BJP British Journal of Pharmacology

DOI:10.1111/bph.12287
www.brjpharmacol.org

RESEARCH PAPER

A single channel mutation alters agonist efficacy at 5-HT$_3$A and 5-HT$_3$AB receptors

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Keywords
serotonin; ligand-gated ion channel; Cys-loop; heteromeric; binding; gating

Received
8 March 2013
Revised
27 May 2013
Accepted
14 June 2013

BACKGROUND AND PURPOSE

5-HT$_3$ receptors are composed of 5-HT$_3$A subunits (homomeric receptors), or combinations of 5-HT$_3$A and other 5-HT$_3$ receptor subunits (heteromeric receptors, the best studied of which are 5-HT$_3$AB receptors). Here we explore the effects of partial agonists at 5-HT$_3$A and 5-HT$_3$AB receptors, and the importance of a channel-lining residue in determining the efficacy of activation.

EXPERIMENTAL APPROACH

Wild type and mutant 5-HT$_3$A and 5-HT$_3$AB receptors were expressed in *Xenopus* oocytes and examined using two-electrode voltage-clamp, or expressed in HEK293 cells and examined using [^{3}H]granisetron binding.

KEY RESULTS

Dopamine, quipazine and VUF10166 were partial agonists at wild type 5-HT$_3$A and 5-HT$_3$AB receptors, with quipazine and VUF10166 causing a long-lived (>20 min) inhibition of subsequent agonist responses. At 5-HT$_3$A receptors, *m*CPBG was a partial agonist, but was a superagonist at 5-HT$_3$AB receptors, as it produced a response 2.6× greater than that of 5-HT. A T6′S substitution in the 5-HT$_3$A subunit decreased EC_{50} and increased R_{max} of dopamine and quipazine at both homomeric and heteromeric receptors. The greatest changes were seen with VUF10166 at 5-HT$_3$A$_{T6'S}$B receptors, where it became a full agonist (EC_{50} = 7 nM) with an EC_{50} 58-fold less than 5-HT (EC_{50} = 0.4 μM) and no longer caused inhibition of subsequent agonist responses.

CONCLUSIONS AND IMPLICATIONS

These results indicate that a mutation in the pore lining domain in both 5-HT$_3$A and 5-HT$_3$AB receptors alters the relative efficacy of a series of agonists, changing some (e.g. quipazine) from apparent antagonists to potent and efficacious agonists.

Abbreviations

5-HT, 5-hydroxytryptamine; GABA, g-aminobutyric acid; HEK, human embryonic kidney; mCPBG, m-chlorophenylbiguanide; nACh, nicotinic acetylcholine; PEIm, polyethylenimine; VUF10166, 2-chloro-(4-methylpiperazine-1-yl)quinoxaline

Introduction

5-HT$_3$ receptors belong to a family of membrane-spanning receptors that are responsible for fast synaptic neurotransmission in the peripheral and central nervous systems. Other family members include the nicotinic acetylcholine (nACh), GABA$_A$ and glycine receptors. These share a common structure consisting of five subunits that surround a central ion-conducting pore. The receptors contain an extracellular domain that binds ligand, a transmembrane domain that allows ion movements across the cell membrane and an intracellular domain that is responsible for receptor trafficking, modulation and channel conductance (Thompson *et al.*, 2010). The agonist binding site is located in the extracellular domain at the interface of two adjacent subunits, and is formed by residues located in three loops from one subunit

(termed principal or +) and three β–sheets from the adjacent subunit (complimentary or –). Binding of agonists to this site propagates a series of conformational changes that result in movements of channel-lining α-helices that open the pore (Bartos *et al.*, 2009; Cederholm *et al.*, 2009; Miller and Smart, 2012). Residues that line this pore are given a prime (′) notation to facilitate comparison between different subunits, with 0′ being a conserved charged residue at the intracellular end.

The 5-HT_3 receptor is expressed in both central and peripheral nervous systems and to date five different subunit types (5-HT_3A – 5-HT_3E) have been described (Jensen *et al.*, 2008; Barnes *et al.*, 2009; Walstab *et al.*, 2010). They participate in a range of physiological functions and have been implicated in depression, drug and alcohol abuse, rheumatic diseases, migraine and neurological phenomena such as anxiety, psychosis, nociception and cognitive function (Thompson and Lummis, 2007; Walstab *et al.*, 2010). The 5-HT_3A subunits can form homomeric receptors, but the other subunits must combine with 5-HT_3A in order to form functional heteromeric receptors. The 5-HT_3A and 5-HT_3AB receptors are the most thoroughly studied and have differing biophysical properties (Hapfelmeier *et al.*, 2003; Kelley *et al.*, 2003; Hales *et al.*, 2006; Hu and Peoples, 2008a). There are also differences in the potencies of non-competitive antagonists that are likely to result from different pore-lining amino-acids contributed by the different subunits (Das and Dillon, 2005; Hu and Peoples, 2008a; Thompson *et al.*, 2011a; 2012a; Baptista-Hon *et al.*, 2012). In contrast, agonists and competitive antagonists have similar affinities at 5-HT_3A and 5-HT_3AB receptors because both receptors contain the same binding interface, formed by adjacent 5-HT_3A receptor subunits (Brady *et al.*, 2001; Lochner and Lummis, 2010; Thompson *et al.*, 2011b; Thompson *et al.*, 2012b). An exception to this is the ligand VUF10166, which binds both to the orthosteric binding site and to an additional allosteric site in the heteromeric receptor, resulting in a lower affinity when compared to the homomer (Thompson *et al.*, 2012b).

Here, we explore the effects of a range of partial agonists (Figure 1) at both receptor types, and show that that a pore-lining residue is an important determinant of ligand efficacy. The data also reveal that the competitive ligand *m*CPBG is potentially a useful tool to distinguish between homomeric and heteromeric 5-HT_3 receptors.

