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Cysteine modification reveals which subunits form the ligand binding site in human heteromeric 5-HT$_3$AB receptors

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Non-technical summary Nerve signals are transmitted across cell membranes by receptors that can consist of multiple different subunits. The 5-HT$_3$ receptor is a pentamer which can function with A subunits alone, or with a mixture of A and B subunits. As 5-HT activates the receptor by binding at the interface of adjacent subunits, it is important to know which subunits are adjacent. Here we show that in both A-only and A+B receptors there is at least one A–A interface, without which the receptor cannot function. This knowledge is important for understanding the receptor mechanism, and also will allow the design of more specific drugs that act at the 5-HT binding site.

Abstract The ligand binding site of Cys-loop receptors is formed by residues on the principal (+) and complementary (−) faces of adjacent subunits, but the subunits that constitute the binding pocket in many heteromeric receptors are not yet clear. To probe the subunits involved in ligand binding in heteromeric human 5-HT$_3$AB receptors, we made cysteine substitutions to the + and − faces of A and B subunits, and measured their functional consequences in receptors expressed in *Xenopus* oocytes. All A subunit mutations altered or eliminated function. The same pattern of changes was seen at homomeric and heteromeric receptors containing cysteine substitutions at A_{R92} (− face), A_{L126}(+), A_{N128}(+), A_{I139}(−), A_{Q151}(−) and A_{T181}(+), and these receptors displayed further changes when the sulphydryl modifying reagent methanethiosulfonate-ethylammonium (MTSEA) was applied. Modifications of A_{R92C}(−)- and A_{T181C}(+)-containing receptors were protected by the presence of agonist (5-HT) or antagonist (d-tubocurarine). In contrast modifications of the equivalent B subunit residues did not alter heteromeric receptor function. In addition a double mutant, $A_{S206C}(-)_{/E229C}(+)$, only responded to 5-HT following DTT treatment in both homomeric and heteromeric receptors, indicating receptor function was inhibited by a disulphide bond between an A+ and an A– interface in both receptor types. Our results are consistent with binding to an A+A– interface at both homomeric and heteromeric human 5-HT$_3$ receptors, and explain why the competitive pharmacologies of these two receptors are identical.

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Abbreviations AFM, atomic force microscopy; d-TC, d-tubocurarine; MTS, methanethiosulfonate; MTSEA, methanethiosulfonate-ethylammonium; PTX, picrotoxin.

Introduction

5-HT$_3$ receptors are members of the Cys-loop receptor superfamily of ligand-gated ion channels that includes the nicotinic acetylcholine (nACh), GABA$_A$ and glycine receptors (Reeves & Lummis, 2002; Thompson & Lummis, 2007). To date, five 5-HT$_3$ receptor subunits (A–E) have been identified, although only homomeric 5-HT$_3$A and heteromeric 5-HT$_3$AB receptors have been extensively characterised. These studies have shown

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that the B subunit alters single channel conductance, response kinetics, current–voltage relationships, EC_{50}, Hill slope, permeability to Ca^{2+} and the potency of some non-competitive antagonists, but the binding affinities of agonists and competitive antagonists are similar at both homomeric and heteromeric receptors (Davies *et al.* 1999; Dubin *et al.* 1999; Brady *et al.* 2001; Hapfelmeier *et al.* 2003; Kelley *et al.* 2003).

Agonists and competitive antagonists bind at the interface of two adjacent subunits, where three regions from each subunit converge: loops A–C from the principal (+) subunit and loops D–F from the complementary (−) subunit. As the receptors are pentameric, a series of different binding sites are possible in heteromeric receptors. An atomic force microscopy (AFM) study suggested a subunit arrangement of BBABA in human 5-HT_3AB receptors, offering the possibility that ligands bind to A+B–, B+A– and/or B+B– binding sites (Barrera *et al.* 2005). However, these data are difficult to reconcile with a more recent mutagenesis study in mouse 5-HT_3AB receptors (where binding site residues from the A subunit were substituted with their B subunit equivalents, and vice versa) that showed ligand binding only occurs at an A+A– interface (Lochner & Lummis, 2010). Therefore, there may be differences in the stoichiometries of mouse and human heteromeric receptors, or data from either the AFM or mutagenesis study may not represent the situation *in vivo*.

Establishing whether the B subunit is a determinant of binding is a valuable goal, as 5-HT_3 receptor antagonists are widely used to treat emesis, and studies suggest that the efficacy of these drugs may critically depend on the B subunit (Tremblay *et al.* 2003; Thompson & Lummis, 2007). It is important to know whether these differing actions are a result of binding to B subunit-containing binding sites, or allosteric effects due to binding sites elsewhere in the receptor. To clarify this issue in human 5-HT_3AB receptors, we made cysteine substitutions in the two potential binding site interfaces of A and B subunits, and used two-electrode voltage-clamp of *Xenopus* oocytes to measure their functional consequences, probe their accessibility and proximity, and examine the ability of 5-HT_3 receptor ligands to protect against modification by a sulphydryl modifying reagent. The use of a functional assay ensures that only a physiologically relevant population of cell surface receptors is studied and allows easy discrimination between homomeric and heteromeric receptors. The results show that A but not B subunit residues affect receptor function.

Methods

Materials

Methanethiosulfonate (MTS) reagents were obtained from Biotium (Hayward, CA, USA). Serotonin (creatinine sulphate complex), d-tubocurarine chloride (d-TC) and picrotoxin (PTX) were obtained from Sigma-Aldrich (Gillingham, UK). All other reagents were of the highest obtainable grade.

Oocyte maintenance

Xenopus laevis were purchased from NASCO (Fort Atkinson, WI, USA) and maintained according to standard methods (Goldin, 1992). Harvested stage V–VI *Xenopus* oocytes were washed in four changes of Ca^{2+}-free ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5), de-folliculated in 1.5 mg ml^{-1} collagenase Type 1A for approximately 2 h, washed again in four changes of Ca^{2+}-free ND96 and stored in ND96 containing 2.5 mM sodium pyruvate, 0.7 mM theophylline and 100 μg ml^{-1} gentamicin. cRNA was *in vitro* transcribed from linearised pGEMHE cDNA template using the mMessage mMachine T7 kit (Ambion, Austin, TX, USA). 5-HT3A cDNA was linearised using SphI and 5-HT3B cDNA was linearised with NheI. Stage V and VI oocytes were injected with 5 ng cRNA, and currents recorded 1–4 days post-injection. A ratio of 1:3 (A:B) was used for the expression of heteromeric 5-HT_3 receptors, as studies indicate that an excess of the secondary subunit promotes the expression of heteromeric receptors (e.g. Rayes *et al.* 2009). The nomenclature used in this article adopts the recent recommendations of NC-IUPHAR (Collingridge *et al.* 2009).

Site-directed mutagenesis

Mutagenesis reactions were performed using the QuikChange method (Agilent Technologies Inc., CA, USA) using human 5-HT3A (accession number: P46098) and 5-HT3B (O95264, both kindly donated by John Peters). Cysteine residues were substituted for amino acids in binding loops A–F (Fig. 1). To facilitate comparisons with previous work, we have used the numbering of the equivalent residues in the mouse 5-HT_3A subunit; for human 5-HT_3A numbering 5 should be subtracted from the residue number, and for human 5-HT_3B numbering 7 should be subtracted. Residues were chosen because they have been previously shown to be important for ligand binding and/or they are located in the binding site in a 5-HT_3 receptor homology model (Thompson & Lummis, 2006).

