig. 9A and C, upper). In addition, 5-HI pre-incubation also reduced desensitization (Fig. 9C). As seen before, pre-incubation with 5 mM 5-HI activated an inward current in the L293S receptor which gradually decayed in the continued presence of 5-HI. Responses to 250-ms or 10-s applications of 5-HT immediate following 5-HI pre-incubation were almost abolished (Fig. 9A & C, lower). The average responses after 5-HI pre-incubation were $\sim 90\%$ and 5% for the wild-type and L293S receptors, respectively (Fig. 9B and D).

4. Discussion

Previous studies indicated that 5-HI has mixed effects on 5-HT₃ receptor-mediated currents, producing a competitive inhibition and an allosteric potentiation of receptor function; and imply existence of two distinct sites of 5-HI action (Kooyman *et al.*, 1994). In the present study, we investigated the mechanism underlying the actions of 5-HI on the 5-HT_{3A} receptor. Our findings provide strong support for this two-site model. Mutations at the L293 residue in the TM2 domain eliminate the positive allosteric modulation of the receptor by 5-HI, and reveal a partial agonism by this compound. The latter finding provides the most direct evidence to date of an interaction of 5-HI with the agonist binding site, which persists in the absence of the positive allosteric modulatory effect.

5-HI shifts the 5-HT concentration-response relationship leftward (van Hooft *et al.*, 1997). The apparent increase in agonist potency produced by 5-HI might result from increased agonist affinity or enhanced channel gating or both (Colquhoun, 1998). The observation that 5-HI slowed deactivation in the wild-type receptor indicates that the rate of channel closure and 5-HT unbinding after agonist removal are decreased by 5HI. Therefore an enhanced agonist affinity might contribute to 5-HI potentiation in the wild-type receptor. However, the observation of 5-HI potentiation of the response activated by a maximally efficacious agonist concentration indicates that a process other than increased agonist affinity is also involved since an increase in affinity alone should not enhance maximal agonist efficacy (Christopoulos & Kenakin, 2002). An increase in channel conductance could also produce an increase in peak current amplitude. However, single-channel measurements indicate that 5-HI does not alter 5-HT₃ receptor conductance (van Hooft *et al.*, 1997). The observations that 5-HI decreases the rate and extent of desensitization provide evidence supporting the idea that this agent stabilizes the open state of the receptor, which in turn likely contributes to the increase in agonist potency by slowing the rate of agonist unbinding (Chang & Weiss, 1999).

We also observed that 5-HI acts as a partial agonist in the L293S construct that lacks 5-HI-induced potentiation. Examination of this mutant receptor also revealed that the direct activation by 5-HI occurs at drug concentrations higher than those that elicit potentiation in the wild-type receptor. This observation is generally consistent with the previous finding that the 5-HI potentiating effect is non-competitive with respect to the agonist binding site, and that the potency of 5-HI for potentiating receptor function is greater than its potency for altering agonist binding (Kooyman *et al.*, 1994). The observation that 5-HI effects in the presence of a low concentration of agonist are similar to those observed at high agonist concentrations in both the wild-type and L293 mutant receptors supports the idea that the potentiating effect is allosteric.

It is possible that slowing of desensitization by 5-HI could account for most or all of the potentiation produced by the drug. A recent study by Solt *et al.* (2007) indicates that desensitization produced by 5-HT dominates the channel closure process even following agonist removal (i.e. our “deactivation” paradigm). We performed kinetic simulations of receptor function designed to look at current decay in the continuous presence of 30 μ M 5-HT and after agonist removal (Figure 10) to determine if our findings with 5-HI could be completely explained by reduced desensitization rate. We used the allosteric model developed by Solt *et al.* (2007) and the kinetic parameters shown in Table 2. Indeed, we could produce reasonable simulations of the 5-HI-induced prolongation of current decay under either continuous or transient agonist application conditions by decreasing the forward desensitization rate constant (K_{d+}). Changes in unbinding steps (K_2 and K_3) had no additional effect on simulated current, indicating that our experimental results could be due to changes in desensitization alone. Similar results were obtained with a linear kinetic model that we have previously used to describe 5-HT₃ function (Zhou *et al.*, 1998).

