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Structural basis of ligand recognition in 5-HT$_3$ receptors

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The 5-HT$_3$ receptor is a pentameric serotonin-gated ion channel, which mediates rapid excitatory neurotransmission and is the target of a therapeutically important class of anti-emetic drugs, such as granisetron. We report crystal structures of a binding protein engineered to recognize the agonist serotonin and the antagonist granisetron with affinities comparable to the 5-HT$_3$ receptor. In the serotonin-bound structure, we observe hydrophilic interactions with loop E-binding site residues, which might enable transitions to channel opening. In the granisetron-bound structure, we observe a critical cation–π interaction between the indazole moiety of the ligand and a cationic centre in loop D, which is uniquely present in the 5-HT$_3$ receptor. We use a series of chemically tuned granisetron analogues to demonstrate the energetic contribution of this electrostatic interaction to high-affinity ligand binding in the human 5-HT$_3$ receptor. Our study offers the first structural perspective on recognition of serotonin and antagonism by anti-emetics in the 5-HT$_3$ receptor.
Keywords: Cys-loop receptor; pentameric ligand-gated ion channel; serotonin; 5-hydroxytryptamine-3 receptor

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INTRODUCTION

Neuronal 5-hydroxytryptamine (5-HT, serotonin) signalling pathways are some of the most complex in the human body. There are at least 15 5-HT receptors grouped into seven distinct signalling families and these are involved in a diverse array of complex brain functions, such as physiological, emotional and cognitive control, including regulation of appetite, sleep, sexual behaviour, anxiety, learning and memory [1]. All 5-HT receptors are G-protein coupled, except for the 5-HT$_3$ receptor (5-HT$_3$R), which is a pentameric ligand-gated ion channel (pLGIC). This receptor has been implicated in hippocampus-dependent memory formation, acts in anxiety, and contributes to schizophrenia and drug abuse [2,3]. Antagonists of the 5-HT$_3$R, such as granisetron and ondansetron, are in clinical use as anti-emetics to suppress nausea and vomiting induced by general anaesthetics, chemotherapeutics and radiotherapy [2,3].

Key to novel entries into drug design programs at this receptor is to reveal the structural determinants of chemical recognition of serotonin and other ligands at 5-HT$_3$R by supporting X-ray crystal structures. Until now, extensive efforts have focused on purification of pLGIC extracellular domains (ECD), including the expression of truncated ECD or as a fusion protein with carrier proteins, chimeras with enhanced water solubility and covalent linkage as dimers of subunits. However, all these failed to produce pentameric assemblies of LGICs suitable for structural studies. In the absence of full-length pLGICs, structural information has been inferred from high-resolution crystal structures of acetylcholine-binding proteins (AChBPs), which are homologous to the ECD of the nicotinic acetylcholine receptor (nAChR) [4]. Co-crystallization of nicotinic ligands with AChBP critically contributed to the insights of ligand recognition at the nAChR and enabled much of the earlier biochemical data to be reconciled [4]. So far, this high-resolution structural data is missing for ligand-binding sites of the majority of members of the pLGIC family, including the 5-HT$_3$R.
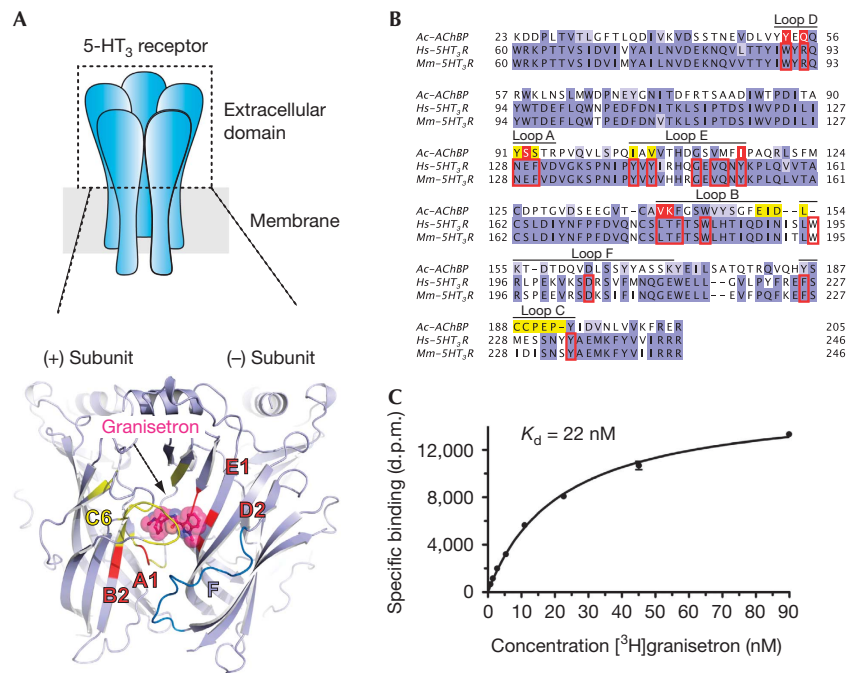
Fig 1 | Protein engineering of 5HTBP. (A) Schematic representation of the 5-HT₃ receptor. The ligand-binding site is located in the extracellular domain at the subunit interface. The inset illustrates the contribution of different binding site loops (A–F) in the acetylcholine-binding protein (AChBP). Loop numbers correspond to the number of residues that were substituted to the corresponding residues of the 5-HT₃ receptor. Granisetron is shown in magenta sticks and transparent spheres. Yellow are tolerated substitutions, red are mutations that show specific [³H]granisetron binding. (B) Sequence alignment of AChBP and 5-HT₃ receptors. Blue colouring indicates BLOSUM62 conservation score. Yellow and red colours in AChBP correspond to those in panel A. Boxed residues indicate positions that are critical for granisetron or serotonin binding in the 5-HT₃ receptor. (C) Example of a radioligand saturation experiment on 5HTBP (construct A1B2D1$_W$E1, see Table 1), which displayed high-affinity [³H]granisetron binding. AChBP, acetylcholine-binding protein; 5-HT₃R, 5-HT₃ receptor.