Electrophysiology

Experiments were performed using either conventional two-electrode voltage-clamp electrophysiology or the Roboocyte (MultiChannel Systems, Reutlingen, Germany), an automated two-electrode voltage-clamp

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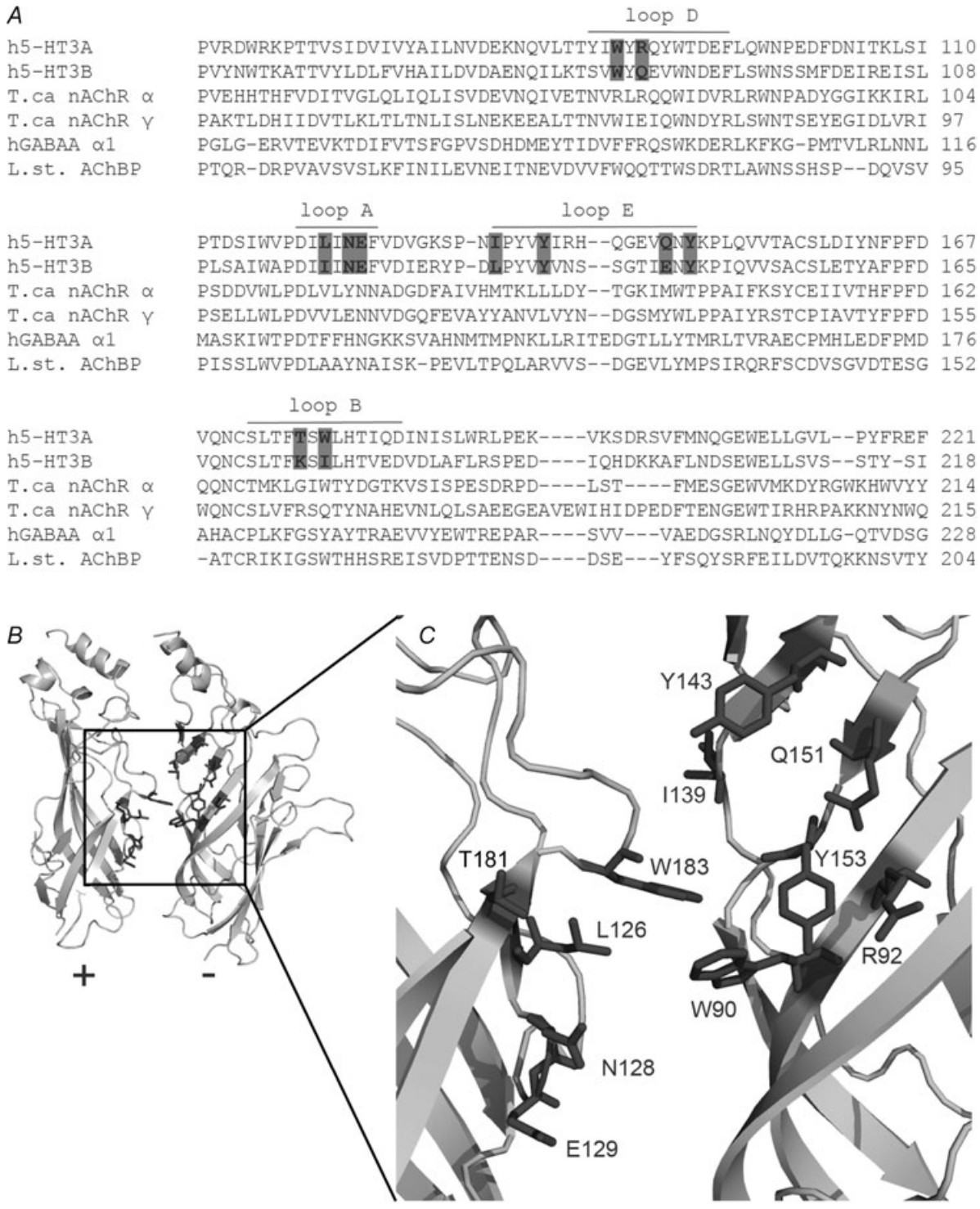

Figure 1. Residues that were mutated in this study
A, sequence alignment of human 5-HT3A and 5-HT3B subunits with *Torpedo californica* nACh receptor α and γ subunits, human GABA$_A$ receptor α1 subunit, and *Lymnaea stagnalis* AChBP. Residues mutated in this study are shown in bold on a grey background. The positions of the six binding loops A–F are indicated by black lines. *B*, a homology model of the 5-HT$_3$A receptor showing the location of the A subunit residues (stick representation) mutated in this study. Note that the numbering of the *A* and *B* residues in panel *A* corresponds to the human 5-HT$_3$ receptor, but the number in panel *B* is according to the mouse numbering used in this paper. Accession numbers for the alignment are: 5-HT3A P46098, 5-HT3B O95264, nACh α P02710, nACh γ P02714, GABA α1 P02710, AChBP P58154.

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system. We found that the two systems gave identical results when parameters from concentration–response curves were compared (eq. 1). For conventional two-electrode voltage clamp electrophysiology, *Xenopus* oocytes were clamped at −60 mV using an OC-725 amplifier (Warner Instruments, LLC, Hamden, CT, USA), Digidata 1322A (Molecular Devices, Sunnyvale, CA, USA) and the Strathclyde Electrophysiology Software Package (Department of Physiology and Pharmacology, University of Strathclyde, UK). Currents were filtered at a frequency of 1 kHz and sampled at 3 kHz. Micro-electrodes were fabricated from borosilicate glass (GC120TF-10, Harvard Apparatus, Edenbridge, UK) using a two stage horizontal pull (P-87, Sutter Instrument Co., Novato, CA, USA) and filled with 3 M KCl. Pipette resistances ranged from 0.5 to 1.5 MΩ. Oocytes were perfused with Ca^{2+}-free ND96 at a rate of 12 ml min^{-1}. Drug application was via a simple gravity fed system calibrated to run at the same rate. Analysis and curve fitting were performed using Prism v. 3.0 (GraphPad Software Inc., La Jolla, CA, USA). Concentration–response and concentration–inhibition data for each oocyte were normalised to the maximum current for that oocyte. The mean ± standard error of the mean (SEM) of the normalised responses for a series of oocytes were plotted against agonist concentration and iteratively fitted to the following equation:

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

where A is the concentration of ligand present; I_A is the current in the presence of ligand concentration A; $I_{\min}$ is the current when $A = 0$; $I_{\max}$ is the current when $A = \infty$, A_{50} is the concentration of A which evokes a current equal to $(I_{\max} + I_{\min})/2$; and n_H is the Hill coefficient. Statistical analysis was performed using a one-way analysis of variance (ANOVA) in conjunction with a Dunnett's *post hoc* test, or Student's unpaired *t* test (GraphPad Prism).

Methanethiosulfonate-ethylammonium (MTSEA) solutions were made up immediately before each experiment, and applied at a concentration of 2 mM for 2 min, followed by a 2 min wash. For protection experiments, pre-treatment values were recorded as two responses at an EC_{50} or maximal concentration of 5-HT. Oocytes were then treated with a maximal concentration of agonist (1 mM 5-HT for 30 s) or competitive antagonist (1 mM d-TC for 60 s) before and during a 2 min treatment with MTSEA or MTSEA–biotin. The responses to two further applications of 5-HT were measured and the amplitude compared to that before treatment. Finally, receptors were subjected to MTSEA or MTSEA–biotin alone, to test for modification in the absence of a ligand. Percentage inhibition was calculated as:

$$(1 - (I_{\max} \text{ after MTSEA} / I_{\max} \text{ before MTSEA})) \times 100 \quad (2)$$

Dithiothreitol (DTT) was used at 10 mM, and made fresh daily from frozen 1 M stocks. For treatment of double cysteine mutants, an initial application of 10 mM DTT for 1 min was used. Thereafter, 10 s treatments were used immediately prior to each of the 5-HT applications.

Radioligand binding

This was performed as described previously (Price & Lummis, 2004). Human embryonic kidney (HEK) 293 cells were maintained on 90 mm tissue culture plates at 37°C and 7% CO_2 in a humidified atmosphere. Cells were transfected using polyethyleneimine (PEI). PEI (30 μl, 1 mg ml^{-1}), 5 μl DNA (A or 1:3 A:B) and 1 ml DMEM were incubated for 10 min at room temperature, added drop-wise to a 80–90% confluent plate, and incubated for 2–3 days before harvesting. Transfected HEK 293 cells were then harvested, washed with Hepes buffer, and 50 μg of crude cell membranes incubated in 0.5 ml Hepes buffer containing [^{3}H]granisetron in a total volume of 500 μl. Non-specific binding was determined using 1 mM quipazine. Reactions were incubated at least 1 h on ice, and then 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. Data were analysed using Prism.

