

REVIEW

Discriminating between 5-HT₃A and 5-HT₃AB receptors

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E-mail: ajt44@cam.ac.uk**Keywords**5-HT₃; ligand gated; ion channel; 5-HT₃A; 5-HT₃B; 5-HT₃AB; antagonist; Cys-loop; allosteric; ligand**Received**

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The 5-HT₃B subunit was first cloned in 1999, and co-expression with the 5-HT₃A subunit results in heteromeric 5-HT₃AB receptors that are functionally distinct from homomeric 5-HT₃A receptors. The affinities of competitive ligands at the two receptor subtypes are usually similar, but those of non-competitive antagonists that bind in the pore often differ. A competitive ligand and allosteric modulator that distinguishes 5-HT₃A from 5-HT₃AB receptors has recently been described, and the number of non-competitive antagonists identified with this ability has increased in recent years. In this review, we discuss the differences between 5-HT₃A and 5-HT₃AB receptors and describe the possible sites of action of compounds that can distinguish between them.

Abbreviations

5-HT, 5-hydroxytryptamine; AFM, atomic force microscopy; BB, bilobalide; DTZ, diltiazem; ECD, extracellular domain; GB, ginkgolide B; M2, second transmembrane α -helix; nACh, nicotinic ACh; NCA, non-competitive antagonist; PTX, picrotoxin; TMD, transmembrane domain

5-HT₃ receptor subtypes

There are seven families of 5-HT receptor (5-HT₁ to 5-HT₇), several containing multiple receptors, which are classified primarily on amino acid similarities and structural properties. The majority of these are GPCRs, with the 5-HT₃ receptor (5-HT₃R) being the only ligand-gated ion channel. The 5-HT₃R also belongs to the Cys-loop receptor family, a group of membrane proteins that include nicotinic ACh (nACh), GABA and glycine receptors, which are responsible for fast synaptic neurotransmission in the CNS and peripheral nervous system (PNS). The first 5-HT₃R subunit to be cloned was 5-HT₃A, but when the protein was expressed in recombinant systems the functional response did not match those seen in some native tissues (Maricq *et al.*, 1991). Many of the differences were resolved when the 5-HT₃B subunit was later cloned (Davies *et al.*, 1999; Dubin *et al.*, 1999). While this subunit could not form functional receptors when expressed alone, in combination with 5-HT₃A subunits it assembled as functional heteromers with altered properties. Since then, heteromeric receptors containing 5-HT₃C, 5-HT₃D or 5-HT₃E subunits have been studied, but these receptors have 5-HT concentration–response curves and biophysical properties that are similar to homomeric 5-HT₃A receptors, and only

minimal pharmacology has been described to date (Niesler *et al.*, 2007; Holbrook *et al.*, 2009). Consequently, only the 5-HT₃A and 5-HT₃AB receptors will be reviewed here (see Niesler, 2011 and Walstab *et al.*, 2010 for reviews on subunits 5-HT₃C to 5-HT₃E).

Currently, the main therapeutic application of 5-HT₃R antagonists is in the management of chemotherapy-induced, radiation-induced, and post-operative nausea and vomiting, where a range of antagonists exhibit high levels of anti-emetic activity with a low incidence of serious adverse effects. There has been limited use of an antagonist for treating Irritable Bowel Syndrome, and partial agonists are also being explored for the same disorder (Gershon and Tack, 2007; Manning *et al.*, 2011). There is also evidence that the 5-HT₃R is involved in depression, drug and alcohol abuse, pruritis, cognitive and psychotic disorders, and pain (reviewed in Thompson and Lummis, 2007; Walstab *et al.*, 2010). 5-HT₃R ligands therefore have considerable therapeutic potential, and subtype-specific ligands could possibly target different conditions to provide a means for improved clinical benefit. Polymorphisms in *HT3A* and *HT3B* genes have been associated with several of these disorders, and to the clinical response to drug treatments, and therefore genetics may also provide opportunities for diagnostics and improved patient care (reviewed in Walstab *et al.*,

2010). The 5-HT₃R antagonists that are currently used in the clinic freely cross the blood brain barrier, making them suitable for treating neurological disorders, while non-permeant compounds could be used to target 5-HT₃R subtypes in the PNS. To this end, Cappelli and co-workers have modified the physicochemical properties of 5-HT₃R ligands to prevent them from crossing the blood brain barrier (Butini *et al.*, 2009; Morelli *et al.*, 2009; Cappelli *et al.*, 2010; Modica *et al.*, 2010). Ligands with activities at more than one receptor have also been described by the same group, and have the potential for treating disorders with complex aetiologies (Morelli *et al.*, 2009; Cappelli *et al.*, 2011). New ligands are still being discovered, and fragment library screens have recently been used to successfully identify novel allosteric modulators and competitive antagonists, with at least one showing 5-HT₃R subtype selectivity. These studies demonstrate that the search for new 5-HT₃R ligands is still valuable over 30 years after the discovery of the first antagonists, and that there may be several ways of targeting the more recently identified 5-HT₃R subtypes (Thompson *et al.*, 2010b; Trattng *et al.*, 2012; Verheij *et al.*, 2012).

Differences between 5-HT₃A and 5-HT₃AB receptors

5-HT₃AB receptors have been extensively investigated in heterologous systems, and when compared with homomeric 5-HT₃R they have differing 5-HT concentration–response curves (increased *EC*₅₀ values and shallower Hill slopes), increased single channel conductance (5-HT₃A = sub-pS; 5-HT₃AB = 16–30 pS), an increased rate of desensitization, reduced relative Ca²⁺ permeability and different current–voltage relationships (5-HT₃A is inwardly rectifying, 5-HT₃AB is linear) (Davies *et al.*, 1999; Kelley *et al.*, 2003; Livesey *et al.*, 2008; Peters *et al.*, 2010). However, it is worth noting that the extent of some of these differences can vary depending upon the species studied; for example, human 5-HT₃A and 5-HT₃AB receptors have a larger difference in their *EC*₅₀ values than their rat or mouse counterparts (e.g. Hanna *et al.*, 2000; Hayrapetyan *et al.*, 2005; Stevens *et al.*, 2005; Thompson and Lummis, 2008; Lochner and Lummis, 2010).

Early work suggested that the 5-HT₃B subunit was only present in the PNS. Now there is evidence for its distribution in the human CNS, but expression in rodents is still disputed (reviewed in Jensen *et al.*, 2008; Barnes *et al.*, 2009; Walstab *et al.*, 2010). In these studies, subtype-specific ligands could have provided a convenient method of probing the character of receptors, but it was only recently that ligands were identified that could distinguish 5-HT₃A from 5-HT₃AB receptors (Table 1). The majority of these are non-competitive antagonists (NCAs) that bind in the receptor pore, and their differences in potency reflect the differing pore-lining residues of the 5-HT₃A and 5-HT₃B subunits.

