Arginine 222 in the Pre-Transmembrane Domain 1 of 5-HT$_{3A}$ Receptors Links Agonist Binding to Channel Gating

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Running Title: R222 controls gating of 5-HT$_{3A}$R

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SUMMARY

Ligand-gated ion channels are integral membrane proteins that mediate fast synaptic transmission. Molecular biological techniques have been extensively used for determining the structure-function relationships of ligand-gated ion channels. However, the transduction mechanisms that link agonist binding to channel gating remain poorly understood. Arginine 222 (R222), located at the distal end of the extracellular N-terminal domain immediately preceding the first transmembrane domain (TM1), is conserved in all 5-HT$_{3A}$ receptors and $\alpha$7-nicotinic acetylcholine receptors that have been cloned. To elucidate the possible role of R222 in the function of 5-HT$_{3A}$ receptors, we mutated the arginine residue to alanine (A) and expressed both the wild-type and the mutant receptor in human embryonic kidney (HEK) 293 cells. Functional studies of expressed wild-type and mutant receptors revealed that the R222A mutation increased the apparent potency of the full agonist, 5-HT, and the partial agonist, 2-Me-5-HT, 5- and 12-fold, respectively. In addition, the mutation increased the efficacy of 2-Me-5-HT, and converted it from a partial agonist to a full agonist. Furthermore, this mutation also converted the 5-HT$_{3}$ receptor antagonist/very weak partial agonist, apomorphine, to a potent agonist. Kinetic analysis revealed that the R222A mutation increased the rate of receptor activation and desensitization, but did not affect rate of deactivation. The results suggest that the pre-TM1 amino acid residue R222 may be involved in the transduction mechanism linking agonist binding to channel gating in 5-HT$_{3A}$ receptors.
INTRODUCTION

In the nervous system, serotonin type 3 (5-HT₃) receptors can mediate fast excitatory synaptic transmission and modulate neurotransmitter release (1). To date, two 5-HT₃ receptor subunits have been identified: 5-HT₃A and 5-HT₃B (2,3). The 5-HT₃A receptor subunits can form functional channels homomerically (2), whereas the 5-HT₃B receptor subunits are nonfunctional when expressed alone (3). However, the 5-HT₃B receptor subunits can form heteromeric channels with the 5-HT₃A receptor subunits, which results in modified biophysical characteristics compared to homomerically expressed 5-HT₃A receptor subunits (3). 5-HT₃A and 5-HT₃B receptor subunits also have different distribution patterns in the nervous system. The 5-HT₃A receptor subunits are expressed in both central and peripheral neurons, whereas the 5-HT₃B receptor subunits are restricted to peripheral neurons (4). This suggests that homomeric 5-HT₃A receptors play a dominant role in 5-HT₃ receptor-mediated responses in the central nervous system.

5-HT₃ receptors belong to a superfamily of ligand-gated ion channels, which includes nicotinic acetylcholine (nACh) receptors, glycine receptors, γ-aminobutyric acid type A (GABAₐ) receptors (5). The subunits in this superfamily are thought to assemble as pentamers with each subunit containing a large extracellular N-terminal domain, four transmembrane domains (TM1-TM4), a large intracellular loop between TM3 and TM4 and an extracellular C-terminal domain (Fig. 1A) (6). The agonist binding sites are thought to be located in the N-terminal domain at subunit-subunit interfaces and the lining of the ion channel is believed to be formed by the second transmembrane (TM2) domain (6). The binding of agonist to the binding sites in the N-terminal domain

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1 The abbreviations used are: 5-HT₃, serotonin type 3; nACh, acetylcholine; GABA, γ-aminobutyric acid; TM, transmembrane domain; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDG, N-methyl-D-glucamine
presumably results in a conformational change of the channel protein, which is then transduced to the TM2 domain to result in channel opening.

A number of studies have been carried out to determine the sequence elements involved in agonist binding and channel gating of 5-HT3A receptors (7-13). However, the mechanisms that transduce the binding of agonist to the opening of the channel are still poorly understood. Understanding the function of ligand-gated ion channels at the molecular level requires understanding how agonist binding to the receptor is converted to channel opening. Arginine (R) 222 is of particular interest because it is located at the distal end of the extracellular N-terminal domain, immediately adjacent to the first transmembrane (TM1) domain (Fig. 1A), and thus it is between the presumed agonist binding sites in the N-terminal domain and TM2. Sequence alignments reveal that R222 of the mouse 5-HT3A receptor is conserved in all of 5-HT3A receptors and α7-nACh receptors that have been cloned from various species (Fig. 1B). Moreover, some mutations at R222 can alter the sensitivity of 5-HT3A receptors to agonists and produce channels that open spontaneously (14). In an attempt to understand the role of R222 in the function of 5-HT3A receptors, we replaced the arginine residue with an alanine using site-directed mutagenesis. The wild-type and R222A 5-HT3A receptors were transiently expressed in HEK 293 cells and the functional properties of the receptors were studied using the whole-cell patch-clamp recording technique in combination with fast solution exchange. The results suggest that R222 is involved in transducing the signal that couples agonist binding to channel opening in 5-HT3A receptors.
EXPERIMENTAL PROCEDURES

