

Subtle differences between 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors are revealed by partial agonists.

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Abstract

5-HT₃ receptors are members of the Cys-loop family of ligand-gated ion channels, and, like most members of this family, there are multiple subunits that can contribute to functional pentameric receptors. 5-HT_{3A} and 5-HT_{3AB} receptors have been extensively characterised but there are few studies on 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors. Here we explore the properties of a range of partial agonists at 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors following expression in *Xenopus* oocytes. The data show that the characteristics of receptor activation differ in the different heteromeric receptors when they are challenged with 5-HT, m-chlorophenylbiguanide (mCPBG), varenicline, 5-fluorotryptamine (5-FT) or thymol. 5-HT, 5-FT, varenicline and mCPBG activation of 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors yields similar EC₅₀s to homomeric 5-HT_{3A} receptors, but maximal responses differ. There are also differences in the levels of potentiation by thymol, which is greater at 5-HT_{3A} receptors than 5-HT_{3AB}, 5-HT_{3AC}, 5-HT_{3AD} or 5-HT_{3AE} receptors. Docking thymol into the receptor indicates a different residue in the transmembrane domain could provide an explanation for these data. Overall our study suggests that 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} have distinct pharmacological profiles to those of 5-HT_{3A} and 5-HT_{3AB} receptors; this is likely related to their distinct roles in the nervous system, consistent with their differential association with various disorders. Thus these data pave the way for drugs that can specifically target these proteins.

Keywords: ligand-gated ion channel, Cys-loop receptor, serotonin receptor

Introduction

5-HT₃ receptors belong to the Cys loop family of pentameric ligand-gated ion channels, a family which includes nicotinic acetylcholine (nACh), GABA and glycine receptors¹. They play important roles in fast neurotransmission in the central and peripheral nervous systems. 5-HT₃ receptors are widely distributed, especially in the central and peripheral nervous system and the gastrointestinal tract, where they mediate fast excitatory neurotransmission and their malfunction contributes to a range of pathophysiological disorders.

In humans there are five 5-HT₃ receptor subtypes, 5-HT_{3A}-5-HT_{3E} (Figure 1). The subunits are encoded by the *HTR3* genes, located on chromosome 11q23 (*HT3A* and *HT3B*) and 3q27 (*HT3C*, *HT3D* and *HT3E*)². As for other Cys-loop receptors, the repertoire of 5-HT₃ receptor subunits is significantly larger than the number of subunit genes due to the presence of multiple promoters, splice variants, single nucleotide polymorphisms and post-translational modifications^{3,4}. Homomeric 5-HT_{3A} receptors and heteromeric 5-HT_{3AB} receptors have been extensively characterized; these proteins have a range of different properties including kinetics, EC₅₀, and the potencies of some agonists and antagonists, but the most dramatic difference is in their single channel conductance which is considerably larger in 5-HT_{3AB} receptors⁵⁻⁷. Heteromeric receptors containing the 5-HT_{3C}, 5HT_{3D} and 5-HT_{3E} subunits have been less well explored despite 5-HT_{3C} and 5-HT_{3E} subunits being implicated in a range of disorders including schizophrenia, autism and nausea^{3,4,8-12}. The 5-HT_{3D} subunit was initially considered to be non-functional, as it lacks the signal sequence and a large region of the N-terminal domain, including the Cys loop and loop D². However it subsequently became apparent that a longer form of this subunit exists, with most of the features expected of a Cys loop receptor subunit⁸. Here we explore the functional parameters of agonists that activate 5-HT_{3A} and 5-HT_{3AB} receptor at 5-HT_{3AC}, 5-HT_{3AD}, and 5-HT_{3AE} heteromeric receptors expressed in *Xenopus* oocytes, and compare them to data from 5-HT_{3A} and 5-HT_{3AB} receptors.