The blockade of the partial agonism of 5-HI at the L293S receptor by a competitive 5-HT₃ receptor antagonist suggests that 5-HI is activating the channel via actions at a site that largely overlaps with the orthosteric agonist/competitive antagonist binding site on the receptor. It is perhaps not surprising that 5-HI can bind to the 5-HT site on the receptor given the structural similarity of the two compounds (Fig. 7A, left). A number of other 5-HT analogs act as 5-HT₃ partial agonists, including tryptamine (van Hooft & Vijverberg, 1996), and the previously untested indole derivatives 5-methoxyindole and 3-(2-hydroxyethyl)indole (Fig. 9). These findings indicate that the orthosteric site of the 5-HT_{3A} receptor may recognize a variety of substituted indole compounds. The previous findings (Kooyman *et al.*, 1994) together with the observations made at present suggest that 5-HI has a low affinity at the orthosteric site.

The binding of agonist to the orthosteric site on a Cys-loop ligand-gated ion channel induces a conformational change in the extracellular N-terminal domain, which is relayed to the TM2-TM3 loop (Lummis *et al.*, 2005). One model of channel activation is that the movement of the TM2-TM3 loop then causes the TM2 domain to rotate leading to channel opening (Miyazawa *et al.*, 2003). Structurally, L293 is in a position to promote communication between the TM2-TM3 loop and the TM2 domain. Certain L293 mutations result in greater agonist potency and efficacy (Hu *et al.*, 2006). The increased dopamine efficacy in the L293S mutation suggests that L293 is critical for the coupling of agonist binding to channel gating in the 5-HT_{3A} receptor. The equivalent position (S270) in the GABA_A receptor has also been found to be an important gating site (Scheller & Forman, 2002). It is likely that the 15' amino acid residue in the TM2 domain of the Cys-loop ligand-gated ion channels constitutes a constraint for receptor activation. Binding of agonist at the orthosteric site releases this intrinsic structural constraint in the receptor protein. 5-HI has no intrinsic agonist activity in the wild-type receptor, suggesting that binding of 5-HI is not sufficient to overcome energy barriers that prevent the channel from opening. The L293S mutation could release the molecular constraints and lower these energy barriers such that 5-HI binding to the mutant receptor is able to produce a sufficient conformational change to open the channel. A similar finding was made with respect to the R222A mutation which converts the antagonist apomorphine into a potent and efficacious agonist by facilitating the conversion between different states of the 5-HT_{3A} receptor (Hu *et al.*, 2003).

Residues in the TM2 domain of Cys-loop ligand-gated channels participate in forming the channel gate, controlling ionic selectivity, and binding agents of physiological and pharmacological importance (Karlin, 2002;Lummis, 2004). Position 15' of the 5-HT_{3A} receptors TM2 domain is a non-pore-facing amino acid residue (Reeves *et al.*, 2001;Panicker *et al.*, 2002). In addition to its role in channel gating, the amino acid residue at 15' also appears to have important roles in channel modulation by alcohol and anesthetics (Mihic *et al.*, 1997;Wick *et al.*, 1998), and binding of these drugs to a hydrophobic pocket involving this residue alters receptor function. In a separate study, we have demonstrated that L293 is critical for alcohol actions on the 5-HT_{3A} receptor; and L293C and L293S mutations abolish alcohol modulation (Hu *et al.*, 2006). Alcohols and 5-HI have similar actions on the 5-HT_{3A} receptor, and mutations at L293 prevent the effects of these agents. Thus, L293 may be part of a common structural element necessary for the modulation by alcohol/anesthetics and 5-HI, although these compounds differ substantially in chemical structure.