Results

5-HT_3A and 5-HT_3AB receptors have distinct characteristics

Wild-type and mutant 5-HT_3 receptors were expressed as either homomeric (A or A_{mut}) or heteromeric (AB, A_{mut}B or AB_{mut}) receptors. 5-HT_3AB receptors differed from 5-HT_3A receptors in their current profiles, EC_{50} values, Hill slopes and picrotoxin (PTX) sensitivity, consistent with previous reports (Davies *et al.* 1999; Hapfelmeier *et al.* 2003; Thompson *et al.* 2007). Current response decay in the presence of 5-HT was more rapid in heteromeric than in homomeric receptors, which showed almost no decay in our buffer (no added Ca^{2+}; Fig. 2*A* and Fig. S1). Heteromeric receptors also had higher EC_{50} values (15-fold) and lower Hill slopes (2.8-fold) than for homomeric receptors (Fig. 2*A* and Fig. S1), and the potency of PTX was less, with IC_{50} values of 9.5 μM ($pIC_{50} = 5.02 \pm 0.09$, $n = 9$) for wild-type A-only receptors, and 55 μM ($pIC_{50} = 4.26 \pm 0.05$, $n = 5$) for wild-type AB receptors (Fig. 2*B*).

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5-HT3A subunit cysteine substitutions affect receptor properties

When expressed alone or in combination with the B subunit, four A subunit mutants (A_{E129C}, A_{Y143C}, A_{Y153C} and A_{W183C}; mouse 5-HT$_3$A subunit numbering) were non-functional and A_{W90C} mutants had an EC_{50} that was too high to be accurately determined (Table 1, Fig. 3 and Fig S1). Receptors containing the A subunit mutants A_{R92C}, A_{L126C}, A_{N128C}, A_{I139C}, A_{Q151C} and A_{T181C} had significant increases in EC_{50}. A similar pattern of changes was seen in oocytes containing mutant A subunits coexpressed with wild-type B subunits.

5-HT3B subunit mutations have no effect on receptor properties

In contrast to mutant A subunits, all mutant B subunits produced functional receptors when co-expressed with wild-type A subunits. With the exception of AB_{W90C}, none of these heteromeric receptors had EC_{50} values that differed significantly from wild-type 5-HT$_3$AB receptors, and all were significantly different from those containing only A subunits, with at least three of the four properties that distinguish 5-HT$_3$AB and 5-HT$_3$A receptors described above (Table 1, Fig. 3 and Fig. S1).

MTSEA treatment of 5-HT3A subunit mutants further affects receptor function

Modification of A_{N128C} and A_{T181C} completely abolished functional responses to 5-HT. There were also changes in EC_{50} values for A_{L126C} and A_{I139C} mutants (Table 1, Fig. 4*A*). Functional A subunit mutants showed reduced maximal current amplitudes (I_{max}) following MTSEA treatment, except for those containing A_{I139C}, where I_{max} was increased ~17-fold (Fig. 4*B*).

The patterns of MTSEA effects on EC_{50} and I_{max} were the same for $A_{mut}B$ receptors (Fig. 4*A* and *B*, Fig. S1). Striking examples of this include the complete block by MTSEA at A_{mut} and $A_{mut}B$ receptors containing A_{N128C} or A_{T181C}, a reduced EC_{50} and increased I_{max} for those containing A_{I139C}, and a reduced I_{max} for A_{R92C}, A_{L126C} and A_{Q151C}.

Application of MTSEA to wild-type 5-HT$_3$A receptors caused no changes to their concentration-response curves (Fig. 2*A*).

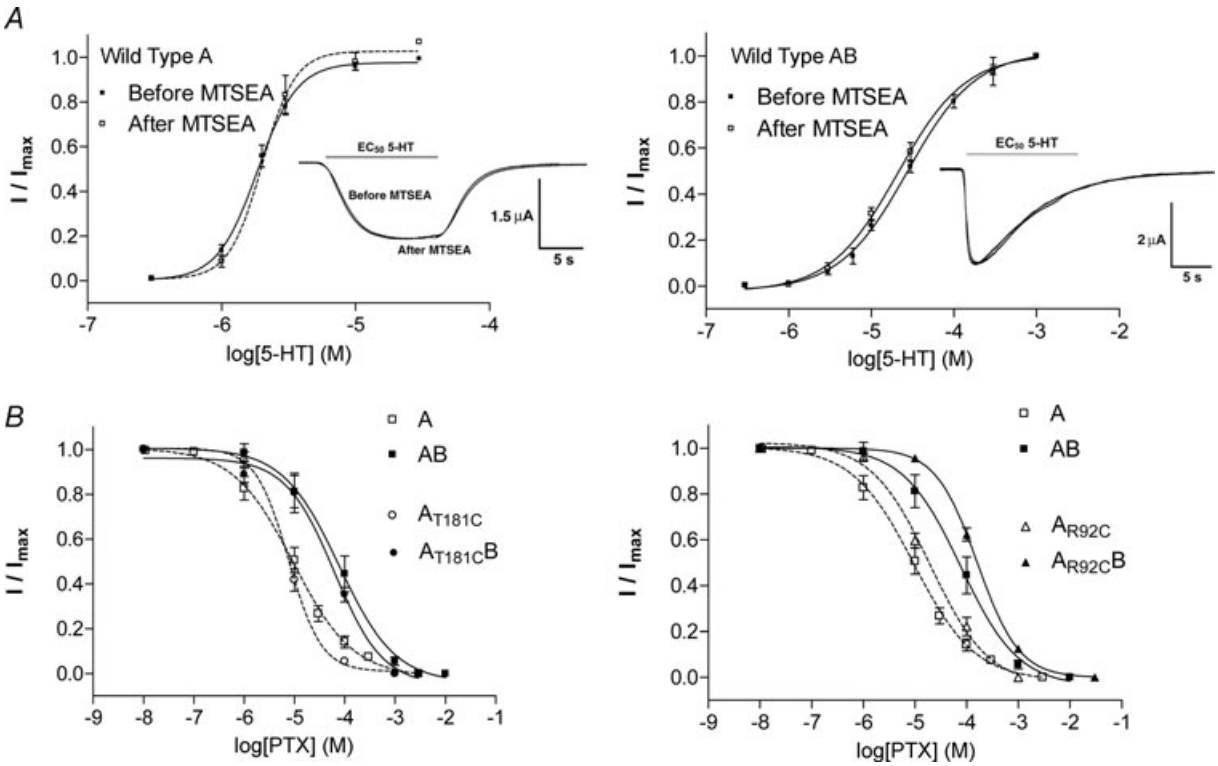

Figure 2. 5-HT concentration–response and PTX concentration–inhibition curves for A and AB receptors
A, 5-HT concentration–response curves. Wild-type receptors were unaltered by MTSEA (comparisons are also shown in Fig. 4). The calculated EC_{50} values and Hill slopes can be found in Table 1. Typical EC_{50} 5-HT responses are shown next to each curve. *B*, PTX concentration–inhibition curves. The presence of the B subunit is confirmed by a rightward shift in the PTX concentration–inhibition curve in heteromeric receptors. The mutants shown are examples, and are the same as those in Fig. 5.

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Table 1. Concentration–response parameters of A, B, D and E loop cysteine mutants