In contrast to the growing list of NCAs that can distinguish between the two 5-HT₃R subtypes, the affinities of competitive antagonists are usually similar at 5-HT₃A and 5-HT₃AB receptors (Brady *et al.*, 2001). The apparent affinities of agonists are slightly reduced at 5-HT₃AB receptors, possibly as a result of their efficacy, but the differences are too small to

be of practical use (Colquhoun, 1998). However, the first reports of competitive antagonist that discriminate between the two receptor subtypes were recently described, and we shall discuss the properties of these below. Between species, the affinities of competitive ligands can vary because of their differing binding site residues. One notable example is 3-(2-hydroxy,4-methoxybenzylidene)-anabasine, which is a partial agonist at the human 5-HT₃R and an antagonist at mouse receptors (Miyake *et al.*, 1995; Hope *et al.*, 1999; Zhang *et al.*, 2006; 2007).

Ligand-binding sites in 5-HT₃R

The 5-HT₃R consists of five subunits that surround a central ion-conducting pore. Each of the subunits contains three functional domains. The extracellular domain (ECD) is responsible for agonist binding, the transmembrane domain (TMD) forms a channel that allows ions to cross the cell membrane, and the intracellular domain influences channel conductance, receptor trafficking and intracellular modulation (Thompson *et al.*, 2010a). The majority of ligands described to date bind at two main regions of the 5-HT₃R: the orthosteric binding site (where the native agonist binds) and the channel (Figure 1).

The orthosteric binding site is located in the ECD at the interface of two adjacent subunits, and is formed by six distinct peptide loops (Figures 1A & 2). These are loops A–C from one subunit (termed the principal or ‘+’ face) and loops D–E from the other (complementary or ‘-’). The molecular determinants of agonists and competitive antagonists that bind here are reviewed in Thompson *et al.* (2010a).

Compounds that interact at locations other than the orthosteric binding site often do so in the TMD. The TMD of each subunit consists of four α -helices (M1–M4), with M2 from each subunit coming together to form the central ion-conducting pore (Figure 1B). To facilitate comparisons between the M2 regions of different subunits, residues are referred to by an index number, with O’ being a conserved positively charged residue at the cytoplasmic end of the α -helices. Many of the NCAs that bind in the pore inhibit the 5-HT₃R by blocking the channel, but allosteric modulators can also influence channel gating by binding to sites such as the inter-subunit region at the extracellular ends of the TMD α -helices (Trattng *et al.*, 2012).

The location of orthosteric binding sites in 5-HT₃AB receptors

The pentameric structure of the 5-HT₃R was first observed using electron microscopy in 1995 (Boess *et al.*, 1995). Homomeric receptors consist of five identical 5-HT₃A subunits, but the stoichiometry of heteromeric 5-HT₃AB receptors has been more difficult to determine. In 2005, it seemed that this problem had finally been solved when atomic force microscopy (AFM) was used to measure angles between antibodies on dual-labelled receptors consisting of co-expressed epitope-tagged human 5-HT₃A-myc and 5-HT₃B-V5 subunits (Barrera *et al.*, 2005). For 5-HT₃AB receptors the results were unequivocal, showing that when samples were labelled with anti-Myc,

Table 1

IC₅₀ values derived from electrophysiological measurements at 5-HT_{3A} and 5-HT_{3AB} receptors

Compound ^a	5-HT _{3A} IC ₅₀ (μM)	5-HT _{3AB} IC ₅₀ (μM)	Mode of action	Reference
5-Hydroxyindole ^b	–	–	Competitive and non-competitive	Deiml <i>et al.</i> , 2004; Hu and Lovinger, 2008
α-Thujone ^b	60	–	Non-competitive	Deiml <i>et al.</i> , 2004
Bilobalide	468	3100	Non-competitive	Thompson <i>et al.</i> , 2011a
Chloroquine	24.3	23.6	Competitive	Thompson and Lummis, 2008
Diltiazem	21.4	302	Competitive and non-competitive	Thompson <i>et al.</i> , 2011a
d-Tubocurarine	3.4	14.2	Competitive	Davies <i>et al.</i> , 1999
Etomidate	180	140	Non-competitive	Rusch <i>et al.</i> , 2007
Ginkgolide B	727	3900	Non-competitive	Thompson <i>et al.</i> , 2011a
Irinotecan	5.37	14.0	Competitive	Nakamura <i>et al.</i> , 2011
Methadone	14.1	41.1	Competitive and non-competitive	Deeb <i>et al.</i> , 2009
Mefloquine	0.66	2.70	Competitive and non-competitive	Thompson and Lummis, 2008
Morphine	0.33	1.15	Competitive and non-competitive	Baptista-Hon <i>et al.</i> , 2012
Pentobarbital	520	270	Non-competitive	Rusch <i>et al.</i> , 2007
Picrotoxin	41.2 ^c	1135 ^c	Non-competitive	Das and Dillon, 2005
Picrotoxinin	10.7	63.1	Non-competitive	Thompson <i>et al.</i> , 2011a
Propofol	370	300	Non-competitive	Rusch <i>et al.</i> , 2007
Quinine	1.06	15.8	Competitive and non-competitive	Thompson and Lummis, 2008
VUF10166	ND	0.04	Competitive and allosteric	Thompson <i>et al.</i> , 2012b
Topotecan	114.1	8.5 ^d	Competitive	Nakamura <i>et al.</i> , 2013

^aThe affinities of the listed compounds may be different if measured by radioligand methods.

^b5-Hydroxyindole and α-thujone have complex actions on 5-HT_{3R} function and concentration dependence has not been enumerated for some subtypes.

^cValues from mouse receptors; all others are from human receptors. ND = inhibition not determined as the dissociation of this ligand is too slow to make equilibrium measurements. It should be noted that several of these compounds also have effects at other receptor types; for example, diltiazem is better known as a voltage-gated calcium channel blocker for use in hypertension; irinotecan is an anti-cancer agent; and chloroquine, mefloquine and quinine are anti-malarial drugs.

^dEC₅₀ value as topotecan potentiates 5-HT_{3AB} receptor responses.

the angle between two antibodies was 144°, with angles of both 72° and 144° being observed with anti-V5 antibodies. These results showed that in heteromeric receptors, more than one copy of both 5-HT_{3A} and 5-HT_{3B} subunits were present in the receptor, that 5-HT_{3A}-myc subunits were always separated by another subunit, and that 5-HT_{3B}-V5 subunits were either adjacent or separated by another subunit. The only possible arrangement of the five subunits was B-B-A-B-A, reminiscent of the α and non-α subunit arrangements in the heteromeric nACh (e.g. δ-β1-α1-ε-α1, β2-β2-α4-β2-α4) and GABA_A (e.g. γ2-β2-α1-2β-α1) receptors.

