Co-expression of the 5-HT$_{3B}$ Serotonin Receptor Subunit Alters the Biophysics of the 5-HT$_3$ Receptor

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ABSTRACT Homomeric complexes of 5-HT$_{3A}$ receptor subunits form a ligand-gated ion channel. This assembly does not fully reproduce the biophysical and pharmacological properties of native 5-HT$_3$ receptors which might contain the recently cloned 5-HT$_{3B}$ receptor subunit. In the present study, heteromeric assemblies containing human 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits were expressed in HEK 293 cells to detail the functional diversity of 5-HT$_3$ receptors. We designed patch-clamp experiments with homomeric (5-HT$_{3A}$) and heteromeric (5-HT$_{3AB}$) receptors to emphasize the kinetics of channel activation and desensitization. Co-expression of the 5-HT$_{3B}$ receptor subunit reduced the sensitivity for 5-HT (5-HT$_{3A}$ receptor: EC$_{50}$ 3 μM, Hill coefficient 1.8; 5-HT$_{3AB}$ receptor: EC$_{50}$ 25 μM, Hill coefficient 0.9) and markedly altered receptor desensitization. Kinetic modeling suggested that homomeric receptors, but not heteromeric receptors, desensitize via an agonist-induced open-channel block. Furthermore, heteromeric 5-HT$_{3AB}$ receptor assemblies recovered much faster from desensitization than homomeric 5-HT$_{3A}$ receptor assemblies. Unexpectedly, the specific 5-HT$_3$ receptor agonist mCPBG induced an open-channel block at both homomeric and heteromeric receptors. Because receptor desensitization and resensitization massively affect amplitude, duration, and frequency of synaptic signaling, these findings are evidence in favor of a pivotal role of subunit composition of 5-HT$_3$ receptors in serotonergic transmission.

INTRODUCTION

The 5-HT$_3$ receptor is a ligand-gated ion channel belonging to the Cys-loop superfamily of pentameric proteins (Maricq et al., 1991; Yakel, 1992). The ion channel is permeable for Na^+- and K^+- (Yang, 1990) and Ca^{2+}- ions (Yang, 1990, Hargreaves et al., 1994), and probably mediates mainly inward currents and membrane depolarization under physiological conditions (for review: Barnes and Sharp, 1999). Activation of postsynaptic 5-HT$_3$ receptors contributes to fast excitatory synaptic transmission in various brain areas, such as the lateral amygdala (Sugita et al., 1992) and the visual cortex (Roerig et al., 1997), whereas presynaptic 5-HT$_3$ receptors modulate the release of various neurotransmitters, e.g., dopamine (for review, van Hooft and Vijverberg, 2000) and GABA (Koyama et al., 2000).

Homo-pentameric complexes of recombinant 5-HT$_{3A}$ receptors function efficiently in heterologous expression systems and share some pharmacological and functional properties with native neuronal 5-HT$_3$ receptors (Maricq et al., 1991; Boess et al., 1995; Green et al., 1995). However, there is evidence from more recent studies that some native 5-HT$_3$ receptors, in both humans and rodents, are heteromeric assemblies of 5-HT$_{3A}$ subunits co-expressed with 5-HT$_{3B}$ subunits, which share 41% amino acid identity with the 5-HT$_{3A}$ subunits (Davies et al., 1999; Hanna et al., 2000). The different recruitment of the 5-HT$_{3B}$ subunit for the formation of heteromeric 5-HT$_3$ receptors might account for some of the heterogeneity of responses after the activation of native 5-HT$_3$ receptors (Yang et al., 1992; Hussy et al., 1994; Fletcher and Barnes, 1998).

The channel closing after activation of an ionotropic receptor channel by an agonist is caused by either agonist unbinding (receptor deactivation) or receptor desensitization (Jones and Westbrook, 1996). Desensitization, which defines the entry into an inactive state, even though the agonist remains bound, is a feature shared by most ligand-gated ion channels. It is assumed to be critically involved in the termination of postsynaptic responses, whereas the rate of recovery from desensitization can determine the ability of synapses to respond to repetitive firing (Huganir et al., 1986; Huganir and Greengard, 1990; Jones and Westbrook, 1996). Thus, desensitization could provide an important mechanism for short-term plasticity of synaptic strength. The molecular basis of ion channel desensitization is still poorly understood. Nevertheless, recent studies have started to unravel some of the molecular steps of desensitization of ionotropic glutamate receptors (Sun et al., 2002).

Desensitization kinetics of 5-HT$_3$ receptors depend on, e.g., receptor subunit amino acid sequence, extracellular calcium concentration (Gunthorpe et al., 2000; Lobitz et al., 2001; Yakel, 1996; Yakel et al., 1993), and the developmental state of cells (Shao et al., 1991). The present study investigates the effect of co-expressed 5-HT$_{3B}$ receptor subunits on 5-HT$_3$ receptor activation, desensitization, and recovery from desensitization.

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MATERIALS AND METHODS

Cell culture

Human embryonic kidney cells (HEK 293, DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were maintained in minimum essential medium, supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin, in an atmosphere of 5% CO_2, 95% air, and 100% relative humidity at 37°C. The cells were harvested two times per week by resuspending in phosphate-buffered saline containing trypsin (100 μg/ml). After washing by centrifugation, and resuspending in fresh medium, the cells were seeded with 20–40% confluency in 20 × 100-mm culture dishes.

Transfection of subunit cDNAs

cDNAs encoding the human 5-HT_{3A} subunit (nucleotides 217–1663, GenBank accession no. D49394) and human 5-HT_{3B} subunit (nucleotides 55–1393, GenBank accession no. AF080582) were cloned into pCDM8 plasmid vectors (Davies et al., 1999). Transfection was performed using an electroporation system (Biotechnologies and Experimental Research, San Diego, CA, USA). HEK 293 cells were transfected with plasmids containing cDNA for the human 5-HT_{3A} subunit or cotransfected with cDNAs for the 5-HT_{3A} and 5-HT_{3B} receptor subunits, respectively. A plasmid encoding the cDNA for green fluorescent protein (GFP), as an expression marker, was cotransfected.

After harvesting from a 20 × 100-mm culture dish, the cells were resuspended in a buffer (2 ml) used for transfection (distilled H_2O containing—in mM—50 K_2HPO_4 × 3 H_2O, 20 K^+-acetate, 25 $MgSO_4$ × 7 H_2O, pH 7.35). Plasmids containing cDNAs for the 5-HT_3 receptor subunits (3 μg for each subunit) and for GFP (6 μg) were added to the cell suspension. Electroporation was performed at 350 V and 1 mF with a pulse time of 30–45 ms. Transfected cells were replaced in 10 × 35-mm culture dishes with supplemented medium and incubated (5% CO_2, 95% air, and 100% relative humidity, 37°C) for 18–24 h before the experiments. After the incubation period, 5–30% of the transfected cells expressed GFP, which is soluble in the cytoplasma, and more than 50% of the green fluorescing cells yielded 5-HT-induced inward currents. The kinetics of 5-HT_3 receptor-mediated currents in HEK 293 cells with cotransfected GFP were identical to those in preparations without GFP cDNA cotransfection. As shown by others (Davies et al., 1999; Dubin et al., 1999), 5-HT (100 μM) did not induce currents in cells transfected with cDNA encoding the 5-HT_{3B} receptor subunit alone.

Electrophysiology

5-HT_3 receptor-mediated currents were measured in GFP-positive cells using the whole-cell patch-clamp technique (Hamill et al., 1981) at room temperature (20–23°C). After forming the whole-cell configuration, the cells could easily be lifted from the bottom of the culture dish. The agonist-induced currents were recorded under voltage-clamp conditions (−40 mV holding potential, if not otherwise indicated) with an Axopatch 200B patch-clamp amplifier, digitized with a sampling rate of 20 kHz, using a digidata 1200 A/D converter, and low-pass filtered at a cutoff frequency of 2 kHz, performed with pClamp 6.0 software (all from Axon Instruments, Foster City, CA, USA).

For the patch-clamp recordings, the medium was replaced by a solution containing (in mM) 140 NaCl, 2.8 KCl, 10 HEPES, pH 7.2 adjusted with NaOH (extracellular solution). Patch pipettes were pulled from thin-walled borosilicate glass tubes with inner filament (outer diameter 1.5 mm, inner diameter 1.17 mm, GC150TF-10, Clark Electromedical Instruments, Pangbourne, Reading, UK) and heat-polished using a two-step horizontal puller (DMZ-Universal Puller, Zeitz-Instruments, Munich, Germany). The pipettes were coated with Sylgard (Dow Corning, Midland, MI, USA). Series resistance of 4–9 MΩ was obtained, when they were filled with a solution containing (in mM) 130 CsCl, 10 EGTA, 10 HEPES, 10 D-glucose, 2 $MgCl_2$, 2 $CaCl_2$, 2 K-ATP, 0.2 Tris GTP, pH 7.2 adjusted with CsOH (intracellular solution). Serial resistance was compensated by >75% by the patch-clamp amplifier. Nonspecific linear leak currents were negligible.