To overcome this limitation, we used a rational approach to engineer ligand recognition properties similar to the 5-HT₃R ECD into the AChBP scaffold. AChBPs provide an excellent starting point because they bind serotonin, albeit with ∼60-fold lower affinity than acetylcholine [4,5], indicating that AChBP and 5-HT₃R ligand recognition sites already share some structural elements. In fact, this property extends to full-length receptors; some pLGICs in invertebrates are activated by acetylcholine as well as serotonin [6]. The similarities are further supported by findings that single-point mutations of the nAChR and 5-HT₃R are sufficient to enable agonist action by serotonin and acetylcholine, respectively [7,8]. In addition, it has been observed that nicotinic ligands, such as epibatidine [9], varenicline [10] and d-tubocurarine [11] also bind with high potency to the 5-HT₃R, and that serotonin acts as an antagonist at neuronal nAChRs [12]. These data indicate that the nAChR and 5-HT₃R share a broadly similar binding site architecture with overlapping ligand recognition characteristics, supporting AChBP as a suitable scaffold for engineering a high-affinity binding site for 5-HT₃R ligands.

RESULTS AND DISCUSSION
Protein engineering of a 5HTBP

First, we used structure-based sequence alignments of *Aplysia californica* acetylcholine-binding protein (Ac-AChBP) seeded with 5-HT₃R sequences to identify homologous ligand-binding residues in the ECD of the 5-HT₃R (Fig 1A,B). Amino-acid residues were prioritized on the basis of their critical contribution to ligand recognition in the 5-HT₃R, which has been studied extensively using both natural and unnatural amino-acid mutagenesis (for a review, see Thompson and Lummis [13] and references therein, summarized in supplementary Table S1 online). Examples include the 5-HT₃R ligand-binding residues W90 and R92 in loop D (homologous to Ac-AChBP-Y53 and Q55). In loop A, residue E129 is essential, and less important are N128 and F130 (Y91, S92 and S93 in Ac-AChBP). Residues in loop B are the highly conserved W183 (W145 in Ac-AChBP), as well as L178, T179 and F180 (V140, K141 and F142 in Ac-AChBP), which were proposed to maintain the local structure of loop B through hydrophobic interactions with the β-sandwich core. Other important residues are Y234 in loop C (Y193 in Ac-AChBP) and Y143, G148, V150 and Y153 in loop E (V106, G111, V113 and I116 in Ac-AChBP). Amino-acid conservation in loop F is poor and aligning pLGICs and AChBP in this region is difficult. However, potential roles in ligand binding have been attributed to W195 and D204.

