Binding Sites for Bilobalide, Diltiazem, Ginkgolide and Picrotoxinin at the 5-HT₃ Receptor.


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Running title: Ginkgolide Binding sites in 5-HT₃R.

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Abbreviations: nACh, nicotinic acetylcholine; AChBP, acetylcholine binding protein; 5-HT, 5-hydroxytryptamine; GABA, gamma-aminobutyric acid; PTX, Picrotoxin; PXN, picrotoxinin; PTN, Picrotin; BB, Bilobalide; GB, Ginkgolide B; GC, Ginkgolide C; DTZ, Diltiazem.
Abstract

Bilobalide (BB), ginkgolide B (GB), diltiazem (DTZ) and picrotoxinin (PXN) are 5-HT3 receptor antagonists whose principal sites of action are in the channel. To probe their exact binding locations 5-HT3 receptors with substitutions in their pore lining residues were constructed (N-4’Q, E-1’D, S2’A, T6’S, L7’T, L9’V, S12’A, I16’V, D20’E), expressed in *Xenopus* oocytes, and the effects of the compounds on 5-HT-induced currents examined. *EC*₅₀s at mutant receptors were less than 6-fold different to wild type, indicating that the mutations were well tolerated. BB, GB, DTZ and PXN had p*IC*₅₀ values of 3.33, 3.14, 4.67 and 4.97 respectively. Inhibition by BB and GB was abolished in mutant receptors containing T6’S and S12’A substitutions, but their potencies were enhanced (42-fold and 125-fold respectively) in S2’A mutant receptors. S2’A substitution also caused GB ligand trap. PXN potency was modestly enhanced (5-fold) in S2’A, abolished in T6’S, and reduced in L9’V (40-fold) and S12’A (7-fold) receptors. DTZ potency was reduced in L7’T and S12’A receptors (5-fold), and DTZ also displaced [³H]granisetron binding, indicating mixed competitive/non-competitive inhibition. We conclude that regions close to the hydrophobic gate of M2 are important for the inhibitory effects of BB, GB, DTZ and PXN at the 5-HT3 receptor; for BB, GB and PXN, the data show that the 6’ channel lining residue is their major site of action, with minor roles for 2’, 9’ and 12’ residues, while for DTZ the 7’ and 12’ sites are important.
Introduction

Bilobalide (BB), ginkgolide B (GB) and picrotoxin (PTX) are non-competitive inhibitors of GABA, glycine and 5-HT₃ receptors (Hadley et al., 2005; Hawthorne et al., 2006; Huang et al., 2004; Pribilla et al., 1992; Sivilotti and Nistri, 1991; Thompson et al., 2011). Diltiazem (DTZ) is primarily a voltage-gated calcium-channel blocker, but also inhibits 5-HT₃ and nACh receptors (Chesnoy-Marchais and Cathala, 2001; Das et al., 2004; Hargreaves et al., 1996; Houlihan et al., 2000). All of these compounds block the receptor channel, and mutations of the channel lining region have indicated specific interactions in GABA and glycine receptors (Thompson et al., 2011; Hawthorne et al., 2006; Heads et al., 2008; Sedelnikova et al., 2006).

The channels in Cys-loop receptors are lined by five (M2) α-helices (one from each subunit), and to simplify comparisons between receptors of this family, the amino acid residues that line this channel are referred to by an index number, with 0' representing the conserved charged residue at the cytoplasmic side of the membrane, (e.g. Imoto et al., 1988). In the glycine receptor GB inhibition is subunit dependent, and a preference for the β-subunit can be attributed to residues at the 2' position of the channel pore (Hawthorne et al., 2006; Hawthorne and Lynch, 2005; Kondratskaya et al., 2005). Experiments with BB and PTX on the same receptor show that the 6' residue is particularly important, and the effects of 6' substitutions on PTX in GABA and 5-HT₃ receptors show that this site of action is conserved across the family (Das and Dillon, 2005; Sedelnikova et al., 2006; Hawthorne and Lynch, 2005). However, there are differences between family members, as 2' mutations in 5-HT₃ receptors have limited effect on PTX inhibition, but a large effect at GABA and glycine receptors (Buhr et al., 2001; Yang et al., 2007). The actions of PTX at GABA and
glycine receptors are further complicated by evidence of multiple actions; residues between 15’ and 19’ also influence the behaviour of PTX in these receptors and either form a second binding site or have a role in the transduction of the PTX inhibitory effect (Dibas et al., 2002 and refs therein).

Here we use two-electrode voltage-clamp to study the effects of M2 amino acid substitutions on BB, GB, DTZ and PXN inhibition of 5-HT3 receptors expressed in *Xenopus* oocytes. To identify potential binding sites for these compounds we made substitutions to nine residues that line the proposed water accessible face of the M2 α-helix. We report the effects that these substitutions have on the potency of the compounds and present a model of their binding locations.

