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Novel mechanism of modulation at a ligand-gated ion channel; action of 5-Cl-indole at the 5-HT$_3$A Receptor

Cryptic Orthosteric Modulation of 5-HT$_3$A

Andrew D. Powell[1,2*], Gillian Grafton[1*], Alexander Roberts[1], Shannon Larkin[1], Nathanael O'Neill[1], Josephine Palandri[1], Reka Otvos[3], Alison J Cooper[1], Chris Ulens[4], Nicholas M. Barnes[1,5]

[1]Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT UK, [2]School of Nursing Midwifery and Social Work, Birmingham City University, Westbourne Rd, Edgbaston, B15 3TN UK, [3]Department of Molecular and Cellular Neurobiology, VU University Amsterdam, Amsterdam, The Netherlands [4]Laboratory of Structural Biology, KU Leuven, Leuven, Belgium, [5]Department of Pharmacology and Therapeutics, School of Biomedical Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia

[*]Joint 1st authors

Correspondence: n.m.barnes@bham.ac.uk

Keywords

5-hydroxytryptamine-3 receptor /5-chloro-indole /ago-allosteric modulator /serotonin /pentameric ligand-gated ion channel / cryptic orthosteric modulator (COM)

Abstract

Background and purpose

The 5-HT_3 receptor is a prototypical member of the Cys-loop ligand-gated ion channel (LGIC) superfamily and an established therapeutic target. In addition to activation via the orthosteric site, receptor function can be modulated by allosteric ligands. We have investigated the pharmacological action of Cl-indole upon the 5-HT_3A receptor and identified that this positive allosteric modulator possesses a novel mechanism of action for LGICs.

Experimental approach

The impact of Cl-indole upon the 5-HT_3 receptor was assessed using single cell electrophysiological recordings and [^{3}H]granisetron binding with HEK293 cells stably expressing the 5-HT_3 receptor.

Key results

Cl-indole failed to evoke 5-HT_3A receptor mediated responses (up to 30 μM) or display affinity for the [^{3}H]granisetron binding site. However, in the presence of Cl-indole, termination of 5-HT application revealed tail currents mediated via the 5-HT_3A receptor that were independent of the preceding 5-HT concentration but were antagonised by the 5-HT_3 receptor antagonist, ondansetron. These tail currents were absent in the 5-HT_3AB receptor. Furthermore, the presence of 5-HT revealed a concentration-dependent increase in the affinity of Cl-indole for the orthosteric binding site of the h5-HT_3A receptor.

Conclusions and implications

Cl-indole acts as both an orthosteric agonist and an allosteric modulator but the presence of an orthosteric agonist (e.g. 5-HT) is a prerequisite to reveal both actions. Precedent for ago-allosteric action is available yet the essential additional presence of an orthosteric agonist is now reported for the first time. This widening of the pharmacological mechanisms to modulate LGICs may offer further therapeutic opportunities.

Tables of Links

Targets
5-HT$_3$ Receptors[a]
5-HT$_3$A
5-HT$_3$AB

Ligands
[^{3}H]-granisetron: IUPAC name 1-methyl-N-(9-methyl-9-azabicyclo[3.3.1]nonan-7-yl)indazole-3-carboxamide
5-hydroxytryptamine: IUPAC name 3-(2-Aminoethyl)-1H-indol-5-ol
5-methylindole: IUPAC name 5-methyl-1H-indole. Not found in ligand database
5-hydroxyindole: IUPAC name 1H-Indol-5-ol
5-chloroindole: IUPAC name 5-chloro-1H-indole. Not found in ligand database
Ondansetron: IUPAC name 9-methyl-3-[(2-methylimidazol-1-yl)methyl]-2,3-dihydro-1H-carbazol-4-one
Phenylbiguanide: IUPAC name 1-(diaminomethylidene)-2-phenylguanidine

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to Pharmacology (Southan et al., 2016), and are permanently archived in The Concise Guide to Pharmacology 2015/16 ([a]Alexander et al., 2015)

Abbreviations

Cl-indole: 5-chloroindole; LGIC: ligand-gated ion channel; nAChR: nicotinic acetylcholine receptor; COM: cryptic orthosteric modulator; 5-HT: 5-hydroxytryptamine; 5OHI: 5-hydroxyindole; 5MeI: 5-methoxyindole