Rapid application and withdrawal of agonists

To mimic the kinetics of synaptic transmitter release, a piezo-driven system for fast exchange of solutions was used (Franke et al., 1987). This system (Fig. 1 *A*) applied the agonist via a liquid filament, i.e., a tiny jet of solution (0.3 ml/min flow rate), discharged from a borosilicate glass tube (inner diameter 150 μm) inside the recording chamber, which was perfused with extracellular solution (2 ml/min). The two solutions form a laminar flow system. The liquid filament consisted of extracellular solution containing indicated concentrations of the respective agonist. The recording chamber and glass tube were connected to a piezo-crystal-driven device (Minitranslator P-249.20, Physik Instrumente, Waldbronn, Germany) that, upon activation, shifted the tube upward by 20 μm to immerse the cell. Upon piezo inactivation, the tube shifted downward to the resting position. The diameter of the HEK 293 cells ranged from 10 to 15 μm, measured by an ocular micrometer. Thus, the cells were clearly smaller than the piezo-driven move. The device allows for a complete exchange of solutions in the vicinity of the cell, held in the whole-cell mode, within 1–5 ms. The time which is required for complete application of the liquid filament (containing the agonist) is, at most, 1.5 ms. This was measured by activation of $GABA_A$ receptors by 1 mM GABA ($\alpha_1\beta_2\gamma_2$ subunit assembly, expressed in HEK 293 cells, whole-cell configuration; for details, see Hapfelmeier et al., 2001). The rise time of the GABA-induced inward current was 1.5 ms (one of 20 representative experiments). The time required for complete withdrawal of the liquid filament is, at most, 4 ms. This was measured by the piezo-driven switch from the liquid filament (GABA solution) to buffer containing the noncompetitive $GABA_A$ receptor blocker picrotoxin (Gurley et al., 1995). The switch to picrotoxin (1 mM) considerably accelerated the decay of the GABA response, revealing a time constant of 4 ms (one of four representative experiments, Fig. 1 *B*). The solution exchange might be faster than the measured times, but cannot be slower. The 10–90% rise and decay times of open pipette responses to this exchange of solutions were below 0.1 ms (see also Heckmann et al., 1996).

Sources of chemicals

5-HT was purchased from Sigma Chemicals (Deisenhofen, Germany), the selective 5-HT_3 receptor agonist m-chlorophenylbiguanide (mCPBG) (Kilpatrick et al., 1990) and the selective 5-HT_3 receptor antagonist [*n*-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4 benzo-xazine-8-carboxamide hydrochloride] (Y25130) (Sato et al., 1992) from TOCRIS (Bristol, UK). The compounds were dissolved in extracellular solution.

Data analysis

Peak current and time to peak (10–90% rise time) were measured using automated detection algorithms (AxoGraph software for MacOS). Receptor desensitization was measured as the decay of the agonist-evoked response in the continued presence of agonist. Receptor deactivation was measured as the decay of the response after the rapid withdrawal of the agonist. The decaying phases of the currents were fitted with AxoGraph software. Biexponential fitting was performed when the monoexponential fit was apparently inadequate. EC_{50} and Hill coefficient were calculated with SigmaPlot 5.00. Data are presented as mean ± SD with the number of experiments indicated.

Computer simulations

Simulations of kinetic schemes and sets of rate constants were performed with a program for macroscopic current modeling (BIOQ-Biochemical Equations software) kindly provided by Prof. Hanna Parnas (Parnas and Parnas

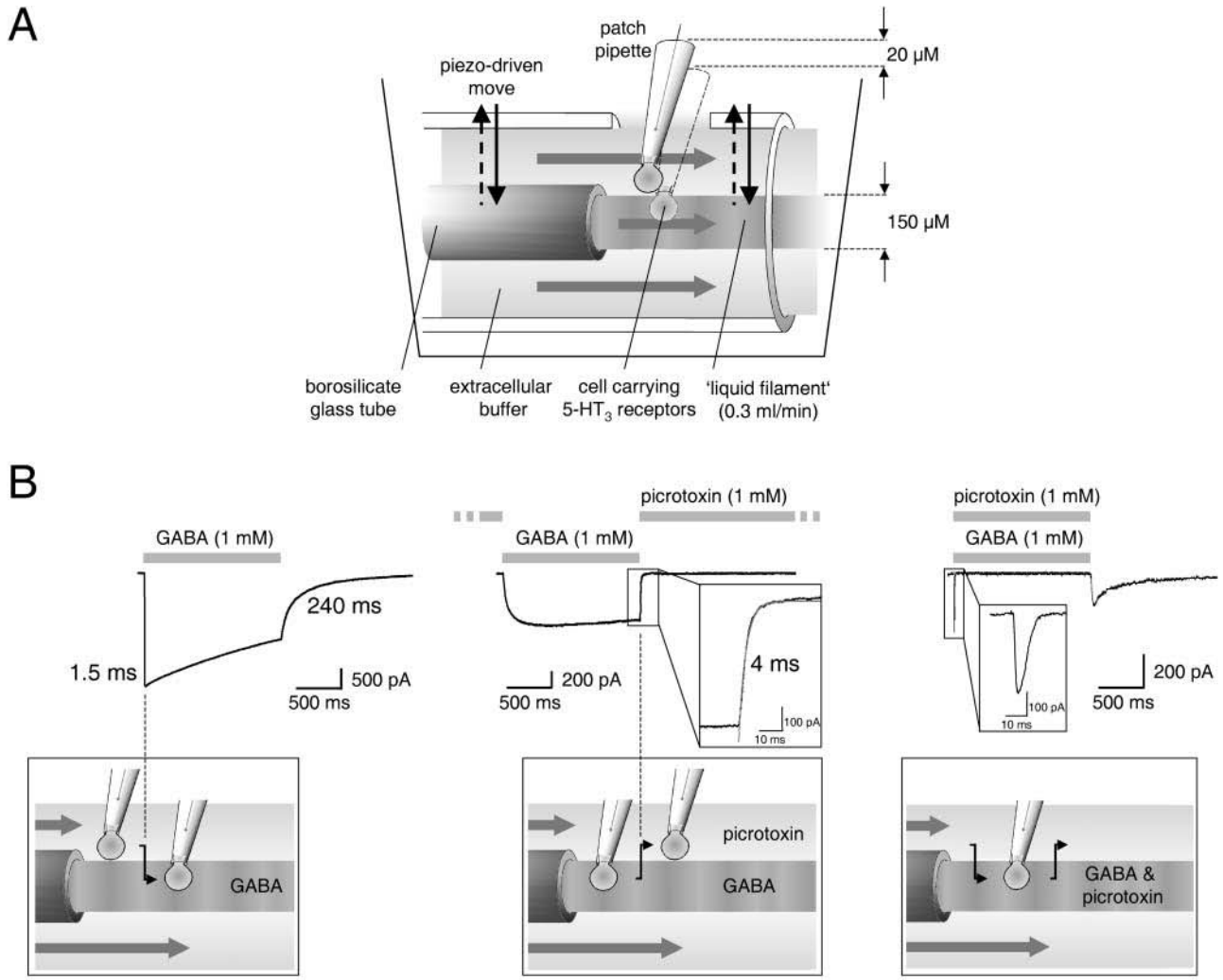

FIGURE 1 (*A*) The ''fast-switch'' system. To mimic the kinetics of synaptic release, a system for rapid exchange of solutions was used. The ''liquid filament'' consists of extracellular solution containing the agonist. Upon piezo-crystal activation, it shifts upward by 20 μm and the whole-cell patch carrying the 5-HT_3 receptors is immersed. Upon piezo inactivation, the liquid filament shifts downward to the resting position. This device allows for a complete exchange of solutions in the vicinity of the cell within 1–5 ms. (*B*) The time required for complete application of the liquid filament is, at most, 1.5 ms: activation of $GABA_A$ receptors by 1 mM GABA reveals a rise time of the GABA-induced current of 1.5 ms (*left*). The time required for withdrawal of the liquid filament is, at most, 4 ms: the piezo-driven switch from the liquid filament (GABA solution) to buffer containing the noncompetitive $GABA_A$ receptor blocker picrotoxin (1 mM) considerably accelerates the decay of the GABA response, revealing a time constant of 4 ms (*mid*). When GABA and picrotoxin are co-applied, the GABA response is rapidly reduced. On rapid withdrawal of the liquid filament, the unblocking of picrotoxin evokes a tail current (*right*).

Neurobiology Lab, Hebrew University, Jerusalem, Israel). This software, for solving a set of differential equations, is a Windows-compatible update based on a UNIX-compatible program for an analytic description of synaptic currents (http://www.ls.huji.ac.il/~parnas/Bioq/bioq.html).

Our experiments were designed to emphasize the 5-HT_3 receptor kinetics of activation, desensitization, and deactivation. The agonist-evoked responses were simulated with kinetic schemes, which are based on the three-state-kinetic-model theory first put forward by del Castillo and Katz (1957). The configuration of the kinetic scheme and the set of rate constants were designed to match simulated and measured responses under the varied experimental conditions.

RESULTS

It has previously been shown that homomeric 5-HT_{3A} and heteromeric 5-HT_{3AB} receptors expressed in HEK 293 cells are equally permeable for Na^+ and K^+, but that 5-HT_{3AB} receptors are less permeable for Ca^{2+} than 5-HT_{3A} receptors (Davies et al., 1999). It is commonly accepted that an increase in extracellular calcium and/or magnesium decreases the current response and accelerates the desensitization of 5-HT_3 receptors (Brown et al., 1998; Maricq et al., 1991; Mochizuki et al., 1999; Yakel, 1992). The accelerating effect of calcium ions on the desensitization of human homomeric 5-HT_{3A} receptors has been reported recently (Lobitz et al., 2001). To eliminate 1), the influence of extracellular calcium and magnesium ions on desensitization and 2), the differences in calcium permeability of homomeric and heteromeric 5-HT_3 receptors, we performed our experiments in calcium- and magnesium-free extracellular solutions, as in

previous studies with heterologously expressed 5-HT$_3$ receptors (Lankiewicz et al., 1998; Lobitz et al., 2001).

Kinetics of 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptors activated by 5-HT

To obtain a kinetic model which simulates the agonist-induced whole-cell currents resulting from the respective application protocol, we designed reaction schemes with sets of rate constants compatible with the present results. The constraints for the chosen rate constants were given by the experimental data, e.g., dose-response curves, time constants of activation, desensitization, and deactivation (see Table 1). Consequently, the kinetic model results in matching the data obtained from the sets of experiments described below.