**Materials and Methods**

*Materials:* All cell culture reagents were obtained from Gibco (Invitrogen Ltd., Paisley, U.K.), except foetal calf serum which was from Labtech International (Ringmer, U.K.). PXN and PTN were separated and purified by recrystallisation following short column vacuum chromatography from PTX purchased from Sigma-Aldrich Australia. BB and GB were isolated from the 50:1 *Ginkgo biloba* leaf extract purchased from Winshing (Australia) Pty Ltd., and purified by short column chromatography and recrystallisation. The 1H and 13C NMR spectra of the purified PXN, PTN, BB and GB were consistent with the published data (Perry et al., 2001; van Beek 2005), and also indicated purity > 98% in all cases. 5-HT3A and 5-HT3B receptor subunit cDNA was kindly donated by J. Peters (University of Dundee, UK).

*Cell culture and Oocyte Maintenance: *Xenopus laevis* oocyte-positive females were purchased from NASCO (Fort Atkinson, Wisconsin, USA) and maintained according to standard methods (Goldin, 1992). Harvested stage V-VI *Xenopus*...
oocytes were washed in four changes of ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5), de-folliculated in 1.5 mg ml⁻¹ collagenase Type 1A for approximately 2 h, washed again in four changes of ND96 and stored in ND96 containing 2.5 mM sodium pyruvate, 50 mM gentamycin, 0.7 mM theophylline.

Human embryonic kidney (HEK) 293 cells were maintained on 90 mm tissue culture plates at 37°C and 7% CO₂ in a humidified atmosphere. They were cultured in Dulbecco’s Modified Eagle’s Medium / Nutrient Mix F12 (1:1) with GlutaMAX™ (Invitrogen, Paisley, UK) containing 10% foetal calf serum. For radioligand binding studies cells in 90 mm dishes were transfected using polyethyleneimine (PEI). 30 µl PEI (1mg / ml), 5 µl cDNA and 1 ml DMEM were incubated for 10 min at room temperature, added dropwise to a 80 - 90% confluent plate, and incubated for 3 - 4 days before harvesting (Reed et al., 2006).

Receptor Expression: Human 5-HT3A (accession number: P46098) and 5-HT3B (AF095264) subunit cDNA were cloned into pGEMHE for oocyte expression (Liman et al., 1992), and pcDNA3.1 (Invitrogen, Paisley, U.K.) for expression in HEK 293 cells. Mouse 5-HT3A (Q6J1J7) and 5-HT3AB (Q9JHJ5) were cloned into pcDNA3.1. cRNA was in vitro transcribed from linearised pGEMHE cDNA template using the mMessage mMachine T7 Transcription kit (Ambion, Austin, Texas, USA). Stage V and VI oocytes were injected with 50 nl of ~500 ng µl⁻¹ cRNA (~25 ng), and currents were recorded 1 - 4 days post-injection. A ratio of 1:3 (A:B) was used for the expression of heteromeric 5-HT₃ receptors, which has been shown to yield good heteromeric expression (Thompson et al., 2007).

Electrophysiology: Using two electrode voltage clamp, Xenopus oocytes were routinely clamped at -60 mV using an OC-725 amplifier (Warner Instruments, Connecticut, USA), Digidata 1322A and the Strathclyde Electrophysiology Software
Package (Department of Physiology and Pharmacology, University of Strathclyde, UK; www.strath.ac.uk/Departments/PhysPharm). Currents were filtered at a frequency of 1 kHz and sampled at 5 kHz. Micro-electrodes were fabricated from borosilicate glass (GC120TF-10, Harvard Apparatus, Edenbridge, Kent, UK) using a two stage horizontal pull (P-87, Sutter Instrument Company, California, USA) and filled with 3 M KCl. Pipette resistances ranged from 1.0 – 2.0 MΩ. Oocytes were perfused with saline at a constant rate of 15 ml min⁻¹. Drug application was via a simple gravity fed system calibrated to run at the same rate. Extracellular saline contained (mM), 96 NaCl, 2 KCl, 1 MgCl₂ and 5 mM HEPES; pH 7.4 with NaOH.

Analysis and curve fitting was performed using Prism v3.02 (GraphPad Software, San Diego, California, USA, www.graphpad.com). Concentration-response data for each oocyte was normalised to the maximum current for that oocyte. A 2 min wash was typically used between drug applications. The mean and SEM for a series of oocytes were plotted against agonist or antagonist concentration and iteratively fitted to the following equation:

\[
I_A = I_{\text{min}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + 10^{nH \cdot (\log A_{50} - \log A)}}
\]  
(Equ. 1)

where \(A\) is the concentration of ligand present; \(I_A\) is the current in the presence of ligand concentration \(A\); \(I_{\text{min}}\) is the current when \(A=0\); \(I_{\text{max}}\) is the maximal current, \(A_{50}\) is the concentration of \(A\) which evokes a current equal to \((I_{\text{max}} + I_{\text{min}})/2\); and \(nH\) is the Hill coefficient. Comparisons, were made by subtracting wild type log \(A\) from mutant log \(A\), (i.e. the ratio of \(EC_{50}\) or \(IC_{50}\)). \(K_B\) was estimated from \(IC_{50}\) values using the Cheng-Prusoff equation with the modification by Leff and Dougall (1993).
where $IC_{50}$ is the concentration of antagonist required to halve the maximal response and $[L]$ is the agonist concentration.

