A single channel mutation alters agonist efficacy at 5-HT3A and 5-HT3AB receptors

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BACKGROUND AND PURPOSE
5-HT3 receptors are composed of 5-HT3A subunits (homomeric receptors), or combinations of 5-HT3A and other 5-HT3 receptor subunits (heteromeric receptors, the best studied of which are 5-HT3AB receptors). Here we explore the effects of partial agonists at 5-HT3A and 5-HT3AB receptors, and the importance of a channel-lining residue in determining the efficacy of activation.

EXPERIMENTAL APPROACH
Wild type and mutant 5-HT3A and 5-HT3AB receptors were expressed in Xenopus oocytes and examined using two-electrode voltage-clamp, or expressed in HEK293 cells and examined using [3H]granisetron binding.

KEY RESULTS
Dopamine, quipazine and VUF10166 were partial agonists at wild type 5-HT3A and 5-HT3AB receptors, with quipazine and VUF10166 causing a long-lived (>20 min) inhibition of subsequent agonist responses. At 5-HT3A receptors, mCPBG was a partial agonist, but was a superagonist at 5-HT3AB receptors, as it produced a response 2.6 times greater than that of 5-HT. A T6'S substitution in the 5-HT3A subunit decreased EC50 and increased Rmax of dopamine and quipazine at both homomeric and heteromeric receptors. The greatest changes were seen with VUF10166 at 5-HT3AT6'SB receptors, where it became a full agonist (EC50 = 7 nM) with an EC50 58-fold less than 5-HT (EC50 = 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-HT3A and 5-HT3AB 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, γ-aminobutyric acid; HEK, human embryonic kidney; mCPBG, m-chlorophenylbiguanide; nACh, nicotinic acetylcholine; PEIm, polyethylenimine; VUF10166, 2-chloro-(4-methylpiperazine-1-yl)quinoxaline

Introduction
5-HT3 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 \(\beta\)-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 \(\alpha\)-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 \(0^\prime\) 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\(_3\)A – 5-HT\(_3\)E) 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\(_3\)A subunits can form homomeric receptors, but the other subunits must combine with 5-HT\(_3\)A in order to form functional heteromeric receptors. The 5-HT\(_3\)A and 5-HT\(_3\)AB 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\(_3\)A and 5-HT\(_3\)AB receptors because both receptors contain the same binding interface, formed by adjacent 5-HT\(_3\)A receptor subunits (Brady et al., 2004; 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 mCPBG is potentially a useful tool to distinguish between homomeric and heteromeric 5-HT\(_3\) receptors.

**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\(_3\)A (accession number: P46098) and 5-HT\(_3\)B (O95264) receptor subunit cDNA were kindly gifted by Prof J. A. Peters (University of Dundee, UK).

**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 humified 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 polyethylenimine (PEI). Thirty microlitres of PEI (1 mg·mL\(^{-1}\)), 5 \(\mu\)L 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 \textit{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 Nhel. Stage V and VI oocytes were injected with 50 nl of $\sim$400 ng $\mu$L$^{-1}$ 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, \textit{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 M$\Omega$. 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 reparameterized Hill equation to calculate the mean $\pm$ SEM:

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

(1)

where $A$ is the concentration of ligand present; $I_0$ is the current in the presence of ligand concentration $A$; $I_{\text{max}}$ is the current when $A = 0$; $I_{\text{min}}$ is the current when $A = \infty$; $K_{\text{50}}$ is the concentration of $A$ which evokes a current equal to $(I_{\text{max}} + I_{\text{min}})/2$; 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 $\mu$g 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 and 5-HT$_3$A receptors differed at 5-HT$_3$A (0.08) and 5-HT$_3$AB receptors (0.19).

At 5-HT$_3$A receptors, mCPBG and 5-HT had similar $EC_{50}$ values (Figure 2C, but at 5-HT$_3$AB receptors the $EC_{50}$ for mCPBG was 16-fold less than 5-HT, and the $R_{\text{max}}$ for mCPBG 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 measurable 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).

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 a UltraVIEW™ LCI Confo 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, mCPBG, 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 ($R_{\text{max}}$) of dopamine compared to 5-HT also differed at 5-HT$_3$A (0.08) and 5-HT$_3$AB receptors (0.19).