Mutagenesis—Point mutation of the mouse 5-HT\textsubscript{3A} receptor was accomplished using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutation was verified by double strand DNA sequencing using an ABI Prism 377 automatic DNA sequencer (Applied Biosystems, Foster City, CA). The cDNAs were then subcloned into the vector pcDNA3.1 (Invitrogen Co., Carlsbad, CA) for expression in HEK 293 cells.

Cell Culture and Transient Receptor Expression—Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were grown in minimum essential medium (MEM, Invitrogen) supplemented with 10% horse serum, and maintained in a humidified incubator at 37°C in 5% CO\textsubscript{2}. The HEK 293 cells were transiently transfected with the wild-type or R222A 5-HT\textsubscript{3A} receptor cDNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Green fluorescent protein (pGreen Lantern, Invitrogen) was co-expressed with the 5-HT\textsubscript{3A} receptor subunits to permit selection of transfected cells under fluorescence optics.

Patch-Clamping Recording—HEK 293 cells were recorded 1-3 day after transfection. Cells were continuously superfused with a solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 5 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH; ~340 mOsm with sucrose). Membrane current was recorded in the whole-cell configuration (15) using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at 20-22°C. Pipettes were pulled from borosilicate glass (TW-150F, World Precision Instruments, Sarasota, FL) using a two-stage puller (Flaming-Brown P-87; Sutter Instruments, Novato, CA) and had resistances of ~5 M\textOmega when filled with
pipette solution containing 140 mM CsCl, 2 mM MgCl₂, 10 mM EGTA, 10 mM HEPES (pH 7.2 with CsOH; ~315 mOsm with sucrose). Cells were held at –60 mV unless otherwise indicated. Data were acquired using pClamp8.0 software (Axon). Currents were filtered at 2 kHz and digitized at 2-10 kHz. Agonists 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 piezoelectric device was driven by TTL pulses from pClamp 8.0 software. Voltage applied to the piezoelectric device produced a rapid lateral displacement of the theta tubing to move the interface between control and agonist solutions. Solution exchange rate was estimated using the potential change induced by switching from the control solution to a 140 mM N-methyl-D-glucamine (NMDG) test solution. The solution exchange time constants were ~0.3 ms for an open pipette tip and ~1.6 ms for whole-cell recording.

Data Analysis—Data are presented as mean ± standard error, unless noted otherwise. Data analysis and curve fitting were performed with Origin6.0 (Microcal Software, Northampton, MA), pClamp8.0 (Axon), Statistica5.5 (StatSoft Inc., Tulsa, OK) or GraphPad InStat3.0 (GraphPad Software Inc., San Diego, CA) software. Concentration-response curves were fitted by the Hill equation, I/I MAX = 1/[1 + (EC₅₀/[Agonist])ⁿ], where I is the current amplitude activated by a given concentration of agonist ([Agonist]), I MAX is the maximum response of the cell, n is the Hill slope and EC₅₀ is the concentration eliciting a half-maximal response. The time constants for activation, deactivation and desensitization were determined with Levenberg-Marquardt algorithms.
RESULTS

Functional Characterization of the Wild-type and Mutant (R222A)5-HT$_{3A}$ Receptors—The wild-type and R222A 5-HT$_{3A}$ receptors were transiently expressed in HEK 293 cells and their responses to the full agonist, 5-HT, or the partial agonist, 2-Me-5-HT, were recorded in the whole-cell configuration with fast solution exchange. Typical responses activated by these agonists are shown in Figs. 2A and 2B. At a holding potential of –60 mV, application of a maximally efficacious concentration of the full agonist, 5-HT (30 $\mu$M), induced a rapidly activating inward current in both the wild-type (Fig. 2A, left) and R222A (Fig. 2B, left) receptors. Application of a maximally efficacious concentration of the partial agonist, 2-Me-5-HT (100 $\mu$M), activated the wild-type receptor more slowly compared to the response activated by 30 $\mu$M 5-HT (Fig. 2A, right). By contrast, the R222A mutation resulted in a more rapidly activating response to 2-Me-5-HT (Fig. 2B, right). In addition, in the wild-type receptor the current activated by 100 $\mu$M 2-Me-5-HT was much smaller in amplitude than the current activated by 30 $\mu$M 5-HT (Fig. 2A), whereas in the R222A mutant receptor the current activated by 100 $\mu$M 2-Me-5-HT was similar in amplitude to that activated by 30 $\mu$M 5-HT (Fig. 2B).