**Radioligand Binding** - Transfected HEK293 were scraped into 1 ml of ice-cold HEPES buffer (10 mM, pH 7.4) and frozen at -20°C. After thawing, they were washed with HEPES buffer, homogenised, and 50 µg of crude cell membranes incubated in 0.5 ml HEPES buffer containing 1 nM $[^3]$Hgranisetron (~ $K_d$) in the presence or absence of BB (2.5 mM - 0.25 nM), GB (2.5 mM - 0.25 nM), DTZ (10 mM – 0.1 nM) or PXN (5 mM - 0.5 nM). Non-specific binding was determined using 1 mM quipazine. Reactions were incubated for at least 1 h at 4°C and terminated by vacuum filtration using a Brandel cell harvester onto GF/B filters pre-soaked in 0.3 % polyethyleneimine. Radioactivity was determined by scintillation counting using a Beckman BCLS6500 (Fullerton, California, USA). Competition binding (8 point) was performed on at least three separate plates of transfected cells from which the average $IC_{50}$ was calculated. Each data set was analyzed by iterative curve fitting using Prism v3.02, according to the equation:

$$B_L = B_{\min} + \frac{B_{\max} - B_{\min}}{1 + 10^{n_H (\log L_{50} - \log L)}}$$

(Equ. 3)

where $L$ is the concentration of ligand present; $B_L$ is the binding in the presence of ligand concentration $L$; $B_{\min}$ is the binding when $L=0$; $B_{\max}$ is the binding when $L=\infty$, $L_{50}$ is the concentration of $L$ which gives a binding equal to $(B_{\max} + B_{\min})/2$; and $n_H$ is the Hill coefficient.
$K_i$ values were estimated from $IC_{50}$ values using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$  (Equ. 4)

where $IC_{50}$ is the concentration of antagonist that blocks half of the specific binding, $[L]$ is the free concentration of radioligand, and $K_d$ is the equilibrium dissociation constant of the radioligand.
Results

To find the molecular determinants of BB, GB, DTZ and PXN binding, a series of mutants were generated at each of the nine channel lining M2 residues of the human 5-HT3A receptor subunit (Fig 1). Residues were mostly mutated to amino acids with similar chemical properties to prevent complications arising from changes in channel gating. Conservative changes minimise the impact on receptor structure and function, but still reveal specific interactions between the receptor and antagonist (e.g. Thompson et al., 2005). Wild type and mutant receptors (except for S2’T and S12’T which were non-functional) responded to 5-HT in a concentration-dependent manner, with $EC_{50}$ and $n_H$ values of mutants differing $<$ 6-fold from wild type (Table 1, Fig 2).

**Effects of compounds** - None of the compounds elicited a response when applied alone. At wild type receptors BB, GB, DTZ and PXN caused a concentration-dependent inhibition of the 5-HT $EC_{50}$ response (Tables 2 - 5, Figs 3). Inhibition was unaltered by pre-treatment of the compounds.

Significant effects on BB inhibition were seen at residues S2’, T6’, L7’, L9’ and S12’ (Table 2, Fig 3A). The most dramatic effects were at T6’S and S12’A, where inhibition by 1 mM BB was completely abolished. Of the remaining mutants that were inhibited by BB, S2’A exhibited the greatest change in potency, with a 42-fold enhancement. L7’T showed a 6-fold enhancement and L9’V a 3-fold reduction in potency.

Inhibition by 1 mM GB was abolished by 6’ and 12’ mutations, but at all other mutants except N-4’ potency was increased (Table 3, Fig 3B). Decreases in $IC_{50}$ were generally small ($<$ 5-fold), with the exception of S2’A, which displayed a decrease of 125-fold.
PXN inhibition was eliminated by T6’S substitution (Table 4, Fig 3C). Significant pIC$_{50}$ changes were also seen at positions -4’, -1’, 2’, 9’ and 12’, but effects on potency were generally small (< 5-fold), with the exceptions of L9’V and S12’A which showed reductions of 38-fold and 6-fold respectively.

The relative changes in DTZ inhibition were less than with the other three compounds (Table 5, Fig 3D). Most of the substitutions had no effect, although mutation of L7’ and S12’ caused 5-fold decreases in potency.

**S2’A mutants must re-open to recover from GB inhibition** - In the S2’A mutant receptor a striking increase in both BB (42-fold) and GB (125-fold) potency was observed. For GB, but not BB, this was accompanied by ligand trap: following a co-application of GB and 5-HT, the next response to 5-HT alone had a reduced current amplitude that only returned to the pre-treatment amplitude with a second 5-HT application. This effect is shown in figure 4, which is a representative example of 8 similar experiments on different oocytes. Peak current were stable with 5-HT alone (Fig 4A), but, following a GB inhibition, recovery was independent of the time interval between the first (5-HT + GB) and second (5-HT alone) applications (Figs 4B - 4E), showing that the effect was not the consequence of prolonged recovery from desensitisation. After the initial increase in current amplitude, further applications of 5-HT did not show any additional increases (Fig 4E). Application of GB in the absence of 5-HT did not change the peak response of subsequent 5-HT applications, but was altered if GB was added at any time during 5-HT application (Figs 4F – FH).