Introduction

The 5-HT_3 receptor is an excitatory ligand-gated ion channel (LGIC) of the Cys-loop family that includes the nicotinic acetylcholine receptors (nAChR), glycine and $GABA_A$ receptors, and Zn^{2+}-activated receptors (Barnes *et al.*, 2009; daCosta *et al.*, 2013). Members of this LGIC family share a common pentameric structure, an observation recently confirmed by the publication of the X-ray structure of the mouse 5-HT_3A receptor (Hassaine et al., 2014). Each subunit of the 5-HT_3 receptor is composed of an extracellular N-terminal domain, four transmembrane domains and a short extracellular C-terminal domain. The orthosteric binding site is located at the interface between two adjacent N-terminal domains (daCosta *et al.*, 2013).

Five human 5-HT_3 receptor subunits have been identified (5-HT3A to 5-HT3E) (Karnovsky *et al.*, 2003; Niesler *et al.*, 2003). The native receptor can exist as a simple homopentamer of 5-HT3A subunits or heteropentamers containing the 5-HT3A subunit and at least one other of the 5-HT3B to E subunits (Niesler, 2011). The pentameric structure gives rise to a potential for five ligand binding sites but recent evidence suggests that just two or three sites are occupied for maximal activation of the channel (Corradi et al., 2009). Studies using heteromeric 5-HT_3AB receptors suggest that orthosteric binding sites are only formed between adjacent 5-HT3A subunits (Lochner *et al.*, 2010; Thompson *et al.*, 2011).

As well as orthosteric agonism, allosteric modulation of the 5-HT_3 receptor has been reported by compounds as diverse as alcohols (Bentley *et al.*, 1998; Downie *et al.*, 1995; Stevens *et al.*, 2005), cannabinoids (Barann et al., 2002), indole derivatives (Newman *et al.*, 2013; Parker *et al.*, 1996; Van Hooft *et al.*, 1997), terpenes (Lansdell *et al.*, 2015; Ziemba *et al.*, 2015), benzamides (Jørgensen *et al.*, 2011) and the class of novel negative allosteric modulators typified by PU02 (Trattnig et al., 2012). A number of these modulators appear to have their effect via a site (or sites) in the transmembrane domain (e.g. for the indole derivatives (Hu *et al.*, 2008)). Allosteric agonism by phenolic monoterpenes has also been reported recently again via an allosteric transmembrane site (Lansdell et al., 2015). An additional potential level of

allosteric modulation has been demonstrated since mutation of the intracellular portal region of the receptor affected gating and co-operativity (e.g. (Kozuska *et al.*, 2014)). This raises the possibility of receptor interactions with intracellular proteins leading to allosteric modulation of receptor function.

Whilst the majority of these allosteric effects are mediated by discrete, non-orthosteric sites, it is becoming apparent that some indole derivatives have a more intriguing and mixed mode of action. In particular, they may have mixed effects depending on their concentration and structure. For instance, the non-selective positive allosteric modulator, 5-hydroxy-indole (5OHI) displays positive allosteric modulation via a transmembrane domain of mouse 5-HT_3A receptors (Hu *et al.*, 2008), and can also display orthosteric agonism and allosteric inverse agonism at a spontaneously active mutated mouse 5-HT_3A receptor. These differing actions were dependent only on concentration. The closely related molecule, 5-methoxy-indole (5MeI) displayed both allosteric agonism and orthosteric inverse agonism at the same receptor, again dependent on concentration (Hu, 2015). Importantly the presence of the orthosteric ligand was not required for these actions.

We have recently described a selective allosteric modulator of the 5-HT_3 receptor, 5-chloro-indole (Cl-indole; (Newman *et al.*, 2013). Our study suggested that Cl-indole slows the decay of 5-HT_3A receptor currents and that it can reactivate desensitised 5-HT_3A receptors in the continued presence of 5-HT (Newman et al., 2013). In the light of the mixed modes of action described for indole derivatives, we undertook a study to further explore the mode of action of Cl-indole on the modulation of the 5-HT_3 receptor. We identified for the first time within the LGIC superfamily, an ago-allosteric modulation in which both agonism and allosteric modulation require the presence of the orthosteric ligand.