		Before MTSEA				After MTSEA			
Loop	Mutant	pEC_{50}	EC_{50} (μM)	n_H	n	pEC_{50}	EC_{50} (μM)	n_H	n
	A	5.73 ± 0.02	1.84	2.91 ± 0.30	9	5.70 ± 0.05	2.00	3.52 ± 0.84	3
	AB	4.56 ± 0.03	27.6	1.05 ± 0.09	12	4.68 ± 0.04	20.9	1.06 ± 0.13	4
	A_{W90C}	—	>300	—	>20	—	—	—	—
D	$A_{W90C}B$	NF	NF	NF	>20	—	—	—	—
	AB_{W90C}	4.98 ± 0.05*	10.5	0.92 ± 0.10	10	4.99 ± 0.03	10.2	1.28 ± 0.11	5
	A_{R92C}	3.99 ± 0.03*	101	3.09 ± 0.58	3	3.90 ± 0.25	126	3.23 ± 0.50	3
D	$A_{R92C}B$	3.74 ± 0.03*	180	1.78 ± 0.16	8	3.58 ± 0.14	265	3.25 ± 0.50	4
	AB_{Q92C}	4.77 ± 0.08	16.9	1.03 ± 0.20	4	4.69 ± 0.12	20.3	0.70 ± 0.15	3
	A_{L126C}	5.27 ± 0.04*	5.32	3.04 ± 0.70	4	4.33 ± 0.12§	47.0	3.10 ± 1.63	4
A	$A_{L126C}B$	4.51 ± 0.05	31.0	1.26 ± 0.15	3	3.75 ± 0.12§	176	0.96 ± 0.19	3
	AB_{I126C}	4.64 ± 0.10	23.0	0.84 ± 0.14	3	4.80 ± 0.07	15.8	1.00 ± 0.16	3
	A_{N128C}	5.25 ± 0.03*	5.63	1.40 ± 0.16	4	Block1†	Block	Block	3
A	$A_{N128C}B$	4.86 ± 0.08*	14.0	0.68 ± 0.10	6	Block†	Block	Block	3
	AB_{N128C}	4.67 ± 0.11	21.1	1.24 ± 0.37	3	4.74 ± 0.08	18.3	1.23 ± 0.27	3
	A_{E129C}	NF	NF	NF	>20	—	—	—	—
A	$A_{E129C}B$	NF	NF	NF	>20	—	—	—	—
	AB_{E129C}	4.73 ± 0.04	18.7	1.07 ± 0.10	7	4.84 ± 0.04	14.3	1.07 ± 0.09	4
	A_{I139C}	4.61 ± 0.04*	24.4	1.36 ± 0.17	3	5.57 ± 0.25§	2.71	1.44 ± 1.11	3
A / E	$A_{I139C}B$	4.41 ± 0.04*	38.8	1.17 ± 0.12	7	5.00 ± 0.15§	10.0	1.15 ± 1.43	3
	AB_{L139C}	4.68 ± 0.05	20.8	1.12 ± 0.14	8	4.65 ± 0.07	22.4	1.08 ± 0.20	6
	A_{Y143C}	NF	NF	NF	>20	—	—	—	—
E	$A_{Y143C}B$	NF	NF	NF	>20	—	—	—	—
	AB_{Y143C}	4.76 ± 0.04	17.5	1.32 ± 0.15	5	4.80 ± 0.04	16.0	1.50 ± 0.17	4
	A_{Q151C}	4.88 ± 0.03*	12.9	1.90 ± 0.25	3	4.71 ± 0.04	16.9	3.13 ± 0.54	4
E	$A_{Q151C}B$	4.18 ± 0.10*	65.5	1.32 ± 0.36	5	4.15 ± 0.16	70.1	1.71 ± 0.90	3
	AB_{E151C}	4.68 ± 0.03	20.1	1.45 ± 0.16	6	4.66 ± 0.04	21.7	1.10 ± 0.12	4
	A_{Y153C}	NF	NF	NF	>20	—	—	—	—
E	$A_{Y153C}B$	NF	NF	NF	>20	—	—	—	—
	AB_{Y153C}	4.78 ± 0.05	16.3	1.04 ± 0.18	5	4.83 ± 0.05	14.9	1.03 ± 0.13	4
	A_{T181C}	4.54 ± 0.02*	28.9	2.38 ± 0.32	7	Block†	Block	Block	3
B	$A_{T181C}B$	4.38 ± 0.03*	41.6	1.51 ± 0.16	7	Block†	Block	Block	8
	AB_{K181C}	4.43 ± 0.09	36.8	0.82 ± 0.11	8	4.51 ± 0.12	30.7	0.76 ± 0.19	4
	A_{W183C}	NF	NF	NF	>20	—	—	—	—
B	$A_{W183C}B$	NF	NF	NF	>20	—	—	—	—
	AB_{I183C}	4.63 ± 0.09	23.4	0.83 ± 0.16	3	4.79 ± 0.07	16.3	1.26 ± 0.24	3

*Significantly different to WT; †significantly different following MTSEA treatment; §Data are means ± SEM.

5-HT3B subunit mutants are not affected by MTSEA modification

MTSEA had no effect on either the EC_{50} or I_{max} of 5-HT_3AB or any 5-HT_3AB_{mut} receptors (Table 1, Figs 2*A* and 4*A* and *B*) indicating that MTSEA modification of B subunit cysteines does not significantly impact on receptor function or that it does not modify these receptors.

Ligands can protect against MTS reagent modification

To determine whether competitive ligands could protect against MTSEA and MTSEA–biotin modification, we examined a residue on the principal (residue 181) and the complementary binding face (residue 92) of each subunit. These residues were chosen as they showed very clear changes in response following the addition of MTSEA. We examined the effects of both an agonist (5-HT) and an antagonist (d-TC) to minimise the risk of making residues inaccessible to MTS modification as a result of ligand-specific changes in receptor structure; studies with AChBP indicate that a conformational change induced by an agonist will be quite distinct from that caused by an antagonist (Hansen *et al.* 2005).

At A_{R92C} and $A_{R92C}B$ receptors MTSEA–biotin almost completely inhibited the 5-HT-induced response (Fig. 5*A*), but inhibition was completely prevented by co-application of 5-HT or d-TC with MTSEA–biotin. Similarly A_{T181C} and $A_{T181C}B$ receptors were inhibited by MTSEA, and protected from inhibition by 5-HT and d-TC

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(Fig. 5*B* and *C*). Protection experiments could not be performed on the equivalent B subunit residues as AB_{Q92C} and AB_{K181C} were unaffected by MTSEA or MTSEA–biotin (Table 1, Fig. 4*A* and *B;* Fig. 5*D*). The results indicate that both A_{R92} (−) and A_{T181} (+) are part of the binding site for 5-HT and d-TC in both homomeric and heteromeric receptors (i.e. ligand binding occurs at A+A– interfaces in both receptor types).

5-HT3A subunit double cysteine mutants respond only after DTT treatment

As an additional test of whether A+A– interfaces are present in heteromeric receptors, a series of double mutants were made with cysteine residues located at opposite sides of the binding site; if these residues are sufficiently close, we would expect disulphide bonds to form between them and modify receptor function. They were engineered into loop C residues on the principal face and either loop E or F residues on the complementary face; in the nAChR these loops come into close proximity upon agonist binding (Gleitsman *et al.* 2008; Mukhtasimova *et al.* 2009). $A_{D204C/S227C}$, $A_{S206C/S227C}$ and $A_{F208C/S227C}$ responded to 5-HT and were unaffected by DTT, indicating that either no disulphide bonds were formed, or that cross-linking here does not affect receptor function (Table 2). In contrast, both $A_{Y153C/S227C}$ and $A_{S206C/E229C}$ only responded to 5-HT following DTT treatment, although the former had responses too small (<100 nA) for further study (Fig. 6*A* and *B*). Responses of $A_{S206C/E229C}$ mutant receptors slowly decreased following removal of DTT suggesting spontaneous reformation of disulphide bonds (Fig. 6*E*). This rate of reformation of bonds followed a single exponential and was complete within 24 min. It was reversed if DTT was re-applied. Expression of the single substitutions showed no DTT effect, demonstrating that the disulphide bond only formed when both substitutions were present. These data indicate that S206C and E229C are sufficiently close to spontaneously form a disulphide bond, and, as they are too far apart to interact within a single subunit, S206C must interact with E229C in the adjacent subunit, cross-linking two subunits across the binding site interface and preventing access or binding of 5-HT (Fig. 6*C*).

We also explored MTSEA treatment of these mutants. Following DTT application, MTSEA treatment of $A_{S206C/E229C}$ receptors caused complete block of the 5-HT response (Fig. 6*A*), although neither of the single mutants alone was affected by MTSEA modification, indicating that two molecules of MTSEA are required to block ligand