However, it is difficult to understand how such a large diversity of competitive antagonists could have similar affinities at 5-HT_{3A} and 5-HT_{3AB} receptor-binding sites when the B-B-A-B-B arrangement does not contain the A+A- interface found in 5-HT_{3A} receptors. Additionally, both homomeric and heteromeric receptors are activated by the same agonists despite the different binding sites this stoichiometry imposes. One proposal suggested that the A+B- interfaces of the heteromeric receptor are equivalent to the A+A- binding sites found in the homomer (Moura Barbosa *et al.*, 2010), but a more likely reason is that a common A+A- binding site exists in both

receptor types. To investigate this, Lochner and Lummis (2010) exchanged residues in mouse 5-HT_{3A} and 5-HT_{3B} subunits to determine the effects these substitutions had on 5-HT_{3R} radioligand binding and function. They found that substitutions in the 5-HT_{3A} subunit altered antagonist binding and 5-HT activation, but equivalent substitutions in the 5-HT_{3B} subunits had no effects, indicating that only 5-HT_{3A} subunits form the orthosteric binding site. Thompson *et al.* (2011c) supported this conclusion using disulphide trapping between cysteines on either side of the binding pocket of the human 5-HT_{3R}. Several residue combinations were tested and pairings between loops C-E and loops C-F were identified. These 5-HT_{3A} subunit double mutants were expressed as both homomeric receptors and in combination with 5-HT_{3B} subunits, and in both receptor types a response to 5-HT was not seen until the disulphide bonds were reduced by DTT. Removal of DTT allowed a gradual reduction in peak current amplitude of subsequent 5-HT responses as the disulphide bonds reformed. These experiments demonstrated that an A+A- interface is essential for agonist activation in both receptor types, an interface that is absent from the B-B-A-B-A stoichiometry that was reported using AFM.

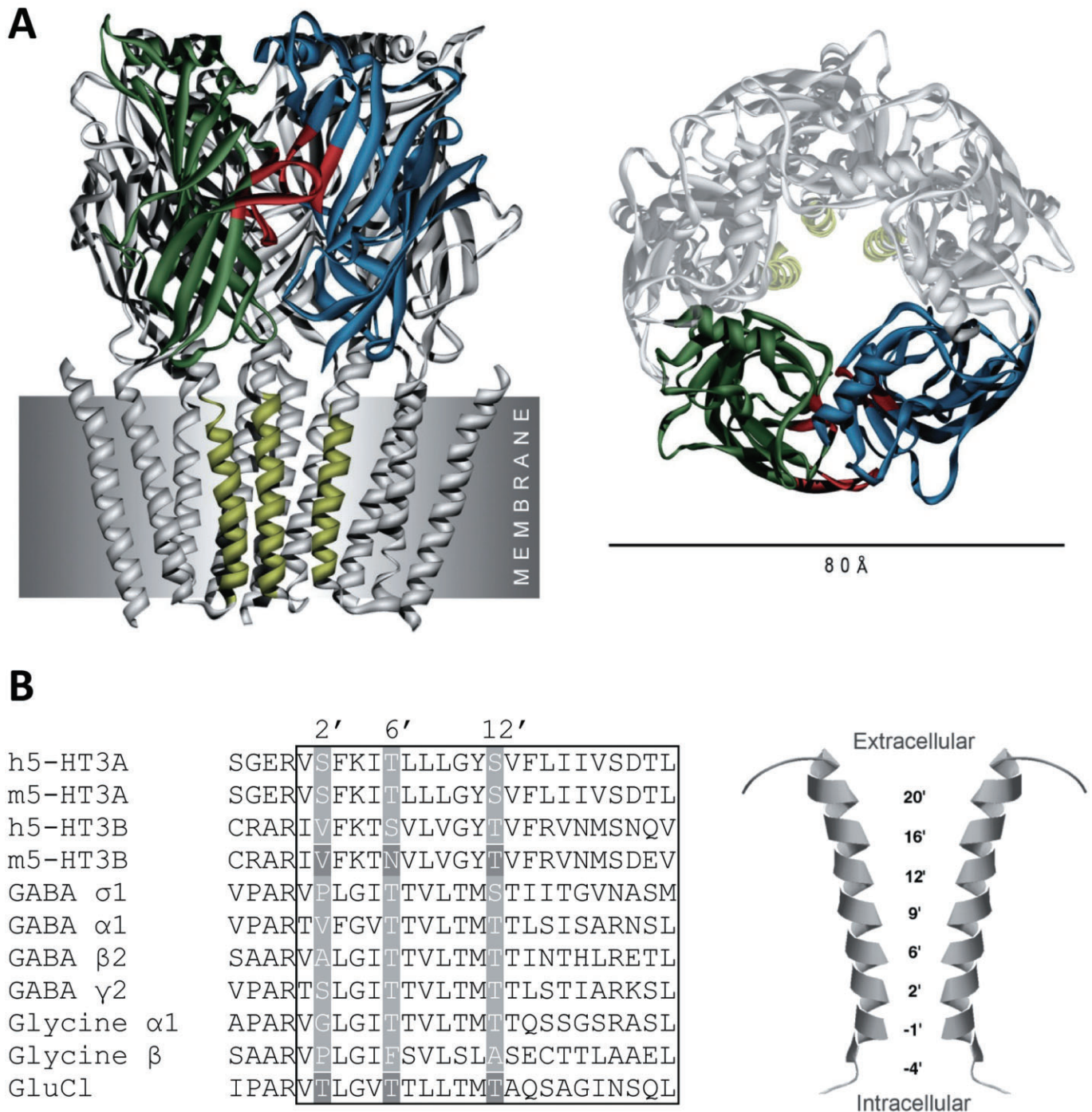


Figure 1

Binding sites in 5-HT₃R. (A) The 5-HT₃R consists of five subunits that surround a central ion-conducting pore that is shown here from the side (left) and from the extracellular side (right) of the cell membrane. The orthosteric binding site (red) is located in the extracellular domain at the interface of two subunits (green and blue). The transmembrane domain consists of four α -helices (M1–M4) from each subunit, and the pore is formed by the convergence of five M2 α -helices (yellow); M1–M4 of the two facing subunits have been removed to view the pore more clearly. Competitive antagonists bind to the orthosteric site and the majority of non-competitive antagonists to the channel. Hydrophobic ligands may bind in inter-subunit cavities at the top of transmembrane domain α -helices. The orthosteric binding site is seen in more detail in Figure 2. (B) An alignment of amino acids that form the M2 α -helices (left) in a range of receptors, and their locations in the pore (right). Channel-lining residues mentioned in the text are highlighted as white on grey. The box shows the extent of the M2 α -helix as described by Hilf and Dutzler (2008). Accession numbers for the alignment are: human 5-HT3A P46098, mouse 5-HT3A Q6J1J7, human 5-HT3B O95264, mouse 5-HT3B Q9JHJ5, human GABA σ P24046, human GABA α 1 P14867, GABA β 2 P47870, GABA γ 2 P18507, Glycine α 1 P23415, Glycine β P48167, GluCl G5EBR3.