The agonist was applied via the fast-switch system (Fig. 1 *A*) to cells carrying 5-HT$_{3A}$ or 5-HT$_{3AB}$ receptors. Fig. 2 *A* shows an inward current through 5-HT$_{3A}$ receptor channels evoked by 5-HT (10 μM). After reaching a peak, the current decayed in the presence of 5-HT, revealing two time constants. Conspicuously, the rapid withdrawal of 5-HT induced a transient increase in the current (*inset*). Such tail currents are typical for the offset of an open-channel block. Pursuant to this, the possible kinetic scheme that best simulated both the biphasic decay of the current (desensitization), and the slow-deactivating tail current, embodies a high-affinity and a low-affinity open-channel block by the agonist as the relevant mechanism of 5-HT$_{3A}$ receptor desensitization (Fig. 2, *B* and *C*).

Fig. 2 *D* depicts a representative whole-cell current evoked by 5-HT (30 μM) via activation of heteromeric 5-HT$_{3AB}$ receptors. In contrast to homomeric 5-HT$_{3A}$ receptors, the 5-HT$_{3AB}$ receptor current desensitized with only one time constant, and the rapid withdrawal of 5-HT from the 5-HT$_{3AB}$ receptors did not induce any tail current. The current rather deactivated rapidly on discontinuation of 5-HT (*inset*). The possible kinetic scheme that reconciles these findings most suitably embodies classical receptor desensitization; i.e., isomerization from an active to an inactive state not requiring any additional binding steps (Fig. 2 *E*). Fig. 2 *F* depicts a simulated 5-HT$_{3AB}$ receptor current that was generated using the provided model (Fig. 2 *E*) with the respective concentration of 5-HT imposed.

Dose-response relationship for receptor activation by 5-HT

Fig. 3 *A* shows 5-HT$_{3A}$ receptor whole-cell currents evoked by stepwise increased 5-HT concentrations. The dose-response relationship for amplitude (EC_{50} 3 $\pm$ 0.6 μM; Hill 1.8 $\pm$ 0.3) and kinetics (10–90% rise time, desensitization, and time course of tail currents) of the currents was best simulated (Fig. 3, *B* and *C*) by the 5-HT$_{3A}$ receptor model provided in Fig. 2 *B*. A mismatch between experiment and simulation was only seen in desensitization kinetics of the current induced by 30 μM 5-HT (see Fig. 3, *A* and *C*, bottom). The model also predicted that channel gating

TABLE 1 Parameters and rate constants with source of their experimental constraints

Parameter	Value	Source of experimental constraint	Figure
5-HT$_{3A}$ receptor			
Binding steps	2	Dose-response curve (Hill coefficient 1.8)	3 *B* (*top*)
Blocking steps	2	Biphasic desensitization, kinetics of tail currents	2 *A*
K_{on} (M^{-1} s^{-1})	2–4 $\times$ 10^6	Dose-response relationship of peak and rise times	3 *B*
K_{off} (s^{-1})	50–70	Rise times, current decay after short agonist pulses	3 *B*, 7 *A*
$K_{on}b_h$ (M^{-1} s^{-1})	1–1.5 $\times$ 10^6	Desensitization (kinetics, degree, dose-dependence)	3 *A*
$K_{off}b_h$ (s^{-1})	0.15–0.25	Tail currents (amplitude, kinetics of decay)	2 *A*, 3 *A*
		Time required for recovery from desensitization	5 *A*
$K_{on}b_l$ (M^{-1} s^{-1})	3–5 $\times$ 10^5	Desensitization (kinetics, degree, dose-dependence)	3 *A*
$K_{off}b_l$ (s^{-1})	4–8	Tail currents (amplitude, kinetics of rise)	
α (s^{-1})	50–500	Implies a mean single-channel open duration of 2–20 ms	2 *A*, 3 *A*
β (s^{-1})	25–35 $\times$ α	Peak amplitudes, rise times	3*A*, 3*B*
		Kinetics and amplitudes of tail currents	3 *A*
5-HT$_{3AB}$ receptor			
Binding steps	1	Dose-response curve (Hill coefficient 0.9)	4 *B* (*top*)
K_{on} (M^{-1} s^{-1})	0.5–2 $\times$ 10^6	Dose-response relationship of peak and rise times	4 *A*, 4 *B*
K_{off} (s^{-1})	10–20	Kinetics of receptor deactivation, peaks, rise times	4 *A*, 4 *B*, 7 *C*
d_{+1} (s^{-1})	8–15	Desensitization (kinetics, degree)	2 *D*, 4 *A*
d_{-1} (s^{-1})	0.1–0.2	Desensitization (kinetics, degree)	2 *D*, 4 *A*
d_{+2} (s^{-1})	0.01 or less	Kinetics of receptor resensitization	6 *A*
d_{-2} (s^{-1})	0.2–0.3	Time required for receptor resensitization	6 *A*
α (s^{-1})	50–500	Implies a mean single-channel open duration of 2–20 ms	
β (s^{-1})	3–4 $\times$ α	Peak amplitudes, rise times	4 *A*, 4 *B*
		Kinetics of receptor deactivation	4 *B* (*bottom*), 7 *C*

Parameters and rate constants of the models were constrained by the experimental data presented in the figures. The value provided for the rate α refers to single-channel data from the literature (e.g., Davies et al., 1999) and own unpublished observations of single-channel events of 5-HT$_{3AB}$ receptors.

requires two agonist binding steps to obtain a (simulated) Hill coefficient of 1.8. A corresponding model without binding steps for channel block (no receptor desensitization; data not shown) yielded a Hill coefficient of 1.95.

The responses evoked by 10 μM 5-HT applied to outside-out patches carrying homomeric 5-HT$_{3A}$ receptors also showed tail currents (Fig. 3 *A*, *inset*). Due to fluctuations, these tail currents were, in part, less prominent compared to the whole-cell responses. Nevertheless, single-channel simulations performed with the same kinetic model and the same set of rate constants (see Fig. 2 *B*) covered this finding. The simulated responses of 1000 channels (Fig. 3 *C*, *inset*) also involved fluctuations and were very similar to the outside-out currents.

Fig. 4 *A* depicts whole-cell currents evoked by 5-HT via activation of heteromeric 5-HT$_{3AB}$ receptors. Here, no tail currents were induced by the withdrawal of 5-HT (tested up to 100 mM; data are shown up to 1 mM). These currents (EC_{50} 25 $\pm$ 4 μM; Hill 0.9 $\pm$ 0.1) were best simulated (Fig. 4, *B* and *C*) by the 5-HT$_{3AB}$ receptor model (provided in Fig. 2 *E*) which requires, in contrast to the 5-HT$_{3A}$ receptor model (see Fig. 2 *B*), only one agonist binding step for channel gating. This 5-HT$_{3AB}$ receptor model also predicted the experimental finding that the time constant of receptor deactivation will moderately increase with increasing agonist concentrations (Fig. 4 *B*, *bottom*).

Kinetics of recovery from receptor desensitization

Repetitive pulses of 5-HT (10 μM) were used to monitor the time course of receptor resensitization (Figs. 5 and 6). Recovery of the 5-HT$_{3A}$ receptor response to >50% and >95% required 7 s and 25 s, respectively (Fig. 5 *A*), closely