The L293 residue could be part of a binding site for 5-HI involved in allosteric potentiation of the receptor. However, testing this hypothesis will have to await the development of technologies to more directly assess drug interactions with this residue. Furthermore, our previous studies suggest that there is little correlation between the physicochemical properties of the amino acid residues substituted at L293 (15') and alcohol action (Hu *et al.*, 2006). It is believed that allosteric agents act by modulating receptor activity through conformational changes in the receptor transmitted from the allosteric to the orthosteric site and/or to the

coupling sites (Christopoulos & Kenakin, 2002). It is possible that L293 is a key residue in regions linking the allosteric site of 5-HI binding to agonist binding and/or channel gating. In this regard, it is interesting to note that amino acid residues in the TM2 domain and TM2-TM3 loop of the GABA_A receptor are the structural determinants for coupling benzodiazepine binding to increased channel gating (Boileau & Czajkowski, 1999; Jones-Davis *et al.*, 2005).

It is possible that the impairment of 5-HI modulation in the L293 mutant receptors is an indirect consequence of the conversion of 5-HI into a partial agonist in the L293S receptor. The decreased 5-HT occupancy at the receptor in the presence of 5-HI in this construct might preclude potentiation. However, this does not appear to be the case. A relatively low concentration of 5-HI (100 μ M) that did not directly activate the L293S receptor produced no potentiation of the function of this construct, despite the fact that this concentration of 5-HI decreases desensitization in the wild-type receptor.

Allosteric modulation of ligand-gated ion channels is a well-known phenomenon. Positive allosteric modulation provides an attractive mechanism for the facilitation of physiologically appropriate receptor activation. Similarly, greater selectivity, potency and/or safety can often be achieved using allosteric modulators, as opposed to orthosteric ligands. Benzodiazepines are widely used central nervous system depressants which allosterically enhance the activity of GABA_A receptor (Sigel & Buhr, 1997). Therefore, the allosteric modulation of the 5-HT_{3A} receptor may hold promise for the development of novel therapeutics in disorders involving this receptor.

In summary, using N1E-115 cells expressing the native 5-HT_{3A} receptor and HEK 293 cells transfected with the recombinant 5-HT_{3A} receptor we have confirmed the positive allosteric action of 5-HI. This positive modulation is accompanied by a decrease in rates of channel desensitization and deactivation. Site-directed mutagenesis reveals that L293 constitutes an important gating site and is crucial for 5-HI positive allosteric modulation. L293 may constitute a common transduction pathway for both agonists and allosteric modulators. In addition, our data support the existence of a second 5-HI binding site at the 5-HT_{3A} receptor that is not involved in the positive modulation, but likely overlaps with the orthosteric site.

Acknowledgements

This work was supported by the NIAAA Division of Intramural Clinical and Basic Research.

Abbreviations

5-HT₃	5-hydroxytryptamine (serotonin) type 3
nACh	nicotinic acetylcholine
TM	transmembrane domain
5-HI	5-hydroxyindole
HEK cells	human embryonic kidney cells
WT	wild-type