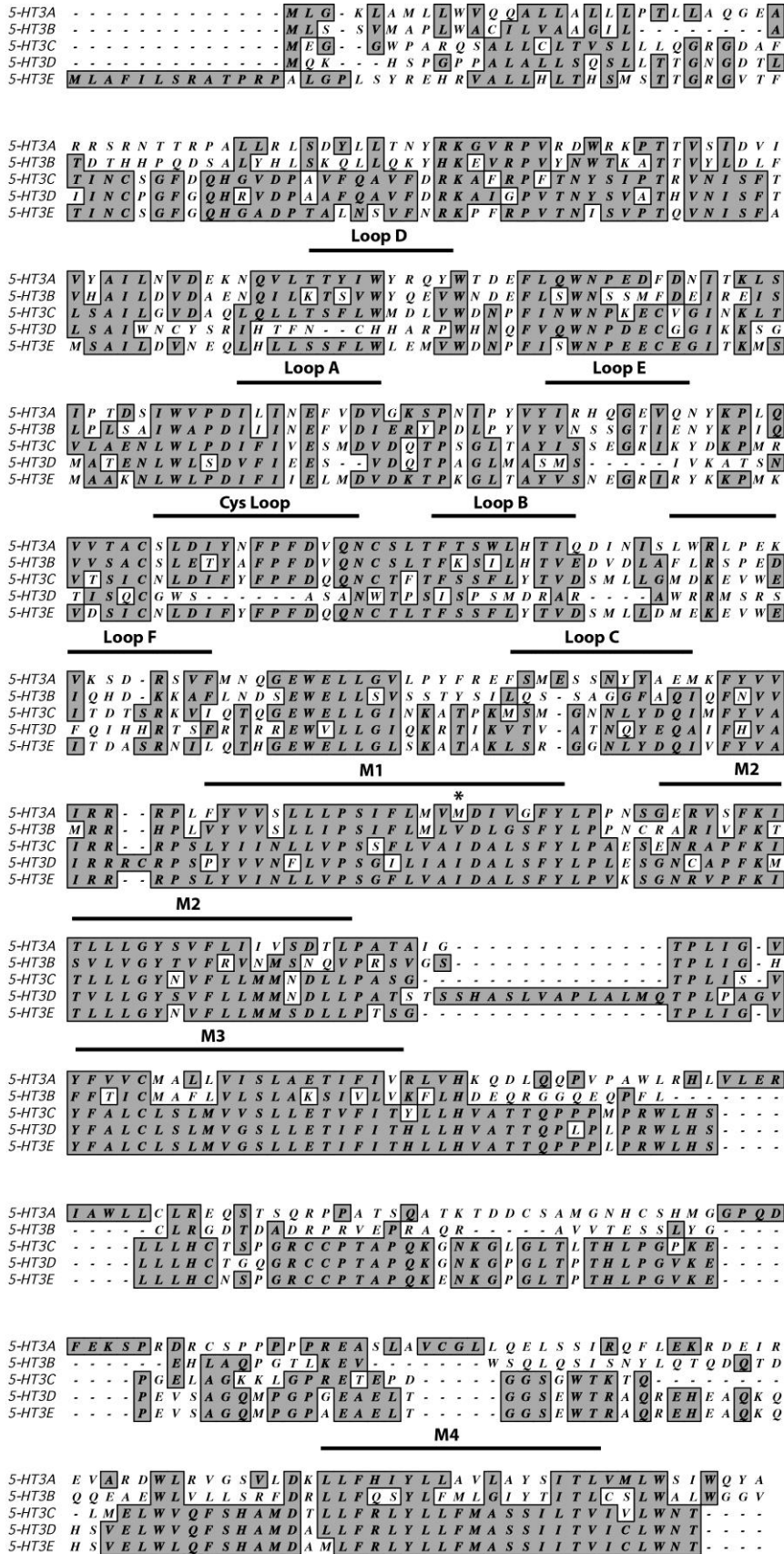


Figure 1. Clustal Omega alignment of human 5-HT3A, B, C, D and E subunits (accession numbers P06498, O95264, Q8WXA8, Q70Z44, A5X5Y0 respectively) showing the approximate positions of binding loops A-F and transmembrane domains 1-4. * represents M259 .

Results

Effect of 5-HT on 5-HT₃ receptors

Application of 5-HT elicited inward currents in oocytes expressing homomeric 5-HT_{3A} receptors and heteromeric 5-HT_{3AB}, 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors. (Figure 2). The EC₅₀ values determined from concentration-response curves for homomeric 5-HT_{3A} receptors were not significantly different to those of 5-HT_{3AB}, 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors (Table 1) similar to previous studies^{4,8,13}.