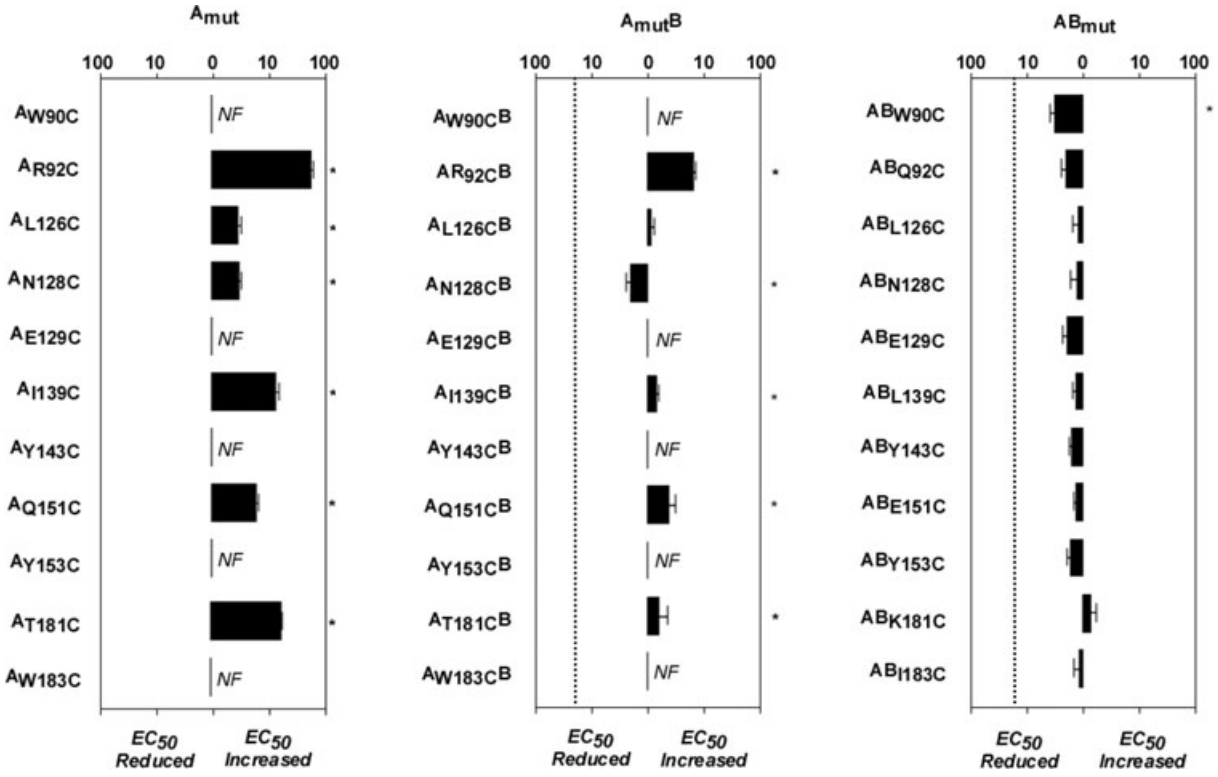

Figure 3. Relative EC_{50} values of wild-type and mutant receptors
Asterisks denote statistically different from wild-type. NF: non-functional at 100 μM 5-HT, or with an EC_{50} too high to be accurately determined. Relative values are shown as the differences ± SED. Data from Table 1. The dotted line represents the difference between wild-type 5-HT$_3$A and wild-type 5-HT$_3$AB responses.

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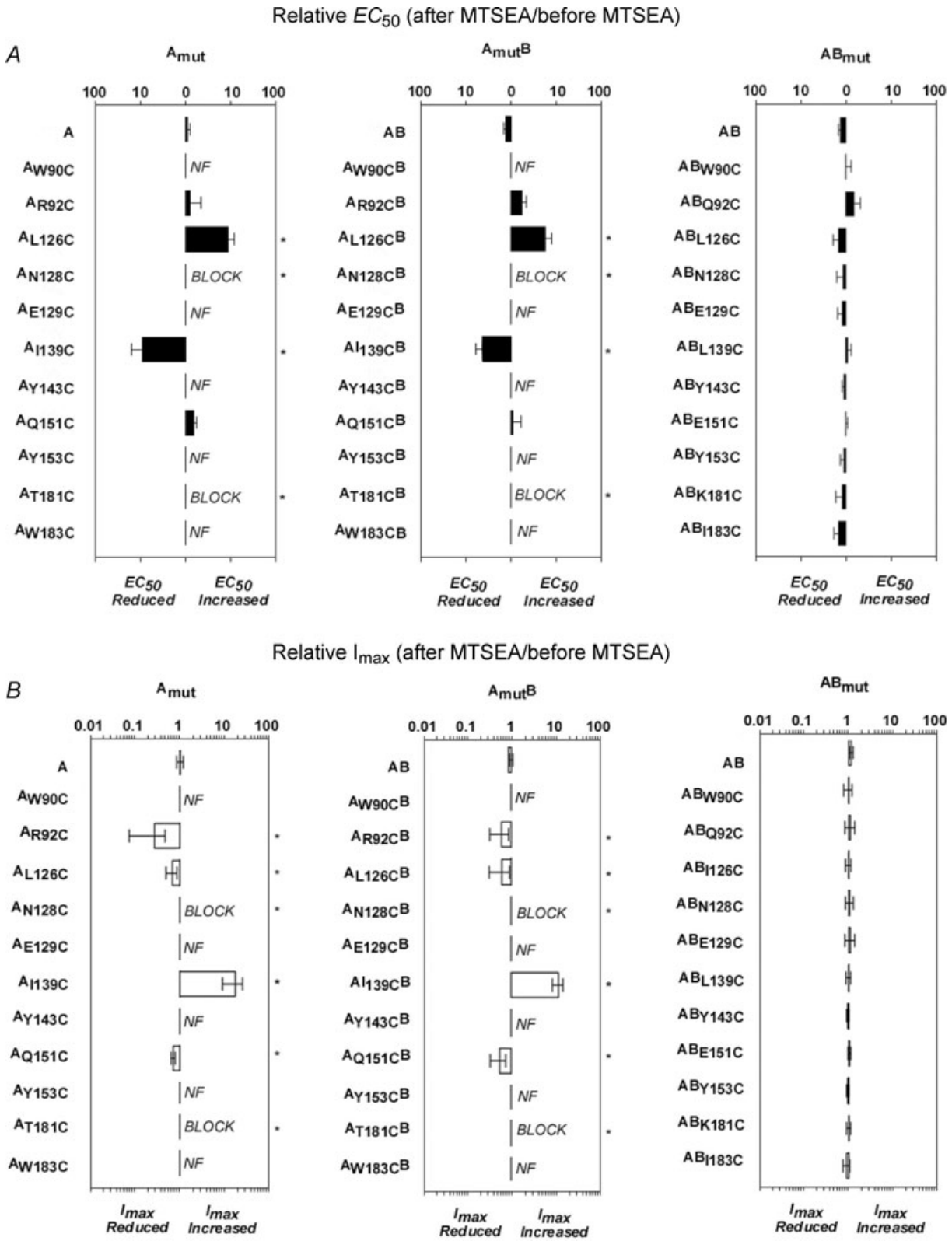

Figure 4. The effect of MTSEA on wild-type and mutant receptors
The EC_{50} or I_{max} obtained after MTSEA treatment is compared to that obtained before treatment. BLOCK: complete inhibition by MTSEA. Asterisks denote statistically different from wild-type. NF: non-functional at 100 μM 5-HT. Relative values are expressed as the difference $\pm$ SED. Data from Table 1.