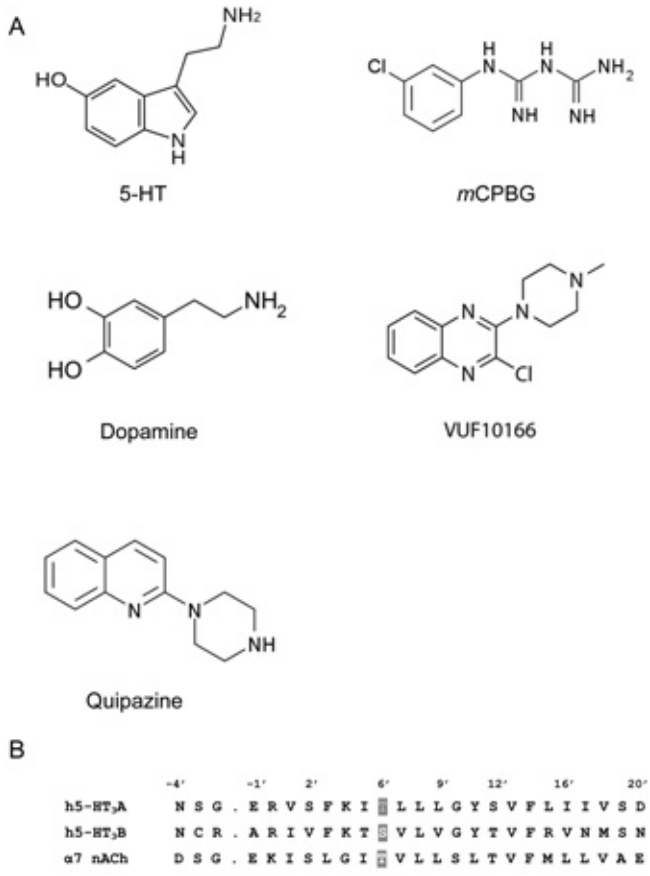

Figure 1
Structures of the ligands used in this study (A) and an alignment (B) of the channel-lining residues of the human 5-HT_3A and 5-HT_3B subunits. Accession numbers for the alignment are 5-HT_3A = P46098; 5-HT_3B = O95264; α7 nACh = P36544.

Methods

Materials

All cell culture reagents were obtained from Gibson (Invitrogen Ltd, Paisley, UK), except fetal calf serum which was from Labtech International (Uckfield, UK). Human 5-HT_3A (accession number: P46098) and 5-HT_3B (O95264) receptor subunit cDNA were kindly gifted by Prof J. A. Peters (University of Dundee, UK).

Oocyte maintenance

Xenopus laevis oocyte-positive females were purchased from NASCO (Fort Atkinson, WI, USA) and maintained according to standard methods (Goldin, 1992). Harvested stage V-VI *Xenopus* oocytes were washed in four changes of ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), de-folliculated in 1.5 mg·mL^{-1} collagenase Type 1A for approximately 2 h, washed again in four changes of ND96 and stored in ND96 containing 2.5 mM sodium pyruvate, 50 mM gentamycin, 0.7 mM theophylline.

HEK293 cell culture

HEK 293 cells were maintained on 90 mm tissue culture plates at 37°C and 7% CO_2 in a humidified atmosphere. They were cultured in DMEM : F12 with GlutaMAX™ I media (DMEM/Nutrient Mix F12 (1:1), Invitrogen, Paisley, UK) containing 10% fetal calf serum. For radioligand binding studies cells in 90 mm dishes were transfected using polyethyleneimine (PEI). Thirty microlitres of PEI (1 mg·mL^{-1}), 5 μL cDNA and 1 mL DMEM were incubated for 10 min at room temperature, added drop wise to an 80–90% confluent plate, and incubated for 2–3 days before harvesting.

Receptor expression

cDNA was cloned into pGEMHE for oocyte expression (Liman *et al.*, 1992), and pcDNA3.1 (Invitrogen) for expression in HEK 293 cells. Mutagenesis was performed using QuikChange

(Agilent Technologies Inc., Santa Clara, CA, USA). cRNA was *in vitro* transcribed from linearised pGEMHE cDNA template using the mMessage mMachine T7 Transcription kit (Ambion, Austin, TX, USA). The 5-HT$_3$A was linearized with SphI and 5-HT$_3$B cDNA with NheI. Stage V and VI oocytes were injected with 50 nl of ~400 ng·μL^{-1} cRNA, and currents were recorded 1–4 days post-injection. A ratio of 1:3 (A : B) was used for the expression of heteromeric receptors.

Electrophysiology

Using two-electrode voltage clamp, *Xenopus* oocytes were clamped at –60 mV using an OC-725 amplifier (Warner Instruments, Hamden, CT, USA), Digidata 1322A and the Strathclyde Electrophysiology Software Package (Department of Physiology and Pharmacology, University of Strathclyde, UK). Currents were recorded at a frequency of 5 kHz and filtered at 1 kHz. Microelectrodes were fabricated from borosilicate glass (GC120TF-10, Harvard Apparatus, Edenbridge, Kent, UK) using a one-stage horizontal pull (P-87, Sutter Instrument Company, Novato, CA, USA) and filled with 3 M KCl. Pipette resistances ranged from 1.0 to 2.0 MΩ. Oocytes were perfused with saline at a constant rate of 12 mL·min^{-1}. Drug application was via a simple gravity fed system calibrated to run at the same rate. Extracellular saline contained (mM), 96 NaCl, 2 KCl, 1 $MgCl_2$ and 5 HEPES; pH 7.4 with NaOH).

Analysis and curve fitting was performed using Prism v4.03 (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). Concentration-response data for each oocyte was normalized to the maximum current for that oocyte. The normalized amplitudes were averaged for a series of oocytes, plotted against agonist or antagonist concentration and iteratively fitted to the following reparametrized Hill equation to calculate the mean ± SEM:

$$I_A = I_{\min} + \frac{I_{\max} - I_{\min}}{1 + 10^{n_H(\log A_{50} - \log A)}} \quad (1)$$

where A is the concentration of ligand present; I_A is the current in the presence of ligand concentration A; $I_{\min}$ is the current when $A = 0$; $I_{\max}$ is the current when $A = \infty$, A_{50} is the concentration of A which evokes a current equal to (I_{max} + I_{min})/2; and n_H is the Hill coefficient. Errors were estimated from residuals of the pooled data.

Radioligand binding

Transfected HEK 293 cells were scraped into 1 mL of ice-cold HEPES buffer (10 mM, pH 7.4) and frozen. After thawing, they were washed with HEPES buffer, resuspended, and 50 μg incubated in 0.5 mL HEPES buffer containing the 5-HT$_3$ receptor antagonist [^{3}H]granisetron. Saturation binding (8 point) assays were performed on at least three separate plates of transfected cells for each mutant using 0.1–20 nM [^{3}H]granisetron. For competition binding (8-point) reactions were incubated for 24 h at 4°C with 0.6 nM [^{3}H]granisetron (~K_d). Non-specific binding was determined using 1 mM quipazine. Reactions were terminated by vacuum filtration using a Brandel cell harvester onto GF/B filters pre-soaked in 0.3% polyethyleneimine. Radioactivity was determined by scintillation counting using a Beckman BCLS6500 (Fullerton, CA, USA). Individual competition binding experiments were analysed by iterative curve fitting using the following equation in Prism v4.03:

$$y = B_{\min} + \frac{B_{\max} - B_{\min}}{1 + 10^{[L] - \log IC_{50}}} \quad (2)$$

where $B_{\min}$ is the non-specific binding, $B_{\max}$ is the maximum specific binding, [L] is the concentration of competing ligand and IC_{50} is the concentration of competing ligand that blocks half of the specific bound radioligand. Values were calculated for separate experiments and reported as mean ± SEM.