For each of the identified residues, we made substitutions to mimic 5-HT₃R residues at the corresponding positions in Ac-AChBP. These substitutions were made to single or several residues (Fig 1A; supplementary Table S1 online) in each of the separate loops and the resultant receptors tested for expression of

Table 1 | Binding constants for different 5HTBP constructs as determined by competition experiments with [³H]granisetron (K_i) and SPR biosensor assays (K_d)

	Mutations in *Aplysia californica* AChBP	[³H]granisetron binding				SPR biosensor assay			
		Granisetron		Serotonin		Granisetron		Serotonin	
		K_i (nM)	Wt/mut	K_i (µM)	Wt/mut	K_d (nM)	Wt/mut	K_d (µM)	Wt/mut
Wild-type		1,049 ± 397		927 ± 181		549 ± 31		693 ± 29	
D2	Y53W, Q55R (D2)	454 ± 71	2.3	159 ± 36	5.8	343 ± 26	1.6	94 ± 11	7.4
A1 B2	S92E (A1)	280 ± 72	3.7	167 ± 28	5.6	307 ± 34	1.8	240 ± 29	2.9
	V140L, K141T (B2)								
A1 B2 C6 D2 E1	S92E (A1)	NB		NB		ND		ND	
	V140L, K141T (B2)								
	C188M, C189E, P190S, E191S, P192N, Y193^{Ins} (C6)								
	Y53W, Q55R (D2)								
	I116Y (E1)								
A1 B2 D2	S92E (A1)	NB		NB		32,050 ± 3,907	0.02	ND	
	V140L, K141T (B2)								
	Y53W, Q55R (D2)								
A1 B2 D1$_W$	S92E (A1)	226 ± 50	4.6	32 ± 7	29	196 ± 33	2.8	18.6 ± 0.3	37
	V140L, K141T (B2)								
	Y53W (D1$_W$)								
A1 B2 D1$_R$	S92E (A1)	50 ± 13	21	218 ± 25	4.3	ND		ND	
	V140L, K141T (B2)								
	Y53W (D1$_R$)								
A1 B2 D1$_W$ E1	S92E (A1)	119 ± 33	8.8	169 ± 23	5.5	ND		ND	
	V140L, K141T (B2)								
	Y53W (D1$_W$)								
	I116Y (E1)								

AChBP, acetylcholine-binding protein; NB, no binding; ND, not determined; SPR, surface plasmon resonance. Values are presented as mean ± s.e.m. of at least three different experiments.

pentameric assemblies by size exclusion chromatography, and by binding with [³H]granisetron, a high-affinity antagonist for the 5-HT$_3$R. Because weaker interactions with high micromolar affinities, such as serotonin at wild Ac-AChBP, are difficult to measure with a radioligand binding assay, we verified our binding experiments with a surface plasmon resonance (SPR) biosensor assay [14] (Table 1). Consistent with the importance of W90 and R92 for ligand binding in the 5-HT$_3$R, their substitution in loop D of Ac-AChBP (construct D2; Y53W, Q55R) yielded detectable [³H]granisetron binding ($K_i = 454 \pm 71$ nM, supplementary Table S1 online). Loop A residues homologous to N128, E129 and F130 gave no protein expression in the triple mutant (construct A3 contains Y91N, S92E, S93F), or the double mutant protein failed to bind [³H]granisetron (construct A2$_{NF}$; Y91N, S92E). However, the single mutant (construct A1; S92E) displayed binding by [³H]granisetron ($K_i = 270 \pm 51$ nM) comparable to construct D2, which is consistent with the essential role of E129

in 5-HT$_3$R ligand recognition [13]. For loop B, constructs B9, B4, B3 did not produce secreted protein, but the double mutant (construct B2; V140L, K141T) displayed [³H]granisetron binding that was similar to constructs A1 and D2 (supplementary Table S1 online).