DA

dopamine

References

- Barnes NM, Sharp T. A review of central 5-HT receptors and their function. *Neuropharmacology* 1999;38:1083–1152. [PubMed: 10462127]
- Bhattacharya A, Dang H, Zhu QM, Schnegelsberg B, Rozengurt N, Cain G, Prantil R, Vorp DA, Guy N, Julius D, Ford AP, Lester HA, Cockayne DA. Uropathic observations in mice expressing a constitutively active point mutation in the 5-HT_{3A} receptor subunit. *Journal of Neuroscience* 2004;24:5537–5548. [PubMed: 15201326]
- Boileau AJ, Czajkowski C. Identification of transduction elements for benzodiazepine modulation of the GABA_A receptor: three residues are required for allosteric coupling. *Journal of Neuroscience* 1999;19:10213–10220. [PubMed: 10575018]
- Chang Y, Weiss DS. Channel opening locks agonist onto the GABA_C receptor. *Nature Neuroscience* 1999;2:219–225.
- Christopoulos A, Kenakin T. G protein-coupled receptor allosterism and complexing. *Pharmacological Reviews* 2002;54:323–374. [PubMed: 12037145]
- Colquhoun D. Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *British Journal of Pharmacology* 1998;125:924–947. [PubMed: 9846630]
- Filatov GN, White MM. The role of conserved leucines in the M2 domain of the acetylcholine receptor in channel gating. *Molecular Pharmacology* 1995;48:379–384. [PubMed: 7565616]
- Findlay GS, Ueno S, Harrison NL, Harris RA. Allosteric modulation in spontaneously active mutant γ -aminobutyric acid_A receptors. *Neuroscience Letters* 2001;305:77–80. [PubMed: 11356312]
- Grosman C, Auerbach A. Asymmetric and independent contribution of the second transmembrane segment 12' residues to diliganded gating of acetylcholine receptor channels: a single-channel study with choline as the agonist. *Journal of General Physiology* 2000;115:637–651. [PubMed: 10779320]
- Grosman C, Salamone FN, Sine SM, Auerbach A. The extracellular linker of muscle acetylcholine receptor channels is a gating control element. *Journal of General Physiology* 2000;116:327–340. [PubMed: 10962011]
- Gunthorpe MJ, Lummis SC. Diltiazem causes open channel block of recombinant 5-HT₃ receptors. *Journal of Physiology* 1999;519:713–722. [PubMed: 10457085]
- Hope AG, Downie DL, Sutherland L, Lambert JJ, Peters JA, Burchell B. Cloning and functional expression of an apparent splice variant of the murine 5-HT₃ receptor A subunit. *European Journal of Pharmacology* 1993;245:187–192. [PubMed: 7683998]
- Hu XQ, Hayrapetyan V, Gadhiya JJ, Rhubottom HE, Lovinger DM, Machu TK. Mutations of L293 in transmembrane two of the mouse 5-hydroxytryptamine_{3A} receptor alter gating and alcohol modulatory actions. *British Journal of Pharmacology* 2006;148:88–101. [PubMed: 16520747]
- Hu XQ, Lovinger DM. Role of aspartate 298 in mouse 5-HT_{3A} receptor gating and modulation by extracellular Ca²⁺. *Journal of Physiology* 2005;568:381–396. [PubMed: 16096341]
- Hu XQ, Zhang L, Stewart RR, Weight FF. Arginine 222 in the pre-transmembrane domain 1 of 5-HT_{3A} receptors links agonist binding to channel gating. *Journal of Biological Chemistry* 2003;278:46583–46589. [PubMed: 12970351]
- Jones-Davis DM, Song L, Gallagher MJ, Macdonald RL. Structural determinants of benzodiazepine allosteric regulation of GABA_A receptor currents. *Journal of Neuroscience* 2005;25:8056–8065. [PubMed: 16135763]
- Karlin A. Emerging structure of the nicotinic acetylcholine receptors. *Nature reviews Neuroscience* 2002;3:102–114.
- Kooyman AR, van Hooft JA, Vanderheijden PM, Vijverberg HP. Competitive and non-competitive effects of 5-hydroxyindole on 5-HT₃ receptors in N1E-115 neuroblastoma cells. *British Journal of Pharmacology* 1994;112:541–546. [PubMed: 8075873]