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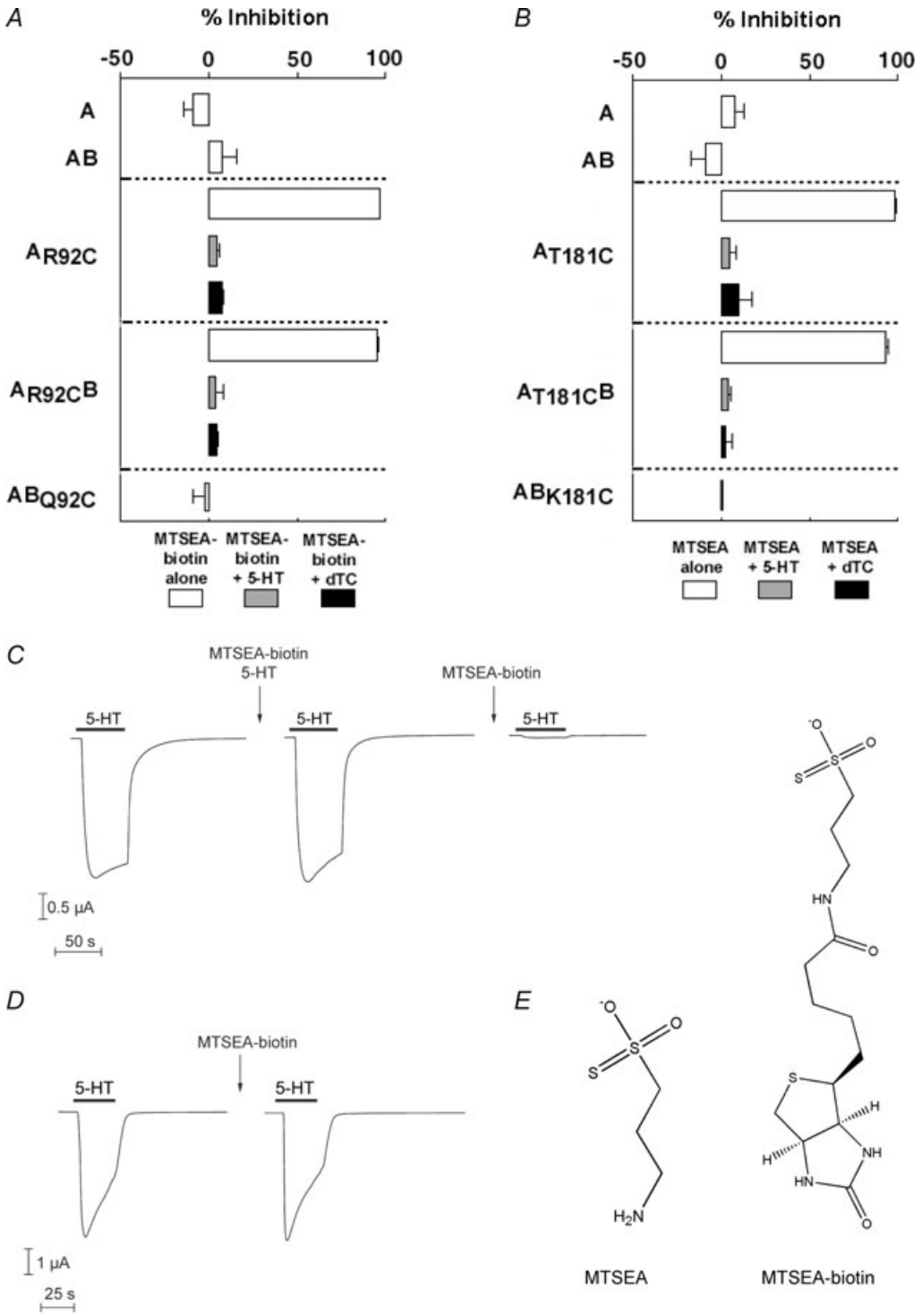

Figure 5. Agonist (5-HT) and antagonist (d-TC) protection of mutant receptors from MTS modification
A, A_{R92C} and $A_{R92C}B$ receptors are completely protected from MTSEA–biotin modification by the presence of either agonist (5-HT) or antagonist (d-TC). There is no effect of MTSEA–biotin on AB_{Q92C} receptors. R92 is a loop D residue located on the A subunit complementary (−) face; Q92 is the equivalent B subunit residue. *B*, A_{T181C} and $A_{T181C}B$ receptors are completely protected from MTSEA modification by the presence of either agonist (5-HT) or antagonist (d-TC). There is no effect of MTSEA on AB_{K181C} receptors. T181 is a loop B residue located on the A subunit principal (+) face; K181 is the equivalent B subunit residue. Typical current traces from oocytes expressing A_{R92C} (*C*) or AB_{Q92C} (*D*) receptors are also shown. 5-HT application (200 μM for A_{R92C} or 30 μM for AB_{Q92C}) is denoted by a black bar above the trace. Arrows indicate applications of MTSEA–biotin (2 mM with or without 1 mM 5-HT) for 2 min, followed by wash for 2 min (see methods for details). *E*, structures of MTSEA and MTSEA–biotin.

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Table 2. Concentration–response parameters of C and F loop cysteine mutants

	Before MTSEA				After MTSEA			
Mutant	pEC_{50}	EC_{50}	n_H	n	pEC_{50}	EC_{50}	n_H	n
A_{S203C}	5.25 ± 0.07	5.66	1.42	3	5.13 ± 0.03	7.48	1.46	3
A_{D204C}	4.04 ± 0.05*	9.07	1.73	3	4.00 ± 0.08	9.92	1.41	3
A_{S206C}	5.31 ± 0.04	4.86	2.43	3	5.19 ± 0.02	6.39	2.44	3
A_{F208C}	4.36 ± 0.05*	44.0	1.71	4	3.55 ± 0.02§	284	1.84	3
A_{S227C}	5.23 ± 0.02	5.86	2.70	3	5.33 ± 0.02	4.64	2.35	3
A_{E229C}	4.56 ± 0.03*	27.5	1.83	6	4.45 ± 0.04	35.2	1.56	4
$A_{Y153C/S227C}$	SR	—	—	9	ND			
$A_{D204C/S227C}$	4.00 ± 0.02*	101	3.43	3	ND			
$A_{S206C/S227C}$	4.60 ± 0.03*	25.4	1.51	3	ND			
$A_{F208C/E227C}$	4.19 ± 0.02*	64.0	3.18	6	ND			
$A_{S206C/E229C}$	4.58 ± 0.02^{*DTT}	26.0	2.15	5	BLOCK†	—	—	5
$A_{S206C/E229C}B$	3.93 ± 0.06^{*DTT}	118	1.04	9	BLOCK†	—	—	4

Data are means ± SEM; *significantly different to WT; †significantly different following MTSEA treatment; ND = not determined; ^{DTT} = Response only seen after DTT treatment; SR = small response (<0.1 μA at 1 mM 5-HT).

access when attached to these particular residues. The 5-HT response could be recovered following application of DTT.

Co-expression of the wild-type B subunit with $A_{S206C/E229C}$ produced heteromeric receptors that similarly only responded to 5-HT after DTT treatment (Fig. 6*B* and *D*). Hill slopes were close to unity and 5-HT concentration–response curves yielded EC_{50} values that were increased compared to both homomeric $A_{S206C/E229C}$ mutants (4.5-fold) and wild-type heteromeric receptors (4-fold), indicating that these were heteromeric and not homomeric receptors. The effect of DTT on heteromeric receptors shows that active A+A– interfaces must be present. The data also show that A+B– and B+A– interfaces do not contribute to receptor activation, as there was no discernable current before DTT treatment, as single A+ or A– Cys mutants do not inhibit function.

Radioligand binding

Saturation binding experiments at 5-HT_3A and 5-HT_3AB receptors expressed in HEK cells revealed similar affinities for [^{3}H]granisetron binding (0.55 and 0.68 nM respectively; Table 3) and similar IC_{50} values for 5-HT displacement (0.09 ± 0.01 and 0.12 ± 0.03 μM, respectively) as reported in previous studies (Davies *et al.* 1999; Dubin *et al.* 1999; Brady *et al.* 2001; Hapfelmeier *et al.* 2003; Kelley *et al.* 2003). These data therefore support the idea of identical binding sites for agonists and antagonists in 5-HT_3A and 5-HT_3AB receptors. We also examined the binding affinities on a range of $A_{mut}B$ and AB_{mut} receptors (Table 3). These data show affinities can be modified or binding ablated in A_{mut}-containing receptors at residues which have previously been shown to contribute to the ligand binding site (e.g. W90 and W183), with no change for those mutant receptors which are more peripheral to the binding pocket (e.g. L126 and Q151). These data also confirm the greater sensitivity of functional *versus* binding assays, e.g. mutation of the A subunit at residues L126 and Q151 revealed changes in function but not binding. This is similar to previous reports, e.g. an alanine scan of 15 consecutive amino acids in loop B of the 5-HT_3 receptor showed that nine of these affected [^{3}H]granisetron binding but all affected the 5-HT EC_{50} (Thompson *et al.* 2008). The contributions to ligand binding of most of the A subunit residues mutated in this study have been previously examined using a range of different amino acids, and their contributions to binding extensively discussed (see Thompson & Lummis, 2006 for review or reports therein).