None of the loop C, E and F mutants displayed [³H]granisetron binding (Fig 1A; supplementary Table S1 online). For loop C, mutant proteins with 12 or 20 substitutions did not express (constructs C12 and C20). Shorter substitutions of the hairpin loop between Y186 and Y193 were tolerated, but did not show detectable [³H]granisetron binding (constructs C5 and C6). Loop E constructs E3 and E1 were expressed, but showed no detectable [³H]granisetron binding. Because loop F sequences are highly variable and alignments are ambiguous, we only tested a mutant protein containing a substitution of the entire loop (construct F13), which failed to express.

Because mutants A1, B2 and D2 contained only single or double mutations that readily displayed high-affinity
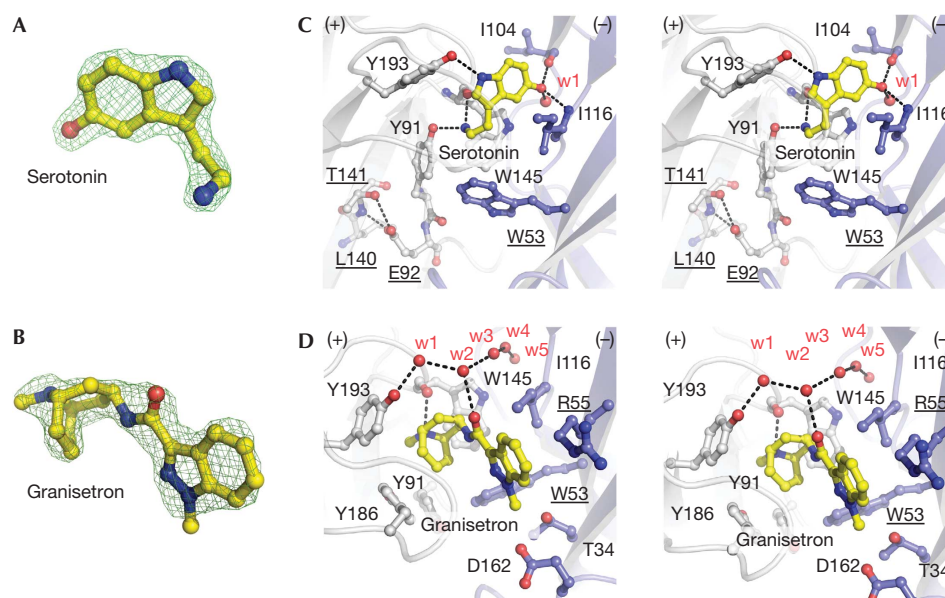
Fig 2 | X-ray crystal structures of 5HTBP in complex with serotonin and granisetron. (**A,B**) $2F_o$-F_c electron density contoured at 1.5σ for serotonin and granisetron, respectively. (**C**) Detailed interactions of serotonin in the ligand-binding site of construct A1B2D1$_W$, which contains mutations Y53W, S92E, V140L and K141T (underlined). The principal (+) subunit is shown in white, the complementary subunit (−) in blue and serotonin in yellow. On the principal face, the aminoethyl nitrogen of serotonin forms hydrogen bonds (shown as dashed lines) with W145 and Y91, as well as a cation–π interaction with W145. On the complementary face, the 5-hydroxy group of serotonin forms hydrogen bonds with a water molecule (shown as a red sphere) in loop E and the backbone atoms of I104 and I116. (**D**) Detailed interactions of granisetron in the ligand-binding site of construct D2, which contains mutations Y53W and Q55R (underlined). Granisetron forms a hydrogen bond with a stretch of water molecules (w1–w5) that extend into the vestibule of the ligand-binding domain. The tropane ring protrudes deep into the binding site and interacts with W53, whereas the indazole moiety points out of the pocket and is stabilized by interactions with R55.