**Competition Binding:** In a previous study we showed that BB, GB and PXN do not compete with granisetron (a high affinity competitive antagonist) at 5-HT$_3$A or 5-HT$_3$AB receptors (Thompson et al., 2011). Here we extended these studies to include DTZ. The pK$_d$ of [H]$^+$_granisetron was $9.04 \pm 0.05$ ($n = 7, K_d = 0.91$ nM) for 5-
HT$_3$A receptors and 9.09 ± 0.05 ($n = 4$, $K_d = 0.80$ nM) for 5-HT$_3$AB receptors, consistent with previous reports (Brady et al., 2001). At concentrations close to $K_d$ [³H]granisetron was displaced by DTZ with pIC$_{50}$ values of 3.52 ± 0.07 ($IC_{50} = 340$ µM, $n = 12$) for 5-HT$_3$A receptors and 3.44 ± 0.01 ($IC_{50} = 366$ µM, $n = 3$) for 5-HT$_3$AB receptors. These yielded $K_i$ values of 171 µM and 183 µM respectively (Equ 4). Hill Slopes were 0.84 ± 0.05 and 1.14 ± 0.20 for 5-HT$_3$A and 5-HT$_3$AB receptors respectively.

To probe whether DTZ has an allosteric effect on the binding site, we also examined its effects on L7′T and S12′A-containing mutant receptors. Neither the affinity for [³H]granisetron (L7′T; $pK_d = 9.00 ± 0.03$, $K_d = 1$ nM , $n = 5$. S12′A; $pK_d = 9.01 ± 0.07$, $K_d = 0.97$ nM, $n = 4$) or the potency with which diltiazem displaces this radioligand (L7′T; $pIC_{50} = 3.37 ± 0.04$, $IC_{50} = 426$ µM , $n = 3$; S12′A: $pIC_{50} = 3.36 ± 0.07$, $IC_{50} = 436$ µM, $n = 4$) were significantly different (Students t-test, $p > 0.05$).

Effects on heteromeric receptors - 5-HT$_3$AB receptors had a $pEC_{50}$ (Table 1) that was similar to values shown elsewhere (Hapfelmeier et al., 2003; Thompson et al., 2007). Inhibition of the $EC_{50}$ response by BB, GB and PXN has been presented by us previously (Thompson et al., 2011) and the values are included in Tables 2 - 4. Here we extended these studies to include DTZ (Table 5). $IC_{50}$ values for all of the compounds were higher (between 5 - 14 fold) at 5-HT$_3$AB receptors than those at 5-HT$_3$A receptors.
Discussion

Mutagenesis is widely used to probe the interactions of ligands with their receptors. Here we made substitutions of nine 5-HT₃ receptor channel-lining residues and studied their effects on BB, GB, DTZ and PXN inhibition. Inhibition by BB, GB and PXN was abolished by T6’S substitution. BB and GB were also highly sensitive to changes at S2’ and S12’, and PXN to changes at L9’. DTZ showed both competitive and non-competitive antagonism, the non-competitive component of which was eliminated by substitutions at L7’ and S12’.