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

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Effects of 5-HT₃AT₆’S substitution

The 5-HT₃B subunits contain a Ser residue at the 6’ channel lining position, and this was introduced into the 5-HT₃A subunit to create the 5-HT₃AT₆’S mutant. At 5-HT₃AT₆’S mutant receptors, the EC₅₀ values of 5-HT and mCPBG were reduced, and the Rₘₐₓ of dopamine was increased (Figure 5A,B). Quipazine no longer caused inhibition of subsequent agonist-evoked responses and instead was an agonist with an EC₅₀ of 27 nM and an Rₘₐₓ 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-HT₃AT₆’S substitution in 5-HT₃AB receptors

When compared to wild type receptors, the effects of the 5-HT₃AT₆’S mutation were greater in heteromeric receptors. 5-HT showed a 67-fold decrease in EC₅₀ at 5-HT₃AT₆’SB receptors when compared to 5-HT₃AB receptors (Figure 5C). Dopamine had a fivefold decrease in EC₅₀ and an Rₘₐₓ of 0.9. mCPBG had a 19-fold decrease in EC₅₀ and no longer acted as a superagonist as it had an Rₘₐₓ similar to that of 5-HT (due either to a reduction in the mCPBG response or an increase in the 5-HT response). Quipazine was a full agonist at 5-HT₃AT₆’SB receptors, with an Rₘₐₓ of 1.2, and an EC₅₀ of 180 nM. It also no longer inhibited subsequent agonist responses. VUF10166 became a potent agonist, with an EC₅₀ of 7 nM (58-fold less than 5-HT; Figure 5D), and an Rₘₐₓ of 1.0. Like quipazine, VUF10166 no longer inhibited subsequent agonist-evoked responses.

Ligand binding

To explore whether the 5-HT₃AT₆’S channel mutation had an effect on ligand binding affinity, we examined saturation binding of the competitive antagonist [³H]granisetron. The Kᵢ of granisetron was not significantly different (P < 0.05) at wild type and mutant receptors (Table 2). Competition of [³H]granisetron binding by the agonists 5-HT, dopamine, mCPBG and quipazine (e.g. Figure 6A) revealed a consistent

Table 1

| Receptor | pEC₅₀ | EC₅₀ (µM) | Hill slope | n  | Rₘₐₓ |
|----------|-------|----------|------------|----|------|
| 5-HT₃A   | 5.79 ± 0.02 | 1.62     | 2.49 ± 0.21 | 7  | –    |
| 5-HT₃AT₆’S | 5.90 ± 0.02* | 1.26     | 2.34 ± 0.20 | 4  | –    |
| 5-HT₃AB  | 4.55 ± 0.03 | 28.1     | 1.03 ± 0.10 | 11 | –    |
| 5-HT₃AT₆’S/B | 6.39 ± 0.02* | 0.41     | 2.09 ± 0.20 | 3  | –    |
| Dopamine | 3.73 ± 0.21 | 186      | 3.42 ± 2.89 | 3  | 0.08 ± 0.01 |
| 5-HT₃AT₆’S | 4.05 ± 0.12 | 89.1     | 1.43 ± 0.12 | 4  | 0.22 ± 0.02* |
| 5-HT₃AB  | 2.96 ± 0.11 | 1096     | 3.68 ± 4.24 | 3  | 0.19 ± 0.03 |
| 5-HT₃AT₆’S/B | 3.63 ± 0.08* | 235      | 1.40 ± 0.30 | 5  | 0.89 ± 0.07* |
| mCPBG    | 5.54 ± 0.07 | 2.88     | 1.70 ± 0.42 | 7  | 0.88 ± 0.04 |
| 5-HT₃AT₆’S | 6.22 ± 0.03* | 0.60     | 3.34 ± 0.69 | 4  | 1.00 ± 0.03 |
| 5-HT₃AB  | 5.76 ± 0.15 | 1.74     | 1.22 ± 0.41 | 5  | 2.58 ± 0.17 |
| 5-HT₃AT₆’S/B | 7.03 ± 0.13* | 0.093    | 1.07 ± 0.13 | 4  | 1.16 ± 0.06* |
| Quipazine| 5-HT₃A   | UD       | –          | 12 | –    |
| 5-HT₃AT₆’S | 7.57 ± 0.10 | 0.027    | 1.37 ± 0.39 | 7  | 0.86 ± 0.06 |
| 5-HT₃AB  | UD       | –        | –          | 8  | –    |
| 5-HT₃AT₆’S/B | 6.75 ± 0.20 | 0.18     | 0.52 ± 0.11 | 6  | 1.21 ± 0.07 |
| VUF10166 | 5-HT₃A   | 5.28 ± 0.14† | 5.20     | 1.24 ± 0.37 | 9  | 0.24 ± 0.02 |
| 5-HT₃AT₆’S | NR       | –        | –          | 5  | –    |
| 5-HT₃AB  | UD       | –        | –          | 3  | –    |
| 5-HT₃AT₆’S/B | 8.15 ± 0.02 | 0.007    | 3.92 ± 0.71 | 6  | 0.99 ± 0.06 |