[³H]granisetron binding, we reasoned that these subtle changes alone are sufficient to mimic the 5-HT₃R-binding site. We then investigated whether these could be combined to produce proteins with improved binding affinities. When the individual loop mutants (A1, B2 and D2) were combined in construct A1B2D2, the new triple mutant no longer bound [³H]granisetron (Table 1). In contrast, constructs A1B2, A1B2D1$_W$ and A1B2D1$_R$ displayed binding properties with improved affinity for 5-HT₃R ligands. Specifically, for construct A1B2D1$_W$, we observe a ~40-fold increase in the affinity of serotonin compared with wild-type Ac-AChBP (K_i A1B2D1$_W$ = 32 ± 7 μM as measured with [³H]granisetron binding and K_d A1B2D1$_W$ = 18.6 ± 0.3 μM as measured with the SPR biosensor assay). Improved affinities were also observed for granisetron, with K_i values of 50 ± 13 nM at A1B2D1$_R$, 226 ± 50 nM at A1B2D1$_W$ and 119 ± 33 nM at A1B2D1$_W$E1 (Table 1). To further validate these constructs as tools to understand ligand binding at the 5-HT₃R, we engineered a series of homologous mutations known to affect granisetron binding in 5-HT₃R [13]. These mutations, namely Y193A, Y193F (homologous to Y234 in loop C of 5-HT₃R), D162A and D162E (homologous to D204 in loop F), W145A and W145Y (homologous to W183 in loop B) were introduced in the background of construct A1B2D1$_R$ and reduce the affinity and/ or level of granisetron binding similar to the 5-HT₃R (supplementary Table S2 online), suggesting that granisetron adopts common binding poses in A1B2D1$_R$ and 5-HT₃R. Together, these results indicate that our engineered binding

proteins (5HTBPs) are valid high-affinity binding models for pharmacological and structural studies of serotonin and granisetron recognition.

Molecular determinants of ligand recognition in 5HTBP

To gain insight into the chemical recognition of serotonergic ligands, we set up crystallization trials with a range of 5-HT₃R agonists, antagonists and partial agonists, in combination with several 5HTBPs. We were able to determine crystal structures for (i) the agonist serotonin in complex with 5HTBP-A1B2D1$_W$ (from here on termed 5HTBP-ser) and (ii) the antagonist granisetron in complex with 5HTBP-D2 (from here on termed 5HTBP-gran). Both structures revealed important aspects of ligand recognition.

The crystal structure of 5HTBP-ser was solved from diffraction data to a resolution of 1.9 Å (see X-ray statistics in supplementary Table S3 online). F_o-F_c omit density clearly revealed the serotonin binding pose and surrounding side-chain interactions at all five binding sites. In Fig 2A, the green mesh shows simple electron density contoured at 1.5σ and serotonin is shown as yellow ball and sticks. Interactions of serotonin with amino-acid residues of the binding pocket are shown in Fig 2C. In the (+) subunit, we find that substitutions in loops A (S92E) and B (V140L and K141T) do not directly interact with serotonin, but position Y91 for optimal hydrogen bonding with the serotonin amine nitrogen, which is equidistant from the W145 carbonyl oxygen (Fig 2A,C). The serotonin aminoethyl nitrogen is ideally placed for a cation–π interaction with W145, consistent with unnatural amino-acid
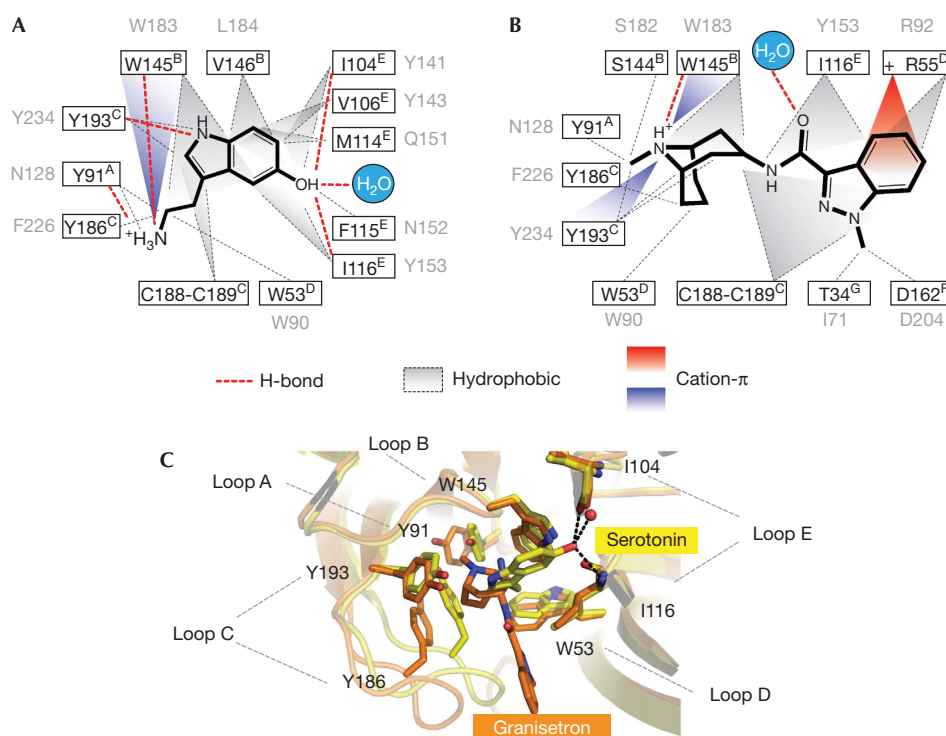
Fig 3 | Molecular blueprint for ligand recognition in the 5-HT₃R. (**A,B**) Structural determinants of serotonin and granisetron recognition, respectively. Boxed residues represent observed contacts in 5HTBP, grey residues are homologous residues in the 5-HT₃ receptor. Superscript letters indicate binding site loops A–F. Red dashed lines indicate hydrogen bonds, grey indicate hydrophobic interactions and blue indicate cation–π interactions. The key cation–π interaction between the indazole moiety of granisetron and R55 is shown in red. (**C**) Superposition of granisetron- and serotonin-bound structures shows side-chain reorientations and a conformational change in the C loop. Serotonin (yellow) adopts a binding pose that partially overlaps with, but is nearly perpendicular to granisetron (orange) and critically depends on interactions with loop E. 5-HT₃R, 5-HT₃ receptor.