Immunofluorescence

This was as described previously (Reeves and Lummis, 2006). Briefly, transiently transfected cells were fixed (4% paraformaldehyde), washed in Tris-buffered saline containing 0.3% Triton (0.1 M Tris pH 7.4, 0.9% NaCl) and incubated overnight at 4°C in pAb77 at 1:1000. Following further washing, biotinylated anti-rabbit IgG (Vector Laboratories, CA, USA) and fluorescein isothiocyanate (FITC) avidin D (Vector Laboratories) were used to detect bound antibody as instructed by the manufacturer. Coverslips were mounted in Vectashield HardSet mounting medium (Vector Laboratories). Immunofluorescence was observed using an Ultra*VIEW*™ LCI Confocal Imaging System (Perkin Elmer, Boston, MA, USA).

Results

Functional properties of wild type 5-HT$_3$A and 5-HT$_3$AB receptors

Inward current responses were recorded for 5-HT, dopamine, *m*CPBG, quipazine and VUF10166 at 5-HT$_3$A and 5-HT$_3$AB receptors. Concentration-response curves yielded the parameters shown in Table 1 and Figures 2–5.

Dopamine was a partial agonist with an EC_{50} higher than that of 5-HT at both 5-HT$_3$A (100-fold, Figure 2A) and 5-HT$_3$AB receptors (40-fold, Figure 2B). The relative maximal current amplitude (R_{max}) of dopamine compared to 5-HT also differed at 5-HT$_3$A (0.08) and 5-HT$_3$AB receptors (0.19).

At 5-HT$_3$A receptors, *m*CPBG and 5-HT had similar EC_{50} and R_{max} values (Figure 2C), but at 5-HT$_3$AB receptors the EC_{50} for *m*CPBG was 16-fold less than 5-HT, and the R_{max} for *m*CBPG was 2.6 (i.e. it was a superagonist; Figure 2D).

Quipazine elicited responses that were too small to accurately determine functional parameters at both 5-HT$_3$A and 5-HT$_3$AB receptors (Figure 3A,B). Its application resulted in inhibition of subsequent agonist responses from which recovery was slow (Figure 3C). Inhibition was also observed at concentrations where no measureable agonist response could be detected (Figure 3D).

With VUF10166, which is structurally similar to quipazine, larger responses allowed determination of functional parameters at 5-HT$_3$A receptors, as previously reported (figure 4A, from Thompson *et al.*, 2012b). Activation of 5-HT$_3$AB receptors was negligible with VUF10166 (figure 4B from Thompson *et al.*, 2012b). Like quipazine, VUF10166 caused inhibition of subsequent agonist-evoked responses. As with 5-HT$_3$A receptors, inhibition of 5-HT$_3$AB receptors was observed following sustained application, but recovery was more rapid (<8 min) as previously reported (figure 4C from Thompson *et al.*, 2012b).

Table 1

Agonist properties at the 5-HT_3R

Receptor	pEC_{50}	EC_{50} (μM)	Hill slope	*n*	R_{max}
5-HT					
5-HT_3A	5.79 ± 0.02	1.62	2.49 ± 0.21	7	–
5-$HT_3A_{T6'S}$	5.90 ± 0.02*	1.26	2.34 ± 0.20	4	–
5-HT_3AB	4.55 ± 0.03	28.1	1.03 ± 0.10	11	–
5-$HT_3A_{T6'S}$B	6.39 ± 0.02*	0.41	2.09 ± 0.20	3	–
Dopamine					
5-HT_3A	3.73 ± 0.21	186	3.42 ± 2.89	3	0.08 ± 0.01
5-$HT_3A_{T6'S}$	4.05 ± 0.12	89.1	1.43 ± 0.12	4	0.22 ± 0.02*
5-HT_3AB	2.96 ± 0.11	1096	3.68 ± 4.24	3	0.19 ± 0.03
5-$HT_3A_{T6'S}$B	3.63 ± 0.08*	235	1.40 ± 0.30	5	0.89 ± 0.07*
mCPBG					
5-HT_3A	5.54 ± 0.07	2.88	1.70 ± 0.42	7	0.88 ± 0.04
5-$HT_3A_{T6'S}$	6.22 ± 0.03*	0.60	3.34 ± 0.69	4	1.00 ± 0.03
5-HT_3AB	5.76 ± 0.15	1.74	1.22 ± 0.41	5	2.58 ± 0.17
5-$HT_3A_{T6'S}$B	7.03 ± 0.13*	0.093	1.07 ± 0.13	4	1.16 ± 0.06*
Quipazine					
5-HT_3A	UD	–	–	12	–
5-$HT_3A_{T6'S}$	7.57 ± 0.10	0.027	1.37 ± 0.39	7	0.86 ± 0.06
5-HT_3AB	UD	–	–	8	–
5-$HT_3A_{T6'S}$B	6.75 ± 0.20	0.18	0.52 ± 0.11	6	1.21 ± 0.07
VUF10166					
5-HT_3A	5.28 ± 0.14[†]	5.20	1.24 ± 0.37	9	0.24 ± 0.02
5-$HT_3A_{T6'S}$	NR	–	–	5	–
5-HT_3AB	UD	–	–	3	–
5-$HT_3A_{T6'S}$B	8.15 ± 0.02	0.007	3.92 ± 0.71	6	0.99 ± 0.06

R_{max} is the maximal current amplitude for the test ligand compared to the maximal current amplitude for 5-HT. NR, no agonist response at 100 μM; UD, undetermined, as responses were too small to accurately determine parameters. Data = mean ± SEM.
*Mutant significantly different to wild type counterpart ($P < 0.05$, Student's *t*-test).
[†]Data from (Thompson *et al.*, 2012b).

Effects of 5-$HT_3A_{T6'S}$ substitution

The 5-HT_3B subunits contain a Ser residue at the 6′ channel lining position, and this was introduced into the 5-HT_3A subunit to create the 5-$HT_3A_{T6'S}$ mutant. At 5-$HT_3A_{T6'S}$ mutant receptors, the EC_{50} values of 5-HT and *m*CPBG were reduced, and the R_{max} of dopamine was increased (Figure 5A,B). Quipazine no longer caused inhibition of subsequent agonist-evoked responses and instead was an agonist with an EC_{50} of 27 nM and an R_{max} of 0.9 (Figure 5B). In contrast, VUF10166 did not elicit an agonist response at up to 100 μM, and the inhibition of subsequent agonist responses was no different to wild type receptors (Figure 5E).

Effects of 5-$HT_3A_{T6'S}$ substitution in 5-HT_3AB receptors

When compared to wild type receptors, the effects of the 5-$HT_3A_{T6'S}$ mutation were greater in heteromeric receptors. 5-HT showed a 67-fold decrease in EC_{50} at 5-$HT_3A_{T6'S}$B receptors when compared to 5-HT_3AB receptors (Figure 5C). Dopamine had a fivefold decrease in EC_{50} and an R_{max} of 0.9. *m*CPBG had a 19-fold decrease in EC_{50} and no longer acted as a superagonist as it had an R_{max} similar to that of 5-HT (due either to a reduction in the *m*CPBG response or an increase in the 5-HT response).