Sensitivity to BB and GB was abolished by substitutions at the 6’ residue, similar to observations at the glycine receptor (Hawthorne et al., 2006). Inhibition was also affected by 2’ substitutions in this receptor, and, in combination with evidence from mutant cycle analysis, suggests that ginkgolides bind close to both positions 2’ and 6’ (Hawthorne et al., 2006). Similar 2’ sensitivity was shown in the present study, with an enhancement of 42-fold for BB and 125-fold for GB. This enhancement of GB sensitivity was accompanied by ligand trap at the 2’ mutant, a phenomenon that has also been described for BB, GA and PXN at glycine receptors (Bali and Akabas, 2007; Hawthorne et al., 2006; Hawthorne and Lynch, 2005; Wang et al., 2007). In our previous work, GB displayed use-dependence at wild type 5-HT₃ receptors, which is similar to the reports of BB at GABAC and GC at glycine receptors (Huang et al., 2006; Ivic et al., 2003; Thompson et al., 2011). These data imply that GB acts as an open-channel blocker of the 5-HT₃ receptor, and, when the channel gate is closed, is trapped in the pore of the high affinity S2’A mutant. The opposite A2’S mutation in insect GABA receptors is responsible for resistance to cyclodiene insecticides and PTX, and in light of this evidence, the binding site of these compounds appears to be broadly conserved across the Cys-loop family (Ffrench-Constant et al., 1993).
In GABA_A receptors the 6’ position is the main (hydrogen bonding) interacting residue and the 2’ position acts as a secondary (hydrophobic) site for PTX binding (Erkkila et al., 2008). Our results support the role of T6’ as a major determinant of PXN binding, but the effect of our conserved mutation (inhibition was abolished) was more pronounced than those reported for the GABA_A receptor, where changing all five of the 6’ Thr residues to Ser only caused a 48-fold shift in the PTX IC_{50} (Erkkila et al., 2008). The effects of the 2’ and 6’ substitutions indicate that BB, GB and PXN can penetrate beyond the central ‘hydrophobic girdle’, which forms the ‘gate’ of the channel (L9’ - V13’; Miyazawa et al., 2003), and our observation that GB is trapped in the high affinity S2’A mutant is consistent with this hypothesis. 2’ residues have been implicated in PTX inhibition in GABA_A, GABA_C, and glutamate-gated Cys-loop receptors, with some small effects at glycine and 5-HT_3A receptors (Das and Dillon, 2005; Etter et al., 1999; Gurley et al., 1995; Shan et al., 2001; Wang et al., 1995; Xu et al., 1995; Zhang et al., 1995). We propose that the decreased IC_{50}s we observed for BB, GB and (to a lesser extent) PXN in the S2’A mutant may arise from the more hydrophobic side-chain of Ala enabling the ligands to adopt a more energetically favourable position within the channel, possibly via interactions with their lipophilic side chains. Ala substitutions at S12’ also had large effects on BB and GB (inhibition abolished), although only moderately effected PXN. If we assume that the 6’ residue is important for ligand interactions, S12’ may act as a secondary binding site, or interact with antagonists as they descend into the pore.

BB, GB, DTZ and PXN inhibition is subunit dependent at the 5-HT_3 receptor, similar to the actions of these compounds at other Cys-loop receptors (Das and Dillon, 2003; Erkkila et al., 2008; Gonzales et al., 2008; Hawthorne et al., 2006; Hawthorne and Lynch, 2006; Thompson et al., 2011). 5-HT_3AB receptors were less sensitive to
PXN than 5-HT₃A receptors, and mutation of the 5-HT₃A 6’ residue to its B subunit counterpart (T6’S) completely abolished inhibition by BB, GB and PXN. As the B subunit 6’S does not abolish inhibition in the heteromeric wild type 5-HT₃ receptor, the other 2-4 A subunit 6’ residues (which combine with 1-3 B subunits to constitute this receptor; Barrera et al, 2005; Lochner and Lummis, 2010) must be sufficient for PXN binding. In the GABAₐ receptor, PTX sensitivity also decreases as the number of mutated subunits is increased from one to five (Erkkila et al., 2008).

BB and PTX have mixed competitive and non-competitive behaviours in a range of Cys-loop receptors, but at least one site of action is in the receptor channel (Huang et al., 2006; Lynch et al., 1995; Wang et al., 1995; Smart and Constanti, 1986). We have previously shown that BB, GB and PXN do not compete with [³H]granisetron, and both these data and our current results are consistent with these compounds binding solely in the 5-HT₃ receptor pore (Thompson et al., 2011). In contrast, the difference between $K_B$ (34 µM) and $K_i$ (171 µM) for DTZ indicates that competitive inhibition cannot completely account for its effects, suggesting DTZ inhibition includes both competitive and non-competitive behaviours. Similar differences have also been reported for DTZ at mouse 5-HT₃ receptors (Gunthorpe and Lummis, 1999; Hargreaves et al., 1996), and mefloquine also has mixed inhibitory behaviour at human 5-HT₃ receptors (Thompson et al., 2007). In our experiments DTZ potency was significantly changed by the 12’ mutation, although Lee et al. (2009) have previously shown that a 13’ mutation in mouse receptors reduces DTZ potency, while the 12’ residue is unaffected. The contrast in our results suggests that there are differences between human and mouse receptors, and this is further demonstrated by the presence of constitutively active 13’ mutants in their study and the absence of such effects at any of the mutants studied here. In contrast to
the homomeric receptors, $K_B$ (149 µM) and $K_i$ (183 µM) values were similar at 5-HT$_3$AB receptors, suggesting that inhibition of the functional response in these receptors can be accounted for entirely by competitive inhibition. For PXN it has been suggested that allosteric effects on the agonist binding site of GABA receptors account for some of inhibitory effects of this compound (Smart and Constanti, 1986), but this is unlikely for DTZ as the potency with which it displaced radioligand binding was similar at wild type, L7’T and S12’A mutant receptors. As all the residue positions studied here differ between A and B subunits, all could potentially disrupt binding in the channel. However, only L7’T and S12’A containing-receptors significantly altered DTZ potency, and $K_B$ values (L7’T = 176 µM; S12’A = 182 µM) were close to the $K_i$ for wild type receptors (5-HT$_3$A = 171 µM; 5-HT$_3$AB = 183 µM). We therefore conclude that the differences in DTZ efficacy at homomeric and heteromeric receptors can be accounted for by differences in their channel properties. This interpretation is also consistent with the long established finding that these two receptor types have identical binding site pharmacologies, but different non-competitive pharmacologies (Brady et al., 2001; Thompson et al., 2007).