studies that identified the same interaction using electrophysiological methods [15]. On the (−) subunit W53 has van der Waals interactions with the aminoethyl group. Serotonin adopts a binding pose that also relies on extensive interactions with loop E. These include hydrophobic interactions with side chains of I104, V106, M114, I116 and backbone atoms of F115 (supplementary Fig S1 online). In addition, several hydrogen bonds are formed between the 5-hydroxy group and the backbone oxygen of I104, the backbone nitrogen of I116 and a water molecule that occupies a pocket deep inside loop E. This mechanism of serotonin recognition offers a likely explanation for the partial agonism of 5-substituted tryptamine analogues, including 5-fluorotryptamine and 5-methoxytryptamine [16]. Owing to the increased size and electronegativity of the substitutions at the 5-position, these tryptamine derivatives are likely to be involved in less favourable interactions with loop E residues, which might limit the efficiency of conformational transitions mediating channel opening of the 5-HT₃R. Finally, the indole nitrogen is involved in a hydrogen bond with Y193 (loop C).

The crystal structure of 5HTBP-gran (complex with granisetron) was solved from diffraction data to a resolution of 2.4 Å. Simple electron density for granisetron is shown as a green mesh (1.5σ, Fig 2B) and side-chain interactions with residues in the binding pocket are shown in Fig 2D. Granisetron adopts a binding

pose that partially overlaps, but is nearly perpendicular to serotonin. The tropane ring of granisetron is buried deep inside the pocket where it mainly contacts residues of loops A and B. The indazole ring points out of the pocket where it interacts with residues of loops D and E. The crystal structure reveals the important contribution of these interactions, as W53 is involved in van der Waals interactions with the ethylene bridge of the tropane ring. R55 is involved in a cation–π interaction between the positive guanidium group of the arginine side chain and the π-electrons of the indazole ring of granisetron. This cation–π dipole is in the opposite direction to the cation–π interaction between the tropane nitrogen of granisetron and the aromatic side chain of W145 (loop B), a residue that is important for granisetron binding in the 5-HT₃R (reviewed in Thompson and Lummis [13]). Similar to the complex with tropisetron (pdb code 2wnc) and nicotinic receptor ligands, the tropane nitrogen of granisetron forms a hydrogen bond with the carbonyl oxygen of W145. The oxygen atom of the granisetron amide bond is involved in a hydrogen bond network, involving five water molecules (w1–w5), which extend into the vestibule of the receptor (Fig 2D).