Concentration-response curves for agonists were constructed by normalizing the amplitude of current activated by a range of 5-HT or 2-Me-5-HT concentrations ($I/I_{\text{MAX}}$ for 5-HT and $I/I_{30\mu\text{M}5\text{-HT}}$ for 2-Me-5-HT), as shown in Figs. 2C and 2D. The amplitude of currents activated by 5-HT or 2-Me-5-HT was concentration-dependent for both wild-type and R222A receptors. In addition, the concentration-response curves for both 5-HT and 2-Me-5-HT were shifted to the left by the R222A mutation (Figs. 2C and 2D). In the R222A receptor, the potency for 5-HT and 2-Me-5-HT were increased 5-fold and 12-
fold, respectively (5-HT, $EC_{50} = 0.5 \pm 0.1 \, \mu M$; 2-Me-5-HT, $EC_{50} = 1.2 \pm 0.2 \, \mu M$), as compared with the wild-type receptor (5-HT, $EC_{50} = 2.7 \pm 0.4 \, \mu M$; 2-Me-5-HT, $EC_{50} = 14.4 \pm 1.4 \, \mu M$). The Hill slope of the concentration-response curve for 5-HT was not significantly changed by the R222A mutation (wild-type, 1.48 $\pm$ 0.08; R222A, 1.44 $\pm$ 0.06; $p > 0.5$), whereas the R222A mutation decreased the Hill slope for 2-Me-5-HT (wild-type, 2.48 $\pm$ 0.10; R222A, 1.20 $\pm$ 0.20; $p < 0.05$). In addition, the maximal response to 2-Me-5-HT was greatly increased by the R222A mutation, from 57.8 $\pm$ 2.3% to 103.3 $\pm$ 5.6% of the current activated by 30 µM 5-HT ($p < 0.01$).

Voltage-independence—To address whether the R222A mutation altered ion permeation, current-voltage (I-V) relationships were obtained by measuring the amplitude of current activated by either 30 µM 5-HT or 100 µM 2-Me-5-HT at holding potentials from –80 to +60 mV (Figs. 3A and 3B). The current evoked by both 5-HT and 2-Me-5-HT reversed at ~5 mV, and the current-voltage relationship exhibited slight inward rectification for both agonists (Figs. 3C and 3D). The R222A mutation had no significant effect on either the reversal potential for both 5-HT (wild-type, 4.90 $\pm$ 0.57; R222A, 4.49 $\pm$ 0.68; $p > 0.5$) and 2-Me-5-HT (wild-type, 5.25 $\pm$ 0.0.62; R222A, 5.19 $\pm$ 0.0.67; $p > 0.5$) or the shape of the I-V relationship (Figs. 3C and 3D).

Antagonist Profile—Since some R222 mutants exhibited spontaneous channel opening when expressed in *Xenopus* oocytes (14), we examined whether the R222A mutation produced such activity in HEK 293 cells. Spontaneous opening of ligand channels can be blocked with antagonists (14;16). As shown in Fig. 4A (left), in cells expressing the wild-type receptors the application of 300 nM MDL 72222, a competitive 5-HT<sub>3</sub> receptor antagonist, for 10 s in the absence of 5-HT did not alter the holding
current. Similarly, in cells expressing the R222A receptors the application of 300 nM MDL 72222 for 10 s did not alter the holding current (Fig. 4A, right). We also tested the effectiveness of MDL 72222 in antagonizing 5-HT-activated currents. MDL 72222 at 300 nM completely blocked the current activated by 30 µM 5-HT in both the wild-type and the R222A receptors (Fig. 4B). Blockade of 5-HT-activated responses by MDL 72222 was readily reversed by washing (data not shown). Apomorphine has been reported to be an antagonist/very weak partial agonist in the 5-HT$_3$ receptors (17). Fig. 4C (left) shows that, in the wild-type receptors, 30 µM apomorphine activated a very small current when compared to the current activated by 30 µM 5-HT. In addition, when co-applied with 5-HT in the wild-type receptors, 30 µM apomorphine inhibited the current activated by 30 µM 5-HT. On the other hand, 30 µM apomorphine activated a significant inward current in the R222A receptors (Fig. 4C, right). The bar graphs in Fig. 4D show the average action of apomorphine in both the wild-type and the R222A receptors. Apomorphine inhibited 5-HT-activated current by 82.1 ± 3.1% in the wild-type receptors ($p < 0.01$). In the wild-type receptors, the average amplitude of the current activated by 30 µM apomorphine was less than 1% of that activated by 30 µM 5-HT; however, in the R222A receptors the average amplitude of the current activated by 30 µM apomorphine was ~ 60% of the amplitude of the current activated by 30 µM 5-HT (wild type, 0.88 ± 0.35; R222A, 60.8 ± 4.3, $p < 0.01$).