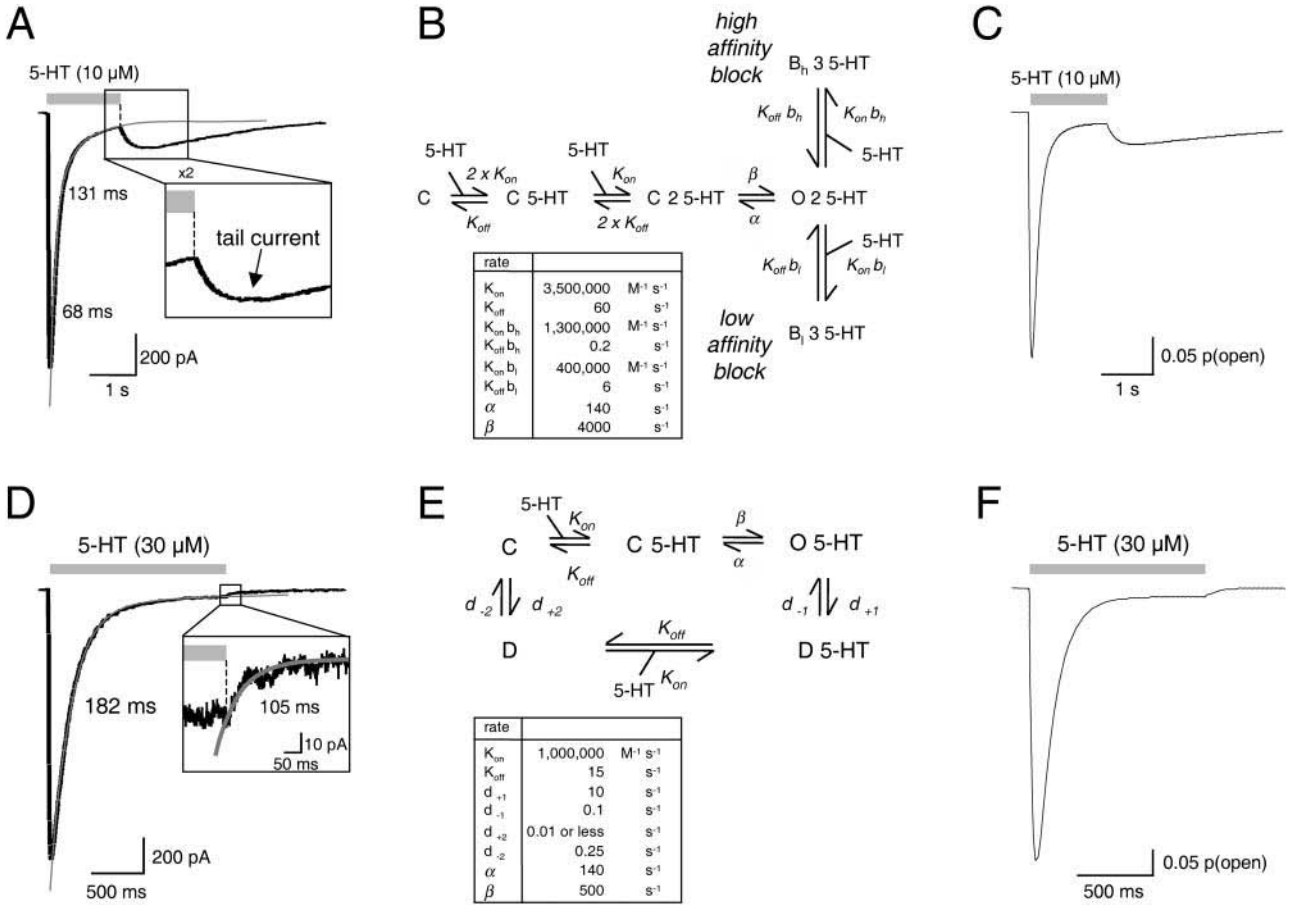

FIGURE 2 (*A*) 5-HT$_{3A}$ receptor response. Application (for 1.5 s, *gray bar*) of 5-HT (10 μM) to a HEK 293 cell carrying 5-HT$_{3A}$ receptors induced an inward current that desensitized, revealing two time constants. The rapid withdrawal of 5-HT (*dashed line*) induced a tail current (*inset*). (*B*) The kinetic model for the 5-HT$_{3A}$ receptor embodies closed *C*, open *O*, and blocked *B* states of the receptor channel. Both the biphasic desensitization and the slow-deactivating tail current were best simulated by a model embodying a high-affinity and a low-affinity open-channel-block state B_h and B_l. (*C*) The simulated current is a plot of the probability of the open state *O* over time. (*D*) A representative 5-HT$_{3AB}$ receptor current, induced by 5-HT (30 μM), desensitized with only one time constant. On the withdrawal of 5-HT, no tail current was induced, and the current rapidly deactivated with a time constant of 105 ms (*inset*). (*E*) The kinetic model for the 5-HT$_{3AB}$ receptor, which best reconciles the experimental data, embodies one agonist binding step and classical receptor desensitization. (*F*) The simulated current matches the measured current.

matching the simulated currents (Fig. 5 *B*). When the repetitive 5-HT application to 5-HT$_{3A}$ receptors coincided with the tail current (re-opened channels), conspicuously, the tail current decayed with a time course similar to the decay of the initial response (Fig. 5 *A*, *top trace*, *inset*). This feature of the current was also predicted by the model (Fig. 5 *B*, *top*) and is well reconciled with the 5-HT-induced channel block. Kinetic schemes that do not embody such an agonist-induced block could not simulate this response (not shown). Within the given model, the rate constant $K_{off}b_h$ (0.2 s^{-1}) defines the 95%-recovery time of 25 s. Fig. 5 *B* (*bottom*) additionally depicts the simulated time course of recovery of the resting state *C* of the receptor.

The recovery of the 5-HT$_{3AB}$ receptor response to >50% (>95%) required 3 s (12 s) (Fig. 6 *A*). Obviously, heteromeric 5-HT$_{3AB}$ receptors resensitized substantially faster than homomeric 5-HT$_{3A}$ receptors, a finding well-predicted by the 5-HT$_{3AB}$ receptor model (Fig. 6 *B*).

Decrease in agonist application time

In the experiments with homomeric 5-HT$_{3A}$ receptors, a stepwise decrease in application time diminished the resulting tail current and accelerated the deactivation phase of the currents (Fig. 7, *A* and *B*). The model also predicted that the current induced by exposure to 5-HT for only 20 ms decayed without any preceding tail current (Fig. 7, *A* and *B*, *bottom*). Under these conditions, the closing of the 5-HT$_{3A}$ receptor channels might occur, due to receptor deactivation, before 5-HT could block the opened channels (open-channel

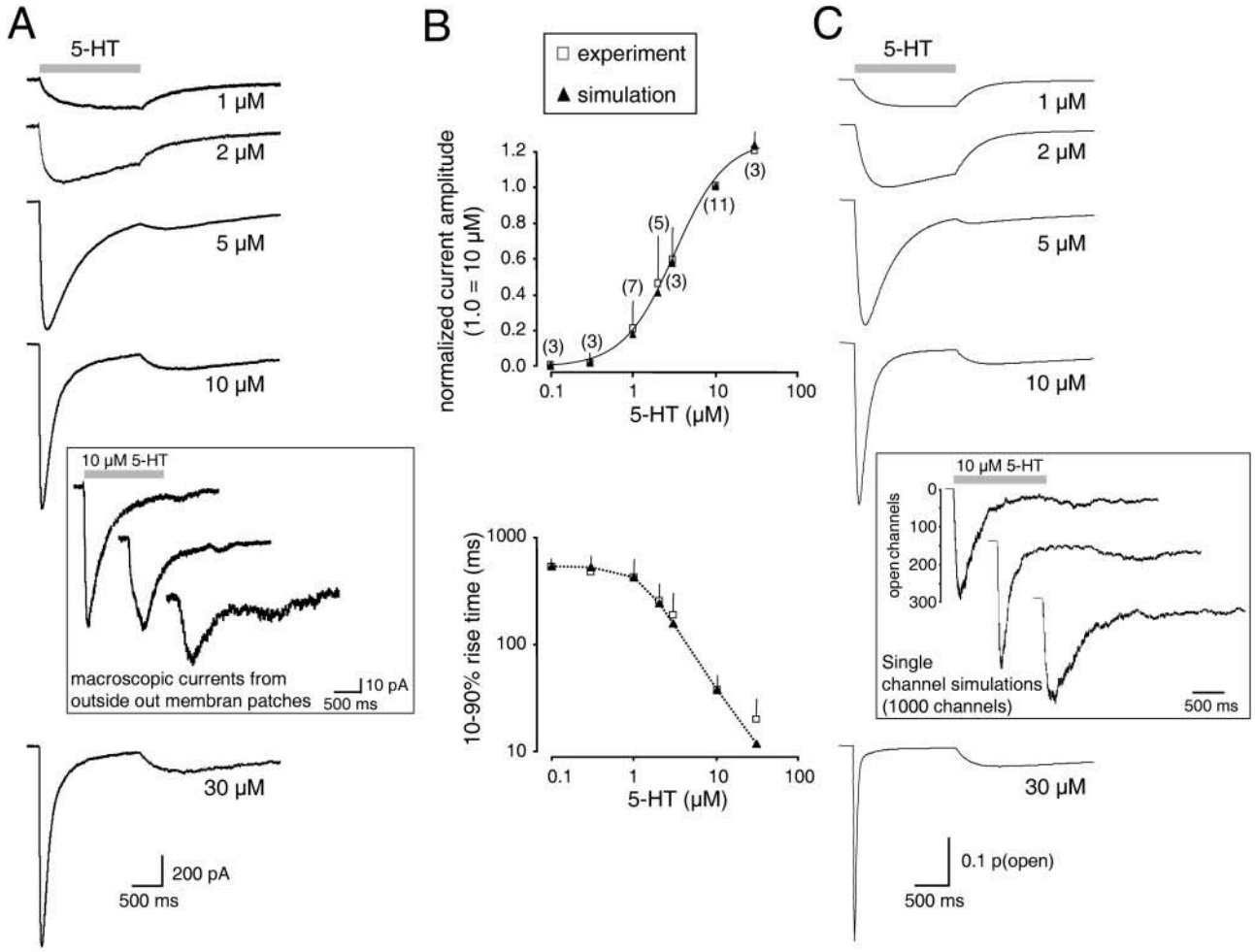

FIGURE 3 Dose-response relationship for 5-HT$_{3A}$ receptor activation by 5-HT. (*A*) Whole-cell currents, induced by stepwise increased concentrations of 5-HT. (*B*) Pooled data of the dose-response relationship for the current amplitude (*top*) and the 10–90% rise time (*bottom*). The measured data (*open squares*, number of experiments in brackets, mean ± SD) are mimicked by the simulated data (*filled triangles*). For receptor activation, the EC_{50} of 5-HT was 3 ± 0.6 μM. The Hill coefficient of 1.8 ± 0.3 suggests two agonist binding steps required for channel gating. (*C*) Corresponding simulated currents generated by the 5-HT$_{3A}$ receptor model (see Fig. 2 *B*) with imposed agonist concentrations. (*A*, *inset*) The responses recorded from outside-out patches also involved tail currents. Due to fluctuations, these tail currents were, in part, less prominent compared to the whole-cell responses. (*C*, *inset*) The single-channel simulations performed with the same kinetic model covered this finding. The simulated responses of 1000 channels were very similar to the outside-out currents.