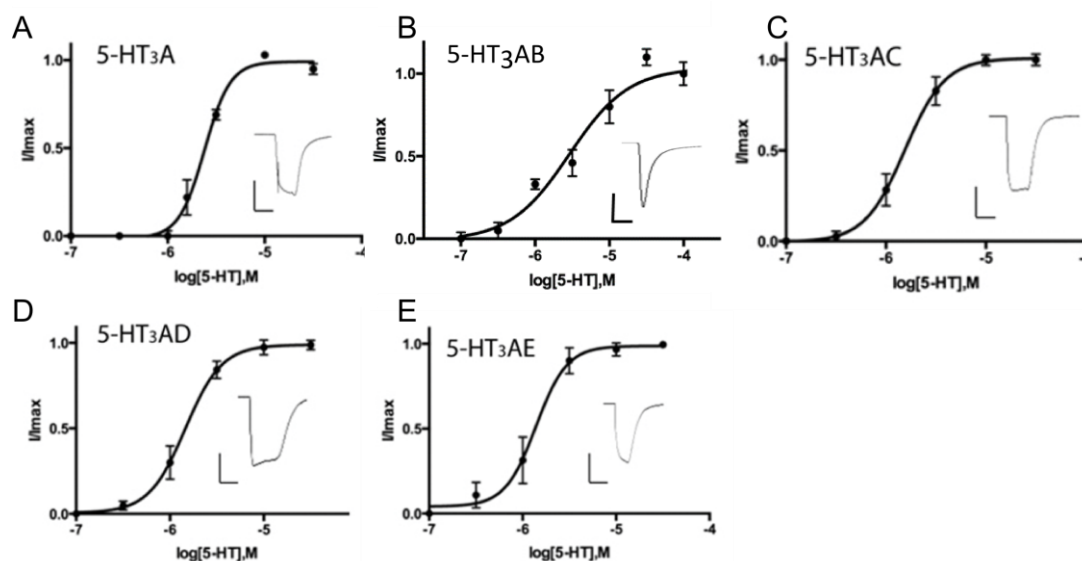


Figure 2: 5-HT concentration response curves for 5-HT₃ receptors expressed in oocytes. Concentration-response curves constructed from responses to 5-HT show 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors have similar EC₅₀s (data in Table 1). Data = mean ± SEM, n= 3-8. Inset: Typical responses to 10 μM 5-HT. Scale bars represent 30s and 5 μA.

Effect of 5-Fluorotryptamine on 5-HT₃ receptors

5-Fluorotryptamine (5-FT) is an agonist at murine 5-HT_{3A} and 5-HT_{3AB} receptors, and in both these receptor populations it is approximately 10 fold less potent than 5-HT, with maximal responses to 5-FT of 64% and 45% respectively when compared to maximal 5-HT responses, demonstrating it is also a partial agonist¹³. Our data presented here from human 5-HT_{3A} and 5-HT_{3AB} receptors indicate it is also a partial agonist in these receptors: we observed maximal responses close to 80% for both 5-HT_{3A} and 5-HT_{3AB} receptors (Figure 3). Application of 5-FT to 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors revealed it was also a partial agonist at these receptors, although maximal responses compared to 5-HT were significantly lower in 5-HT_{3AE} receptors (56 ± 3%, n=5) compared to 5-HT_{3A} receptors (79 ± 4%, n=7). EC₅₀ values were higher when compared to those with 5-HT, but there were no differences between the values obtained in 5-HT_{3A}, 5-HT_{3AB}, 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors (Table 1).