Activation Kinetics—To evaluate the kinetics of 5-HT$_3$A receptor activation, 5-HT or 2-Me-5-HT was applied at different concentrations for a sufficient duration to allow the receptor-agonist interaction to reach equilibrium. Figs. 5A and 5B show normalized responses to application of 2.7 and 30 µM 5-HT (Fig. 5A), and 14.4 and 100 µM 2-Me-5-
HT (Fig. 5B), respectively, from cells expressing the wild-type or the R222A receptors. The records show that high agonist concentrations elicited a faster activation than low agonist concentrations for both the wild-type and the R222A receptors. In addition, the rate of receptor activation was agonist-dependent for both the wild-type and the R222A receptors; fast for 5-HT and slower for 2-Me-5-HT. The R222A mutation significantly increased the rate of receptor activation for both 5-HT and 2-Me-5-HT. The activation of 5-HT$_{3A}$ receptors could be fitted with a single exponential function. The time constants for 2.7 and 30 µM 5-HT were 124.0 ± 7.4 ms and 16.2 ± 0.65 ms, respectively, in the wild-type receptors; and 19.3 ± 1.4 ms and 8.7 ± 0.5 ms, respectively, in the R222A receptors. The time constants for 14.4 and 100 µM 2-Me-5-HT were 753.1 ± 43.4 ms and 288.7 ± 12.4 ms, respectively, in the wild-type receptors; and 19.6 ± 1.4 ms and 13.0 ± 1.0 ms, respectively, in the R222A receptors. Figs. 5C and 5D plot the activation rate (inverse of the activation time constant, $\tau_{\text{ACTIVATION}}$) as a function of 5-HT (Fig. 5C) and 2-Me-5-HT (Fig. 5D) concentration for the wild-type and the R222A receptors. Although the concentration at which the activation rate was half-maximal was 3.7 µM 5-HT for the R222A receptor, 25 µM 5-HT was required to achieve the same activation rate for the wild-type receptor. Similarly, 23 µM 2-Me-5-HT produced the half-maximal activation rate in the R222A receptor, but extrapolation suggests that 1800 µM 2-Me-5-HT would be needed to produce the same activation rate for the wild-type receptor.

Gating—Activation of ligand-gated ion channels involves agonist binding and conformational changes that lead to gating. Previous studies on ligand-gated ion channels suggest that at low agonist concentrations, agonist binding is the rate-limiting step; however, when the agonist concentration is high, gating becomes the rate-limiting
step and the activation rate approximates the channel opening rate (18). In view of this, we examined the activation of both the wild-type and the R222A receptors with saturating concentrations of 5-HT. The increase in activation rate with increasing agonist concentrations appeared to reach a plateau at ~300 µM 5-HT for both the wild-type (Fig. 6A) and R222A (Fig. 6B) receptors, since the activation rate did not appear to be accelerated by increasing 5-HT concentration above 300 µM (up to 3 mM). On the other hand, the activation rate was faster in R222A receptors than in wild-type receptors (Fig. 6C). In the wild-type receptors, for 5-HT concentrations ≥300 µM, averaged activation rates (Fig. 6D) were not significantly different (ANOVA, $p > 0.5$). Similarly, in the R222A receptors, average activation rates (Fig. 6D) were not significantly different for 5-HT concentration ≥300 µM (ANOVA, $p > 0.3$). However, the R222A mutation significantly accelerated the activation rate for 300 µM ($p < 0.01$), 1 mM ($p < 0.001$) and 3 mM 5-HT ($p < 0.001$), compared with the wild-type receptors (Fig. 6D).

Deactivation Kinetics—Deactivation is the process of agonist unbinding and channel closing, and it is generally considered as an index of agonist affinity (19;20). To determine whether the R222A mutation alters the rate of deactivation, we examined the current deactivation kinetics after rapid removal of the agonist. The records in Figs. 7A and 7B illustrate the current deactivation after rapid removal of 1 mM 5-HT (Fig. 7A) and 100 µM 2-Me-5-HT (Fig. 7B) in cells expressing the wild-type and R222A 5-HT$_{3A}$ receptors. 5-HT was removed after a 2 ms application, while 2-Me-5-HT was removed before desensitization was observed. When the agonist was rapidly washed-out, the currents decayed back to baseline as a mono-exponential function for both the wild-type and the R222A receptors. The R222A mutation did not alter the deactivation rate
(inverse of deactivation time constant, $\tau_{DEACTIVATION}$) for either 5-HT (wild-type, 0.34 ± 0.03 s$^{-1}$; R222A, 0.34 ± 0.03 s$^{-1}$; $p > 0.5$) or 2-Me-5-HT (wild-type, 0.57 ± 0.02 s$^{-1}$; R222A, 0.58 ± 0.02 s$^{-1}$; $p > 0.5$). However, the deactivation rate was agonist-dependent and, on average, 2-Me-5-HT had a significantly faster deactivation rate than 5-HT ($p < 0.01$) for both the wild-type and the R222A receptors, Fig. 7C).