- Kooyman AR, van Hooft JA, Vijverberg HP. 5-Hydroxyindole slows desensitization of the 5-HT₃ receptor-mediated ion current in N1E-115 neuroblastoma cells. *British Journal of Pharmacology* 1993;108:287–289. [PubMed: 7680589]
- Lummis SC. The transmembrane domain of the 5-HT₃ receptor: its role in selectivity and gating. *Biochemical Society Transactions* 2004;32:535–539. [PubMed: 15157179]
- Lummis SC, Beene DL, Lee LW, Lester HA, Broadhurst RW, Dougherty DA. Cis-trans isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. *Nature* 2005;438:248–252. [PubMed: 16281040]
- Mannaioni G, Carpenedo R, Moroni F. 5-hydroxyindole causes convulsions and increases transmitter release in the CA1 region of the rat hippocampus. *British Journal of Pharmacology* 2003;138:245–253. [PubMed: 12522096]
- Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL. Sites of alcohol and volatile anaesthetic action on GABA_A and glycine receptors. *Nature* 1997;389:385–389. [PubMed: 9311780]
- Miller C. Genetic manipulation of ion channels: a new approach to structure and mechanism. *Neuron* 1989;2:1195–1205. [PubMed: 2483110]
- Miyazawa A, Fujiyoshi Y, Unwin N. Structure and gating mechanism of the acetylcholine receptor pore. *Nature* 2003;423:949–955. [PubMed: 12827192]
- Neijt HC, te Duits I, Vijverberg HP. Pharmacological characterization of serotonin 5-HT₃ receptor-mediated electrical response in cultured mouse neuroblastoma cells. *Neuropharmacology* 1988;27:301–307. [PubMed: 3374770]
- Ortells MO, Lunt GG. Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *Trends in Neurosciences* 1995;18:121–127. [PubMed: 7754520]
- Panicker S, Cruz H, Arrabit C, Slesinger PA. Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. *Journal of Neuroscience* 2002;22:1629–1639. [PubMed: 11880493]
- Placzek AN, Grassi F, Papke T, Meyer EM, Papke RL. A single point mutation confers properties of the muscle-type nicotinic acetylcholine receptor to homomeric $\alpha 7$ receptors. *Molecular Pharmacology* 2004;66:169–177. [PubMed: 15213309]
- Reeves DC, Goren EN, Akabas MH, Lummis SC. Structural and electrostatic properties of the 5-HT₃ receptor pore revealed by substituted cysteine accessibility mutagenesis. *Journal of Biological Chemistry* 2001;276:42035–42042. [PubMed: 11557761]
- Reeves DC, Lummis SC. The molecular basis of the structure and function of the 5-HT₃ receptor: a model ligand-gated ion channel. *Molecular membrane biology* 2002;19:11–26. [PubMed: 11989819]
- Scheller M, Forman SA. Coupled and uncoupled gating and desensitization effects by pore domain mutations in GABA_A receptors. *Journal of Neuroscience* 2002;22:8411–8421. [PubMed: 12351715]
- Selina Mok MH, Kew JN. Excitation of rat hippocampal interneurons via modulation of endogenous agonist activity at the $\alpha 7$ nicotinic ACh receptor. *Journal of Physiology* 2006;574:699–710. [PubMed: 16690715]
- Sigel E, Buhr A. The benzodiazepine binding site of GABA_A receptors. *Trends in pharmacological sciences* 1997;18:425–429. [PubMed: 9426470]
- Solt K, Ruesch D, Forman SA, Davies PA, Raines DE. Differential effects of serotonin and dopamine on human 5-HT_{3A} receptor kinetics: interpretation within an allosteric kinetic model. *Journal of Neuroscience* 2007;27(48):13151–13160. [PubMed: 18045909]
- van Hooft JA, van der Haar E, Vijverberg HP. Allosteric potentiation of the 5-HT₃ receptor-mediated ion current in N1E-115 neuroblastoma cells by 5-hydroxyindole and analogues. *Neuropharmacology* 1997;36:649–653. [PubMed: 9225290]
- van Hooft JA, Vijverberg HP. Selection of distinct conformational states of the 5-HT₃ receptor by full and partial agonists. *British Journal of Pharmacology* 1996;117:839–846. [PubMed: 8851499]
- Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, Ye Q, Harrison NL, Harris RA. Mutations of gamma-aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor? *Proceedings of the National Academy of Sciences of the United States of America* 1998;95:6504–6509. [PubMed: 9600996]

- Zhang L, Hosoi M, Fukuzawa M, Sun H, Rawlings RR, Weight FF. Distinct molecular basis for differential sensitivity of the serotonin type 3A receptor to ethanol in the absence and presence of agonist. *Journal of Biological Chemistry* 2002;277:46256–46264. [PubMed: 12368287]
- Zhou Q, Verdoorn TA, Lovinger DM. Alcohols potentiate the function of 5-HT₃ receptor-channels on NCB-20 neuroblastoma cells by favouring and stabilizing the open channel state. *Journal of Physiology (London)* 1998;507:335–352. [PubMed: 9518697]
- Zwart R, De Filippi G, Broad LM, McPhie GI, Pearson KH, Baldwinson T, Sher E. 5-Hydroxyindole potentiates human $\alpha 7$ nicotinic receptor-mediated responses and enhances acetylcholine-induced glutamate release in cerebellar slices. *Neuropharmacology* 2002;43:374–384. [PubMed: 12243767]