Discussion

The ligand binding site of the 5-HT_3 receptor is formed by the convergence of amino acid loops from two adjacent subunits, termed principal (+) and complementary (−). Binding sites in the homomeric 5-HT_3A receptor are composed of A+A– interfaces, but binding sites in the heteromeric 5-HT_3AB receptor could be located at A+A–, A+B–, B+A– or B+B– interfaces. Given the inconsistencies of 5-HT_3AB stoichiometries in the literature, we have performed a range of experiments (including disulphide trapping and the substituted cysteine accessibility method or SCAM) on cysteine-substituted residues to clarify which subunit interfaces are present in the human heteromeric 5-HT_3AB receptor ligand binding site. The data show no effect of B subunit mutations, no rescue of non-functional A subunit

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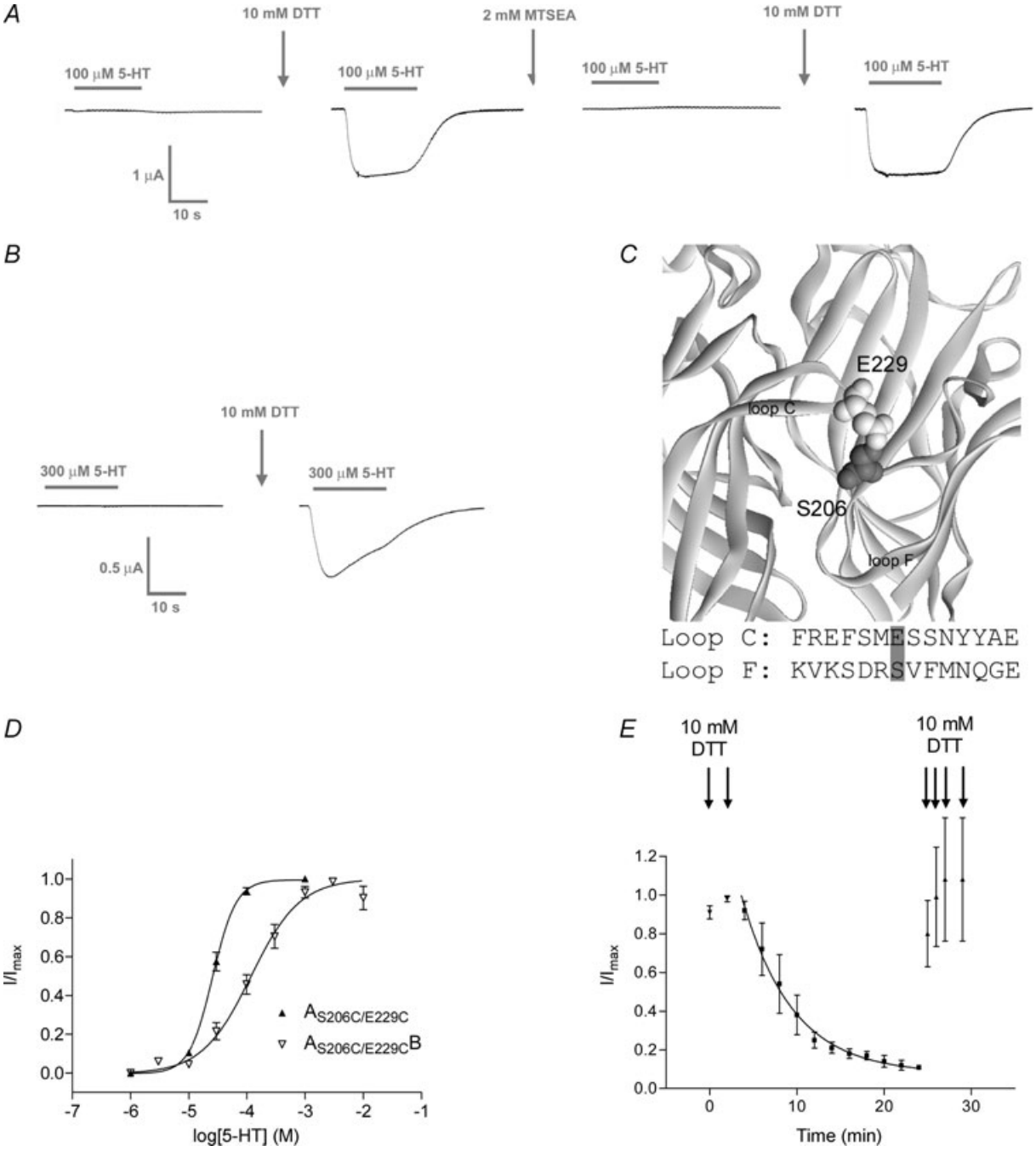

Figure 6. The effects of DTT on homomeric and heteromeric receptors containing A subunit double cysteine mutations ($A_{S206C/E229C}$) in the C and F loops
A, 5-HT-induced currents are only seen after application of DTT (10 mM for 1 min). A subsequent application of MTSEA (2 mM for 1 min) inhibits this response; this inhibition can be reversed by DTT. *B*, DTT treatment is also required for 5-HT-induced responses in heteromeric $A_{S206C/E229C}$B receptors. *C*, the locations of S206 and E229 on a 5-HT$_3$ receptor homology model (template PDB ID; 2PGZ). *D*, Concentration–response curves for homomeric and heteromeric receptors containing the A subunit double cysteine mutant. Parameters derived from these curves (Table 2) are consistent with those expected for homomeric and heteromeric responses. *E*, Disulphide bonds spontaneously reform in $A_{S206C/E229C}$B mutant receptors. Following removal of DTT, peak current responses to 5-HT (100 μM) decline with an exponential time course ($\tau = 0.17 \pm 0.01$ min^{-1}), but recover following several 10 s DTT applications (arrows). All traces are representative of $\geq$4 separate experiments.

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Table 3. [^{3}H]granisetron binding affinities at $A_{mut}B$ and AB_{mut} 5-HT$_3$ receptors

Mutant	K_d (nM)	Mutant	K_d (nM)
A	0.55 ± 0.12	A	0.55 ± 0.12
AB	0.68 ± 0.22	AB	0.68 ± 0.22
$A_{W90C}B$	NB	AB_{W90C}	0.57 ± 0.35
$A_{R92C}B$	3.8 ± 0.5*	AB_{Q92C}	0.64 ± 0.52
$A_{L126C}B$	0.35 ± 0.01	AB_{I126C}	0.57 ± 0.14
$A_{N128C}B$	2.0 ± 0.7*	AB_{N128C}	0.84 ± 0.33
$A_{E129C}B$	NB	AB_{E129C}	0.56 ± 0.17
$A_{I139C}B$	1.9 ± 0.6*	AB_{L139C}	0.65 ± 0.21
$A_{Y143C}B$	NB	AB_{Y143C}	0.80 ± 0.20
$A_{Q151C}B$	0.44 ± 0.02	AB_{E151C}	0.64 ± 0.37
$A_{Y153C}B$	2.9 ± 0.6*	AB_{Y153C}	0.41 ± 0.22
$A_{T181C}B$	3.0 ± 0.3*	AB_{K181C}	0.73 ± 0.25
$A_{W183C}B$	NB	AB_{I183C}	0.50 ± 0.20

Data are means ± SEM, $n = 3$–5. *Significantly different to WT, $P < 0.05$; NB, no binding

mutations by B subunits, and cross linking of A residues located on either side of the binding pocket in both homomeric and heteromeric receptors. These results are only consistent with the binding sites of both homomeric and heteromeric human 5-HT$_3$ receptors being located at A+A– interfaces.

Cysteine substitution of A subunit residues, and covalent modification of these with MTSEA, confirmed the importance and accessibility of residues that have been previously identified in mutagenesis and modelling studies (Venkataraman *et al.* 2002; Reeves *et al.* 2003; Thompson *et al.* 2005, 2008; Yan *et al.* 2006). MTSEA had significant effects on receptors containing cysteine-substituted residues in both the principal and complementary faces of A subunits. Residues in each face were protected by both agonist and antagonist, confirming their location in the binding site. Similar effects were seen in homomeric and heteromeric receptors indicating that an A+ and an A– face are required for binding at both receptor types. Further evidence from disulphide trapping experiments revealed cross-linking of cysteine-substituted A+ and A– residues in both homomeric and heteromeric receptors. These data show that residues on opposite sides on the binding interface can covalently interact in both these receptor types, and therefore demonstrate that adjacent A subunits must be present in heteromeric receptors.

The 5-HT$_3$B subunit only forms functional receptors when expressed in combination with the A subunit, as when expressed alone it does not appear to traffic to the cell surface (Boyd *et al.* 2002). 5-HT$_3$AB receptors have distinct biophysical properties and altered potencies of channel blocking ligands, properties which can be explained by differing residues within the transmembrane and intracellular domains (Davies *et al.* 1999; Brady *et al.* 2001; Das & Dillon, 2003; Hapfelmeier *et al.* 2003; Holbrook *et al.* 2009). The affinities of agonists and competitive antagonists, however, are almost identical at homomeric and heteromeric receptors, implying that they share a common binding site at an A+A– interface.