A structural framework for ligand interactions in 5-HT₃R

A molecular blueprint for recognition of serotonin and granisetron, as observed in 5HTBP crystal structures, is shown in Fig 3A–C. The strong conservation of residues between 5HTBP
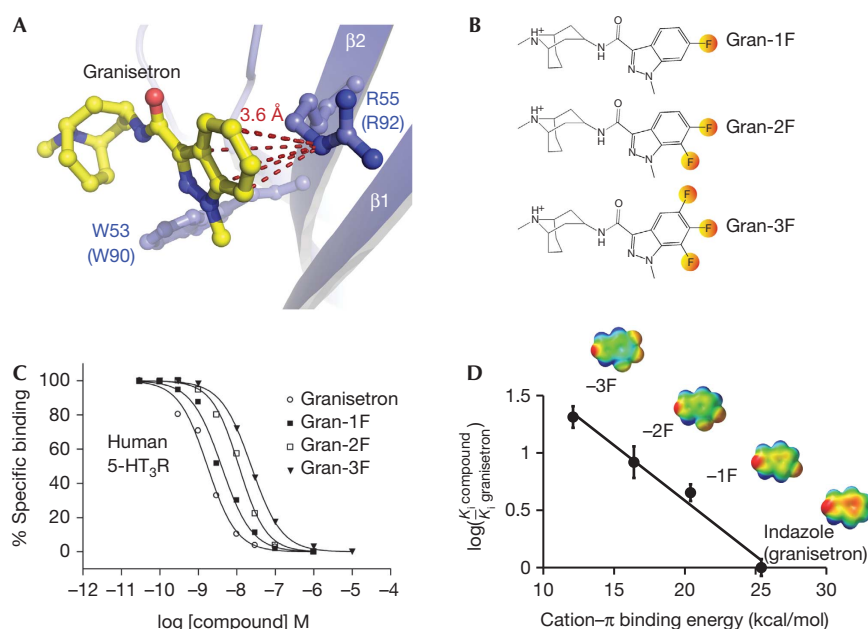
Fig 4 | A cation–π interaction probed in the human 5-HT₃R with chemically tuned granisetron analogues. (**A**) In the crystal structure of granisetron-bound 5HTBP, we observe a cation–π interaction between the indazole moiety of granisetron (shown in yellow) and the positively charged R55 (homologous to R92 in 5-HT₃R) of loop D in the binding site. (**B**) Chemical structures of gran-1F, -2F and -3F. (**C**) Typical concentration–response curves for displacement of [³H]granisetron binding by fluorinated granisetrons at the human 5-HT₃R. These plots were used to determine K_i values and subsequently create the fluorination plot shown (**D**; data = mean ± s.e.m., $n = 4$–6). Electrostatic potentials surfaces for indazole and fluorinated derivatives are shown near each average data point. Electrostatic potentials span a range of − 100 (red) to + 100 (blue) kJ/mol. 5-HT₃R, 5-HT₃ receptor.

and the 5-HT₃R allow us to make reliable predictions on the nature of ligand interactions in the 5-HT₃R and help us to understand previous mutagenesis studies in the 5-HT₃ [13].

For serotonin, we observe interactions with the conserved aromatic residues W145 (W183 in loop B of 5-HT₃R), Y186 and Y193 (F226 and Y234 in loop C of 5-HT₃R) and the engineered W53 (W90 in loop D of 5-HT₃R). For Y193, a critical hydrogen bond is formed with the indole nitrogen of serotonin, in agreement with the effects on channel activation that have been reported at this position in 5-HT₃R. Unlike granisetron, serotonin has extensive interactions with residues in loop E (Fig 3A,C), including hydrogen bonds with the backbone atoms of I104 and I116 (homologous to Y141 and Y153 in 5-HT₃R) and hydrophobic interactions with V106, M114 and F115 (homologous to Y143, Q151 and N152 in 5-HT₃R). In the 5-HT₃R, Y143, Q151 and Y153 have been implicated in agonist binding and channel gating, as substitutions increase the serotonin EC₅₀, but do not affect granisetron binding. This is consistent with a critical role of loop E in agonist recognition and a ligand-binding pose of serotonin that is nearly perpendicular to granisetron. The hydrogen bond between Y91 (loop A) and the serotonin amine nitrogen is also reflected in the 5-HT₃R, where a hydrogen bond between the equivalent E129 and serotonin has been demonstrated [17].