Figure 1. Effects of 5-HI on 5-HT₃ receptor-mediated currents

A, traces show current activated by 30 μ M 5-HT from individual N1E-115 cells (upper) and HEK 293 cells transiently expressing the wild-type receptor (WT, lower) in the absence and presence of 5 mM 5-HI. Bar indicates the time of agonist application. Cells were first exposed to 30 μ M 5-HT for 10 s followed by complete washout of the agonist, followed by exposure to the same concentration of agonist in the presence of 5-HI. B, traces show alteration of desensitization by 5 mM 5-HI in N1E-115 cells (upper) and HEK 293 cells transfected with the WT receptor (lower). Current amplitudes were normalized to peak current and superimposed for comparison. Bar indicates the time of agonist application. Traces in the absence of 5-HI are in black, whereas those in the presence of 5-HI are in grey. Data are summarized in Table 1.

Figure 2. Effects of selected mutations in TM2 domain and the TM2-TM3 loop on 5-HI modulation
A, sequence alignment of the TM2 domains of selected Cys-loop ligand-gated ion channels. Asterisks denote putative channel-lining amino acid residues. The open box indicates the residues in the GABA_A and glycine receptors that are thought to be part of anesthetic binding site. *B*, traces show modulation of current amplitude by 5-HI in the L293C and L293S receptors. *C*, traces show alteration of desensitization by 5 mM 5-HI in HEK 293 cells expressing the L293C and L293S receptors. *D*, traces show modulation of current amplitude by 5-HI in the T284F and D298A receptors. *E*, traces show alteration of desensitization by 5 mM 5-HI in HEK 293 cells expressing the T284F and D298A receptors. Traces in the absence of 5-HI are in black, whereas those in the presence of 5-HI are in grey. The bar indicates the time of agonist application. Currents were normalized to peak current and superimposed for comparison in *C* and *E*. Data are summarized in Table 1.

Figure 3. Effects of L293S mutation on modulation of 5-HT_{3A} receptor desensitization across a range of 5-HI concentrations

A, traces show alterations of desensitization by various concentrations of 5-HI in HEK 293 cells expressing the WT (upper) and L293S (lower) receptors. The bar indicates the time of agonist application. Currents were normalized to peak current and superimposed for comparison. Time constants were estimated by fitting with either a mono-exponential (WT) or a bi-exponential (L293S) function. **B**, averaged data show effects of 5-HI on desensitization of currents activated by 30 μ M 5-HT in the WT and L293S receptors. Each bar represents mean $\pm$ S.E.M. from 5–10 cells. ** $p < 0.01$. The traces obtained in the absence of 5-HI are in black, whereas those obtained in the presence of 5-HI are in grey.

Figure 4. Effects of 5-HI on current activated by low concentrations of agonist

A, traces show current activated by 1 μM 5-HT in the WT receptor in the absence (left) and presence (right) of 5 mM 5-HI. Inset depicts the superimposed traces in the absence and presence of 5-HI on an expanded time scale to emphasize the initial current activation phase. **B**, traces show current activated by 1 μM 5-HT in the L293C (left) and L293S (right) receptors in the absence and presence of 5 mM 5-HI. Similar responses were obtained from 6–8 cells. **C**, traces show current activated by 3 μM 5-HT in the WT receptor in the absence (left) and presence (right) of 5 mM 5-HI. Inset depicts the superimposed traces in the absence and presence of 5-HI on an expanded time scale to emphasize the initial current activation phase. **D**, traces show current activated by 3 μM 5-HT in the L293S receptor in the absence and presence of 5 mM 5-HI. Similar responses were obtained from 7–8 cells. The traces obtained in the absence of 5-HI are in black, whereas those obtained in the presence of 5-HI are in grey.

Figure 5. Effects of L293S mutation on 5-HI modulation of 5-HT_{3A} receptor deactivation
 A, traces show alterations of deactivation by 5 mM 5-HI in HEK 293 cells expressing the WT (upper) and L293S (lower) receptors. Arrow indicates the brief (2 ms) application of 5-HT. Currents were normalized to peak current and superimposed for comparison of deactivation. Time constants were estimated by fitting with either a mono-exponential (WT) or a bi-exponential (L293S) function. The traces obtained in the absence of 5-HI are in black, whereas those obtained in the presence of 5-HI are in grey. B, Averaged data show effects of 5-HI on deactivation of currents activated by 30 μ M 5-HT in the WT and mutant receptors. Each bar represents mean $\pm$ S.E.M. from 6–8 cells. ** $p < 0.01$.