Materials and Methods

Nomenclature

The molecular target nomenclature conforms to the British Journal of Pharmacology's Concise Guide to Pharmacology (Alexander *et al.*, 2015).

Cell Culture

HEK293 cells stably expressing the human 5-HT3A subunit (HEK293-5-HT3A cells (Brady et al., 2001)) or the heteromeric 5-HT3A/5-HT3B receptor (HEK293-5-HT3A/3B cells) (Dubin *et al.*, 1999) were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) penicillin/streptomycin (10,000 U ml^{-1} penicillin and 10 mg ml^{-1} streptomycin) and G418 (250 µg ml^{-1}) or G418 plus zeocin (250 µg ml^{-1} each, HEK293-5-HT3A/3B cells) and maintained at 37°C, 5% CO_2, 95% air at 95% relative humidity.

Radioligand binding

Radioligand binding assays were performed similar to our previous studies (*e.g.* (Monk et al., 2004)). Briefly, cells were homogenised (Polytron) and resuspended in Tris buffer (in mM; Tris 25, pH 7.4 adjusted with NaOH). Radioligand binding assays were performed in triplicate; binding tubes contained 500 µl of competing drug(s) or vehicle (Tris buffer), and 100 µl of [^{3}H]-granisetron (~1 nM); ~3 TBqmmol^{-1}; Perkin-Elmer). An aliquot (100 µl) of the cell homogenate was added to initiate binding, which was allowed to proceed at room temperature for 60 min before termination by rapid filtration and washing with Tris buffer under vacuum through Whatman GF/B filters, followed by assay of the radioactivity remaining on the filters.

5HT-Binding Protein

The A1B2D1$_W$ 5HT-binding protein was produced and binding experiments with the protein were performed exactly as described by (Kesters *et al.*, 2013). Radioactivity was determined using a Top Count Scintillation and Luminescence reader (Packard).

Single cell electrophysiology

Approximately 18h prior to electrophysiology assays, cells were seeded directly onto 13 mm diameter glass coverslips coated with poly-L-lysine and fibronectin, at a density of 2 x 10^4 cells per coverslip. Macroscopic currents were recorded in the whole-cell recording mode of the patch-clamp technique from HEK293-5-HT3A cells or HEK293-5-HT3A/3B cells cultured on coverslips using an inverted microscope. Cells were superfused at ~4 ml min^{-1} with an extracellular solution (in mM; NaCl 140, KCl 2.8, $CaCl_2$ 1.0, glucose 10, HEPES 10, pH 7.4 adjusted with NaOH). Patch electrodes were pulled from borosilicate glass (O.D. 1.2 mm, I.D. 0.69 mm; Harvard Apparatus, Edenbridge, UK) using a P-97 puller (Sutter, Novato, CA) and filled with intracellular solution consisting of (in mM) 135 CsCl, 2 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Mg-ATP and 0.3 Na-GTP; pH adjusted to 7.3 with KOH (osmolarity ~285 mOsm). Patch electrodes typically had open tip resistances of 4-7 MΩ. Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Wokingham, UK), low-pass Bessel filtered at 1 kHz (NL-125, Digitimer Ltd, Welwyn Garden City, UK) and digitized at 10 kHz by a digidata 1302 (Molecular Devices). Experiments were performed at room temperature with the cells voltage-clamped at -60 mV.

Agonist-evoked currents were elicited by pressure ejection (20-kPa; Picospritzer II; General Valve, Fairfield, NJ) of agonist (5-HT 1.0-10 µM) from modified patch pipettes placed ~30 µm from the recorded cell.

Data and Statistical Analysis

Values are expressed as mean±SEM. Curve fitting and data analysis were performed in Origin 8 (Silverdale Scientific, Stoke Mandeville, UK). Experimenters were not blinded to the experimental conditions. Experiments were randomised where appropriate (eg for the paired pulse experiments, interpulse interval was

randomised using a latin square design). ANOVA with subsequent unpaired t-tests (with Bonferonni correction for multiple comparisons) were used to evaluate differences; a Shapiro-Wilk tested normality. Post-hoc tests were only conducted if F was <0.05; the variance of the data was homogenous. Significance criterion was $p<0.05$.