*Desensitization Kinetics*—In the continued presence of agonist, the activated current exhibited a marked decrease in amplitude after reaching a peak, indicating receptor desensitization. Examples of the desensitization resulting from the application of 30 µM 5-HT and 100 µM 2-Me-5-HT are shown in Figs. 8A and 8B, respectively. For the wild-type 5-HT$_{3A}$ receptors, the desensitization of the current was relatively slow and well described by a mono-exponential function. In addition, the desensitization time constant for 2-Me-5-HT in the wild-type receptors was significantly slower than for 5-HT ($p < 0.04$). For the R222A receptors, a bi-exponential function was required to adequately fit the desensitization of the current activated by 5-HT or 2-Me-5-HT. Both the fast and slow desensitization components of the R222A receptors were similar for 5-HT and 2-Me-5-HT ($p > 0.50$). The fast component contributed ~60% of the total current decay for both agonists (Figs. 8C and 8D). The slow component of the decay in the R222A receptor for either 5-HT or 2-Me-5-HT was faster than the current decay in the wild-type receptor ($p < 0.01$).
DISCUSSION

In the present study, we investigated the role of amino acid residue R222 in the function of 5-HT$_{3A}$. We found that the R222A mutation increased the apparent potency of both the agonist, 5-HT, and the partial agonist, 2-Me-5-HT. The R222A mutation also increased the efficacy of the partial agonist, 2-Me-5-HT, and converted the antagonist/very weak partial agonist, apomorphine, to a potent agonist. In addition, the R222A mutation accelerated the rate of 5-HT$_{3A}$ receptor activation and desensitization, but it did not alter the rate of 5-HT$_{3A}$ receptor deactivation.

Ligand-gated ion channels exist in at least three interconvertible states - resting, open and desensitized - and the function of these channels is thought to be determined by the transitions among these states (6). Activation of ligand-gated ion channels involves the binding of agonist to the receptor that results in a conformational change of the protein that opens the channel. In the present study, how the R222A mutation affects 5-HT$_{3A}$ receptor activation is examined.

Since deactivation is defined as the process of the unbinding of agonist from the open state of the receptor and the channel returning to the closed resting state, it is expected that receptors with a higher affinity for agonist in the open state would decrease the probability of agonist unbinding from the receptor. Thus, agonist affinity could be assessed from agonist unbinding rate (19;20). If the R222A mutation increases agonist affinity, the deactivation rate of the receptor would be expected to be slower. We found, however, that the R222A mutation did not change the rate of deactivation, as currents for both wild-type and R222A receptors decayed with similar kinetics upon removal of agonist. The observation that the mutation did not alter the deactivation rate for either 5-
HT or 2-Me-5-HT is thus inconsistent with an increase in agonist affinity for R222A receptors. This is in consistent with the observation in a single-oocyte binding assay (14). However, we can not exclude the possibility from this study that R222 is part of the agonist binding pocket.

2-Me-5-HT is a partial agonist at mouse wild-type 5-HT3A receptors (21). Since the relative efficacy of an agonist is dependent, in part, upon the gating process (22), the conversion of the partial agonist, 2-Me-5-HT, into a full agonist suggests the possibility of an enhanced gating efficacy in the R222A receptors. The increase in efficacy by R222A mutation is not peculiar to 2-Me-5-HT, as this mutation also converted the extremely weak 5-HT3 receptor partial agonist, apomorphine, to a potent agonist. In addition, the R222A mutation was found to enhance the rate of 5-HT3A receptor activation at saturating concentrations of 5-HT, which is also consistent with a facilitation of the gating process. For the ligand-gated ion channels, it has been found that a mutation that facilitates gating may only cause a shift to the left of the agonist concentration-response curve for a full agonist (22). The observations that the R222A mutation accelerated activation of 5-HT3A receptors by 5-HT and increased the apparent potency of 5-HT suggest that the efficacy of 5-HT to gate the channel is also enhanced by the mutation. Alterations in apparent potency and efficacy of agonists by mutations have been observed for other ligand-gated ion channels. For example, point mutations in α7-nACh receptors at L247 (23) and AMPA receptors at A636 (24) converted an antagonist into an agonist and increased the apparent potency of agonists. On the other hand, mutations in the NR1 subunit of NMDA receptors at D732 (25) and the α2 subunit of
nACh receptors at D200 (26;27) decreased apparent agonist potency, and converted partial agonists into competitive antagonists.