Rₘₐₓ 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).
Figure 2
Concentration-responses curves for 5-HT, dopamine and mCPBG at wild type homomeric and heteromeric 5-HT₃ receptors. Dopamine was a partial agonist at both 5-HT₃A receptors (A) and 5-HT₃AB receptors (B). mCPBG-evoked responses were similar to 5-HT (C), but were greater at 5-HT₃AB 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.
trend of lower $K_i$ values; analysis of agonist $pK_i$ values paired for 5-HT$_3$A and 5-HT$_3$AB receptors revealed a statistically significant decrease (paired t-test, $P < 0.05$). Similar pairing of 5-HT$_3$AB and 5-HT$_3$AT6'S receptors also revealed a decrease in $K_i$ in the mutant receptors (paired t-test, $P < 0.05$). VUF10166 was anomalous in having a larger $K_i$ value in 5-HT$_3$AB compared to 5-HT$_3$A receptors, as previously reported (Thompson et al., 2012b). VUF10166 had a large decrease in $K_i$ at 5-HT$_3$AT6'SB compared to 5-HT$_3$AB receptors, but there was no decrease in the $K_i$ value for 5-HT$_3$AT6'S as compared to 5-HT$_3$A receptors.

**Immmunofluorescence**

As the 5-HT$_3$AT6'S mutant receptors had $K_i$ values more similar to wild type 5-HT$_3$A then wild type 5-HT$_3$AB receptors, the expression of the mutant 5-HT$_3$B subunit was confirmed.

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**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$_3$A and 5-HT$_3$AB 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$_3$A and 5-HT$_3$AB 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.
by immunofluorescent labelling with a 5-HT₃B-specific antibody (Figure 6B; pAB77, Reeves and Lummis, 2006). Cells that were transfected with wild type and mutant 5-HT₃A subunits alone showed no specific labelling. In contrast, co-expression of 5-HT₃A and 5-HT₃B 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-HT₃A and 5-HT₃AB receptors, and that a T6'S substitution in the 5-HT₃A 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 EC₅₀ value 230-fold lower than the EC₅₀ for 5-HT in wild type receptors.

Increased sensitivity of 5-HT₃ 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 GABAₐ receptors containing M2 mutations (Ueno et al., 2000; Zhang et al.,...
Figure 5
Effects of a 5-HT$_3$A$_{T6'S}$ 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$_3$A$_{T6'S}$ receptors. (B) Quipazine, dopamine and mCPBG also have decreased EC$_{50}$ values, and R$_{max}$ was increased for dopamine and quipazine. (C) Incorporation of the 5-HT$_3$B subunit also resulted in decreased EC$_{50}$ values and R$_{max}$ of dopamine and quipazine compared to wild-type receptors. mCPBG 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$_3$A$_{T6'S}$B receptors VUF10166 was a full and potent agonist. (E) The slow recovery from VUF10166 inhibition was not altered at 5-HT$_3$A$_{T6'S}$ mutant receptors, but was no longer seen at 5-HT$_3$A$_{T6'S}$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.
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 mCPBG at low concentrations will also inhibit 5-HT3 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 [3H]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$_3$AT6’S 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$_3$AT6’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$_3$AT6’S subunit mutation alters the influence of the allosteric binding site in the heteromer. In support of this hypothesis,