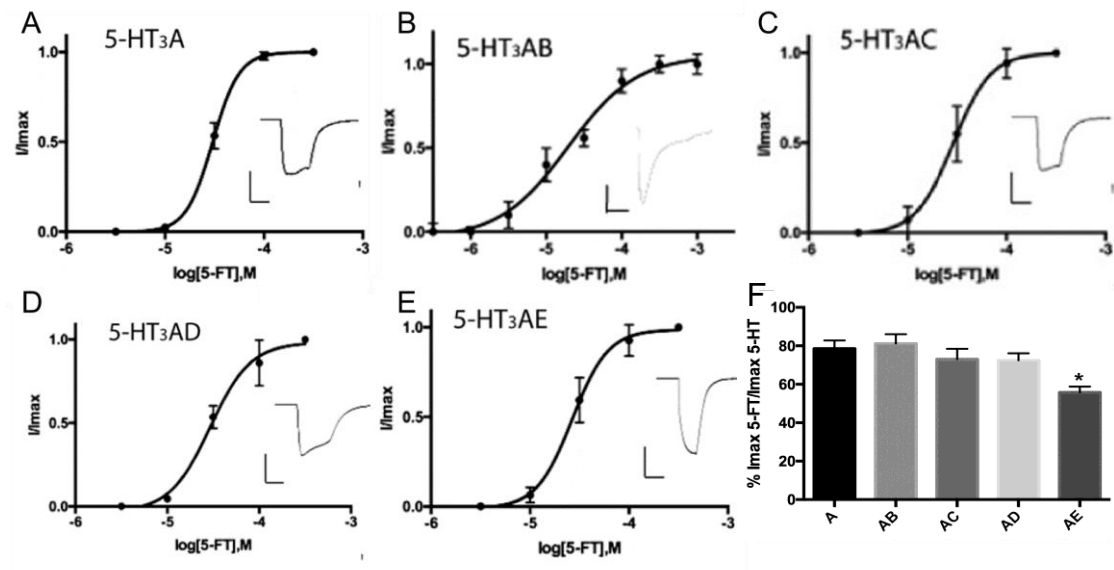


Figure 3: 5-FT activation of 5-HT₃ receptors. Concentration-response curves constructed from responses to 5-FT show 5-HT_{3A} (A), 5-HT_{3AB} (B), 5-HT_{3AC} (C), 5-HT_{3AD} (D) and 5-HT_{3AE} (E) receptors have similar EC₅₀s (data in Table 1). Data = mean ± SEM, n= 3-8. Inset: Typical responses to 100 μM 5-FT; scale bars represent 30s and 5 μA. F: Maximum responses to 5-FT compared to a maximum response to 5-HT show it is a partial agonist with a lower maximal response in 5-HT_{3AE} receptors. Data = mean ± SEM, n= 3-8; *= significantly different to 5-HT_{3A} receptors.

Effect of mCPBG on 5-HT_{3AB}, 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} heteromeric receptors

mCPBG is a more potent agonist than 5-HT in both 5-HT_{3A} and 5-HT_{3AB} receptors, as previously reported^{6,13}, and, as in similar studies, we observed no significant differences in mCPBG EC₅₀s between 5-HT_{3A}, 5-HT_{3AB}, 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors (Table 1). However the data again revealed different efficacies (I_{max} mCPBG/I_{max} 5-HT) for mCPBG at 5-HT_{3AB}, 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} heteromeric receptors when compared to 5-HT_{3A} receptors (Table 1, Figure 4). mCPBG is a super-agonist at 5-HT_{3AB} receptors (as previously reported^{14,18}) with maximal responses significantly greater than maximal responses with 5-HT. However at 5-HT_{3AC}, 5-HT_{3AD} and 5-HT_{3AE} receptors mCPBG is a partial agonist, with mCPBG I_{max} values significantly lower than maximal responses with 5-HT in 5-HT_{3A} receptors (Figure 4).

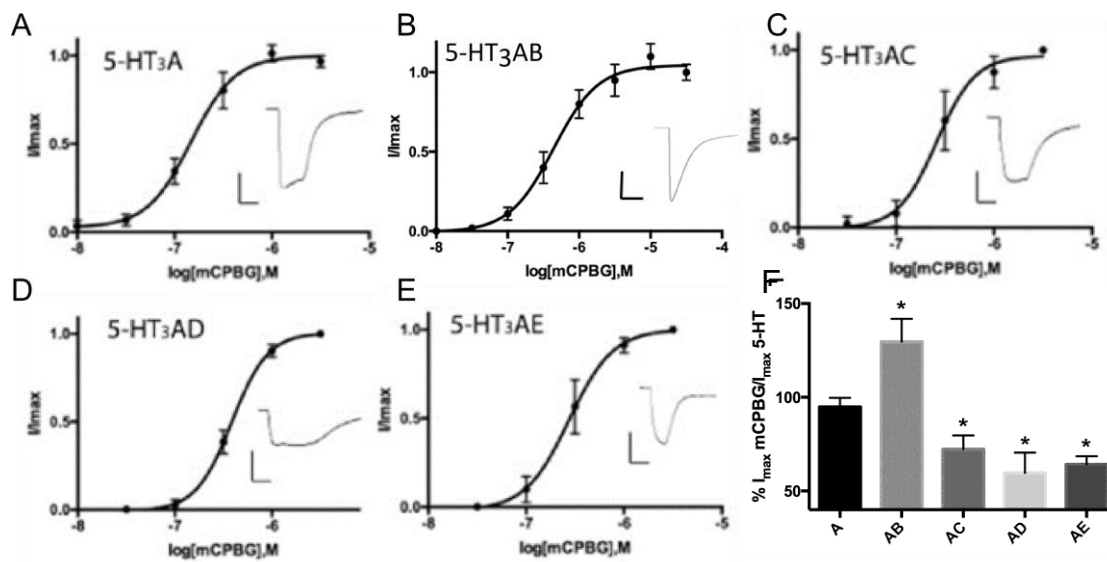


Figure 4: mCPBG activation of 5-HT₃ receptors. A-E: Concentration-response curves constructed from responses to mCPBG show 5-HT₃AB, 5-HT₃AC, 5-HT₃AD, and 5-HT₃AE receptors have similar EC₅₀s (data in Table 1); data = mean ± SEM, n= 3-8. Inset: Typical responses to 1 μM mCPBG; Scale bars represent 30s and 5 μA. F: Maximum responses to mCPBG compared to maximal responses with 5-HT show it is a super agonist at 5-HT₃AB receptors and a partial agonist with a lower maximal response relative to the maximal response with 5-HT in 5-HT₃AC, 5-HT₃AD, and 5-HT₃AE receptors when compared to 5-HT₃A receptors. Data = mean ± SEM, n= 3-8; *= significantly different to 5-HT₃A receptors.