In summary, we have shown that BB, GB and PXN inhibit 5-HT$_3$ receptor function by interacting with the 6’ and (to a lesser extent) the 2’ residue in the receptor channel, consistent with their sites of action being conserved across the Cys-loop family. For BB and GB the 12’ residue was identified as a new site of interaction, and may represent another binding region, or may simply indicate there is an interaction with the compounds as they descend into the channel. In contrast, DTZ inhibition of the 5-HT$_3$ receptor contains both competitive (homomeric and heteromeric) and non-competitive behaviours, the later of which can be attributed to
the 7’ region of channel. The data are consistent with the binding sites shown on the cartoon in fig 5.

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**Authorship Contributions**

Participated in study design: Thompson, Lummis

Contributed experiments: Thompson

Contributed materials: Duke

Performed data analysis: Thompson, Lummis

Wrote or contributed to the writing of the manuscript: Thompson, Lummis, Duke

Other: Lummis acquired funding for this project
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Footnotes

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Figure Legends

Figure 1. M2 channel lining residues of mouse and human 5-HT₃ receptors. The probable ‘gate’ region of the channel (~ 8 Å long) is boxed (Miyazawa et al., 2003). The M2 amino acid sequence for the murine A subunit (accession number Q6J1J7) is shown, with residues that are water accessible highlighted in grey; ¹ Kaneez and White, 2004, ² Reeves et al., 2001, ³ Panicker et al., 2002. m = mouse, h = human.

Figure 2. Wild type and mutant 5-HT₃ receptor concentration-response curves. 5-HT pEC₅₀ values were determined from concentration-response curves (Panel A; data = mean ± SEM, n can be found in Table 1). The difference between wild type and mutant 5-HT₃ receptors is shown in Panel B. Data = mean ± SED.

Figure 3. Concentration-inhibition of BB, GB, DTZ and PXN at wild type and mutant 5-HT₃ receptors. The difference between wild type and mutant pIC₅₀ values are shown for each of the compounds (mean ± SED). n can be found in Tables 2 - 5.

Figure 4. GB is trapped in the closed state of the S2’A mutant receptor. Applications of EC₅₀ 5-HT at 1 min intervals have the same current amplitude (Panel A). Following co-application of GB and 5-HT, the next 5-HT response is reduced in amplitude, and only returns to its pre-drug amplitude with a second application of agonist (Panel B). This effect is independent of the recovery period between applications (Panels B - E), and is only present when GB is applied in the open state.
of the receptor (Panels F - H). Traces are representative of 8 similar experiments performed on different oocytes. Ligand trap was not seen for BB, DTZ or PXN.

Figure 5. A cartoon of potential binding regions for BB, DTZ, GB and PXN at the 5-HT₃ receptor. The 12’ residue is located in the upper region of the channel (see Fig 1) and its substitution reduces the potency of all four of the ligands tested. Given its location, we speculate that it may affect the passage of compounds as they pass to their binding sites lower down. The 6’ residue is particularly critical for BB, GB and PXN binding as substitution abolishes inhibition (open circle). At S2’A mutant receptors, the potencies of BB, GB and PXN were all increased, which may be the result of the smaller Ala substitute allowing the ligands to adopt a more energetically favourable position. DTZ potency is altered by mutation at the 7’ position (grey circles) suggesting it binds here, in addition to acting at the agonist binding site.
Table 1. Changes in the parameters of 5-HT concentration-response curves caused by M2 substitutions.

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<th>Receptor</th>
<th>pEC\textsubscript{50}</th>
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<th>n</th>
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<td>L9'V</td>
<td>5.31 ± 0.02</td>
<td>4.9</td>
<td>1.76 ± 0.15</td>
<td>4</td>
</tr>
<tr>
<td>L7'T</td>
<td>5.09 ± 0.02</td>
<td>8.0</td>
<td>2.11 ± 0.18</td>
<td>5</td>
</tr>
<tr>
<td>T6'S</td>
<td>5.88 ± 0.03</td>
<td>1.3</td>
<td>2.15 ± 0.30</td>
<td>4</td>
</tr>
<tr>
<td>S2'T</td>
<td>NF</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>S2'A</td>
<td>5.66 ± 0.03</td>
<td>2.2</td>
<td>2.37 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>E-1'D</td>
<td>5.61 ± 0.02</td>
<td>2.5</td>
<td>2.79 ± 0.30</td>
<td>5</td>
</tr>
<tr>
<td>N-4Q</td>
<td>5.60 ± 0.02</td>
<td>2.5</td>
<td>3.31 ± 0.38</td>
<td>5</td>
</tr>
<tr>
<td>5-HT\textsubscript{3}AB\textsuperscript{E}</td>
<td>4.55 ± 0.05</td>
<td>28</td>
<td>1.00 ± 0.12</td>
<td>8</td>
</tr>
</tbody>
</table>