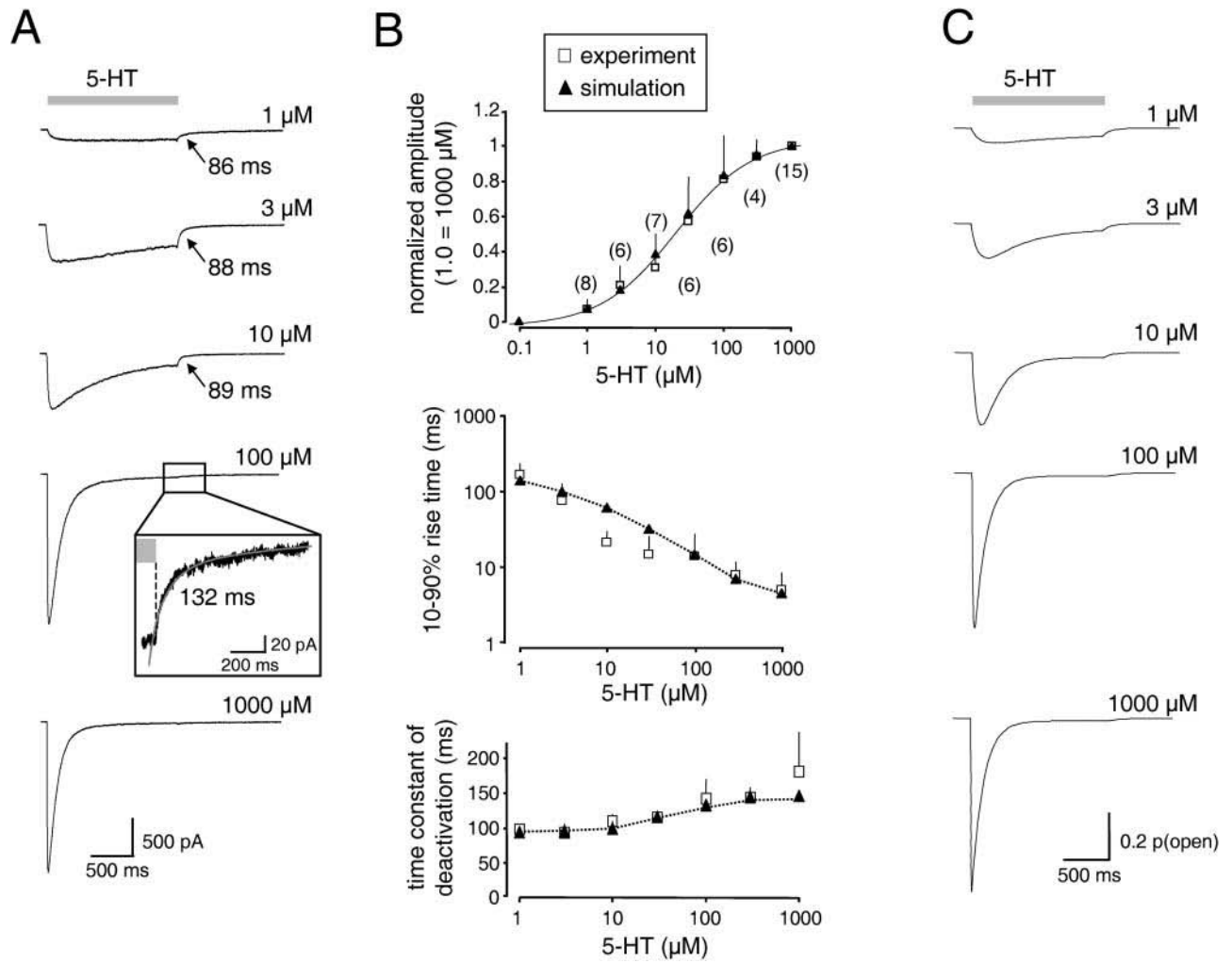

FIGURE 4 Dose-response relationship for 5-HT_{3AB} receptor activation by 5-HT. (*A*) Whole-cell currents, induced by different concentrations of 5-HT. (*B*) Pooled data of the dose-response relationship for the current amplitude (*top*), the 10–90% rise time (*middle*), and the time constant of current deactivation (*bottom*). The measured data (*open squares*, number of experiments in brackets, mean ± SD) are matched by the simulated data (*filled triangles*). For receptor activation, the EC_{50} of 5-HT was 25 ± 4 μM. The Hill coefficient of 0.9 ± 0.1 suggests that only one agonist binding step is required for channel gating. (*C*) Corresponding simulated currents generated by the 5-HT_{3AB} receptor model (see Fig. 2 *E*).

block). The 5-HT_{3A} receptor response decreased in amplitude (Fig. 7, *A* and *B*, *bottom*), because the application time (20 ms) was shorter than the time required to reach the peak current (10–90% rise time: 38 ± 13 ms with 10 μM 5-HT).

In the experiments with heteromeric 5-HT_{3AB} receptors, a stepwise decrease in application time from 1500 ms to 100 ms decreased the fraction of current decay due to desensitization and increased the fraction due to deactivation (Fig. 7, *C* and *D*). In line with our 5-HT_{3AB} receptor model, the time constant of deactivation became shorter when the application time was decreased (Fig. 7, *C* and *D*). In agreement with others (Jones and Westbrook, 1995), this model predicts that receptors in a desensitized state can prolong agonist-induced responses. Obviously, this mechanism is even more effective when desensitization results from an agonist-induced channel block (see Fig. 7, *A* and *B*).

Current-voltage relationship for receptor activation by 5-HT

The I-V curve of 5-HT_{3A} receptor responses showed a slight inward rectification (Fig. 8 *A*). However, the holding potential did not influence the kinetics of the 5-HT-induced currents. The induction of the tail currents was voltage-independent suggesting that 5-HT induced a voltage-independent open-channel block at the homomeric 5-HT_{3A} receptors. The selective 5-HT_3 receptor antagonist Y25130 (10 nM) reduced the 5-HT_{3A} receptor currents (Fig. 8, *B*). A complete block of the currents was achieved by 300 nM Y25130 (not shown).

The I-V curve determined for the heteromeric 5-HT_{3AB} receptors (Fig. 8 *C*) also showed a slight inward rectification (seen at >30 mV). As in homomeric 5-HT_{3A}

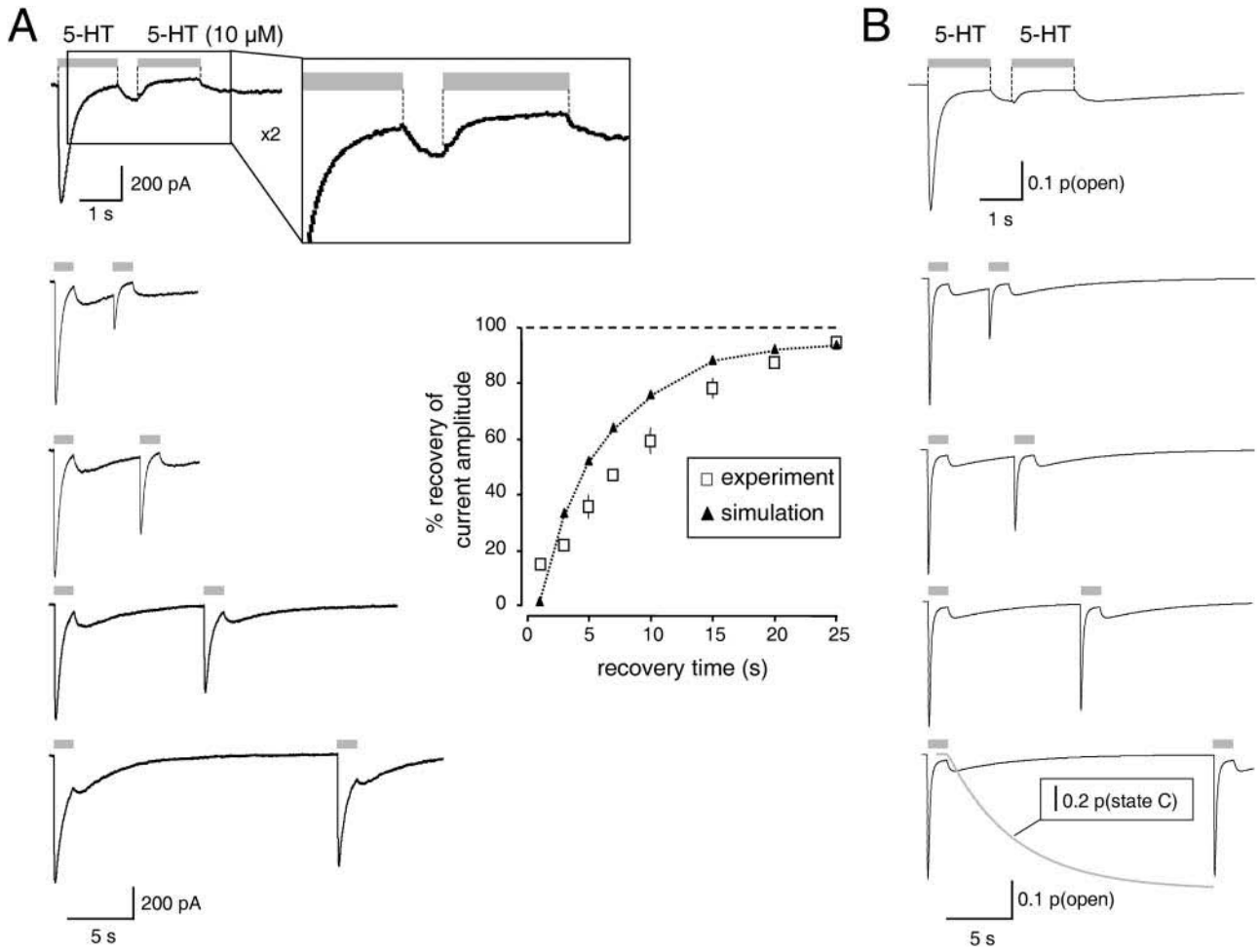

FIGURE 5 Kinetics of 5-HT$_{3A}$ receptor resensitization. Repetitive pulses (*gray bars*) of 5-HT (10 μM) were applied. (*A*) Current traces from a representative cell. (*B*) Simulated currents (model provided in Fig. 2 *B*). Recovery of the response to >50% (>95%) required 7 s (25 s, mean ± SD, $n = 3$). An interval of 0.5 s between the two agonist pulses resulted in an overlap of the second pulse and the tail current (*A, top, inset*) resulting in a current decay within the tail current. This effect, which is reconciled with an agonist-induced open-channel block, was also predicted by the model (*B, top*).

receptors, the kinetics of these currents were voltage-independent and the responses were reduced by Y25130 (10 nM) (Fig. 8 *D*).

Receptor activation by the specific 5-HT$_3$ receptor agonist mCPBG

Activation of homomeric 5-HT$_{3A}$ receptors by increasing concentrations of the agonist mCPBG resulted in a bell-shaped dose-response curve. The withdrawal of mCPBG (>3 μM) evoked a prominent tail current (Fig. 9 *A*). Receptor resensitization to repetitive mCPBG pulses was rather slow. The recovery of the response to >50% (>95%) required 20 s (50 s) (Fig. 9 *B*).