The distance between the agonist binding sites and the channel pore of the nACh receptor is estimated to be ~30 Å (28). The agonist binding sites of the 5-HT$_{3A}$ receptor are thought to be comparable with those of the nACh receptor (29). Since the agonist binding sites are at a distance from the channel gating mechanism, the binding signal must involve a transduction mechanism to open the channel. The binding of agonist to the receptor is thought to provide the driving force to open the channel (30;31). It is proposed for nACh receptors that the binding of agonist triggers a localized disturbance at the binding sites that transmits to transmembrane domains of the receptor through a small rotation of the N-terminal domain (32). Our observations on the role of R222 in the gating of 5-HT$_{3A}$ receptors suggest that this amino acid residue may be involved in the transduction of the signal from agonist binding to channel gating.

The pre-TM1 region and TM2-TM3 loop of several ligand-gated ion channels have been found to be critical in the coupling of agonist binding to channel gating. Certain residues of the TM2-TM3 loop in nACh receptors (33;34), glycine receptors (35), and GABA$_A$ receptors (36) have been found to participate in the transduction of agonist binding to channel gating. For NMDA receptors, the pre-TM1 segment was found to link agonist binding to channel gating and to affect entry into open and desensitized states (37). In addition, a recent study suggests that an interaction of residues in TM1 of nACh receptors, which are near R222 in 5-HT$_{3A}$ receptors, and TM2 contributes to the gating process (38). Our data suggest that R222 located in pre-TM1 of 5-HT$_{3A}$ receptors may also play a crucial role in the transduction mechanism coupling agonist binding to
channel opening and entry into the desensitized state. In this context, R222 may serve as a constraint to gating of the 5-HT\textsubscript{3A} receptors by maintaining the wild-type receptors in the resting closed state. The R222A mutation may release this constraint and reduce the energy barrier for channel opening, increasing apparent agonist potency and partial agonist efficacy, as a result of enhanced coupling between agonist binding and gating.

In the present study, it appears that the R222A mutation reduced the energy barrier for agonist to open the channel. In this regard, it should be noted that arginine and alanine residues differ in their size, polarity, and hydrophobicity. Facilitated coupling resulting from the R222A mutation might be due to the decreased side-chain size, the increased hydrophobicity or the reduced charge. Any of these changes may reduce the energy barrier for channel opening and thus facilitate the transition from a closed to an open state. Mutations at V385 of the \(\alpha\) subunit of muscle type nACh receptors revealed that both volume and stereochemistry contribute to channel gating but not to binding affinity (39). The observation that some 5-HT\textsubscript{3A} receptor mutants, such as R222F and R222I, exhibits spontaneous channel opening (14), is consistent with the notion that R222 mutations can reduce the energy barrier for channel opening.

It has been proposed that the closed resting state is the most stable conformation in the absence of agonist, whereas the closed desensitized state is the most stable conformation in presence of agonist (6). Desensitization is a widespread phenomenon among ligand-gated ion channels (40). Our observation that the R222A receptors exhibited a significantly faster desensitization than the wild-type receptors suggests that although the R222A mutation facilitated gating of the receptor, it also increased the proportion of receptors entering into desensitized state from the open state. It has been
proposed for the nACH receptor that gating and desensitization are energetically coupled events (41). Our observation that an increase in gating efficacy is associated with an enhanced desensitization of 5-HT\textsubscript{3A} receptors is consistent with such a notion and suggests that desensitization of 5-HT\textsubscript{3A} receptors is positively coupled to gating.

In summary, we have found that the R222A mutation can enhance the apparent potency of agonists and the efficacy of partial agonists in 5-HT\textsubscript{3A} receptors. Kinetic analysis indicates that these alterations are associated with faster activation and desensitization of this receptor-channel. The unique position of R222 may allow it to couple the binding of agonist to the opening of the channel, since the R222A mutation appears to reduce the energy barrier for gating. A reduced energy barrier may speed up activation, which in turn may increase the apparent potency of agonists and the efficacy of partial agonists. Thus, our data suggest that R222 has a functional role in the signal transduction mechanism of 5-HT\textsubscript{3A} receptors.
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FIGURE LEGENDS

Fig.1. **Putative 5-HT$_{3A}$ receptor topology and partial sequence alignment of 5-HT$_{3A}$ receptors and $\alpha$7-nACh receptors.** A, putative 5-HT$_{3A}$ receptor topology with four transmembrane domains (TM1-TM4), a large extracellular N-terminal domain, a large intracellular loop between TM3 and TM4, and a short extracellular C-terminal domain. C-C denotes cys-loop. B, partial amino acid sequence alignment of the pre-TM1/TM1 of the cloned 5-HT$_{3A}$ receptors and $\alpha$7-nACh receptors. Numbering corresponds to the mouse 5-HT$_{3A}$ receptor sequence. Note that the mouse 5-HT$_{3A}$ receptor arginine (R) 222, labeled with an asterisk, is conserved in all the 5-HT$_{3A}$ and $\alpha$7-nACh receptors.