Data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

Cl-Indole modulates 5-HT-induced responses but is not an agonist

A brief application of Cl-indole (30 μM, 100 ms) did not evoke a current in HEK293 cells expressing the human 5-HT_3A receptor (Figure 1) whereas bath application of 5-HT (10 μM) produced a large inward current. As the 5-HT-induced current began to decay, a second brief application of Cl-indole (30 μM, 100 ms) reactivated the current (Figure 1). These data demonstrate the Cl-indole is not an agonist in its own right, but in the presence of 5-HT, it is capable of modulation of the 5-HT3A receptor. The reactivation of the current suggested that Cl-indole might act via an effect on either desensitisation or resensitisation of the receptor.

Cl-Indole does not modulate 5-HT_3A receptor desensitisation

In order to establish if Cl-indole modulates receptor desensitisation, we tested the effect of Cl-indole on the rate of receptor desensitisation evoked by prolonged receptor activation. Twenty second applications of 5-HT (10 μM) produced large currents (1.53±0.25 nA, n=11) that displayed profound desensitisation (12.4±3.23% of peak; Figure 2Ai). The current decay was best fit with a double exponential function, with time constants of 1.02±0.09 s and 9.79±1.09 s (Figure 2Bi & Bii). Superfusion of Cl-indole (10 μM) did not alter the amplitude of 5-HT induced currents (1.37±0.27 nA, n=11, p=0.54); nor did it alter the degree (13.9±3.9 % of peak, p=0.79) or rate (τ_1 1.22±0.20 s, p=0.31; τ_2 7.78±0.41 s, p=0.21) of receptor desensitisation (Figure 2Biii). These data demonstrate that Cl-indole does not modulate 5-HT_3A receptor desensitisation.

Cl-indole does not modulate 5-HT_3A receptor resensitisation

We previously demonstrated that when 5-HT_3A receptors were completely desensitised by prolonged application of 5-HT, subsequent application of Cl-indole generated an inward current indicative of Cl-indole being able to reactivate desensitised receptors (Newman et al., 2013) (see also Figure 1). We therefore

tested whether Cl-indole could modulate receptor resensitisation using a dual pulse protocol; a single prolonged application of 5-HT (10 μM, 20 s) induced receptor desensitisation (Figure 3A). After removal of the 5-HT, a second test pulse of 5-HT (10 μM, 1 s) was applied with a delay between 5 s and 2 minutes. Recovery from desensitisation was estimated by the fractional recovery of the current elicited by the second 5-HT pulse and was plotted against the inter-pulse interval. Cl-indole did not modulate the rate of receptor resensitisation (control τ_r=18.4±2.0 s; Cl-indole τ_r 17.7±3.1 s; n=5, p=0.8; Figure 3B).

Cl-indole induces tail currents

In the absence of Cl-indole, removal of 5-HT resulted in the evoked currents decaying back to the resting holding current level (see Figure 3A and 4A, *black traces*). In contrast, removal of 5-HT in the continued presence of Cl-indole resulted in a relatively large, slowly developing inward tail current which peaked at 158.3±30.1 pA (13.3±1.8%) of the 5-HT-induced current amplitude (Figure 4A, red trace; n=13; see also Figure 3A, red trace). The rise time of the tail current was best fit by a single time constant (τ 2.77±0.35 s) and the tail current decayed slowly back to baseline (time to reach 50% level 11.06±1.7 s). The effect of Cl-indole on the presence of a tail current was completely reversed upon wash (Figure 4A, *grey trace)*. As the tail current arose immediately after the removal of the 5-HT, we examined if the tail current amplitude was correlated with the current remaining at the end of the agonist application; we also used lower concentrations of 5-HT (1 - 3 μM and 1 μM) as these lower 5-HT concentrations evoked currents with reduced desensitisation (Figure 4B). The tail current amplitude did not correlate with the amount of current remaining (Figure 4Bi, n=27, ANOVA p=0.32). In contrast, the amplitude of the tail current correlated positively with the 5-HT_3A receptor current density (Figure 4Bii, n=13, ANOVA p<0.001).