Figure 6. L293S mutation alters gating of the 5-HT_{3A} receptor

A, chemical structure of 5-HT, dopamine (DA) and 5-HI (left) and their agonist activity in the WT (middle) and L293S (right) receptors. Cells expressing the WT and L293S receptors were first exposed to 30 μ M 5-HT. After complete washout of the agonist, the cells were challenged with either 3 mM DA or 5 mM 5-HI. The arrow indicates the application of 5-HT for 250 ms. Bars indicate the application times for DA and 5-HI. B, traces show current activated by 30 μ M 5-HT and various concentrations of 5-HI in the L293S receptor. Cells expressing the L293S receptor were first exposed to 30 μ M 5-HT. After complete washout of the agonist, the cells were challenged with 5-HI concentrations ranging from 0.1 to 10 mM. The trace activated by 5-HT is in grey, whereas those activated by 5-HI are in black. Inset depicts currents activated by 5-HI with expanded current and time scales. C, concentration-response curves for dopamine and 5-HI in the WT and mutant receptors. The responses elicited by dopamine or 5-HI were normalized to peak current activated by 30 μ M 5-HT for each cell expressing either the wild-type or L293S receptor. Each data point represents mean $\pm$ S.E.M. from 5–9 cells.

Figure 7. Properties of 5-HT-activated current in the L293S receptor

A, traces show the inability of TMB-8 (middle) and MDL 72222 (right) to alter the holding current in a cell expressing the L293S receptor. Cells expressing the L293S receptor were first exposed to 30 μ M 5-HT. After complete washout of the agonist, the cells were challenged with 100 μ M TMB-8 or 300 nM MDL 72222 for 10 s. Similar responses were observed in 5 cells. B, traces show current activated by 5 mM 5-HT in the absence (left) and presence of 300 nM MDL 72222 (right). The bar indicates the application time for 5-HT (black) and MDL 72222 (grey). Similar responses were observed in 6 cells. C, voltage ramps obtained at the peak of current activated by 3 μ M 5-HT in the WT receptor and 5 mM 5-HT in the L293 receptor. Currents activated by the voltage protocol in the absence of 5-HT and 5-HT have been subtracted. Data are normalized to the current amplitude obtained at -80 mV. Similar results were obtained from 6–8 cells. D, trace shows the effects of holding potential on the rebound current observed upon removal of 5-HT. Similar responses were observed in 3 cells.

Figure 8. Agonist activity of 5-HT analogs in the WT receptor

A, chemical structure of 5-HT, 5-methoxyindole (5-MI) and 3-(2-hydroxyethyl)indole (3-2-HEI) (left) and traces showing the agonist activity of 5-MI and 3-2-HEI (right). Cells expressing the WT receptor were first exposed to 30 μ M 5-HT. After complete washout of the agonist, the cells were challenged with either 5 mM 5-MI or 3-2-HEI. **B**, averaged data show the efficacy of 5-HT analogs in the WT receptor. The relative efficacy of the partial agonists was determined by normalizing the current amplitude activated by partial agonists as a percentage of that activated by 30 μ M 5-HT. Each bar represents mean $\pm$ S.E.M. from 7–10 cells. **C**, voltage ramps obtained at the peak of current activated by 5 mM 5-MI or 3-2-HEI in the WT receptor. Currents activated by the voltage protocol in the absence of agonists have been subtracted. Data are normalized to the current amplitude obtained at –80 mV. Similar results were obtained from 5–9 cells.