* values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be directly compared. NF = Non-functional. Values are mean ± SEM.
Table 2. Bilobalide concentration-inhibition at mutant 5-HT$_3$ receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pIC$_{50}$</th>
<th>IC$_{50}$ (µM)</th>
<th>Hill Slope</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT$_3$A$^2$</td>
<td>3.33 ± 0.03</td>
<td>468</td>
<td>1.48 ± 0.16</td>
<td>6</td>
</tr>
<tr>
<td>D20'E</td>
<td>3.21 ± 0.04</td>
<td>600</td>
<td>1.25 ± 0.12</td>
<td>4</td>
</tr>
<tr>
<td>I16'V</td>
<td>3.12 ± 0.04</td>
<td>200</td>
<td>1.43 ± 0.15</td>
<td>5</td>
</tr>
<tr>
<td>S12'A</td>
<td>NI*</td>
<td>NI</td>
<td>NI</td>
<td>3</td>
</tr>
<tr>
<td>L9'V</td>
<td>2.79 ± 0.06*</td>
<td>1600</td>
<td>0.84 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td>L7'T</td>
<td>4.11 ± 0.09*</td>
<td>78</td>
<td>0.96 ± 0.09</td>
<td>5</td>
</tr>
<tr>
<td>T6'S</td>
<td>NI*</td>
<td>NI</td>
<td>NI</td>
<td>3</td>
</tr>
<tr>
<td>S2'A</td>
<td>4.94 ± 0.04*</td>
<td>11</td>
<td>0.86 ± 0.04</td>
<td>3</td>
</tr>
<tr>
<td>E-1'D</td>
<td>3.20 ± 0.12</td>
<td>630</td>
<td>0.67</td>
<td>4</td>
</tr>
<tr>
<td>N-4Q</td>
<td>3.33 ± 0.10</td>
<td>470</td>
<td>1.09 ± 0.20</td>
<td>5</td>
</tr>
<tr>
<td>5-HT$_3$AB$^6$</td>
<td>2.51 ± 0.15*</td>
<td>3100</td>
<td>0.68 ± 0.13</td>
<td>4</td>
</tr>
</tbody>
</table>

* Sig Dif. to wild type (ANOVA with Dunnett’s Post-Test; p < 0.05). NI = No Inhibition at 1 mM. $^6$ values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be compared. Values are mean ± SEM.
Table 3. Ginkgolide B concentration-inhibition at mutant 5-HT3 receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pIC_{50}</th>
<th>IC_{50} (µM)</th>
<th>Hill Slope</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT3A^2</td>
<td>3.14 ± 0.05</td>
<td>727</td>
<td>1.10 ± 0.13</td>
<td>4</td>
</tr>
<tr>
<td>D20'E</td>
<td>3.78 ± 0.05*</td>
<td>170</td>
<td>1.49 ± 0.23</td>
<td>4</td>
</tr>
<tr>
<td>I16'V</td>
<td>3.79 ± 0.07*</td>
<td>160</td>
<td>1.18 ± 0.15</td>
<td>5</td>
</tr>
<tr>
<td>S12'A</td>
<td>NI*</td>
<td>NI</td>
<td>NI</td>
<td>4</td>
</tr>
<tr>
<td>L9'V</td>
<td>3.63 ± 0.08*</td>
<td>240</td>
<td>0.79 ± 0.12</td>
<td>5</td>
</tr>
<tr>
<td>L7'T</td>
<td>3.71 ± 0.07*</td>
<td>210</td>
<td>1.46 ± 0.29</td>
<td>4</td>
</tr>
<tr>
<td>T6'S</td>
<td>NI*</td>
<td>NI</td>
<td>NI</td>
<td>3</td>
</tr>
<tr>
<td>S2'A</td>
<td>5.24 ± 0.08*</td>
<td>5.8</td>
<td>0.75 ± 0.10</td>
<td>5</td>
</tr>
<tr>
<td>E-1'D</td>
<td>3.82 ± 0.04*</td>
<td>150</td>
<td>1.07</td>
<td>4</td>
</tr>
<tr>
<td>N-4Q</td>
<td>3.18 ± 0.08</td>
<td>660</td>
<td>0.87 ± 0.08</td>
<td>5</td>
</tr>
<tr>
<td>5-HT3AB^&amp;</td>
<td>2.41 ± 0.22*</td>
<td>3900</td>
<td>0.52 ± 0.14</td>
<td>4</td>
</tr>
</tbody>
</table>