Effect of varenicline on 5-HT₃AB, 5-HT₃AC, 5-HT₃AD and 5-HT₃AE heteromeric receptors

Varenicline is a nACh receptor agonist with different potencies and efficacies at different subtypes of nACh receptors¹⁹. Varenicline is also an agonist at 5-HT₃A receptors¹⁵. In our hands varenicline also activated 5-HT₃A, 5-HT₃AB, 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors with no difference in EC₅₀ values (Table 1, Figure 5) but again there were some differences in relative I_{max} values compared to maximal responses with 5-HT. 5-HT₃AB and 5-HT₃AD receptors have lower I_{max} varenicline/I_{max} 5-HT compared to 5-HT₃A receptors, while values for 5-HT₃AC and 5-HT₃AE receptors were similar (Figure 5F).

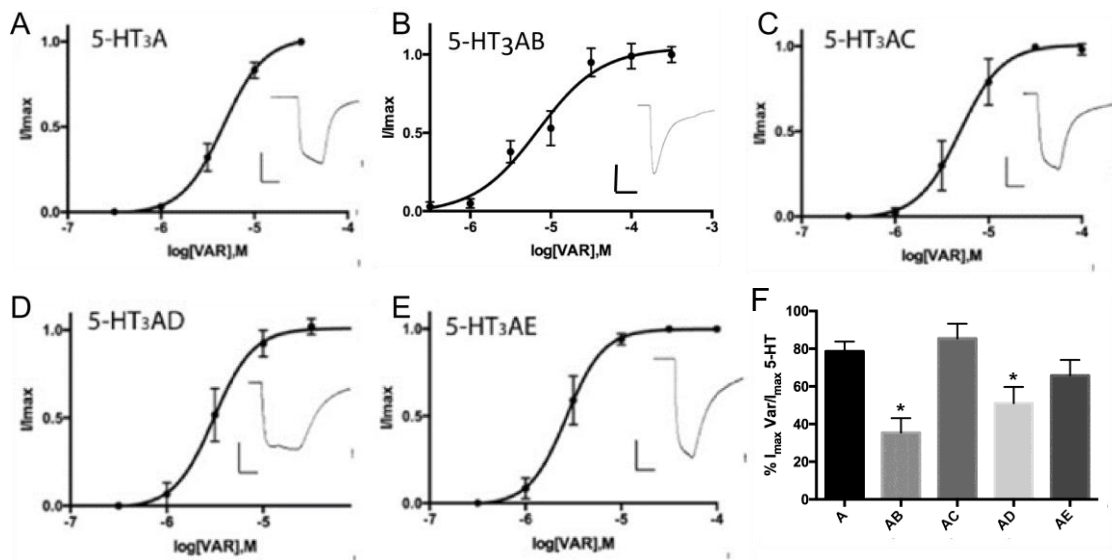


Figure 5: Varenicline activation of 5-HT₃ receptors. A-E: Concentration-response curves constructed from responses to varenicline show 5-HT₃A, 5-HT₃AB, 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors have similar EC₅₀s (data in Table 1); data = mean ± SEM, n= 3-8. Inset: Typical responses to 10 μM varenicline. F: Maximum responses to varenicline show it is a partial agonist with lower maximal responses in 5-HT₃AB and 5-HT₃AD receptors. Data = mean ± SEM, n= 3-8; *= significantly different to 5-HT₃A receptors.

Effect of thymol on 5-HT₃AB, 5-HT₃AC, 5-HT₃AD and 5-HT₃AE heteromeric receptors

Thymol, a plant-derived monocyclic terpene, is an allosteric agonist of 5-HT₃A receptors and also potentiates responses at sub-maximal 5-HT concentrations¹⁶. We observed enhancement of 5-HT elicited responses in all the heteromeric receptors but this level of enhancement was reduced in 5-HT₃AB, 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors compared to 5-HT₃A receptors (Figure 6).