Our current data support the hypothesis that the ligand binding site in both homomeric and heteromeric receptors is located at the interface of adjacent A subunits, and is consistent with a recent study on mouse 5-HT$_3$AB receptors where residues from the B subunit were substituted with the aligning residues of the A subunit and *vice versa* (Lochner & Lummis, 2010); mutations in the A, but not the B, subunit caused changes in the 5-HT-elicited response and the affinity (K_d) of the radiolabelled competitive antagonist [^{3}H]granisetron. In our current work we used human and not mouse receptors, and probed a wider range of potential binding site residues throughout loops A–F. We observed large functional changes at all homomeric and heteromeric receptors containing modified A subunit residues, but none at those with B subunit modifications. Similarly, there were changes in [^{3}H]granisetron binding affinity to receptors with A subunit mutations, and no changes to those containing B subunit mutations. If B subunits were part of the binding pocket we would expect to observe at least some changes, as previous reports have shown that the binding site is highly sensitive to changes of both ligand-interacting and non-interacting residues (Spier & Lummis, 2000; Beene *et al.* 2004; Price & Lummis, 2004; Thompson *et al.* 2005; Sullivan *et al.* 2006; Price *et al.* 2008). Equivalent locations of the A and B subunit residues can only be confirmed when we have high resolution structural data, but strong evidence to support similar secondary structures of these subunits comes from crystal structures in homologous proteins: e.g. AChBP and ELIC have only ∼18% sequence identity and yet the structures of these receptors overlay with only 1.5 Å root mean square deviation (Hilf & Dutzler, 2008). Given the considerably higher sequence similarity between the A and B extracellular domains, we would expect that the residues in the B subunit binding site to be positioned similarly to those in A subunits. Indeed it seems inconceivable, given the large number of substitutions that were made to both the principal and complementary faces of the B subunit, that *none* would have had an effect if this subunit contributed to the binding site, particularly as *all* A subunit modifications significantly altered receptor responses.

We also consider it unlikely that responses from oocytes injected with both A and B subunits are contaminated by homomeric currents, as neither we nor other researchers in this field have seen evidence of homomers when heteromers are expressed (e.g. Barrera *et al.* 2005). This may be because B subunits cannot express alone and therefore strongly interact with any A subunits

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that have been produced, and some support for this speculative hypothesis comes from studies showing that co-transfection with B subunits results in the function of some non-responding mutant A subunits (Wu *et al.* 2010). We have taken great care in this study to demonstrate that the properties of the receptors are consistent with heteromers and not homomers whenever we express both subunits; the altered EC_{50} values, Hill slopes, characteristic response profiles and PTX sensitivity, provide strong evidence that we have heteromeric and not homomeric receptors.

Our data demonstrate an A+A– interface in heteromeric receptors, and although this agrees with a previous study on mouse receptors, it conflicts with a study using AFM that defined the subunit arrangement around the receptor rosette as BBABA, i.e. no A+A– interfaces (Barrera *et al.* 2005). Although it is difficult to explain the discrepancy between these results, there are several possible explanations. For example, the stoichiometry of Cys-loop receptors can be influenced by external factors such as temperature or the ratios of subunit DNA transfected (Zwart & Vijverberg, 1998; Nelson *et al.* 2003), and expression systems and added tags may also be critical. It may be that differences in endogenous levels of chaperones, e.g. 14-3-3 and RIC-3, affect stoichiometry and expression of nACh and 5-HT$_3$ receptors (Exley *et al.* 2006; Walstab *et al.* 2010). The location of the receptors being examined may also be important and a possible explanation for the differences may be simply that we sampled only functional cell surface receptors, whilst AFM detects both intracellular and cell surface receptors, the former of which may be non-functional.

Several of our A subunit cysteine mutants were non-functional (W90, E129, Y143, Y153 and W183), which confirms the critical importance of these residues as reported elsewhere (Spier, 2000; Yan *et al.* 1999; Venkataraman *et al.* 2002; Beene *et al.* 2004; Price & Lummis, 2004; Thompson *et al.* 2005, 2008; Sullivan *et al.* 2006; Price *et al.* 2008). The other A subunit mutants were all modified by MTSEA, which places them on a solvent accessible surface. These data, and the proximity of S206 and E229 on loops C and F, support the homology models we originally used to identify the target residues (Fig. 1; Reeves *et al.* 2003; Thompson *et al.* 2005). Residues we studied here also show similarities in accessibility and protection to the aligning residues of other members of the Cys-loop family. For example, the residues equivalent to the A subunit R92 in nACh (*Torpedo* γE57; Sullivan & Cohen, 2000) and GABA$_A$ (α1R66; Boileau *et al.* 1999) receptors are modified by MTS compounds and protected by competitive ligands, demonstrating that they line the ligand binding site. Residues that align with Y143 and Y153 in the nACh (γL109C) and GABA$_C$ (S168C) receptors can also be protected from MTS modification (Sullivan & Cohen, 2000; Sedelnikova *et al.* 2005), although Y143 and Y153 could not be tested in our study as the cysteine substitutions generated non-functional receptors.

Residues that face into the binding site and directly contact the ligand may be less likely to produce gross structural changes that alter receptor gating than residues which point into the protein interior (Ward *et al.* 1990; Chen *et al.* 1998; Brams *et al.* 2011). However, gating effects may explain the MTSEA-dependent potentiation that was displayed by receptors containing the A subunit mutation I139C, which was the only residue in this study that was not predicted to be in the binding site. None of the B subunit mutations displayed this (or any other) effect, suggesting that the B subunit residues we investigated do not have a role in gating. This contrasts with B subunit residues in the pore and the intracellular domain, the former of which are responsible for the differing potencies of bilobalide, ginkgolide B, chloroquine, mefloquine and PTX at homomeric and heteromeric receptors, and the latter for the dramatic increase in single channel conductance observed when B subunits are incorporated into heteromeric receptors (Kelley *et al.* 2003; Das & Dillon, 2005; Thompson *et al.* 2007, 2010*a*, 2010*b*; Thompson *et al.* 2011).

Our data are insufficient to define a stoichiometry for the heteromeric receptor as there are four possible stoichiometries that are compatible with our results: AAAAB, AABAB, AAABB and AABBB. The first is less likely as a 4:1 stoichiometry is unknown in the Cys-loop receptor superfamily, although there are many examples of a single non-agonist binding subunit being incorporated into receptors containing three subunit types (e.g. GABA$_A$ $\alpha\beta\gamma$). The reduced Hill coefficient for activation of heteromeric receptors by 5-HT may indicate that there is only one agonist binding site, supporting the possibility of an AABAB or AABBB stoichiometry (Rayes *et al.* 2009). As the B subunit can confer spontaneous opening to heteromeric receptors, AABAB or AABBB receptors may be able to convert to the open state upon binding of a single agonist (Bhattacharya *et al.* 2004; Hu & Peoples, 2008). Homomeric receptors, however, require two or three agonist molecules to open, indicating an AAABB stoichiometry may also be possible (Rayes *et al.* 2009). There is also the possibility that different stoichiometries can occur, e.g. $\alpha4\beta2$ nACh receptors can exist as $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ (Nelson *et al.* 2003). Thus the discrepancy between our and the AFM data may purely be that their conditions and tags favoured a 2A:3B ratio, whereas ours favoured a 3A:2B ratio, although our data indicate that a BBABA stoichiometry would be non-functional, and therefore is less physiologically relevant.

In summary, we describe methods for probing the subunits that contribute to binding and activation at heteromeric Cys-loop receptors. The effects of (a) cysteine mutations in the 5-HT$_3$ receptor A subunit, and absence

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of changes in the modified B subunit, (b) MTSEA modification of only A subunits, (c) protection of A subunit residues from MTS-modification, and (d) the presence of disulphide bonds between A+ and A– substituted residues in homomeric and heteromeric receptors, provides compelling evidence that ligands bind to a common A+A– interface in both receptor types, a location supported by their identical competitive pharmacologies.

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Conception and design of the study: A.J.T., K.L.P. and S.C.R.L. Collection and analysis of data: A.J.T. and K.L.P. Interpretation of data: A.J.T., K.L.P. and S.C.R.L. Drafting the article or revising it critically for important intellectual content: A.J.T., K.L.P. and S.C.R.L. Obtaining funding for the study: S.C.R.L. All authors approved the final version of the manuscript.

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