A

		LoopD	
5-HT3A	VSIDVIVYAILNVDEKNQVLTYYI	WYRQYWTDEFLQWNPEDFDNITKLSIPTDSIWVPDI	
5-HT3B	VYLDLDFVHAILDVAENQILKTSV	WYQEVWNDEFLSWNSMFMDEIREISLPLSAIWAPDI	
	LoopA	LoopE	LoopB
5-HT3A	L	INEFVDVGKSPNIPYVYIRHQGEVQNYKPLQVVTACSLDIYNFPFDVQNC	SLTFTSWLH
5-HT3B	L	INEFVDIERYPDLPYVYVNSSGTIENYKPIQVVSACSLETYAFPFVQNC	SLTFKSI LH
		LoopF	LoopC
5-HT3A	TIQDINISLWRLPEKVK	SDRSVFMNQGEWELLGVLPHYFRE	FSMESSNYAEMKFYVIRR
5-HT3B	TVEDVDLAFLRSPEDIQ	HDKKAFLNDSEWELLSVSSYSTYSI	LQS-SAGGFAQIQFNVMRR
	M1	M2	
5-HT3A	RPLFYVVS	LLLPSIFLMVMDIVGFYLPENSGERVSFKITLLGLYSVFLIIVSDTL	PATAI
5-HT3B	HPLVYVVS	LLIPSIFLMLVDLGSFYLPENCRARIVFKTSVLVGYTVFRVNMNSQV	PRSVG
	M3		
5-HT3A	GTPLIGVYFV	VCMA LLVVISLAETIFIVRLVHKQDLQQPVPAWLRHLVLERIAWLLCLREQ	
5-HT3B	STPLIGHFFT	ICMAFLVLSLAKSIVLVKFLHDEQRGG-----	QEQPFLCLRGD
5-HT3A	STSQRP--	PATSQATKTDDCSAMGNHCSHMGGPQDFEKSPRDRCSPPPPPREASLAVCGL	
5-HT3B	TDADRPRVEPRAQRAVVT	ESSLYGEHLAQP-----	TLKEV
		M4	
5-HT3A	LQELSSIRQFLEKR	DEIREVAR	DWLRVGSVLDKLLFHIYLLAVLAYSITLVMLWSIWQYA
5-HT3B	WSQLQSISNYLQT	QDQTDQQEAEWLVL	LSRFDRLLFQSYLFMLGIYITITLCSLWALWGGV

B

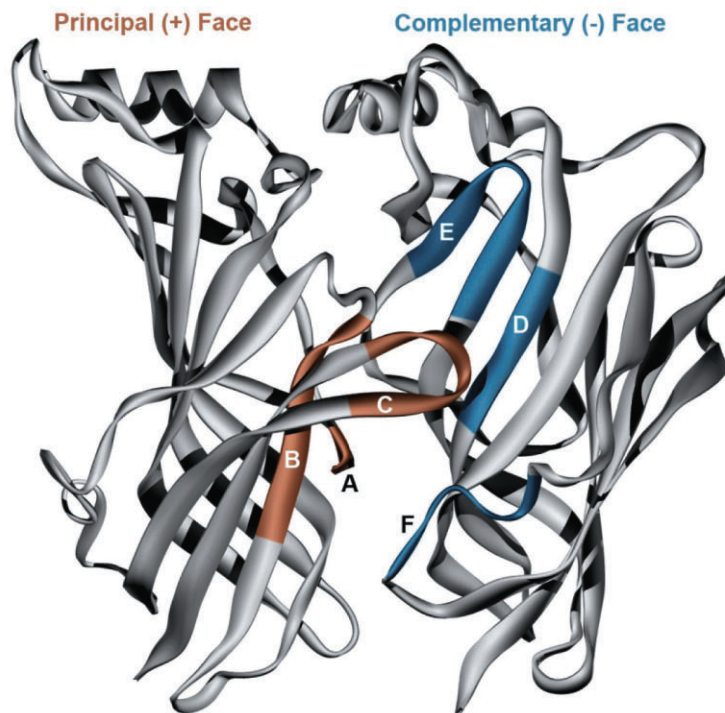


Figure 2

Human 5-HT3A and 5-HT3B subunits. (A) A protein sequence alignment highlighting the binding loops of the ECD and the α -helices of the transmembrane domain (M1–M4). The orange and blue colours show the residues that are shown in (B); a description of specific ligand–receptor interactions in the orthosteric binding site of the 5-HT₃R can be found in a review by Thompson *et al.* (2010a). The channel can also be seen in Figure 1B. Accession numbers for the human sequences are 5-HT3A P46098, 5-HT3B O95264. (B) A homology model of the 5-HT₃A receptor extracellular domain, showing binding loops A–C of the principal (orange) face and D–F of the complementary (blue) face. Only two of the five subunits are shown for clarity.

However, there are several other possible reasons for the differences between these studies, including external factors such as temperature, ratios of subunit DNA transfected, expression systems, added epitope tags and differences in endogenous levels of chaperones. It is also possible that while the studies of Lochner and Lummis (2010) and Thompson *et al.* (2011c) examined only functional cell-surface receptors, AFM may have detected both intracellular and cell-surface receptors that could differ in their stoichiometries.