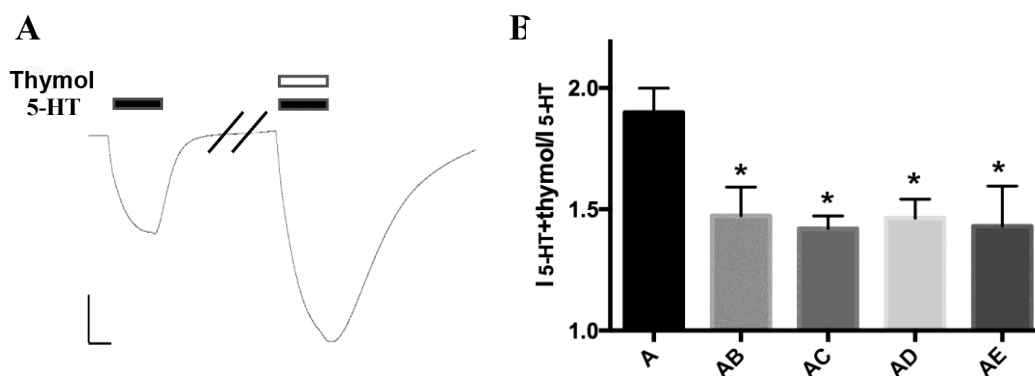


Figure 6: Thymol enhancement of 5-HT₃ receptors. A: Typical response to thymol. Here 5-HT₃A receptors were stimulated with 3 μM 5-HT and then with 3 μM 5-HT plus 10 μM thymol. Scale bars represent 30s and 5 μA. B: Similar experiments in heteromeric receptors revealed lower levels of enhancement. Data = mean ± SEM, n= 3-8; *= significantly different to 5-HT₃A receptors.

Docking of Thymol

Thymol was docked into the transmembrane region of a model of the human 5-HT₃A receptor. The data show that it was located close to M2 and M1 and in particular is within 2.5 Å of Met259 in M1 (Figure 7, see Figure 1 for its location in the amino acid sequence). These data support previously published docking data, which indicate the importance of this Met residue and are supported by functional studies¹⁵. As the equivalent residue in 5-HT₃C, 5HT₃D and 5-HT₃E subunits is Ileu, we generated this mutation *in silico*, which revealed the distance from thymol is increased to 3.5 Å; this could have a significant effect on its stability on the binding pocket (Figure 7C and D).

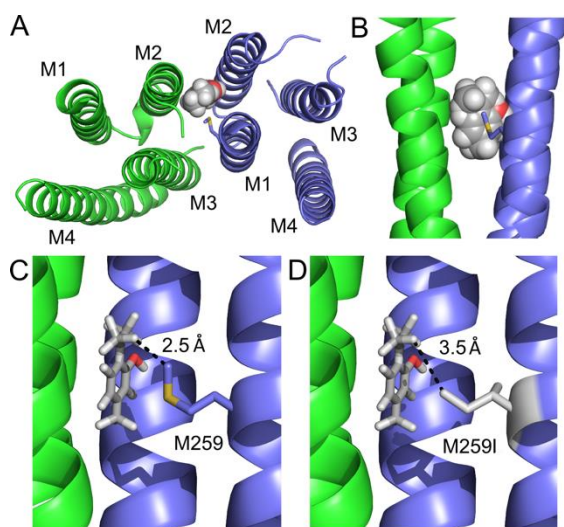


Figure 7. Thymol docked into a model of the human 5-HT₃A receptor showing its proximity to M259 and the consequence of a M259I mutation. The location of M259 in the 5-HT₃A subunit sequence is shown in Figure 1

Discussion

The human 5-HT₃ subunits 5-HT₃C, 5-HT₃D and 5-HT₃E have been little studied, yet there is evidence that they are involved in a range of disorders^{3,4,8-12}. Here we explore characteristics of these subunits, and show there are subtle but significant differences in their response to different 5-HT₃ receptor agonists, indicating different pharmacological profiles for each. This is consistent with the fact that these subunits have different expression patterns in humans, suggesting distinct physiological roles^{4,8}. The data are discussed in more detail below.

5-HT₃ receptors are typical Cys-loop receptors in having multiple subunits. Subunits 5-HT₃C, 5-H3TD and 5-HT₃E have to date been identified in humans and in some other species (e.g. dog and chimp) but are lacking in rodents². There is still some controversy about the role of the 5-HT₃D subunit: expression has been demonstrated in multiple locations including the human colon, but some data suggests that the 5-HT₃D subunit does not contribute to functional receptors and could be a pseudogene in some species^{2-4,8}.