Also the heteromeric 5-HT$_{3AB}$ receptors were sensitive to mCPBG (Fig. 10 *A*). In contrast to 5-HT, a tail current was evoked by high concentrations of mCPBG (100 μM) (Fig. 10 *A*, *bottom*). This finding suggests that mCPBG can induce an open-channel block also at 5-HT$_{3AB}$ receptors. The induction of the tail currents was voltage-independent and occurred without rectification (Fig. 10 *B*).

DISCUSSION

We compared the different activation and inactivation kinetics of homomeric and heteromeric human 5-HT$_3$ receptors expressed in HEK 293 cells. The time constants of 5-HT$_3$ receptor activation and desensitization and the EC$_{50}$ and Hill values obtained resemble those published previously (for review, see Reeves and Lummis, 2002). The differences between native and heterologously expressed homomeric 5-HT$_3$ receptors were attributed to a factor associated to native receptors (Hooft et al., 1997) until the 5-HT$_{3B}$ receptor subunit was identified and cloned (Davies et al., 1999).

5-HT$_{3A}$ receptor desensitization

Based on the main finding that rapid withdrawal of 5-HT from 5-HT$_{3A}$ receptors evoked tail currents, we designed a

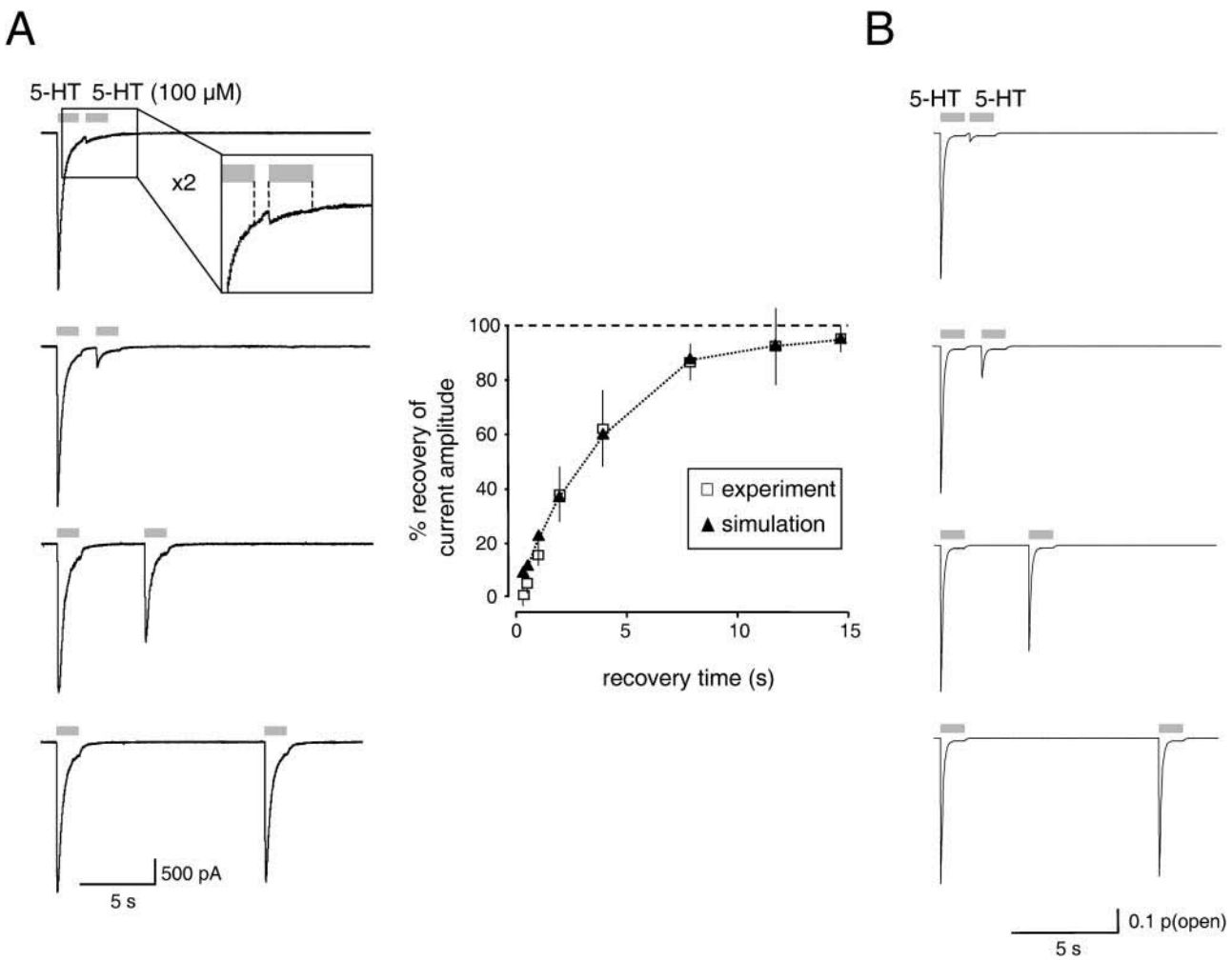

FIGURE 6 Kinetics of 5-HT_{3AB} receptor resensitization. Repetitive pulses (*gray bars*) of 5-HT (100 μM) were applied. (*A*) Current traces from a representative cell. (*B*) Simulated currents (5-HT_{3AB} receptor model, see Fig. 2 *E*). Recovery of the response to >50% (>95%) required 3 s (12 s, mean ± SD, $n = 5$). After shorter intervals between the agonist pulses, a small current with kinetics similar to those of the initial response was induced by the second pulse (see also *inset*). This effect is reconciled with classical receptor desensitization and was also predicted by the model.

model for receptor desensitization due to an agonist-induced open-channel block. We attributed the double-exponential time course of desensitization to the overlapping of a low-affinity and a high-affinity block (see Fig. 2, *A–C*).

The 5-HT_{3A} receptor currents depicted in various recent papers (Lankiewicz et al., 1998; Brown et al., 1998; Davies et al., 1999; Dubin et al., 1999, Gunthorpe and Lummis, 2001) most commonly do not display tail currents on agonist discontinuation. Instead, a rather slow receptor deactivation is typically seen in these recordings. The most parsimonious explanation for this finding is that the slow withdrawal of the agonist by the devices employed for application suppresses the induction of a perspicuous tail current. Mott et al. (2001) employed a rapid switching system to apply agonists to murine homomeric 5-HT_{3A} receptors. The authors observed deactivation kinetics similar to or slower than desensitization, which is also in line with our findings. In this study tail currents are not reported, but it is conspicuous to us that the time course of whole-cell currents after a prolonged agonist application is not depicted in detail. The authors provide a rather complex model for receptor desensitization to match their data. Our aim was to suggest a simplified model including a self-block by the agonist to match the characteristic time course of the responses of human homomeric 5-HT_{3A} receptors.

The fast-switch system (Fig. 1 *A*) employed in the present study allows the discernment of tail currents as a kinetic property of ligand-gated ion channels featuring the offset of an open-channel block (Hapfelmeier et al., 2001). 5-HT_{3A} receptor-associated tail currents have apparently been recorded, but have not been detailed any further (Lankiewicz et al., 1998; Dubin et al., 1999). These findings also involved ion-selectivity-converted 5-HT_{3A} receptors (Gunthorpe and Lummis, 2001). In a previous study, Gunthorpe and Lummis (1999) attributed the reappearance of the inward current (which we termed tail current) through murine 5-HT_{3A} re-