In order to further explore the origin of the tail current, we examined the current-voltage relationship of the tail current. Similar to the IV curve for the 5-HT_3A receptor current, the IV curve for the tail current reversed at approximately zero mV (Figure 5). These data suggest that the tail current arises from activation of 5-HT_3A

channels (see also figure 7), and that channel opens in a manner consistent with orthosteric receptor activation.

The tail current recorded in the presence of Cl-indole was qualitatively similar to tail currents that arise following relief from open channel block (Rossokhin et al., 2014). Given that 5-HT is known to induce channel block at concentrations above 5 µM (Corradi et al., 2009), we explored further if Cl-indole could induce tail currents after application of lower concentrations of 5-HT. Cl-indole did not potentiate 3 µM 5-HT-induced currents (Control = 0.73±0.11 nA; Cl-indole = 0.68±0.17 nA; n=6, p=0.91), yet the presence of Cl-indole induced a tail current upon removal of the lower concentration of 5-HT (Figure 6). Following removal of 5-HT at 3 µM, the tail current peaked at 135±29.8 pA (18.7±3.3 % of the agonist induced current; n=6, p=0.15), with a similar decay time (8.54±1.1 s; p=0.65). The rise time in the tail current (5.42±0.78 s) following removal of 5-HT at 3 µM was significantly slower than for tail currents evoked following removal of 10 µM 5-HT (p=0.01, Figure 6).

Cl-indole (10 µM) potentiated 1.0 µM 5-HT-induced currents (Control = 149.0±50.7 pA; Cl-indole = 473.5±146.0 pA; n=9, p=0.03) but not currents arising from higher concentrations of 5-HT. In the presence of Cl-indole (10 µM), tail currents were also observed following removal of 5-HT at 1 µM; the peak amplitude of the tail current was 197.5±50.6 pA, the rise time was significantly slower (7.37±0.81 s; p<0.001) and the decay time was unaltered (13.73±1.0 s; n=9, p=0.26; Figure 6). Although the peak amplitude of the tail current evoked by removal of 1 µM 5-HT did not significantly differ from those evident following removal of higher 5-HT concentrations, the Cl-indole-induced tail currents evoked following removal of 1 µM 5-HT were significantly larger *relative* to the 5-HT-induced peak current (108.2±37.3 %; n=9, p=0.003).

Does Cl-indole function as an ago-allosteric modulator?

The homomeric 5-HT_3A receptor has 5 potential orthosteric binding sites, but various studies have shown that even when fully activated only 3 molecules of 5-HT are bound per receptor leaving unbound orthosteric sites (Corradi *et al.*, 2009; Mott *et al.*, 2001; Solt *et al.*, 2007). Given the structural similarity between Cl-indole and the

natural ligand 5-HT, we hypothesised that Cl-indole may function as an ago-allosteric modulator, a mode of action which has previously been described in members of the GPCR superfamily (Schwartz *et al.*, 2006). We tested this in two ways: firstly, we examined if the competitive antagonist, ondansetron, which interacts with the orthosteric site, could block the Cl-indole-induced tail current. Secondly, we examined if 5-HT could modulate the ability of Cl-indole to interact with the orthosteric site in radioligand binding studies.

In the continual presence of Cl-indole, ondansetron (10 µM) was applied via a second picospritz pipette one second prior to the withdrawal of the 5-HT. In the absence of ondansetron, tail current amplitude was 250±18 pA, whereas effectively no tail current was observed in the presence of ondansetron (11±3 pA; n= 5, $p<0.001$ Figure 7A). In a parallel set of experiments, we tested the effect of ondansetron (10 µM) applied after development of the tail current. Ondansetron applied at the peak of the tail current immediately reduced the current back to baseline levels (Figure 7B, n=5). The duration of the tail current (in the order of seconds) would suggest that it is occurring without the presence of the orthosteric ligand (which would be washed away). As ondansetron copmpetively binds at the orthosteric site (Duffy *et al.*, 2012) this strongly argues that Cl-indole interacts with the orthosteric site.
These data demonstrate that antagonism of the orthosteric site prevents Cl-indole-evoked tail currents.