Other experiments have also supported the presence of an A+A⁻ interface in both receptor types (Thompson *et al.*, 2011c). Single cysteine substitutions to binding-site residues on the principal or complementary faces of 5-HT₃A subunits showed that the majority affected the 5-HT EC₅₀ and the binding affinity of the 5-HT₃-specific competitive antagonist [³H]granisetron, regardless of whether they were expressed as homomeric or heteromeric receptors. Further changes were seen when (2-aminoethyl)methanethiosulfonate hydrobromide (MTSEA) was applied to these mutant receptors, a reagent that covalently modifies cysteine residues, adding bulk and thereby limiting access to the ligand-binding site; co-applying MTSEA with 5-HT₃R ligands protected the residues, confirming their location in the binding site. In contrast, the 5-HT₃B subunit substitutions did not alter 5-HT function or granisetron binding, suggesting that neither the principal nor the complementary faces of this subunit bind ligand or activate the receptor. These results support a stoichiometry that contains an A+A⁻ interface in heteromeric receptors. However, to provide further support for this stoichiometry, evidence from other techniques is needed, such as that from high-resolution structural data Förster resonance energy transfer, total internal reflection fluorescence microscopy and reporter mutations that have been used on other Cys-loop receptors (Chang *et al.*, 1996; Boorman *et al.*, 2000; Durisic *et al.*, 2012; Srinivasan *et al.*, 2012).

Competitive antagonists with differing properties at 5-HT₃A and 5-HT₃AB receptors

The majority of competitive ligands have similar affinities at 5-HT₃A and 5-HT₃AB receptors (Table 1, Figure 3; Brady *et al.*, 2001; Low *et al.*, 2001). The ligand VUF10166, however, is unusual because it distinguishes between the two receptor types via their binding sites. VUF10166 displaces [³H]granisetron with sub-nanomolar affinity at 5-HT₃A receptors, and shows surmountable effects on [³H]granisetron saturation binding curves, indicating competitive antagonism (Thompson *et al.*, 2012b). Dissociation of [³H]granisetron in the presence of excess VUF10166 is best fit by a single exponential decay, suggesting that there is a single population of binding sites. When the 5-HT₃B subunit is co-expressed these properties are changed. The affinity of VUF10166 is lower at 5-HT₃AB receptors, and [³H]granisetron saturation-binding curves are insurmountable, indicating a non-competitive behaviour. [³H]granisetron dissociation in the presence of excess VUF10166 is also altered; 5-HT₃AB receptors have two rates, one that is similar to 5-HT₃A receptors and another that is more rapid. The faster dissociation rate is eliminated when

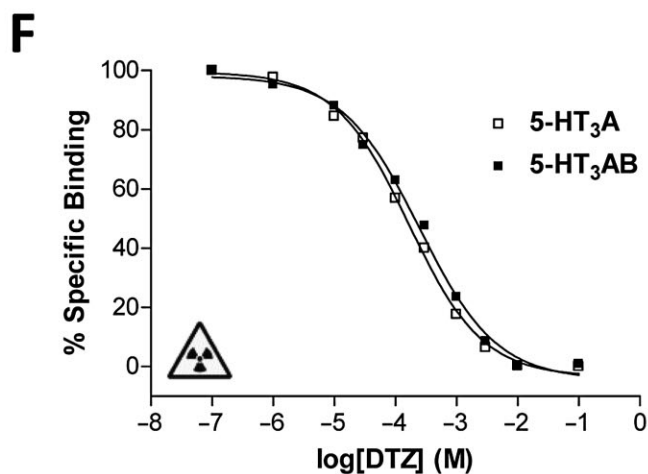
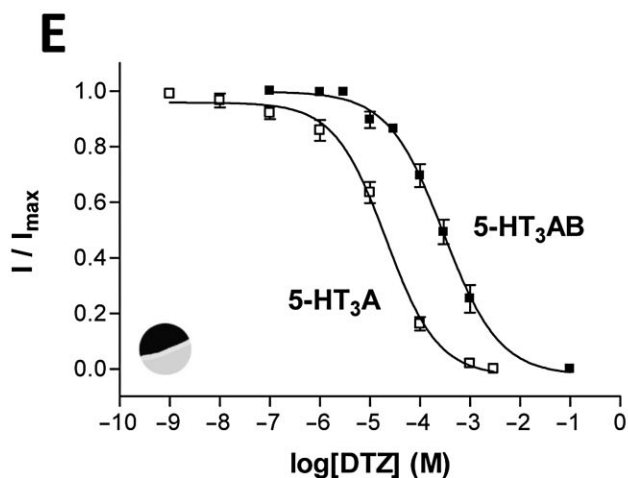
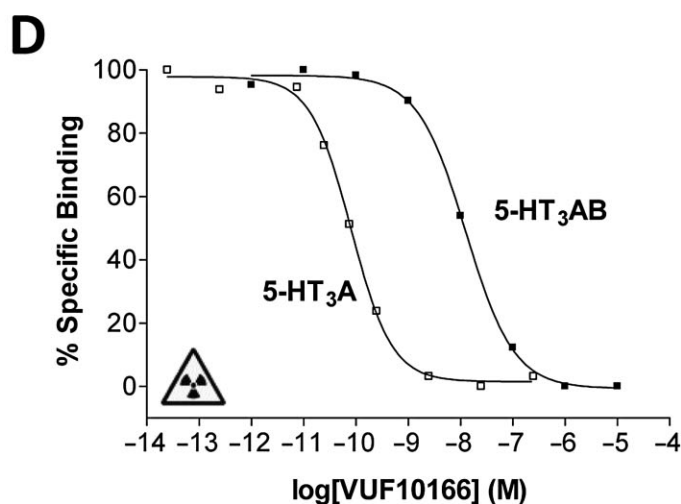
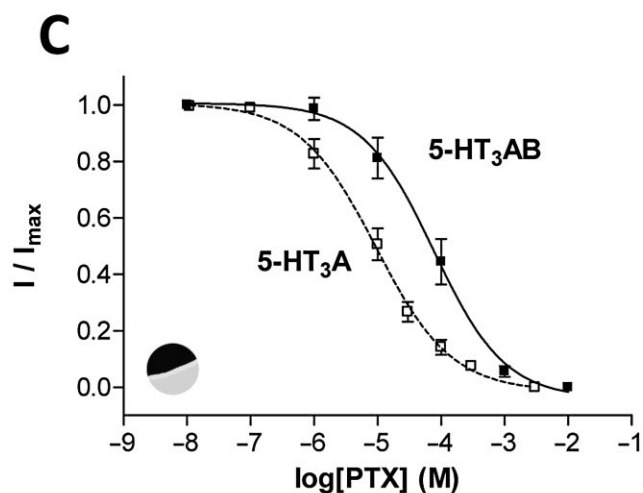
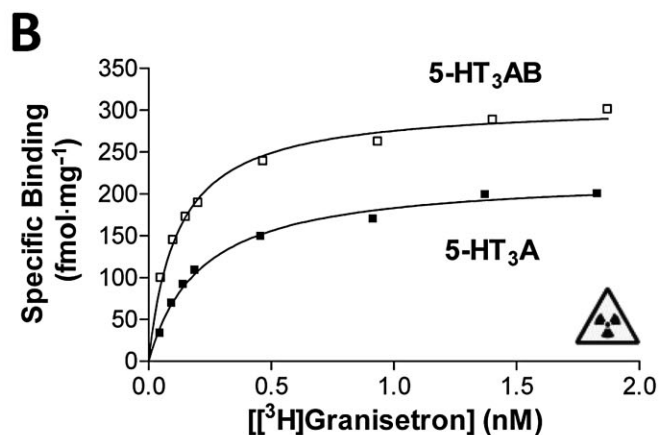
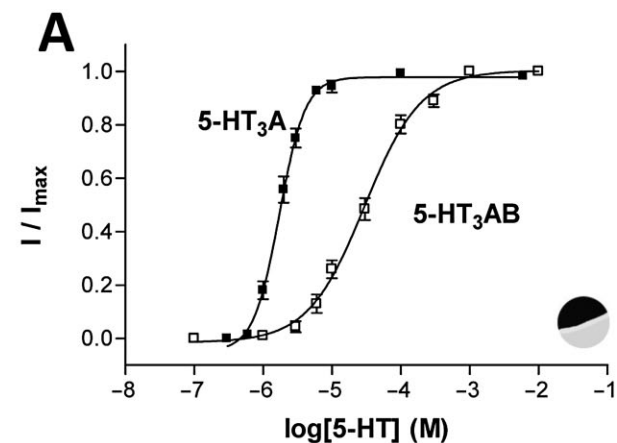
substitutions are made to the complementary face of the 5-HT₃B subunit (B⁻), but is unaffected by substitutions to the principal face (B⁺), indicating an interaction of VUF10166 at an A+B⁻ interface.