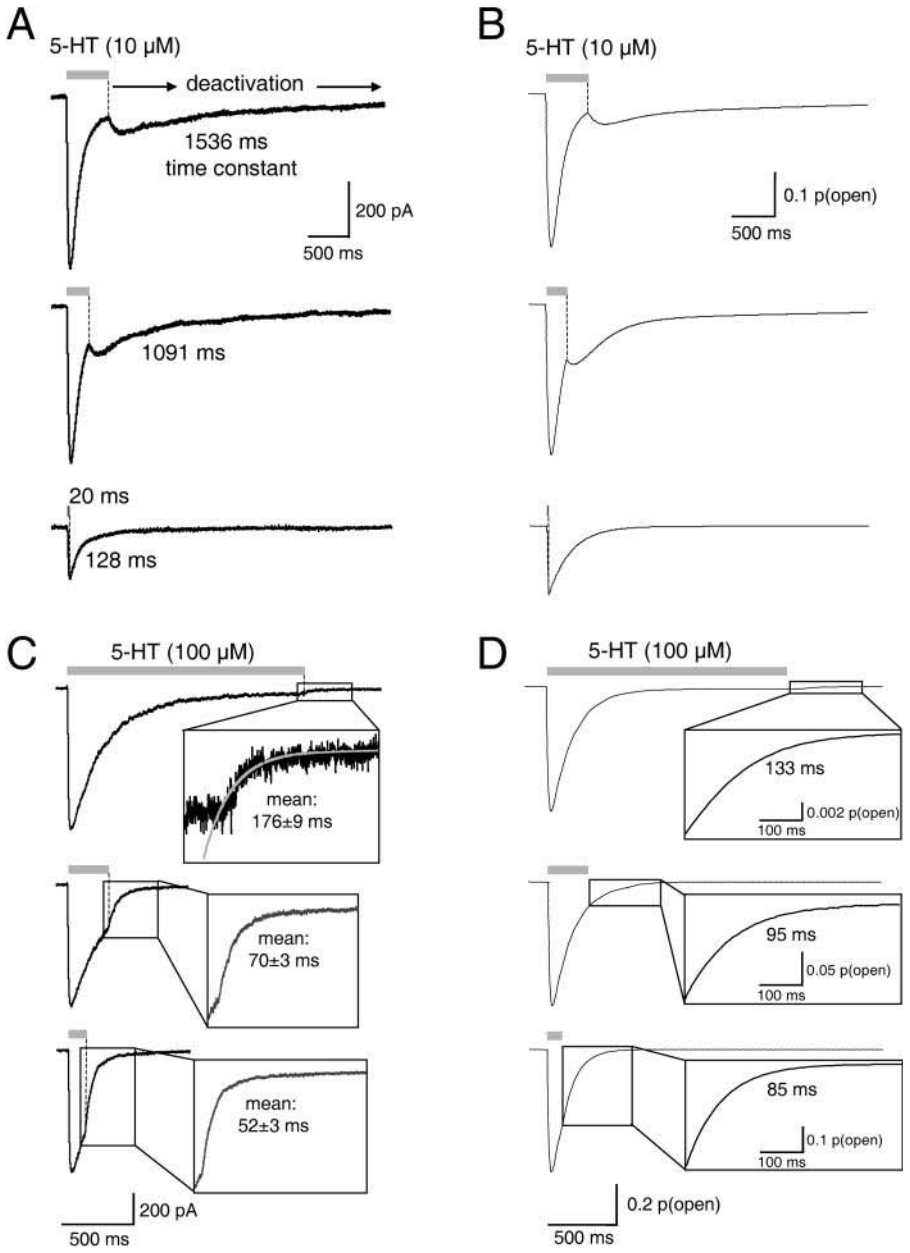

FIGURE 7 (*A*) 5-HT$_{3A}$ receptor whole-cell currents, evoked by 5-HT (10 μM) pulses of different duration (one representative of three cells). On the 5-HT pulse of 20 ms, the response was reduced and no tail current occurred (*bottom trace*). (*B*) Simulated currents. (*C*) 5-HT$_{3AB}$ receptor whole-cell currents, evoked by 5-HT pulses of different duration (one representative of five cells). Shortening the 5-HT pulse reduced the time constant of receptor deactivation (*insets*). (*D*) Simulated currents.

ceptors to the offset of an open-channel block by diltiazem, and provided a simple scheme for this mechanism. High concentrations of 5-hydroxyindole also block 5-HT$_3$ receptors and induce tail currents on washout, whereas low concentrations potentiate the 5-HT-induced currents (Kooyman et al., 1994). These findings are in line with our present data which suggest two distinct binding sites for 5-HT, which convey opposite effects on the 5-HT$_{3A}$ receptor. A binding site in the channel pore (involved in open-channel block) is suggested by the finding that mutations in putative channel lining domains of cloned 5-HT$_3$ receptors strongly determined the time course of desensitization, especially under calcium-free conditions (Yakel et al., 1993; Gunthorpe et al., 2000; Lobitz et al., 2001).

5-HT$_{3AB}$ receptor desensitization

The desensitization phase of 5-HT$_{3AB}$ receptor currents was not followed by a tail current, even at rather high 5-HT concentrations, excluding a channel block as the underlying mechanism for receptor desensitization. We used a reaction scheme based on classical receptor desensitization, a mechanism first put forward by Katz and Thesleff (1957), and emphasized also in recent studies (Jones and Westbrook, 1996; Sun et al., 2002). Our cyclic scheme is based on the finding that the rate d_{-1} has to be 0.1 s^{-1} or less to cover the kinetics of receptor desensitization. However, in a linear model, this value of d_{-1} would not allow for recovery from desensitization within ~12 s, as was found in the experi-

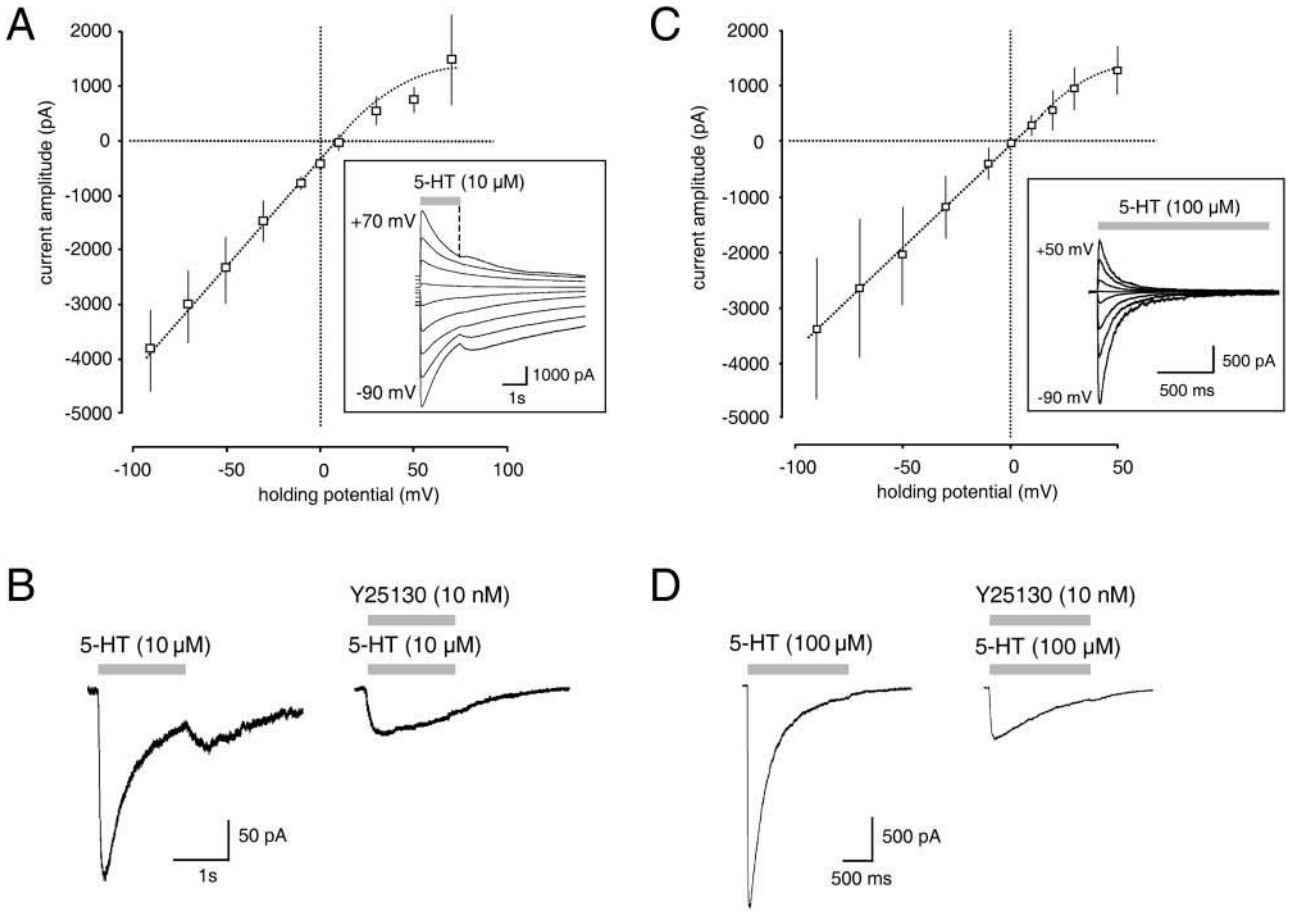

FIGURE 8 (*A*) The current-voltage plot of 5-HT_{3A} receptor currents induced by 5-HT reveals slight inward rectification (mean ± SD, $n = 3$). The inset shows a series of currents recorded at holding potentials from −90 to +70 mV. The induction of tail currents was voltage-independent. (*B*) The selective 5-HT_3 receptor antagonist Y25130 (10 nM) reduced the 5-HT_{3A} receptor-mediated current. (*C*) The current-voltage plot of 5-HT_{3AB} receptor currents induced by 5-HT also reveals slight inward rectification (mean ± SD, $n = 3$). The inset shows a series of currents, recorded from a representative cell at holding potentials from −90 to +50 mV. The kinetics of the 5-HT_{3AB} receptor currents were voltage-independent. (*D*) The selective 5-HT_3 receptor antagonist Y25130 (10 nM) reduced the 5-HT_{3AB} receptor-mediated current.

ments. Under these conditions, (simulated) resensitization would require more than 60 s. To account for faster resensitization, we must consider a cyclic model which provides an alternate, faster return path to the resting state (*C*) of the receptor. In this cyclic scheme, the rate constant d_{-2} ($0.25\ s^{-1}$) then results in the desired recovery time of 12 s. The value of the rate constant d_{+2} can hardly be determined by experiments, because this rate represents the desensitization of ''sleeping channels''. The fraction of desensitized to active receptors in the absence of the agonist (d_{+2}/d_{-2}) was suggested to be on the order of 0.1 or less (Dilger and Liu, 1992). We set the value of d_{+2} to $0.01\ s^{-1}$, because if d_{+2} considerably exceeds this value, the kinetics of resensitization (see Fig. 6) cannot be simulated well. The provided kinetic model (see Fig. 2 *E*) simulated the activation, desensitization, deactivation, and resensitization of the 5-HT_{3AB} receptor-mediated currents.

Our model also predicted that desensitized states can prolong receptor responses by slowing receptor deactivation (Jones and Westbrook, 1995; 1996). This effect would preferentially affect synaptic transmission at serotonergic synapses carrying homomeric 5-HT_{3A} receptors, which might desensitize due to an open-channel block and, thus, deactivate rather slowly (see Fig. 7 *A*). Recently, an enhancement of neuromuscular transmission in zebrafish has been attributed to an agonist-induced, open-channel block of acetylcholine receptors (Legendre et al., 2000).

The effect of mCPBG on 5-HT_3 receptors

Like 5-HT, the 5-HT_3 agonist mCPBG induced a concomitant open-channel block at homomeric 5-HT_{3A} receptors. The self-block by mCPBG even resulted in a bell-shaped dose-response curve for current amplitude. Furthermore, receptor resensitization under mCPBG was clearly slower than under the natural ligand, an effect which has been also reported for native mouse 5-HT_3 receptors (van Hooft and Vijverberg, 1996).