We have previously demonstrated that Cl-indole (in the absence of 5-HT) at concentrations up to 100 µM does not compete with [^{3}H]-granisetron binding at the 5-HT$_3$A receptor (Newman et al., 2013), which was confirmed in the present study (Figure 8). However, in the presence of sub-maximal concentrations of 5-HT, Cl-indole competed for [^{3}H]-granisetron binding sites, with the affinity of Cl-indole increasing for the [^{3}H]-granisetron-labelled orthosteric site of the 5-HT$_3$A receptor binding site with increasing concentrations of 5-HT (Figure 8, Table 1). In contrast, parallel experiments demonstrated that sub-maximal concentrations of 5-HT failed to modify the affinity of the partial agonist phenylbiguanide (Figure 8, Table 1).

In complementary experiments we tested the ability of Cl-indole to bind to the 5-HT binding protein. This protein, formed by mutation of the acetylcholine binding protein,

is able to bind 5-HT albeit with reduced affinity compared to the native 5-HT_3 receptor (Kesters *et al.*, 2013). At higher concentrations, Cl-indole competed with [^{3}H]-granisetron but did not show the same increase in affinity in the presence of 5-HT as for the homomeric receptor (supplementary data figure 1), which suggests the 5-HTBP does not fully replicate the interaction of Cl-indole with the h5-HT_3A receptor.

Cl-indole does not induce tail currents in the heteromeric 5-HT_3AB receptor

In contrast to the 5-HT_3A receptor, the heteromeric 5-HT_3AB receptor has been reported to have only one orthosteric site, formed at the interface of two A subunits (Miles *et al.*, 2013). Since the action of Cl-indole requires the presence of the orthosteric agonist, we hypothesised that the agonist activity of Cl-indole would not be apparent at the heteromeric receptor. Unlike the homomeric receptor, the presence of Cl-indole (10 μM) did not induce a tail current after removal of 5-HT; it did however potentiate the peak current amplitude (control 786.7±108.3 pA, Cl-indole 1111.9±192.5 pA, n=6, p=0.03, Figure 9B). As subtle differences exist in the pharmacology of the heteromeric receptor, we tested if a higher concentration of Cl-indole (30 μM) would induce tail currents. Cl-indole did not induce a tail current but did again potentiate the peak current amplitude (control 585.1±56.2 pA, Cl-indole 984.4±136.5 pA, n=5, p=0.02, Figure 9A, B). Consistent with the lack of tail current in the heteromeric receptor, the presence of submaximal concentrations of 5-HT did not modulate the affinity of Cl-indole binding at the 5-HT_3AB receptor (Table 2).

Discussion

Our previous studies demonstrated that Cl-indole was a potent and selective positive allosteric modulator of the 5-HT_3 receptor (Newman et al., 2013). Cl-indole slowed the rate at which 5-HT_3A receptor-mediated currents decayed back to baseline following a brief application of 5-HT and appeared able to reactivate desensitised receptors (Newman et al., 2013). In the present study we demonstrated that Cl-indole alone fails to activate the 5-HT_3A receptor, although the presence of 5-HT reveals an action of Cl-indole once desensitisation had commenced (Figure 1). Based on the present and our previous studies, we therefore hypothesised that Cl-indole may modulate the desensitised state of the receptor. In the present studies the mechanism of action of Cl-indole was explored more fully. We used prolonged applications of the endogenous orthosteric agonist, 5-HT, to induce robust desensitisation of the 5-HT_3A receptor but Cl-indole did not alter either the rate or magnitude of receptor desensitisation (Figure 2Biii) or the rate at which 5-HT_3A receptors recovered following desensitisation (Figure 3). Given that some other allosteric modulators of 5-HT_3A receptors have a profound effect on desensitisation rate (Deiml *et al.*, 2004; Kooyman *et al.*, 1993; Van Hooft *et al.*, 1997), our data suggest that Cl-indole functions via an alternate mechanism.