### Table 2

| Receptor     | $pK_i$     | $K_i$   | $n$  |
|--------------|------------|---------|------|
| **Granisetron** |            |         |      |
| 5-HT$_3$A    | 9.11 ± 0.05| 0.77 nM | 8    |
| 5-HT$_3$AT6’S| 9.28 ± 0.09| 0.52 nM | 5    |
| 5-HT$_3$AB   | 9.13 ± 0.09| 0.74 nM | 4    |
| 5-HT$_3$AT6’SB| 9.09 ± 0.04| 0.81 nM | 6    |
| **5-HT**     |            |         |      |
| 5-HT$_3$A    | 6.38 ± 0.35| 0.42 μM | 5    |
| 5-HT$_3$AT6’S| 6.89 ± 0.39| 0.13 μM | 5    |
| 5-HT$_3$AB   | 5.57 ± 0.06| 2.69 μM | 3    |
| 5-HT$_3$AT6’SB| 5.67 ± 0.06| 2.14 μM | 4    |
| **Dopamine** |            |         |      |
| 5-HT$_3$A    | 4.26 ± 0.20| 55.0 μM | 6    |
| 5-HT$_3$AT6’S| 4.53 ± 0.07| 23.4 μM | 6    |
| 5-HT$_3$AB   | 4.04 ± 0.15| 91.2 μM | 6    |
| 5-HT$_3$AT6’SB| 4.59 ± 0.27| 25.7 μM | 4    |
| **mCPBG**    |            |         |      |
| 5-HT$_3$A    | 6.82 ± 0.14| 0.15 μM | 8    |
| 5-HT$_3$AT6’S| 7.10 ± 0.24| 0.08 μM | 3    |
| 5-HT$_3$AB   | 6.37 ± 0.12| 0.43 μM | 3    |
| 5-HT$_3$AT6’SB| 6.64 ± 0.12| 0.16 μM | 5    |
| **Quipazine**|            |         |      |
| 5-HT$_3$A    | 8.83 ± 0.02| 1.48 nM | 5    |
| 5-HT$_3$AT6’S| 9.06 ± 0.16| 0.87 nM | 5    |
| 5-HT$_3$AB   | 8.31 ± 0.12| 4.90 nM | 4    |
| 5-HT$_3$AT6’SB| 8.76 ± 0.13| 1.73 nM | 4    |
| **VUF10166** |            |         |      |
| 5-HT$_3$A    | 9.82 ± 0.26| 0.15 nM | 5    |
| 5-HT$_3$AT6’S| 9.11 ± 0.13| 0.78 nM | 14   |
| 5-HT$_3$AB   | 7.45 ± 0.12| 35.5 nM | 10   |
| 5-HT$_3$AT6’SB| 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).
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-HT3 receptors, causing both a decreased in agonist EC50 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-HT3 receptor L9′A, L9′F, L9′V and L9′T mutations have small or no effects on 5-HT EC50 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-HT3A subunit causes significant hypersensitivity (~70-fold decrease in EC50) to 5-HT and enhanced constitutive activity when co-expressed with 5-HT3B; in addition there is a minor increase in EC50 (~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-HT3 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-HT3 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 mCPBG at 5-HT3 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 mCPBG has a maximal current response similar to that of 5-HT. In contrast, at human 5-HT3 receptors, the maximum current response to mCPBG is 2.6-fold greater than that for 5-HT. Therefore, a comparison of responses to 5-HT and mCPBG is a simple and effective method of determining expression of 5-HT3 subunits in human 5-HT3 receptors; for example at 30 μM 5-HT and mCPBG current amplitudes at homomeric receptors are equal, but mCPBG-induced responses are fivefold larger in 5-HT3 receptor subtypes.

In summary, we have shown that a channel-lining Thr6′ residue found in the 5-HT3A subunit is a determinant of agonist efficacy; when a T6′S substitution is made in the 5-HT3A subunit, activation by partial agonists is enhanced, with currents being evoked at lower concentrations and with increased maximal amplitudes in both 5-HT3A and 5-HT3B 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.

VUF10166 is only a full agonist at 5-HT3A5′S and not 5-HT3A7′S receptors, and a S6′T substitution in the 5-HT3B 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 EC50, maximal current or agonist/antagonist character of 12 ligands that
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