Functional studies also reveal differences. VUF10166 potently inhibits 5-HT-induced responses at 5-HT₃A and 5-HT₃AB receptors expressed in oocytes, but recovery from inhibition is much faster at 5-HT₃AB receptors, consistent with the more rapid dissociation seen in radioligand-binding studies (Table 1). At homomeric receptors, VUF10166 also elicits a partial agonist response ($R_{max} = 0.24$) at micromolar concentrations, followed by a long-lived inhibition of subsequent responses, possibly due to receptors slowly accumulating in a ligand-bound desensitized state, as has been observed for other 5-HT₃R agonists (van Hooft and Vijverberg, 1996). Similar to the binding described above, substitutions to the complementary face of the 5-HT₃B subunit (B⁻) produce receptors with recovery rates more similar to those from 5-HT₃A receptors containing only A+A⁻ binding sites, supporting the hypothesis that the interaction of VUF10166 at an A+B⁻ interface is responsible for the observed differences between the homomeric and heteromeric receptors. Therefore, at 5-HT₃A and 5-HT₃AB receptors, VUF10166 binds in the orthosteric binding site formed at A+A⁻ interfaces, but at 5-HT₃AB receptors it also binds to an A+B⁻ binding site from where it may allosterically increase the dissociation of ligands bound to the A+A⁻ binding site (Thompson *et al.*, 2012b). Differing effects of topotecan at 5-HT₃A and 5-HT₃AB receptors were also reported during the preparation of this review. At high micromolar concentrations 5-HT₃A receptors currents are competitively inhibited by topotecan while 5-HT₃AB receptor currents are potentiated, a property that is influenced by a 5-HT₃B subunit mutation (Y129C) that lies outside of the binding site (Nakamura *et al.*, 2013).

Other ligands that may bind to sites other than the orthosteric binding site include *d*-tubocurarine and azasetron. These ligands inhibit 5-HT₃A receptor currents with differing potencies than those from 5-HT₃AB receptors, but radioligand binding shows they have the same affinities at both 5-HT₃A and 5-HT₃AB receptors (Davies *et al.*, 1999; Dubin *et al.*, 1999; Brady *et al.*, 2001). As the binding and functional studies give different results, it is possible that these ligands also bind elsewhere or they are slow to reach equilibrium, meaning that current responses desensitize before the full antagonist effects are seen, a property that could particularly influence inhibition at the more rapidly desensitizing 5-HT₃AB receptor.

Non-competitive antagonists

A range of NCAs can discriminate between 5-HT₃A and 5-HT₃AB receptors (Table 1, Figure 3). Picrotoxin (PTX) is a well-known GABA_AR channel blocker that blocks many other Cys-loop receptors, and was one of the first to be studied in detail at the 5-HT₃R (Das *et al.*, 2003a). Its potency at 5-HT₃AB receptors is lower than at 5-HT₃A receptors, and substitution of 5-HT₃A subunit channel-lining residues has shown that PTX binds close to the 6' position of M2 (Das and Dillon, 2003; Thompson *et al.*, 2011a). PTX binding is also influenced by substitutions at the 9' and 12' residues, which may affect the



passage of this NCA as it descends through the narrowest region (9'-13') of the pore to its binding site at the 6' position (Thompson *et al.*, 2011a). In GABA_A, glycine and glutamate-gated chloride channels (GluCl), PTX acts at or close to the -2', 2' and 6' residues, demonstrating that it can reach below the channel gate (9') to exert its actions in all PTX-sensitive Cys-loop receptors (Ffrench-Constant *et al.*, 1993; Gurley *et al.*, 1995; Hawthorne *et al.*, 2006; Hibbs and Gouaux, 2011).

Other channel-blocking compounds might similarly be expected to distinguish 5-HT₃A and 5-HT₃AB receptors. This is indeed the case for bilobalide (BB) and ginkgolide B (GB) that have binding sites that overlap with the structurally related PTX and are ~6-fold less potent at 5-HT₃AB than at 5-HT₃A receptors (Thompson *et al.*, 2011b). It is possible that differing residues in the channel of the 5-HT₃B subunit are responsible for the lower potency at heteromers because sub-