Fig. 2. **The R222A mutation increases the potency of agonists and converts the partial agonist, 2-Me-5-HT, into a full agonist.** A and B, traces show current activated by 5-HT and 2-Me-5-HT in HEK 293 cells expressing wild-type (WT) (A) and R222A (B) receptors. C, concentration-response curves for 5-HT from WT (○) and R222A (●) receptors. I and $I_{MAX}$ are the current at a given agonist concentration and the maximal current, respectively. The average $I_{MAX}$ values for 5-HT were 937 ± 114 pA for the WT receptors and 522 ± 44 pA for the R222A receptors. D, concentration-response curves for 2–Me-5-HT from WT (○) and R222A (●) receptors. I and $I_{30 \mu M 5-HT}$ are the current at a given agonist concentration and the current activated by 30 μM 5-HT, respectively. The average $I_{MAX}$ values for 2-Me-5-HT were 544 ± 38 pA for the WT receptors and 560 ± 54 pA for the R222A receptors. The difference in the maximal currents evoked by 5-HT may reflect the expression efficiency of R222A receptors. Each data point represents mean ± S.E. from 6-9 cells.
Fig. 3. The R222A mutation does not alter the current-voltage (I-V) relationship for 5-HT or 2-Me-5-HT. A and B, traces show current activated by 30 µM 5-HT (A) or 100 µM 2-Me-5-HT (B) at various holding potentials in cells expressing WT or R222A receptors. C and D, current–voltage relationships for 5-HT-(C) and 2-Me-5-HT- (D) activated current from WT (○) and R222A (●) receptors. Each data point represents the mean ± S.E. from 6 cells.

Fig. 4. The R222A mutation does not alter the antagonist profile of MDL 72222, but it converts the antagonist/very weak partial agonist, apomorphine, to a potent agonist. A, traces show that the 5-HT3 antagonist, MDL 72222 (300 nM), does not alter the holding current in WT (left) or R222A (right) receptors. Bar indicates time of MDL 72222 application. Similar responses were observed in at least 5-7 cells. B, traces show current activated by 30 µM 5-HT in the absence (○) and presence of 300 nM MDL 72222 (●). MDL 72222 was pre-applied for 1.5 min before co-application with 5-HT. Bar indicates time of 5-HT application. Similar responses were observed in at least 5-8 cells. C, traces show the current activated by 30 µM 5-HT or 30 µM apomorphine in WT (left) and R222A (right) receptors, and inhibition of the current activated by 30 µM 5-HT by 30 µM apomorphine in WT receptors (left). The traces in the inset show the current activated by 30 µM apomorphine in WT receptors on an expanded amplitude scale. D, average action of apomorphine in WT (left) and R222A (right) receptors. Each bar represents mean ± S.E. from 5-8 cells. ** p < 0.01.

Fig. 5. The R222A mutation accelerates the activation rate of 5-HT3A receptors in response to 5-HT and 2-Me-5-HT. A and B, traces show receptor activation elicited by 2.7 µM 5-HT (EC50 at WT) and 30 µM 5-HT (A), and 14.4 µM 2-
Me-5-HT (EC$_{50}$ at WT) and 100 µM 2-Me-5-HT ($B$) in WT and R222A receptors. Agonist-activated responses were normalized to peak current for comparison. Bar indicates time of agonist application. $C$ and $D$, plots of the activation rate (inverse of $\tau_{\text{ACTIVATION}}$) versus 5-HT concentration ($C$) and 2-Me-5-HT concentration ($D$) in WT (○) and R222A (●) receptors. Each data point represents mean ± S.E. from 10-24 cells.

Fig. 6. **The R222A mutation accelerates opening rate of 5-HT$_{3A}$ receptors.** $A$ and $B$. Normalized and superimposed receptor activation in response to supermaximal concentrations of 5-HT (100, 300, 1000 and 3000 µM) in WT ($A$) and R222A ($B$) receptors. Activation rate appears similar when 5-HT concentration is greater than 300 µM in both WT and R222A receptors. Bar indicates time of 5-HT application. $C$, superimposed traces from $A$ and $B$ show the difference of activation rate for WT and R222A receptors in response to 1000 µM 5-HT, with a faster time scale. $D$, average activation rate at supermaximal concentrations of 5-HT in WT (○) and R222A (■) receptors. Each bar represents mean ± S.E. from 7-8 cells. **$p < 0.01$.