* Sig Dif. to wild type (ANOVA with Dunnett’s Post-Test; p < 0.05). NI = No Inhibition at 1 mM. & values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be compared. Values are mean ± SEM.
Table 4. Picrotoxinin concentration-inhibition at mutant 5-HT₃ receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pIC₅₀</th>
<th>IC₅₀ (µM)</th>
<th>Hill Slope</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₃A²</td>
<td>4.97 ± 0.12</td>
<td>11</td>
<td>0.68 ± 0.12</td>
<td>13</td>
</tr>
<tr>
<td>D20'E</td>
<td>4.73 ± 0.06</td>
<td>18</td>
<td>0.78 ± 0.07</td>
<td>4</td>
</tr>
<tr>
<td>I16'V</td>
<td>5.03 ± 0.11</td>
<td>9.3</td>
<td>0.70 ± 0.11</td>
<td>5</td>
</tr>
<tr>
<td>S12'A</td>
<td>4.18 ± 0.11*</td>
<td>66</td>
<td>0.69 ± 0.09</td>
<td>5</td>
</tr>
<tr>
<td>L9'V</td>
<td>3.38 ± 0.20*</td>
<td>420</td>
<td>0.91 ± 0.25</td>
<td>3</td>
</tr>
<tr>
<td>L7'T</td>
<td>5.36 ± 0.15</td>
<td>4.4</td>
<td>0.51 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td>T6'S</td>
<td>NI*</td>
<td>NI</td>
<td>NI</td>
<td>4</td>
</tr>
<tr>
<td>S2'A</td>
<td>5.70 ± 0.07*</td>
<td>2.0</td>
<td>0.61 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>E-1'D</td>
<td>4.40 ± 0.06*</td>
<td>40</td>
<td>1.00 ± 0.11</td>
<td>5</td>
</tr>
<tr>
<td>N-4Q</td>
<td>4.37 ± 0.21*</td>
<td>43</td>
<td>0.80 ± 0.22</td>
<td>4</td>
</tr>
<tr>
<td>5-HT₃AB&amp;</td>
<td>4.20 ± 0.11*</td>
<td>62</td>
<td>0.68 ± 0.09</td>
<td>4</td>
</tr>
</tbody>
</table>

* Sig Dif. to wild type (ANOVA with Dunnett’s Post-Test; p < 0.05). NI = No Inhibition at 1 mM. & values taken from Thompson et al. (2011) were recorded at the same time as the current results and can be compared. Values are mean ± SEM.
### Table 5. Diltiazem concentration-inhibition at mutant 5-HT₃ receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>( pIC_{50} )</th>
<th>( IC_{50} ) (µM)</th>
<th>Hill Slope</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₃A</td>
<td>4.67 ± 0.07</td>
<td>21</td>
<td>0.84 ± 0.11</td>
<td>7</td>
</tr>
<tr>
<td>D20'E</td>
<td>4.59 ± 0.05</td>
<td>26</td>
<td>1.4 ± 0.20</td>
<td>4</td>
</tr>
<tr>
<td>I16'V</td>
<td>4.67 ± 0.07</td>
<td>21</td>
<td>0.99 ± 0.17</td>
<td>5</td>
</tr>
<tr>
<td>S12'A</td>
<td>3.82 ± 0.05*</td>
<td>150</td>
<td>1.64 ± 0.33</td>
<td>4</td>
</tr>
<tr>
<td>L9'V</td>
<td>4.91 ± 0.09</td>
<td>12</td>
<td>0.86 ± 0.15</td>
<td>4</td>
</tr>
<tr>
<td>L7'T</td>
<td>3.80 ± 0.06*</td>
<td>160</td>
<td>0.83 ± 0.08</td>
<td>5</td>
</tr>
<tr>
<td>T6'S</td>
<td>4.89 ± 0.07</td>
<td>13</td>
<td>0.96 ± 0.15</td>
<td>7</td>
</tr>
<tr>
<td>S2'A</td>
<td>4.89 ± 0.17</td>
<td>13</td>
<td>0.60 ± 0.15</td>
<td>5</td>
</tr>
<tr>
<td>E-1'D</td>
<td>4.88 ± 0.07</td>
<td>13</td>
<td>1.01 ± 0.17</td>
<td>3</td>
</tr>
<tr>
<td>N-4Q</td>
<td>4.96 ± 0.12</td>
<td>11</td>
<td>1.08 ± 0.14</td>
<td>3</td>
</tr>
<tr>
<td>5-HT₃AB</td>
<td>3.52 ± 0.10*</td>
<td>300</td>
<td>0.79 ± 0.11</td>
<td>5</td>
</tr>
</tbody>
</table>

* Sig Dif. to wild type (ANOVA with Dunnett’s Post-Test; \( p < 0.05 \)). Values are mean ± SEM.
Figure 1

A

| m5-HT₃A¹ | D S G . E R V S F K I T L L L G Y S V F L I I V S D |
| m5-HT₃A² | D S G . E R V S F K I T L L L G Y S V F L I I V S D |
| m5-HT₃A³ | D S G . E R V S F K I T L L L G Y S V F L I I V S D |
| h5-HT₃A  | N S G . E R V S F K I T L L L G Y S V F L I I V S D |

B

diltiazem

picrotoxinin

bilobalide

ginkgolide B
Figure 2

A

B

WT pEC50 - Mutant pEC50

5-HT

[5-HT] (M)

D20E

T6'S

WT

S2'A

E-1'D

N-4Q

I16V

L9V

L7'T

S12A

< -2

-1

0

1

2

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Figure 4