Figure 3

Examples of electrophysiological (●) and radioligand binding (△) measurements at human 5-HT₃A and 5-HT₃AB receptors. (A) Concentration–response curves differ at human 5-HT₃A and 5-HT₃AB receptors. Higher concentrations of 5-HT are needed to elicit a current response at 5-HT₃AB receptors and the slope of the curves differs. Parameters derived from these curves are: 5-HT₃A, $pEC_{50} = 5.76 \pm 0.03$, $EC_{50} = 1.74 \mu\text{M}$, $n_H = 2.3$, $n = 6$ and 5-HT₃AB, $pEC_{50} = 4.53 \pm 0.04$, $EC_{50} = 29.5 \mu\text{M}$, $n_H = 1.0$, $n = 6$. (B) Saturation binding with the radioligand [³H]granisetron shows that like many other competitive ligands it has the same affinity at 5-HT₃A and 5-HT₃AB receptors. K_d values for these representative curves were 0.21 and 0.19 nM for 5-HT₃A and 5-HT₃AB receptors respectively. (C) Like many other non-competitive antagonists, the sensitivity of 5-HT₃R currents to PTX differs at the two receptor types. Parameters derived from these curves are: 5-HT₃A, $pIC_{50} = 5.02 \pm 0.09$, $IC_{50} = 9.55 \mu\text{M}$, $n_H = 0.7$, $n = 9$ and 5-HT₃AB, $pIC_{50} = 4.26 \pm 0.06$, $IC_{50} = 55.0 \mu\text{M}$, $n_H = 0.7$, $n = 5$. (D) VUF10166 is unusual as this competitive antagonist has differing affinities at 5-HT₃A and 5-HT₃AB receptors. K_d values for these representative curves were 0.08 and 12.6 nM for 5-HT₃A and 5-HT₃AB receptors respectively. (E) Similar to many other NCAs, the sensitivity of 5-HT₃R currents to DTZ also differs at 5-HT₃A and 5-HT₃AB receptors. Mutagenesis has shown that DTZ has a pore-binding site in the 5-HT₃A receptor that is responsible for its increased potency relative to 5-HT₃AB receptors. Parameters derived from these curves are: 5-HT₃A, $pIC_{50} = 4.68 \pm 0.07$, $IC_{50} = 20.9 \mu\text{M}$, $n_H = 0.8$, $n = 7$ and 5-HT₃AB, $pIC_{50} = 3.53 \pm 0.01$, $IC_{50} = 295 \mu\text{M}$, $n_H = 0.8$, $n = 5$. (F) In contrast to the electrophysiological measurements shown in panel (E), radioligand competition binding studies show that the binding affinity of DTZ is the same at 5-HT₃A and 5-HT₃AB receptors. This is consistent with the majority of other competitive antagonists that also have similar binding affinities at the two receptor types. K_i values for these representative curves were 180 μM for 5-HT₃A receptors and 169 μM for 5-HT₃AB receptors.

stitution of the 6' and 12' positions of the 5-HT₃A abolishes BB and GB inhibition. Additionally, an alanine substitution at the 2' position in 5-HT₃A receptors causes GB to be trapped in the channel following pore closure, and is only relieved following several subsequent agonist applications. Similar ligand trap has been reported for BB, GA and PTX at glycine receptors, highlighting the similarities of action that NCAs often share at other members of the Cys-loop family (Hawthorne and Lynch, 2005; Bali and Akabas, 2007).

Diltiazem (DTZ) is another inhibitor that has a lower potency (~14-fold) at 5-HT₃AB receptors than 5-HT₃A receptors in functional studies. It has mixed (competitive and non-competitive) antagonism, and probing the competitive component using [³H]granisetron binding reveals similar DTZ affinity at both receptor types, consistent with most other competitive antagonists. The non-competitive component is only present in the 5-HT₃A receptor and is due to DTZ acting in the pore at higher concentrations. This component is voltage dependent and substitution of the 7' and 12' positions abolishes the non-competitive antagonism found in homomeric receptors (Gunthorpe and Lummis, 1999; Thompson *et al.*, 2011a).

Morphine also shows mixed antagonism consisting of a low-affinity (μM) competitive component and higher affinity non-competitive component (Baptista-Hon *et al.*, 2012). At both 5-HT₃A and 5-HT₃AB receptors, inhibition of 5-HT currents is surmountable when morphine is co-applied, and radioligand competition reveals similar binding affinities (Brady *et al.*, 2001; Baptista-Hon *et al.*, 2012). The non-competitive component becomes apparent when morphine is pre-applied, and its potency is reduced ~4-fold in the presence of the 5-HT₃B subunit. However, the binding site for the non-competitive component is unlikely to be in the channel because inhibition is not voltage dependent and substitution of the whole of the 5-HT₃A subunit M2 region with the aligning 5-HT₃B sequence does not affect morphine potency. Therefore, the location of the binding site for the non-competitive component is still unknown. Methadone is an analogue of morphine that is also fourfold less potent at 5-HT₃AB receptors than at 5-HT₃A receptors in functional studies. Its inhibition at 5-HT₃A receptors is surmountable, but at 5-HT₃AB receptors it is insurmountable and voltage dependent, suggesting that unlike morphine, methadone

may act in the pore of heteromeric receptors (Deeb *et al.*, 2009).

The antimalarial compounds quinine and mefloquine also show mixed antagonism (Thompson and Lummis, 2008). Quinine is a competitive antagonist at 5-HT₃A receptors, but its potency is 15-fold less at 5-HT₃AB receptors where it also has non-competitive actions (Table 1). In comparison, mefloquine has mixed competitive and non-competitive effects at both 5-HT₃A and 5-HT₃AB receptors, but is fourfold less potent at the heteromer. For both quinine and mefloquine the non-competitive activity is dependent upon pre-application, and for mefloquine a small voltage dependence in the 5-HT₃A receptor (5-HT₃AB was not tested) suggests channel binding (Thompson *et al.*, 2007). The closely related antimalarial chloroquine is solely competitive at both receptor types, and does not display differences in potency, consistent with competitive antagonists having similar affinities at both receptor types.

The fact that NCAs can distinguish between 5-HT₃A and 5-HT₃AB receptors provides a practical means of discriminating between the 5-HT₃R subtypes in functional studies, such as electrophysiological experiments in transfected cells (e.g. Thompson *et al.*, 2011c), and their relatively low potency means that agonist responses often recover quickly following inhibition. These compounds, however, are unlikely to have therapeutic applications as many also affect other members of the Cys-loop family, or entirely different receptor classes and cellular pathways.

Modulators of 5-HT₃R function

Allosteric modulators bind to regions that are distinct to the orthosteric binding site and can alter agonist sensitivity, agonist efficacy and channel kinetics. There are a number of allosteric modulators that affect the 5-HT₃R including *n*-alcohols, anaesthetics, antidepressants, cannabinoids, opioids, steroids and natural compounds, many of which also modulate other Cys-loop receptors (see reviews by Davies, 2011, Machu, 2011 and Walstab *et al.*, 2010). Specific effects on different 5-HT₃R subtypes have not been widely explored, although alcohols and inhaled anaesthetics have been shown to have reduced sensitivity at 5-HT₃AB receptors, and the

potencies of the intravenous anaesthetics etomidate, propofol and pentobarbital, and the modulatory compound PU02, are similar at the two receptor types (Table 1; Hayrapetyan *et al.*, 2005; Solt *et al.*, 2005; Stevens *et al.*, 2005; Rusch *et al.*, 2007). All of these compounds are likely to bind in an intersubunit binding cavity at the top of the TMD which imposes an upper size limit upon them (Stevens *et al.*, 2005; Nury *et al.*, 2011). Although we still do not have high-resolution structural information on 5-HT₃R, we can hypothesize that incorporation of the 5-HT₃B subunit is likely to alter the size, shape and number of these binding cavities. This is supported by the finding that mutating this region has significant effects on the properties of some 5-HT₃R allosteric modulators (Trattnig *et al.*, 2012).