The identification of multiple subunits has important implications; each functional receptor is pentameric, and the specific subunits that constitute it will define the properties of the receptor e.g. the second transmembrane domain (M2) of each receptor contributes to the pore, influencing ion flux, and the orthosteric binding pocket is located between two adjacent subunits, thus each has the potential to contribute differently to the binding properties of one or all ligands that bind to this site. Our data indicate all the subunits can be incorporated with 5-HT₃A subunits to form functional 5-HT₃ receptors, consistent with previous studies, although there is some discrepancy as to whether 5-HT₃B, 5-HT₃C, 5-H3TD and 5-HT₃E subunits reach the membrane if expressed alone. One study suggest that they do⁸, while two indicate that they

do not^{4,17}. The reasons for this discrepancy are not yet clear, but it is possible that differences in expression systems (e.g. HEK versus CHO) and/or intracellular components (e.g. glycosylation machinery, chaperones) are important for single subunit trafficking or expression.

Activation of 5-HT₃AC, 5-HT₃AD and 5-HT₃AE by 5-HT reveals similar parameters to those obtained with 5-HT₃A receptors, as in earlier studies, although we did not specifically probe the kinetics and current-voltage relationships of 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors as they have been previously investigated and reported to show no differences compared to 5-HT₃A receptors^{4,8}. This is in contrast to 5-HT₃AB receptors, where we and others have shown a range of differences^{5,6,18}. The most recent studies suggest that 5-HT binds at an AA interface in 5-HT₃AB receptors, which likely have an AABAB stoichiometry, but in which there is only one of compared to 5 in homomeric receptors²⁰. Differences in other regions of the 5-HT₃B subunit also contribute to the functional differences, including the pore regions and the intracellular domain⁵⁻⁷.

The agonist mCPBG also has been known for some years to distinguish between 5-HT₃A and 5-HT₃AB receptors, as it acts as a super-agonist in 5-HT₃AB receptors, with responses up to 2.5 times larger than for 5-HT^{6,14,18}. A detailed exploration of the action of mCPBG at 5-HT₃AB receptor suggest its unusual action here is likely due to it being able to bind at non AA interfaces; it is also capable of allosterically modulating the activity of 5-HT from these sites¹⁴. Our data show that mCPBG can also distinguish 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors from those of 5-HT₃A and 5-HT₃AB as maximal responses in these 3 heteromers are lower than in 5-HT₃A receptors. Thus, as mCPBG is not a super agonist, we suggest it binds to the same interface as 5-HT (AA) to activate the receptor, with the difference in I_{max} values in 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors being due to less efficient transduction from the binding site to the pore and/or less effective flux through the receptor following incorporation of these subunits.

Varenicline is an interesting compound which has proved to be a popular smoking cessation agent. This is thought to be through its action as a partial agonist of $\alpha 4\beta 2$ nACh receptors¹⁹, but it has also been shown to be a relatively potent and nearly full agonist at 5-HT₃A receptors (I_{max}/I_{max}5-HT = 80%)¹⁵. However it has a lower relative maximal response at rodent 5-HT₃A receptors (I_{max}/I_{max}5-HT = 35%), suggesting only minor changes are necessary in the structure of the protein to decrease the efficacy of varenicline¹⁵. This hypothesis is supported by the data from the heteromeric receptors tested here, as varenicline has similar efficacy to 5-HT₃A receptors at 5-HT₃AC and 5-HT₃AE receptors (~80%), but significantly less at 5-HT₃AB and 5-HT₃AD receptors (38% and 50% respectively). These different efficacies show the different subunits can differentially effect receptor responses.

Thymol is one of an increasing number of compounds that have been shown to modulate 5-HT₃ receptors^{3,16}. We show here that thymol can enhance the response to 5-HT-elicited responses in all our heteromeric 5-HT₃ receptors, but to differing levels. Data from Lansdell et al¹⁶ indicate that thymol binds between the M1 and M2 transmembrane domains with the lowest energy docked conformations of thymol in close proximity to the side-chain of M259 in M1. When M259 was mutated to Val, 5-HT₃A receptors no longer responded to thymol. The equivalent residue is Ile in the M1 of 5-HT₃AC, 5-HT₃AD and 5-HT₃AE receptors (Figure 1). We therefore created a 5-HT₃A receptor homology model where this critical residue was mutated *in silico* to Ile. We were still able to observe docking of thymol but the distance between thymol and Ile was 3.5Å, in comparison to 2.5 Å for M259 (Figure 7). This increase in distance may be responsible for the difference in the ability of thymol to potentiate responses.