Quipazine was a full agonist at 5-$HT_3A_{T6'S}$B receptors, with an R_{max} of 1.2, and an EC_{50} of 180 nM. It also no longer inhibited subsequent agonist responses. VUF10166 became a potent agonist, with an EC_{50} of 7 nM (58-fold less than 5-HT; Figure 5D), and an R_{max} of 1.0. Like quipazine, VUF10166 no longer inhibited subsequent agonist-evoked responses.

Ligand binding

To explore whether the 5-$HT_3A_{T6'S}$ channel mutation had an effect on ligand binding affinity, we examined saturation binding of the competitive antagonist [^{3}H]granisetron. The K_d of granisetron was not significantly different ($P < 0.05$) at wild type and mutant receptors (Table 2). Competition of [^{3}H]granisetron binding by the agonists 5-HT, dopamine, *m*CPBG and quipazine (e.g. Figure 6A) revealed a consistent

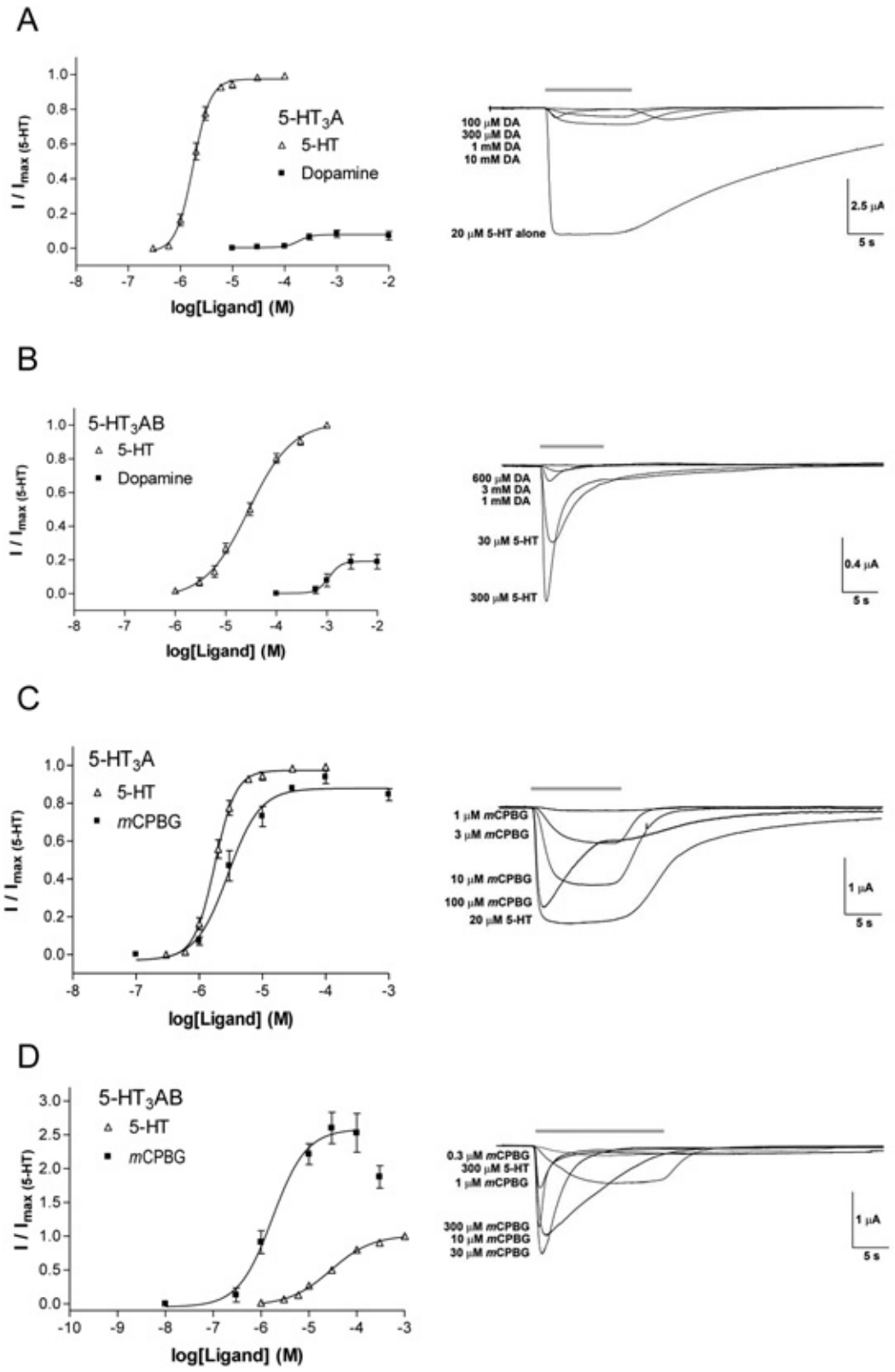

Figure 2

Concentration-responses curves for 5-HT, dopamine and *m*CPBG at wild type homomeric and heteromeric 5-HT$_3$ receptors. Dopamine was a partial agonist at both 5-HT$_3$A receptors (A) and 5-HT$_3$AB receptors (B). *m*CPBG-evoked responses were similar to 5-HT (C), but were greater at 5-HT$_3$AB receptors (D). Example traces are shown besides each curve. Data are normalized to the maximum 5-HT response in each oocyte and plotted as the mean ± SEM for a series of oocytes. Parameters derived from these curves are in Table 1.