Fig. 7. **The deactivation rate of 5-HT$_{3A}$ receptors is not altered by the R222A mutation.** $A$ and $B$, traces show deactivation after termination of the application of 1 mM 5-HT ($A$) or 100 µM 2-Me-5-HT ($B$) from cells expressing WT (left) or R222A (right) receptors. The agonist-activated responses are normalized to peak current for comparison. The arrow indicates the start of agonist washout. 5-HT was applied for 2 ms and was then quickly removed, whereas 2-Me-5-HT washout was started before desensitization (current decay) was observed. $C$, average deactivation kinetics for 5-HT (left) and 2-Me-5-HT (right) from the WT (○) and R222A (■) receptors. Each bar represents mean ± S.E. from 13-24 cells.
Fig. 8. **The R222A mutation accelerates the desensitization rate of 5-HT$_{3A}$ receptors.** *A* and *B*, traces show desensitization of WT and R222A receptors to 10 sec application of 30 µM 5-HT (*A*) or 100 µM 2-Me-5-HT (*B*). The agonist-activated currents are normalized to peak current and superimposed for comparison. Bar indicates time of agonist application. The desensitization is best fit with one exponential function in the WT receptors, whereas two exponential functions best fit the decay of current in the R222A receptors. *C* and *D*, average desensitization kinetics for 5-HT (*C*) and 2-Me-5-HT (*D*) in WT and R222A receptors. Each bar represents mean ± S.E. from 8-10 cells.
### Figure 1

#### A

![Diagram showing the structure of a receptor](image)

#### B

| Species          | Sequence                        |
|------------------|---------------------------------|
| **Mouse 5-HT₃₄R**| FYV I RRRPFL YAVSLLLPS IFLMVVDIVGFLPP |
| **Rat 5-HT₃₄R** | FYVV I RRRPFL YAVSLLLPS IFLMVVDIVGFLPP |
| **Human 5-HT₃₄R** | FYVV I RRRPFL YAVSLLLPS IFLMVMDIVGFLPP |
| **Ferret 5-HT₃₄R** | FYVV I RRRPFL YAVSLLLPS IFLMVMDIVGFLPP |
| **Guinea-pig 5-HT₃₄R** | FYVV I RRRPFL YAVSLLLPS IFLMVMDIVGFLPP |
| **Mouse α₇ nAChR** | YTV TMRRRRTLYGLNNL I PCVL I S A L ALLVFL LPA |
| **Rat α₇ nAChR** | YTV TMRRRRTLYGLNNL I PCVL I S A L ALLVFL LPA |
| **Human α₇ nAChR** | FTV TMRRRRTLYGLNNL I PCVL I S A L ALLVFL LPA |
| **Bovine α₇ nAChR** | FTV SMRRRRTLYGLNNL I PCVL I S A L ALLVFL LPA |
| **Chick α₇ nAChR** | FTV TMRRRRTLYGLNNL I PCVL I S A L ALLVFL LPA |
Figure 2
Figure 3
Figure 5

A

2.7 µM 5-HT

WT

2.7 µM 5-HT

R222A

100 ms

50 ms

30 µM 5-HT

WT

30 µM 5-HT

R222A

25 ms

25 ms

B

14.4 µM 2-Me-5-HT

WT

14.4 µM 2-Me-5-HT

R222A

1000 ms

50 ms

100 µM 2-Me-5-HT

WT

100 µM 2-Me-5-HT

R222A

500 ms

25 ms

C

Activation Rate (s⁻¹)

0 25 50 75 100 125

0.1 1 10 100

5-HT (µM)

WT

R222A

D

Activation Rate (s⁻¹)

0 25 50 75

1 10 100

2-Me-5-HT (µM)

WT

R222A
Figure 6

A
5-HT (100, 300, 1000 & 3000 μM)
WT

B
5-HT (100, 300, 1000 & 3000 μM)
R222A

C
5-HT (1000 μM)
WT
R222A

D

| 5-HT (μM) | Activation Rate (s⁻¹) |
|-----------|----------------------|
| 100       |                     |
| 300       |                     |
| 1000      |                     |
| 3000      |                     |

** **
Figure 7

A

WT

5-HT

2 s

B

WT

2-Me-5-HT

2 s

C

Deactivation rate (s⁻¹)

5-HT

2-Me-5-HT

WT

R222A
Figure 8

A

30 µM 5-HT

R222A
WT

B

100 µM 2-Me-5-HT

R222A
WT

C

30 µM 5-HT

Desensitization Rate (s⁻¹)

Fast
Slow

100% 37%

WT R222A

D

100 µM 2-Me-5-HT

Desensitization Rate (s⁻¹)

Fast
Slow

100% 41%

WT R222A
Arginine 222 in the pre-transmembrane domain 1 of 5-HT3A receptors links agonist binding to channel gating
Xiang-Qun Hu, Li Zhang, Randall R. Stewart and Forrest F. Weight

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