A surprising observation from the desensitisation studies was that the presence of Cl-indole revealed tail currents following removal of the 5-HT. Our unpublished data show that mutation of the 5OHI binding site in the human 5-HT_3A receptor used in this study, had no effect on the ability of Cl-indole to modulate the 5-HT response. A tail current has previously been described following the treatment of mouse 5-HT_3A receptors with 5OHI (Kooyman *et al.*, 1993). High concentrations of 5OHI (10 to 50 mM) evoked a tail current superficially similar to that evoked by Cl-indole in our study. However unlike Cl-indole, high concentrations of 5OHI inhibited the 5-HT response and the tail currents could be ascribed to relief of 5OHI-mediated block. Similarly, 10 mM trichloroethanol also induced a tail current at mouse 5-HT_3A receptors, which was ascribed to relief from a blocked state (Zhou *et al.*, 1996). Indeed, tail currents are most commonly ascribed to the relief from non-competitive open channel block. For example, penicillin is an open-channel blocker at the $GABA_A$ receptor, where it binds within the pore and locks the receptor into an open but blocked conformation (Rossokhin et al., 2014). Removal of penicillin generates

large tail-currents as receptors transition from an open-blocked to an open, ion-permeant state (Rossokhin et al., 2014). Open channel block of the 5-HT_3A receptor by 5-HT has been described by Hapfelmeier *et al* (Hapfelmeier et al., 2003) in macroscopic currents and at the single channel level by Corradi *et al* (Corradi et al., 2009). It has been proposed that 5-HT can occupy a non-agonist binding site and block conductance through the receptor in an open-channel like manner. It is therefore possible that Cl-indole may enhance 5-HT-mediated open-channel block, thereby causing a larger proportion of receptors to exist in a blocked state during continued agonist exposure. Following agonist removal, this population of receptors would transition from blocked to the open-conductive state and produce the observed tail currents. However, our data indicates that Cl-indole does not function in this manner. Previous reports of 5-HT-mediated channel block have shown that it is only evident at concentrations exceeding 5 μM (Corradi *et al.*, 2009; Hapfelmeier *et al.*, 2003). In the present studies tail currents were associated with lower concentrations of 5-HT, the amplitude of tail currents was not altered across the range of 5-HT concentrations investigated (1-10 μM), and tail currents were not observed in the absence of Cl-indole. Taken together, these data indicate that Cl-indole-induced tail currents do not arise from promotion of channel block.

We propose that Cl-indole potentiates 5-HT mediated responses by a novel mechanism where in the presence of the orthosteric ligand (5-HT), Cl-indole can bind to orthosteric sites leading to increased receptor activity. Several observations support this hypothesis: (1) the concentration-dependent presence of 5-HT reveals an ability of Cl-indole to compete for the [^{3}H]granisetron-labelled orthosteric binding, (2) the orthosteric 5-HT_3 receptor antagonist, ondansetron, effectively blocks the Cl-indole-dependent tail current and (3) the IV curve of the tail current is indistinguishable from that for the 5-HT_3A receptor. Additional support is lent by the observation that in a heteromeric 5-HT_3AB receptor with potentially only one available orthosteric site, no tail current was observed. Our interpretation is therefore that Cl-indole acts as both a positive allosteric modulator and an orthosteric agonist requiring the presence of an orthosteric ligand such as 5-HT.

Ago-allosteric modulators have been defined as ligands that can function both as an agonist in their own right and as an allosteric modulator of the orthosteric ligand (Schwartz *et al.*, 2007). This class of modulators has been well described for GPCRs

(Smith *et al.*, 2011a; Smith *et al.*, 2011b; Yamazaki *et al.*, 2012) but has also been described for cys-loop LGICs. Thus phenobarbitol, 4BP-TQS and thymol and carvacrol have all been described as ago-allosteric modulators for $GABA_A$, nicotinic $\alpha7$ and 5-HT_3A receptors, respectively (Amin *et al.*, 1993; Gill *et al.*, 2011; Lansdell *et al.*, 2015). However, all these modulators act as allosteric agonists in the absence of the orthosteric ligand. Interestingly, 4BP-TQS acts via a site in the TM2 domain of the nicotinic $\alpha7$ receptor (Gill et al., 2011), while thymol and carvacrol display agonism via a site in TM1 of the 5-HT_3A receptor (Lansdell et al., 2005).

The proposed mechanism of action of Cl-Indole shows some similarity to compounds that modulate nAChRs by acting through 'non-canonical' extracellular agonist binding sites (Short *et al.*, 2015) or as 'accessory site-selective agonists' (Wang *et al.*, 2015). These compounds act at heteromeric receptors with the mechanism of action explained by compounds interacting with sites distinct from the conventional orthosteric site. Importantly, this study examined the effect of Cl-Indole on homomeric 5-HT_3A receptors, which are expected to have five identical agonist binding sites.