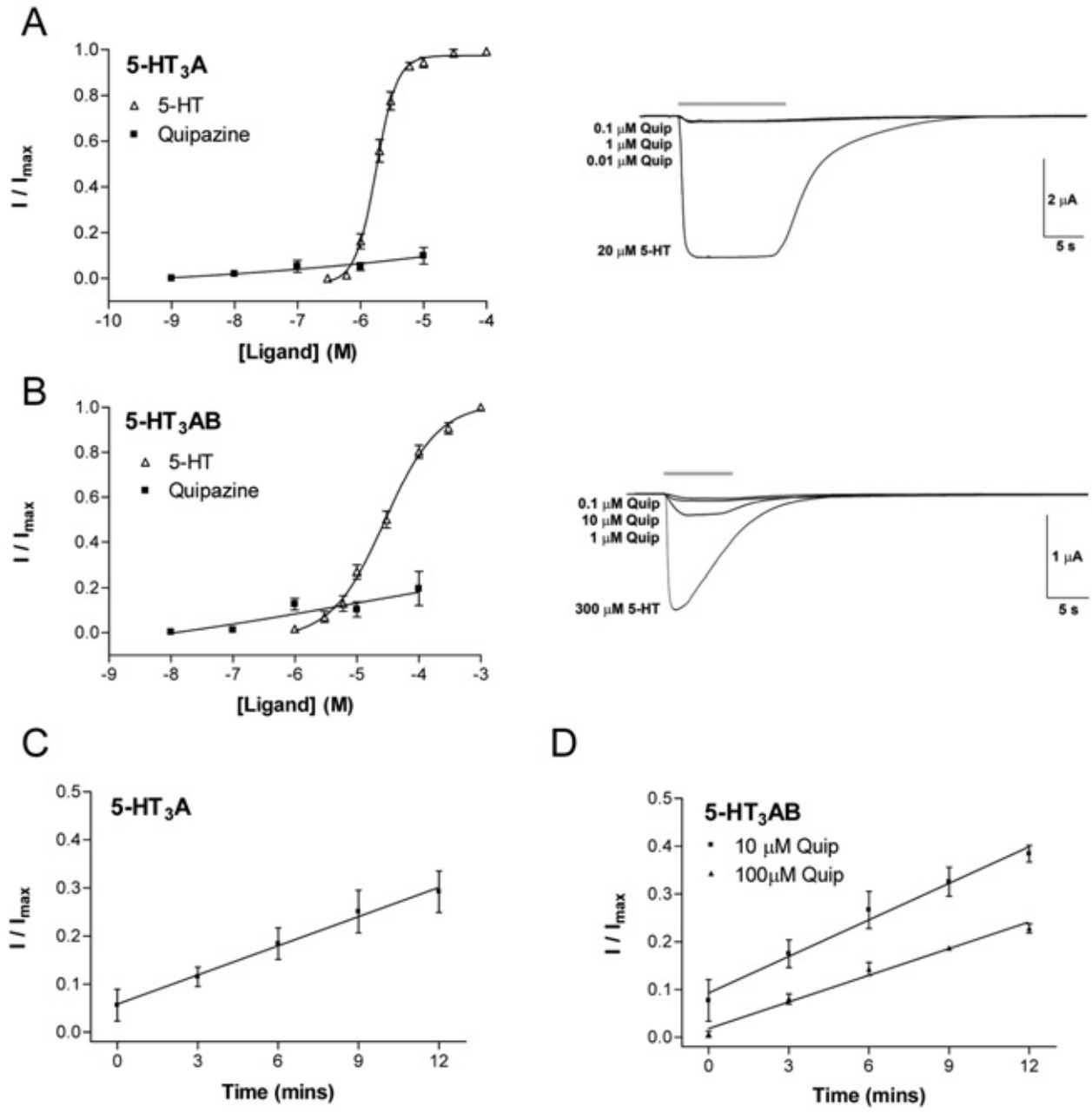

Figure 3

Concentration-response curves and the long-lived inhibition of subsequent 5-HT_3 receptor agonist responses by quipazine. Partial agonist responses are seen with quipazine at both 5-HT_3A and 5-HT_3AB receptors (A & B). Example traces are shown besides each curve. Application of quipazine causes inhibition of subsequent 5-HT responses, and recovery at both 5-HT_3A and 5-HT_3AB receptors is slow (C & D). Data are normalized to the maximum 5-HT response in each oocyte and plotted as the mean ± SEM for a series of oocytes. The small size of the quipazine responses meant it was not possible to make accurate measurements of the EC_{50} values.

trend of lower K_i values; analysis of agonist pK_is paired for 5-HT_3A and 5-$HT_3A_{T6'S}$ receptors revealed a statistically significant decrease (paired t-test, $P < 0.05$). Similar pairing of 5-HT_3AB and 5-$HT_3A_{T6'S}$B receptors also revealed a decrease in K_is in the mutant receptors (paired t-test, $P < 0.05$). VUF10166 was anomalous in having a larger K_i value in 5-HT_3AB as compared to 5-HT_3A receptors, as previously reported (Thompson *et al.*, 2012b). VUF10166 had a large decrease in K_i at 5-$HT_3A_{T6'S}$B compared to 5-HT_3AB receptors, but there was no decrease in the K_i value for 5-$HT_3A_{T6'S}$ as compared to 5-HT_3A receptors.

Immmunofluorescence

As the 5-$HT_3AB_{T6'S}$ mutant receptors had K_i values more similar to wild type 5-HT_3A then wild type 5-HT_3AB receptors, the expression of the mutant 5-HT_3B subunit was confirmed