For granisetron, interactions are also observed with highly conserved aromatic residues, namely W145 (homologous with W183 in loop B), the engineered W53 (homologous with W90 in

loop D) and Y186 and Y193 (homologous with F226 and Y234 in loop C). Conserved interactions also occur with S144 (S182 in loop B of 5-HT₃R), D162 (D204 in loop F of 5-HT₃R) and the engineered R55 (R92 in loop D of 5-HT₃R), which crucially contributes to a cation–π interaction with the indazole moiety of granisetron. The critical contribution of these residues in ligand recognition is consistent with mutagenesis studies showing lack of granisetron binding in W90A, W183A and Y234A mutants of 5-HT₃R [13].

Chemical tuning of a unique cation–π interaction in 5-HT₃R

The critical role of a cation–π interaction between the cationic nitrogen of ligands and the conserved loop B aromatic side chain of the 5-HT₃R-binding site has been studied extensively [15,18]. However, one of the remarkable features in the 5HTBP crystal structures is that the binding of granisetron also relies on a novel cation–π interaction, involving the indazole moiety of granisetron and the cationic R55 in loop D of the binding site (homologous to R92 in the 5-HT₃R, Fig 4A). This interaction is unique to 5HTBP and has not been observed in more than 50 AChBP crystal structures solved so far [19]. To investigate whether this cation–π interaction is recapitulated in high-affinity binding of granisetron to 5-HT₃R, we synthesized a series of fluorinated granisetron analogues (termed gran-1F, -2F and -3F, Fig 4B) and determined their affinities for the human 5-HT₃R. Adding small, highly electronegative fluorine atoms allowed us to fine-tune the electrostatic surface potential of the indazole moiety

of granisetron (Fig 4D) and investigate the energetic contribution of the cation–π interaction with R92. We observed that each additional fluorine atom produces a step-wise shift of the binding curve (Fig 4C), showing that the electronegative surface potential of the indazole moiety contributes to a cation–π interaction, in agreement with the interaction observed in the 5HTBP-gran structure. The plot in Fig 4D formalizes the cation–π interaction through the linear relationship between the experimentally determined binding constants (K_i) and the calculated cation–π binding ability of the fluorinated granisetron analogues. Such a correlation does not exist when the granisetron analogues were tested on the R92A mutant of 5-HT₃R (supplementary Fig S2 online). Together, these results illustrate the relevance of the 5HTBP crystal structures for understanding ligand recognition in the 5-HT₃R and demonstrate that a cation–π interaction involving a cationic centre in the binding site of the 5-HT₃R critically contributes to high-affinity binding.

Conformational changes on ligand binding

Comparison of the granisetron- and serotonin-bound structures shows that loop C undergoes a conformational change (Fig 3C), characterized by a contraction around serotonin (6.90 ± 0.03 Å distance W145-C188) and an extension on binding of granisetron (8.7 ± 0.2 Å distance W145-C188). This is similar to observations made for nicotinic agonists and antagonists [19], and indicates that serotonin binding affecting loop C is part of a conserved mechanism of signal transduction among different members of the pLGIC family [19]. In addition, the differing side-chain orientations of Y91 (loop A) and W53 (loop D) are consistent with their role as structural switches in other pLGIC family members [19].

CONCLUSION

5-HT₃R are critical for fast synaptic excitation in the mammalian brain and are the target for clinically important anti-emetics such as granisetron. Our study, which reveals the critical elements for serotonin and granisetron recognition in 5HTBP, brings closer a longstanding goal to understand ligand recognition by the 5-HT₃R at high structural resolution. Although there might be subtle differences between the interactions of these ligands with 5HTBP and 5-HT₃R, the general interactions are likely to be conserved and are in agreement with a wealth of mutagenesis studies performed on 5-HT₃R. We expect that 5HTBPs will be useful tools for further structural studies, screening of fragment libraries and the rational design of new drugs targeting the 5-HT₃R.

METHODS

All AChBP mutants were purified by affinity chromatography with nickel sepharose (GE Healthcare) and size exclusion chromatography on a Superdex 200 column (GE Healthcare). 5HTBP-Gran was crystallized in the presence of 0.5 mM granisetron and 200 mM Na₂SO₄, bistrispropane pH 8.5, 18% PEG3350. 5HTBP-Ser was crystallized in the presence of 5 mM serotonin creatinine sulphate and 400 mM (NH₄)H₂PO₄. Full methods are described in supplementary information online and include details on radioligand binding assays, SPR biosensor experiments and the synthesis of fluorinated granisetron analogues.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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