Other compounds reported to modulate the 5-HT₃R may have distinct modes of action. For example, the convulsant α -thujone shows a subunit-dependent inhibition of 5-HT₃R responses. Interpretation of its effects is complicated as α -thujone may alter 5-HT₃A and 5-HT₃AB receptor desensitization rates, which are already quite different at the two receptor types (Hölld *et al.*, 2000; Deiml *et al.*, 2004). However, it does not compete with the 5-HT₃R-selective antagonist [³H]GR65630 at 5-HT₃A receptors, showing that it does not bind to the orthosteric binding site, and as tail currents (brief currents that appear after removal of high concentrations of 5-HT in functional studies) are not inhibited by the continued presence of α -thujone it is thought to be not directly blocking the channel (Hapfelmeier *et al.*, 2001). It has been suggested that it increases the likelihood of auto-inhibition by 5-HT channel blockade rather than block by α -thujone itself, but further work is needed to clarify this. The binding site of α -thujone is unknown, but similar changes in desensitization are seen in the presence of 5-hydroxyindole, where mutation of the 15' position in 5-HT₃A receptors abolishes the potentiating effects of 5-OHi (Kooymann *et al.*, 1994; Hu and Lovinger, 2008). Mutation of the 15' position in 5-HT₃A receptors abolishes the potentiating effect of 5-OHi, suggesting an action of this compound at the 15' position (Hu and Lovinger, 2008). At higher concentrations, 5-OHi competes with [³H]GR65630, which is unsurprising given that 5-OHi shares much of its molecular structure with 5-HT, and could also provide an explanation why 5-OHi can elicit agonist responses in some mutant receptors (Hu and Lovinger, 2008). However, caution may be needed when interpreting the effects of the 15' mutation as they are conspicuously similar to those of alcohols and channel mutations that enhance ligand efficacy rather than directly affect a specific binding site (Lovinger and White, 1991; Downie *et al.*, 1995; Palma *et al.*, 1996). Therefore, the similar properties of α -thujone and 5-OHi at lower concentrations could reflect a common binding site at the channel 15' position, but further work is needed to rule out the possibility of broader effects on channel gating.

5-HT₃R homology models

Without a crystal structure of the 5-HT₃R, researchers have used homology models based on crystal structures of homologous proteins. At 5-HT₃A receptors, these have been used to support mutagenesis, to identify residues important

for ligand binding, and for studies that used mutant cycle analysis and molecular rulers to define the geometry of the binding region (Yan and White, 2002; 2005; Nyce *et al.*, 2010; Thompson *et al.*, 2010a). The similar effects that 5-HT₃A subunit mutations have in homomeric and heteromeric receptors have shown that these homology models of A+A- binding sites are relevant to both receptor types (Thompson *et al.*, 2011c). For homology models containing 5-HT₃B subunits, Lochner and Lummis (2010) predicted possible ligand interactions at A+B-, B+A- and B+B- interfaces, which were subsequently tested using mutagenesis and shown to be unlikely to exist. The same study found that mutation of identified residues in the 5-HT₃A subunit altered 5-HT activation and [³H]granisetron, supporting the proposal that heteromeric receptors are activated via an A+A- interface. Moura Barbosa *et al.* (2010) and De Rienzo *et al.* (2012) provided comprehensive computational validations of their homology models, and known binding site interactions for 5-HT and granisetron were present when these ligands were docked into the A+A- binding site of their models. However, experimental validation is still required for their binding sites containing the 5-HT₃B subunit as many of the residues with predicted ligand interactions in B+A- binding sites are known to not effect 5-HT activation or granisetron binding (Lochner and Lummis, 2010; Thompson *et al.*, 2011c). The finding that VUF10166 binds to the A+B- interface presents another opportunity for evaluating interactions at the A+B- interface (Thompson *et al.*, 2012b).

At other Cys-loop receptors, homology models have also been used to probe ligand interactions in the pore (e.g. Zhorov and Bregestovski, 2000; Jensen *et al.*, 2010). At 5-HT₃R, there has been limited use of similar homology models, and these have been restricted to 5-HT₃A receptors alone (Thompson *et al.*, 2012a). With the identification of increasing numbers of channel-binding ligands and allosteric compounds, we anticipate these heteromeric models will see further utility in the future and could help us understand the differences between the properties of ligands at 5-HT₃A and 5-HT₃AB receptors.

Therapeutic implications

The first 5-HT₃R antagonists were described in the 1950s, but it was several decades before that the first antagonists were licensed for clinical use. These competitive antagonists are now widely used for the treatment of nausea and vomiting arising from chemotherapy, radiotherapy and general anaesthesia. There is also potential for other therapeutic applications as genetic and physiological evidence indicates that the 5-HT₃R may be associated with several other disorders (Walstab *et al.*, 2010). These drugs target the A+A- orthosteric binding site shared by 5-HT₃A and 5-HT₃AB receptors, but as we report in this review, there are now competitive and non-competitive ligands that can distinguish between 5-HT₃A and 5-HT₃AB receptors. As yet, none of these are clinically used to target 5-HT₃R, and NCAs are unlikely to be of therapeutic value because they lack specificity. However, competitive antagonists and allosteric modulators are more selective, and the recent descriptions of competitive ligands with receptor subtype specificity show it is possible to target

the different 5-HT₃R subtypes. Allosteric ligands provide further possibilities, and with a growing number of subunit variants (including those containing the more recently identified 5-HT₃C-E subunits) there is a now wider range of potential sites at which these modulators could bind. The remaining challenge is to determine the physiological roles of the different 5-HT₃R subtypes, develop specific ligands and then determine their therapeutic value. High throughput assay has recently been used to identify novel, potent and selective 5-HT₃R ligands (Thompson *et al.*, 2010b; Trattinig *et al.*, 2012; Verheij *et al.*, 2012), showing that there is still chemical space in which 5-HT₃R ligands reside, and given the widespread distribution of these receptors, there are still considerable therapeutic opportunities for 5-HT₃R ligands that are waiting to be realized.

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Conflict of interest

None.

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