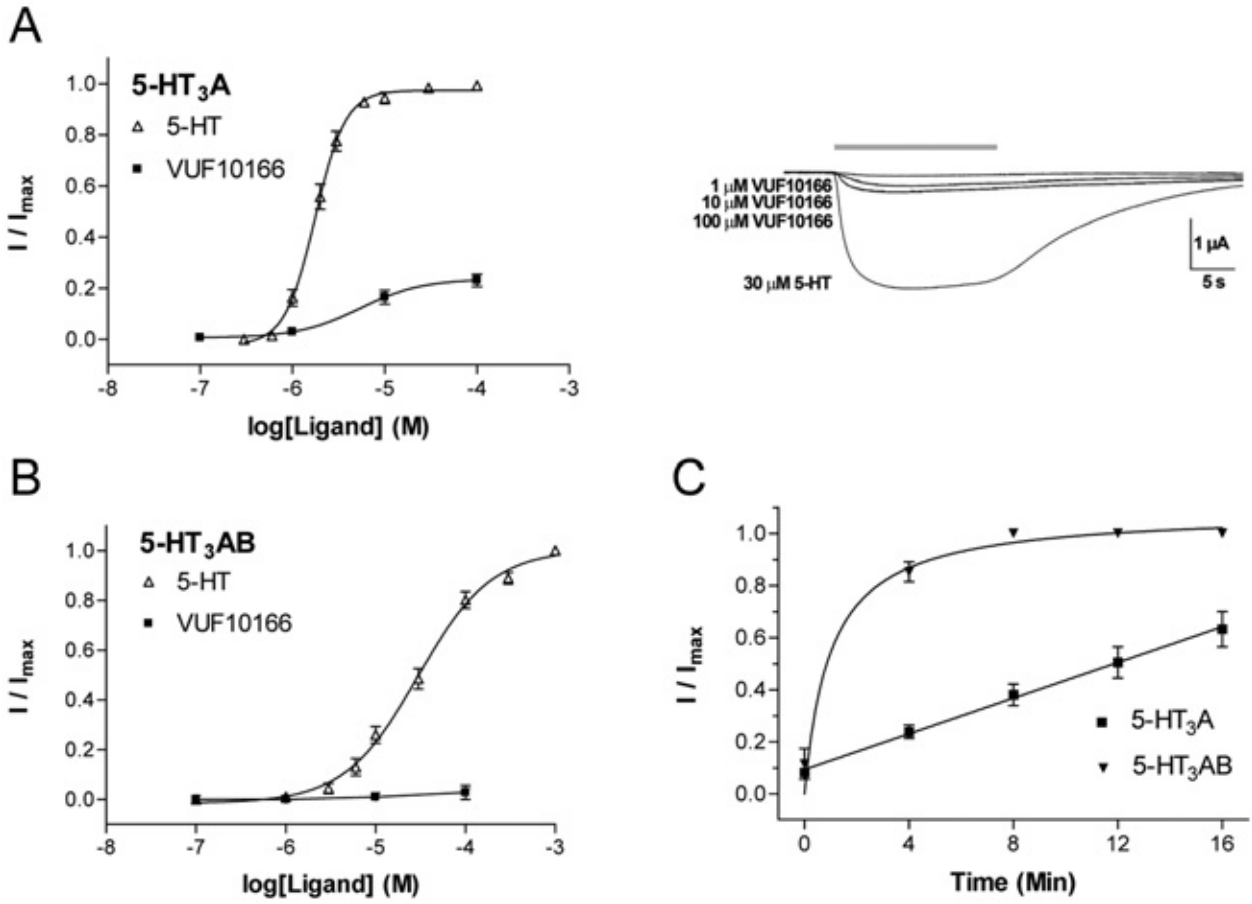

Figure 4

Concentration-response curves and long-lived inhibition of 5-HT$_3$ receptor responses by VUF10166. (A) Concentration-response curves showing the agonist responses to 5-HT and VUF10166 at 5-HT$_3$A receptors. Examples VUF10166 agonist responses are compared with a maximal (30 μM) 5-HT response at 5-HT$_3$A receptors. (B) VUF10166 activation of 5-HT$_3$AB receptors was negligible (R_{max} = 0.03 ± 0.02, n = 3). (C) Recovery from VUF10166 inhibition was slower at 5-HT$_3$A than at 5-HT$_3$AB receptors; at these concentrations 5-HT$_3$AB receptors recovered from inhibition within ~8 min, whereas recovery for 5-HT$_3$A receptor responses was ~25 min. Parameters from these curves are in Table 1. Data in this figure are duplicated from Thompson *et al.* (2012b) with kind permission.

by immunofluorescent labelling with a 5-HT$_3$B-specific antibody (Figure 6B; pAB77, Reeves and Lummis, 2006). Cells that were transfected with wild type and mutant 5-HT$_3$A subunits alone showed no specific labelling. In contrast, co-expression of 5-HT$_3$A and 5-HT$_3$B subunits was detected as a halo of fluorescence at the cell surface, indicating that receptors were correctly assembled and trafficked to the plasma membrane.

Discussion

This study has shown that partial agonists have different properties at 5-HT$_3$A and 5-HT$_3$AB receptors, and that a T6′S substitution in the 5-HT$_3$A subunit can increase the efficacy of partial agonists at both receptor types. This resulted in quipazine changing from being effectively an antagonist in wild type receptors, to being a potent and efficacious agonist in mutant receptors, and VUF10166 becoming an agonist in mutant heteromeric receptors with an EC_{50} value 230-fold lower than the EC_{50} for 5-HT in wild type receptors.

Increased sensitivity of 5-HT$_3$ receptors to partial agonists has been achieved in previous studies by treatment with allosteric modulators or by mutation (Downie *et al.*, 1995; Solt *et al.*, 2005; Hu *et al.*, 2006; Hu and Peoples, 2008b). Quipazine, for example, was originally considered an antagonist because of the apparent absence of inwards currents (later reported to be <1.6% the size of maximal 5-HT currents) and the long-lived inhibition of subsequent agonist responses; it was more recently shown to be a partial agonist by co-applying the allosteric modulator trichloroethanol (Downie *et al.*, 1995). Mutation of the pre-TM1 region similarly increases the maximal current evoked by the partial agonist dopamine, and enables allosteric modulators such as *n*-alcohols to activate the receptor when applied alone (Zhang *et al.*, 2002; Hu and Peoples, 2008b); similar direct activation by ethanol has also been seen at GABA$_A$ receptors containing M2 mutations (Ueno *et al.*, 2000; Zhang *et al.*,