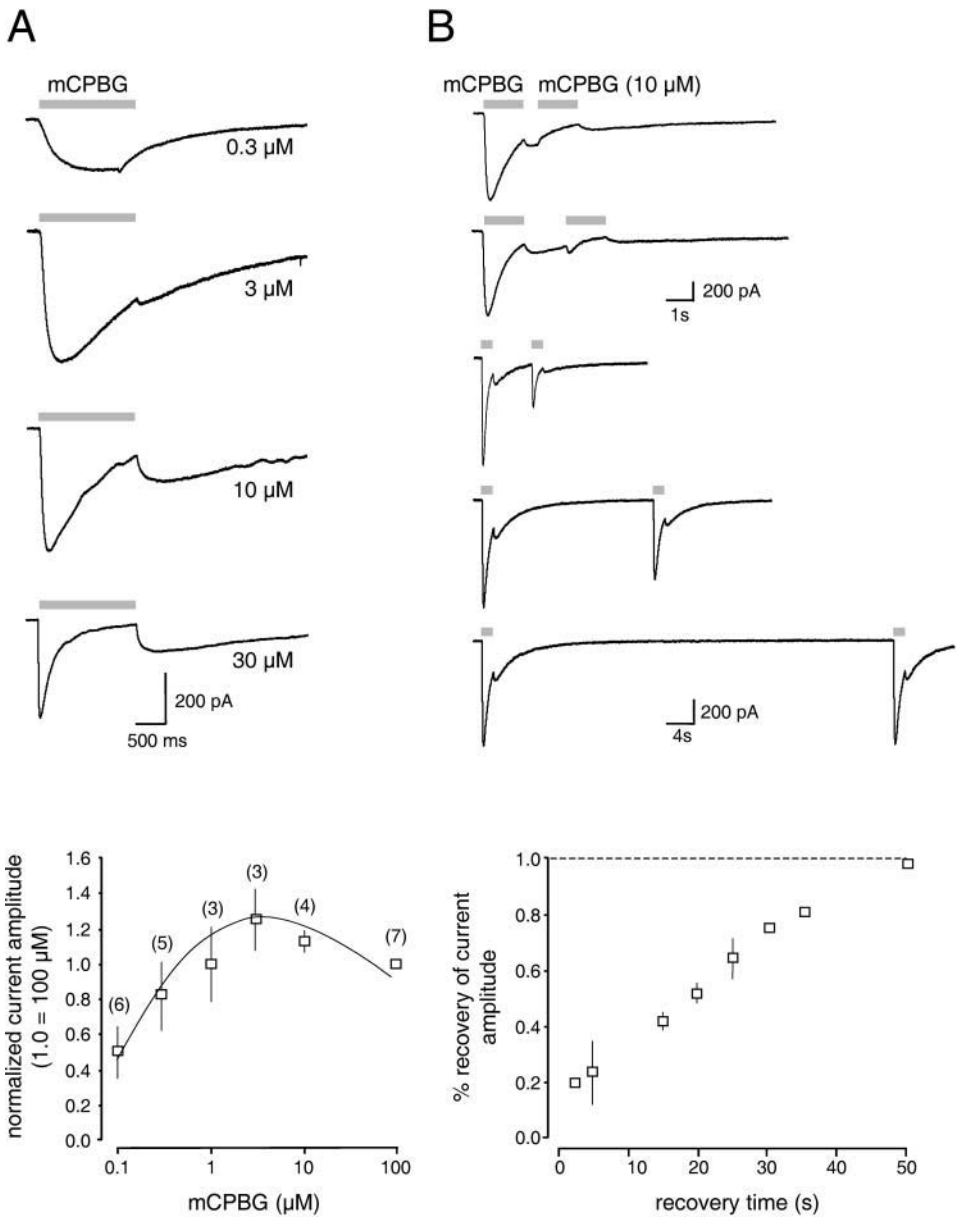

FIGURE 9 (*A*) The application (*gray bar*) of the selective 5-HT$_3$ agonist mCPBG to cells carrying homomeric 5-HT$_{3A}$ receptors evoked inward currents. The dose-response curve for mCPBG-induced receptor activation is bell-shaped (mean ± SD, number of experiments in brackets, *bottom*). The withdrawal of mCPBG (10 μM, 30 μM) induced prominent tail currents. (*B*) Repetitive pulses of mCPBG (10 μM) were used to monitor the time course of receptor resensitization. The data are given as mean ± SD ($n = 3$).

In the present study, mCPBG also acted as a potent agonist at the heteromeric 5-HT$_{3AB}$ receptors. Unexpectedly, mCPBG, but not 5-HT, also evoked tail currents at the 5-HT$_{3AB}$ receptors (see Fig. 10), suggesting that, in contrast to 5-HT, mCPBG can induce an open-channel block at these receptors. We conclude that the mechanisms of desensitization depend on both receptor subunit composition and ligand properties.

Does kinetic modeling help to unravel 5-HT$_3$ receptor-mediated signaling?

The sigmoid time course of the initial part of resensitization of homomeric 5-HT$_{3A}$ receptors (see Fig. 5), which resembles that reported for murine 5-HT$_3$ receptors (van Hooft and Vijverberg 1996), is an experimental finding not predicted by our kinetic model. A multistep mechanism for resensitization, suggested by van Hooft and Vijverberg (1996), or the introduction of dynamic rates instead of rate constants, would match these experimental data. Interestingly, Mott et al. (2001) report simple-exponential time course of murine 5-HT$_{3A}$ receptor resensitization, which would be in line with our 5-HT$_{3A}$ receptor model.

Some of our recordings showed double-exponential time course of receptor deactivation after brief agonist pulses. This was also seen by Mott et al. (2001). However, we surrendered to expand our model to match these findings in detail, because our aim was to provide simplified models to

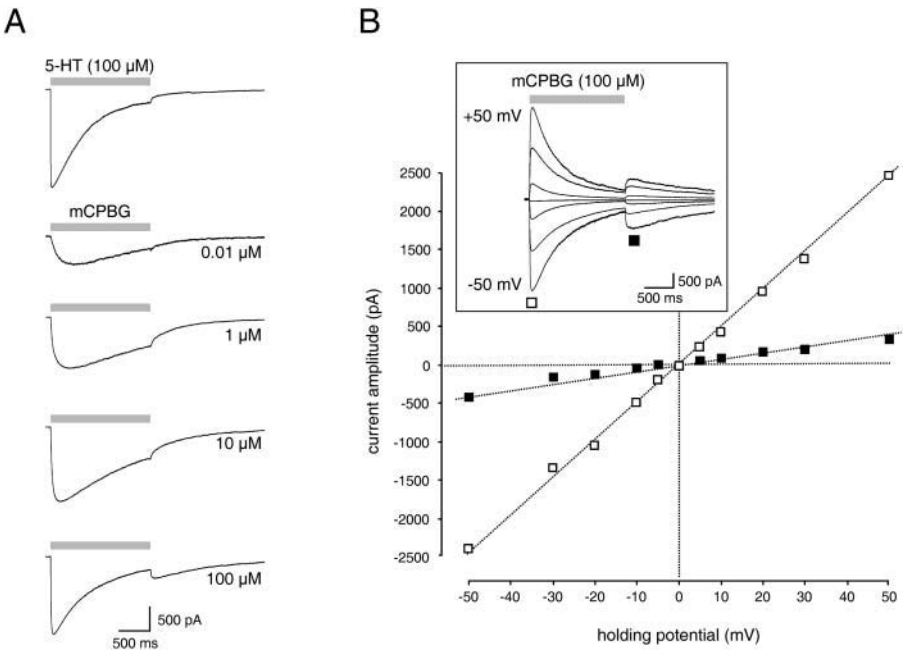

FIGURE 10 Application of mCPBG to cells carrying heteromeric 5-HT_{3AB} receptors. (*A*) 5-HT (100 μM) induced an inward current without tail current (*top*). mCPBG also induced inward currents. In contrast to 5-HT, the withdrawal of mCPBG (100 μM) induced a tail current (*bottom*). (*B*) The mCPBG-induced responses did not show any rectification.

focus on the different properties of homomeric and heteromeric 5-HT_3 receptors.

Data from various ligand-gated ion channels provide evidence for strikingly different patterns by which receptor-specific kinetics of desensitization can determine the size, time course, and frequency of synaptic transmission (for review, see Jones and Westbrook, 1996). The present study shows that homomeric and heteromeric 5-HT_3 receptors substantially differ in the mechanisms underlying desensitization, sensitivity for the endogenous agonist, and time required for resensitization. These findings might help to unravel the complexity of serotonergic synaptic transmission. Furthermore, our findings address the problem that the homopentameric 5-HT_3 receptor assemblies do not fully reproduce the biophysical characteristics of some native receptors (for review, see Fletcher and Barnes, 1998). In line with others (Mott et al., 2001), we found that, in homomeric receptors, the kinetics of deactivation are slower or similarly slow compared to the desensitization phase. In heteromeric receptors, the kinetics of deactivation are significantly faster than those of desensitization, a finding which is typically seen in native 5-HT_3 receptors (Zhou and Galligan, 1999). It also remains to be shown if the agonist-induced channel block could be relevant for the very small single-channel conductance values obtained in homopentameric receptors (for review, see Fletcher and Barnes, 1998). In heteromeric and native receptors, distinct single-channel events with relatively high conductances were recorded (Davies et al., 1999; Zhou and Galligan, 1999). Nevertheless, neither the homomeric nor the heteromeric assembly can be considered as the ''more nativelike'' receptor, since recent findings suggest the differential expression of both homomeric and heteromeric 5-HT_3 receptors in sensory neurons (Morales et al., 2001).

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