Variants of 5-HT₃ receptor genes have long been suspected of playing a role in the etiology of gastrointestinal (GI) disorders^{3,4}. 5-HT₃ receptors are involved in GI motility and have been implicated in a range of GI diseases including anorexia and bulimia, and, most particularly, irritable bowel syndrome (IBS) with variants in both 5-HT₃A and 5-HT₃C genes being associated with the diarrhoea predominant form of IBS (IBS-D); this is also consistent with the many reports of beneficial effects of 5-HT₃ receptor antagonists on this condition. The widespread distribution of 5-HT₃C, 5-HT₃D and 5-HT₃E subunits in the central and peripheral nervous systems is consistent with an important physiological role for these subunits,

and thus understanding their pharmacology is an important first step. Our data show that it is possible to distinguish between these heteromeric receptors with different agonists, and thus it is also likely there are differential effects of antagonists. In addition heteromeric receptors containing more than 2 types of subunit may prove a fertile research area for future studies.

Conclusions

In conclusion, we show here that partial agonists can distinguish between 5-HT₃AB, 5-HT₃AC, 5-HT₃AD and 5-HT₃AE heteromeric receptors. These data suggest that it is possible to distinguish between these different heteromeric 5-HT₃ receptors physiologically, and the design of drugs that specifically target receptors containing these subunits is possible.

Methods

Oocyte maintenance and RNA preparation - *Xenopus laevis* oocytes were purchased from Ecocyte Bioscience (Austin, TX) and stored in ND 96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES pH 7.5) containing 2.5 mM sodium pyruvate, 50 mM gentamicin, and 0.7 mM theophylline. RNA was transcribed *in vitro* from Sph I - linearized plasmid cDNA template using the mMessage mMachine T7 transcription kit (Ambion, Austin, TX). Oocytes were injected with 5-20 ng of cRNA, and currents recorded 1-2 days post injection.

Electrophysiology - Oocytes were clamped at -60 mV using the Roboocyte (Multi Channel Systems), an automated two-electrode voltage clamp workstation. They were continually perfused with Ca-free ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 10 mM HEPES pH 7.5) at 2 ml/min. For partial agonist experiments each oocyte was tested with 4-6 concentrations of each agonist, with a test maximal concentration of 5-HT at the start and end of the experiment to monitor stability of the response. A few oocytes showed rundown during the experiment and these data were not included. Concentration-response data for each oocyte were normalized to the maximum 5-HT-induced current for that oocyte. The mean and S.E.M. for a series of oocytes were plotted against agonist concentration and iteratively fitted to the four-parameter logistic equation using Prism. Values are presented as mean ± S.E.M. Statistical significance (P<0.05) was calculated using a one-way ANOVA and Dunnett's post-test.

Modelling and docking- Models of the human 5-HT₃R subunits based on the mouse 5-HT₃A receptor structure¹⁰ (4pir) were created using MODELLER 9.14²² and used as templates for the *in silico* docking of thymol using GOLD Suite v 5.3 (Cambridge Crystallographic Data Centre). The binding site was defined as a sphere with radius 10Å, with M259 at the centre.

Abbreviations: 5-HT: 5-hydroxytryptamine; nACh receptor: nicotinic acetylcholine; GABA: gamma-aminobutyric acid; HEK: human embryonic kidney; AChBP: acetylcholine binding protein.

Author contributions: Participated in research design: SCRL, KLP. Conducted experiments: YH, KLP, SCRL. Performed data analysis: YH, KLP, SCRL. Wrote or contributed to the writing of the manuscript: KLP, SCRL.

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Tables

Table 1: EC₅₀ values for 5-HT₃ agonists

		5-HT_{3A}	5-HT_{3AB}	5-HT_{3AC}	5-HT_{3AD}	5-HT_{3AE}
5-HT	pEC ₅₀	5.69 ± 0.06	5.36 ± 0.05	5.74 ± 0.04	5.77 ± 0.05	5.89 ± 0.02
	EC ₅₀ (μM)	2.0	4.4	1.8	1.7	1.3
5-FT	pEC ₅₀	4.51 ± 0.03	5.62 ± 0.04	4.60 ± 0.06	4.66 ± 0.07	4.72 ± 0.06
	EC ₅₀ (μM)	31	24	25	22	19
mCPBG	pEC ₅₀	6.42 ± 0.05	6.29 ± 0.05	6.62 ± 0.03	6.52 ± 0.05	6.60 ± 0.03
	EC ₅₀ (μM)	0.38	0.51	0.24	0.30	0.25
Varenicline	pEC ₅₀	5.32 ± 0.04	5.42 ± 0.03	5.29 ± 0.03	5.36 ± 0.04	5.37 ± 0.04
	EC ₅₀ (μM)	4.7	3.8	5.1	4.4	4.3

Data= mean ± SEM, n= 4-8. No values were significantly different (p < 0.05) to WT 5-HT_{3A} receptors.