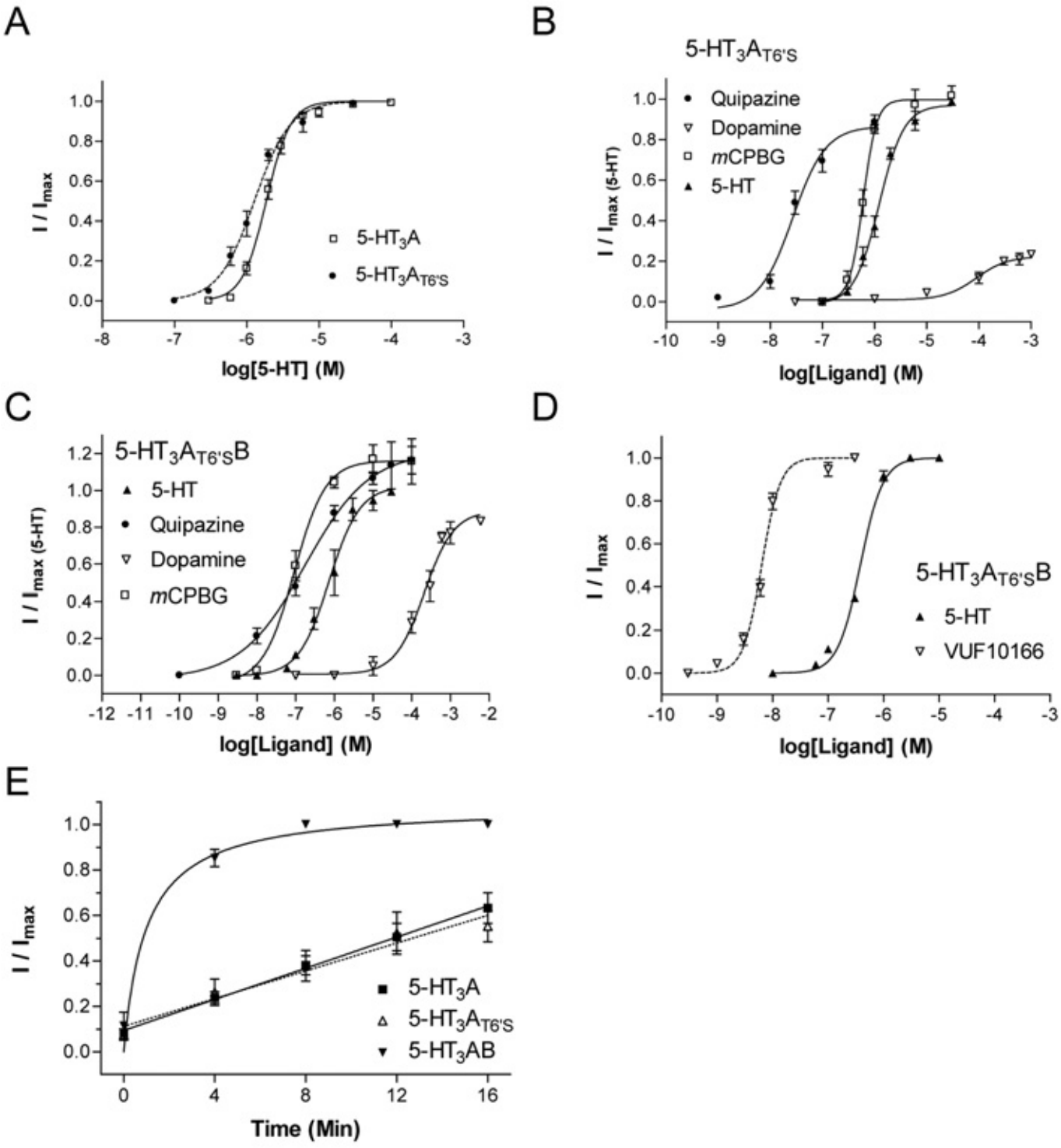

Figure 5

Effects of a 5-$HT_3A_{T6'S}$ mutation on the responses of homomeric and heteromeric 5-HT_3 receptors. (A) Relative to wild type responses, a decrease in 5-HT EC_{50} was seen at 5-$HT_3A_{T6'S}$ receptors. (B) Quipazine, dopamine and *m*CPBG also have decreased EC_{50} values, and R_{max} was increased for dopamine and quipazine. (C) Incorporation of the 5-HT_3B subunit also resulted in decreased EC_{50} values and R_{max} of dopamine and quipazine compared to wild-type receptors. *m*CPBG showed an apparent decrease in maximal current, but it is possible that this is due to the relative increase in 5-HT efficacy. (D) At 5-$HT_3A_{T6'S}B$ receptors VUF10166 was a full and potent agonist. (E) The slow recovery from VUF10166 inhibition was not altered at 5-$HT_3A_{T6'S}$ mutant receptors, but was no longer seen at 5-$HT_3A_{T6'S}B$ receptors. Data are normalized to the maximum 5-HT response in each oocyte and plotted as the mean ± SEM for a series of oocytes. Parameters from these curves are in Table 1.

Table 2

Ligand binding parameters at 5-HT$_3$R

	Receptor	pK$_i$	K$_i$	*n*
Granisetron[$]	5-HT$_3$A	9.11 ± 0.05	0.77 nM	8
	5-HT$_3$A$_{T6'S}$	9.28 ± 0.09	0.52 nM	5
	5-HT$_3$AB	9.13 ± 0.09	0.74 nM	4
	5-HT$_3$A$_{T6'S}$B	9.09 ± 0.04	0.81 nM	6
5-HT	5-HT$_3$A	6.38 ± 0.35	0.42 μM	5
	5-HT$_3$A$_{T6'S}$	6.89 ± 0.39	0.13 μM	5
	5-HT$_3$AB	5.57 ± 0.06	2.69 μM	3
	5-HT$_3$A$_{T6'S}$B	5.67 ± 0.06	2.14 μM	4
Dopamine	5-HT$_3$A	4.26 ± 0.20	55.0 μM	6
	5-HT$_3$A$_{T6'S}$	4.53 ± 0.07	23.4 μM	6
	5-HT$_3$AB	4.04 ± 0.15	91.2 μM	6
	5-HT$_3$A$_{T6'S}$B	4.59 ± 0.27	25.7 μM	4
mCPBG	5-HT$_3$A	6.82 ± 0.14	0.15 μM	8
	5-HT$_3$A$_{T6'S}$	7.10 ± 0.24	0.08 μM	3
	5-HT$_3$AB	6.37 ± 0.12	0.43 μM	3
	5-HT$_3$A$_{T6'S}$B	6.64 ± 0.12	0.16 μM	5
Quipazine	5-HT$_3$A	8.83 ± 0.02	1.48 nM	5
	5-HT$_3$A$_{T6'S}$	9.06 ± 0.16	0.87 nM	5
	5-HT$_3$AB	8.31 ± 0.12	4.90 nM	4
	5-HT$_3$A$_{T6'S}$B	8.76 ± 0.13	1.73 nM	4
VUF10166	5-HT$_3$A	9.82 ± 0.26	0.15 nM	5
	5-HT$_3$A$_{T6'S}$	9.11 ± 0.13	0.78 nM	14
	5-HT$_3$AB	7.45 ± 0.12	35.5 nM	10
	5-HT$_3$A$_{T6'S}$B	8.84 ± 0.02*	1.44 nM	8

[$]Values are K$_d$ from saturation binding experiments. Data = mean ± SEM.
*Mutant significantly different to wild type counterpart ($P < 0.05$, Student's t-test).

2002). These mutations and modulators most likely modify the rates of the open-closed state transitions, which is the probable effect of the T6'S mutation described here.

At wild type receptors, the structurally related compounds quipazine and VUF10166 are partial agonists with slow off rates, explaining why they inhibit subsequent agonist applications when pre-applied (Downie *et al.*, 1995; Thompson *et al.*, 2012b). 5-HT, 2-methyl-5-HT and *m*CPBG at low concentrations will also inhibit 5-HT$_3$ receptor responses, but recovery from these ligands is much more rapid (Bartrup and Newberry, 1996). As these agonists cross-desensitise one another, it is likely that they share a common mechanism of activation and desensitization (van Hooft and Vijverberg, 1996; Hu and Peoples, 2008b).

Our radioligand binding data showed that wild type and mutant receptors had similar K_d values for [^{3}H]granisetron, which suggests that the binding site is not significantly altered by the channel mutation. For agonists, it is not possible to accurately determine the K_i using radioligand binding, as differences in gating efficacy can influence the apparent affinity (Colquhoun, 1998; Purohit and Grosman, 2006). Such differences are thought to underlie the finding that the affinities of 5-HT$_3$ agonists are slightly lower at wild type 5-HT$_3$A than at 5-HT$_3$AB receptors (Peters *et al.*, 2009). Our data revealed a decrease in agonist K_i values when mutant receptors were compared to their wild type counterparts, which we propose are due to increased efficacy of the ligands at the mutant receptors; detailed single channel studies, however, would be needed to confirm this.

VUF10166 behaved differently to the other agonists: it had a lower K_i (155-fold) at 5-HT$_3$AB receptors than at 5-HT$_3$A receptors, due to the influence of an additional allosteric binding in the heteromer (Thompson *et al.*, 2012b). At 5-HT$_3$A$_{T6'S}$B receptors, however, the K_i of VUF10166 resembled that found at homomeric receptors, rather than the lower value of wild type heteromers (Table 2). A possible explanation is that the 5-HT$_3$B subunit does not express with 5-HT$_3$A$_{T6'S}$ subunits, but high levels of immunofluorescence with a 5-HT$_3$B receptor-specific antisera suggests that this is not the case. An alternative possibility is that the 5-HT$_3$A$_{T6'S}$ subunit mutation alters the influence of the allosteric binding site in the heteromer. In support of this hypothesis,