Figure 9. Effects of pre-incubation with 5-HI on 5-HT-activated currents in the WT and L293S receptors

A and C, cells expressing the WT or L293S receptors were first exposed to 30 μ M 5-HT for 250 ms. After complete washout of the agonist, the cells were exposed to 5 mM 5-HI for 20 s before challenging with 30 μ M 5-HT alone for 250 ms (A) or 10 s (C). B and D, averaged data show the effect of 5-HI pre-incubation on 5-HT-activated responses. The current amplitude activated by 5-HT immediately followed by 5-HI pre-incubation was normalized as a percentage of that activated by 30 μ M 5-HT. Each bar represents mean $\pm$ S.E.M. from 7–10 cells.

Figure 10. Slowing of desensitization alone recapitulates effects of 5HI in simulations of current
A Allosteric kinetic model (from Solt et al. 2007) used to simulate 5-HT activation of the receptor and potential 5-HI actions. **B** Simulated current produced using the allosteric model assuming continuous application of 30 μ M 5-HT for 10 sec with k_{d+} set at an estimated normal level for wild-type 5-HT_{3A} receptor (black trace) and reduced by ~ 1 order or magnitude to simulate the effect of 5-HI (Table 2). Note the similarity in current decay profile to the data presented in Figure 1B lower traces. **C** Simulated current produced using the allosteric model in a “deactivation” protocol (20 sec duration) in which agonist is set at 0 and all channels are assumed to be in the A3O state at the start of the trace. Black trace shows current with estimated

WT k_{d+} , grey trace with reduced k_{d+} as in A and Table 2. Note the similarity in time course to actual current traces in Figure 5A (top trace).

Table 1
Effects of selected mutations in TM2 and TM2-TM3 loop on modulation of desensitization kinetics by 5-HT

	5-HT alone					5-HT + 5-HT					n
	τ_{FAST} (ms)	% Fast	τ_{SLOW} (ms)	% Slow	$\tau_{WEIGHTED}$ (ms)	τ_{FAST} (ms)	% Fast	τ_{SLOW} (ms)	% Slow	$\tau_{WEIGHTED}$ (ms)	
NIE-115	295 ± 36	73 ± 3	2899 ± 457	27 ± 3	718 ± 118	613 ± 71**	20 ± 3	1119 ± 819**	80 ± 3	9092 ± 817**	6
WT	--	--	3700 ± 353	100	3700 ± 353	--	--	11153 ± 867**	100	11153 ± 867**	9
L293C	382 ± 87	68 ± 5	2646 ± 77	32 ± 5	1103 ± 162	313 ± 79	70 ± 5	2498 ± 153	30 ± 5	1093 ± 177	6
L293S	508 ± 46	66 ± 2	3013 ± 300	34 ± 2	1375 ± 159	392 ± 35	65 ± 3	2716 ± 193	35 ± 3	1212 ± 110	9
T284F	269 ± 21	73 ± 2	2155 ± 287	27 ± 2	783 ± 80	--	--	3297 ± 206**	100	3297 ± 206**	15
D298A	200 ± 26	81 ± 2	1353 ± 133	19 ± 2	425 ± 33	602 ± 48**	49 ± 1**	3174 ± 187	52 ± 1**	1931 ± 148	5

Desensitization time constants and percent contributions were derived from exponential fits to the current decay. To facilitate comparisons, the desensitization data were also transformed to weighted desensitization (see Materials and Methods). Data shown represent mean ± S.E.M. from n cells.

** $p < 0.01$.

Table 2

Kinetic parameters for current simulations

	5-HT alone	5-HT + 5-HT
5-HT*	30 μ M	30 μ M
k_1	$1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	$1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
k_2	10 s^{-1}	10 s^{-1}
k_3	0.01 s^{-1}	0.01 s^{-1}
β	400 s^{-1}	400 s^{-1}
α	1 s^{-1}	1 s^{-1}
k_{d+}	0.5 s^{-1}	0.04 s^{-1}
k_{d-}	0.01 s^{-1}	0.01 s^{-1}
k_{DR}	0.7 s^{-1}	0.7 s^{-1}
L_0	3×10^6	3×10^6

5-HT concentration and rate constants used to generate simulated 5-HT_{3A} receptor-mediated currents (Figure 10). Note that 5-HT concentration applies only to current simulation of response to continuous agonist application (Figure 10B), while current in simulated “deactivation” protocol run in agonist-free condition (Figure 10C).