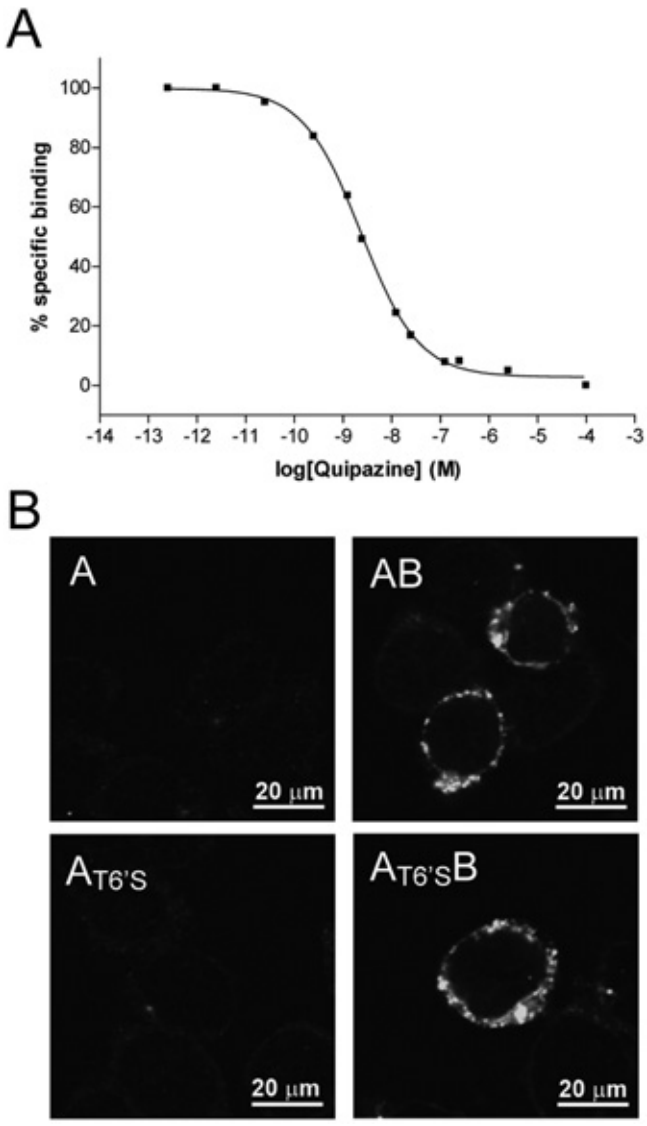

Figure 6

Radioligand binding and 5-HT$_3$B subunit expression in HEK293 cells. (A) Example data for competition of [^{3}H]granisetron binding with quipazine at wild type 5-HT$_3$AB receptors transiently expressed in HEK293 cells. The curve is representative of four similar experiments and is typical of the curves for all of the ligands studied here. Parameters derived from curves like these were averaged and the mean ± SEM values are shown in Table 2. (B) Typical immunofluorescent labelling (pAb77; Reeves and Lummis, 2006) of 5-HT$_3$B subunits expressed with either wild type or mutant 5-HT$_3$A subunits in HEK293 cells.

VUF10166 is only a full agonist at 5-HT$_3$A$_{T6'S}$B and not 5-HT$_3$A$_{T6'S}$ receptors, and a S6'T substitution in the 5-HT$_3$B subunit, creating a heteromeric receptor with a ring of five Thr residues similar to wild type receptors, leaves the inhibitory properties of VUF10166 unaltered (Thompson *et al.*, 2012b).

A homologous T6'S mutation has been studied in α7 nACh receptors, but it had little effect on the EC_{50}, maximal current or agonist/antagonist character of 12 ligands that were tested (Placzek *et al.*, 2005). A better studied α7 nACh mutation is L9'T, the effects of which are more similar to those of our T6'S mutant 5-HT$_3$ receptors, causing both a decreased in agonist EC_{50} and a conversion of antagonists to agonists (Bertrand *et al.*, 1992; Palma *et al.*, 1996; 1998; Demuro *et al.*, 2001; Fucile *et al.*, 2002). In contrast, 5-HT$_3$ receptor L9'A, L9'F, L9'V and L9'T mutations have small or no effects on 5-HT EC_{50} values, although they do significantly affect desensitization (Yakel *et al.*, 1993; Thompson *et al.*, 2011a). The 13' residue has also been explored and V13'S in the 5-HT$_3$A subunit causes significant hypersensitivity (~70-fold decrease in EC_{50}) to 5-HT and enhanced constitutive activity when co-expressed with 5-HT$_3$B; in addition there is a minor increase in EC_{50} (~3 fold) when V13' is substituted with Cys (Dang *et al.*, 2000; Reeves *et al.*, 2001; Panicker *et al.*, 2002; Bhattacharya *et al.*, 2004). Ser and Thr substitutions at V13' also result in hypersensitivity in a variety of nACh receptor subtypes and it therefore seems that polar residues can be incorporated at this location in the pore with comparable effects (Briggs *et al.*, 1999; Dash and Lukas, 2012). The hypersensitivity that the substituted residues confer has enabled researchers to isolate the roles of specific nACh receptor subtypes, mimic clinical disorders, and help with the development of therapeutics (reviewed in Drenan and Lester, 2012). A similar exploitation of the hypersensitive V13'S 5-HT$_3$ receptor mutant was used to study uropathy, and in the future this or other hypersensitive mutants might prove useful to establish the role of the 5-HT$_3$ receptor in disorders such as depression, drug and alcohol abuse, pruritis, cognitive and psychotic disorders and in pain (Bhattacharya *et al.*, 2004; Thompson and Lummis, 2007; Walstab *et al.*, 2010).

The effects of *m*CPBG at 5-HT$_3$A receptors have been reported by several groups, and indeed in one study it was changed from an agonist into an antagonist by mutations in the orthosteric binding site (Spang *et al.*, 2000; Price *et al.*, 2008; Verheij *et al.*, 2012). These studies were performed in rodent receptors where *m*CPBG has a maximal current response similar to that of 5-HT. In contrast, at human 5-HT$_3$AB receptors, the maximum current response to *m*CPBG is 2.6-fold greater than that for 5-HT. Therefore, a comparison of responses to 5-HT and *m*CPBG is a simple and effective method of determining expression of 5-HT$_3$B subunits in human 5-HT$_3$ receptors; for example at 30 μM 5-HT and *m*CPBG current amplitudes at homomeric receptors are equal, but *m*CPBG-induced responses are fivefold larger in 5-HT$_3$AB receptors.

In summary, we have shown that a channel-lining Thr6' residue found in the 5-HT$_3$A subunit is a determinant of agonist efficacy; when a T6'S substitution is made in the 5-HT$_3$A subunit, activation by partial agonists is enhanced, with currents being evoked at lower concentrations and with increased maximal amplitudes in both 5-HT$_3$A and 5-HT$_3$AB receptors. Pharmacologically, this emphasizes that despite some apparent functional similarities between antagonists and low efficacy partial agonists, it is possible to distinguish them by introducing a gain-of-function mutation. The changes we see are consistent with an effect on gating efficacy that has been observed in other mutant Cys-loop receptors, and emphasizes the importance of the 6' channel location in influencing the equilibrium of the open and closed states.

Acknowledgements

This work was supported by a grant from the Wellcome Trust [081925] to SCRL. SCRL is a Wellcome Trust Senior Research Fellow in Basic Biomedical Science. We thank Linda Silvestri for excellent technical assistance.

Conflict of interest

None.

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