Therefore Cl-indole fundamentally differs from previously described modulators by its agonist action at the orthosteric site, as well as its allosteric action, both of which require the additional presence of an orthosteric ligand such as 5-HT. Since the action of Cl-indole is hidden, requiring the presence of an orthosteric agonist to be revealed, we propose that it forms a new class of action that we term cryptic orthosteric modulation (COM).

The 5-HT_3A receptor has five orthosteric sites (Barnes et al., 2009) of which two to three are believed to be occupied for maximal receptor activation (Corradi et al., 2009). The question therefore arises whether Cl-indole binds to unoccupied orthosteric sites or does it compete with 5-HT for occupied sites? Both in this study and previously (Newman *et al.*, 2013) we have shown that Cl-indole potentiates responses induced by sub-maximal but not maximal 5-HT concentrations. Studies investigating receptor stoichiometry using nAChR suggest that agonist binding to a single binding site within the LGIC complex can activate the channel (Andersen *et al.*, 2013; Rayes *et al.*, 2009). As the number of binding sites engaged increases, the channel can become maximally activated (Corradi et al., 2009). Therefore at low

agonist concentrations it would be predicted that relatively few binding sites are occupied and the current amplitude is consequently lower. Cl-indole can then interact with the free orthosteric sites to potentiate the response; this action is only revealed by the presence of 5-HT. In contrast at higher 5-HT concentrations, multiple orthosteric binding sites are occupied, effectively blocking Cl-indole access to the orthosteric site. As a result Cl-indole cannot potentiate the response. Once the 5-HT molecules begin to dissociate, the orthosteric binding sites become sequentially available, allowing Cl-indole to bind and re-activate the channel, thus generating the evident tail current. This proposes that Cl-indole can only interact with unoccupied orthosteric sites. Our previous study demonstrated that when Cl-indole was applied to desensitised receptors, a current was evoked (Newman *et al.*, 2013), which in the light of the present findings suggests that this current may have resulted from generation of a tail current.

The heteromeric 5-HT_3AB receptor has been reported to have the subunit order of A-A-B-A-B ((Miles *et al.*, 2013) Since the orthosteric site is formed from an A-A interface, this stoichiometry suggests the presence of just a single orthosteric site in the heteromeric 5-HT_3AB receptor. Our hypothesis is that the tail current is due to agonism of the receptor via the orthosteric site in the presence of the orthosteric ligand. This is supported by the absence of a tail current and lack of Cl-indole competition in the presence of 5-HT for the heteromeric 5-HT_3AB receptor. Interestingly, the peak 5-HT_3AB heteromeric receptor current was potentiated by Cl-indole. We interpret this to mean that Cl-indole may have an allosteric action via the A-B and B-A interfaces. The potential for allosteric action through an A-B or B-A site is supported by Miles et al (Miles *et al.*, 2015) who showed that mcPBG can be both an agonist and allosteric modulator at the heteromeric receptor. These observations are consistent with our proposal that Cl-indole forms a new class of molecule exhibiting both agonist and allosteric actions requiring the presence of the orthosteric ligand.

The novel mechanism of action of Cl-indole at the 5-HT_3 receptor may offer therapeutic advantages for ligands with this mechanism at LGICs. Thus for instance a potentiation of 5-HT_3 receptor function may benefit patients with constipation-predominant irritable bowel syndrome. Furthermore, if the novel mechanism translates to other members of the LGIC receptor superfamily, then such potentiation

of $GABA_A$ receptors for example may offer benefits for the treatment of a variety of conditions such as anxiety. Alternatively, a potentiation of nicotinic acetylcholine receptors may benefit patients with cognitive decline.

In summary, using the 5-HT_3A receptor as a model, this study identifies a previously unknown mode of activation of LGICs. We demonstrate that Cl-indole represents a modulator requiring the presence of the orthosteric ligand for its action – a Cryptic Orthosteric Modulator. The retained physiological control via the endogenous agonist to reveal both actions may offer therapeutic advantages for drugs targeting LGICs via this mechanism.

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Author Contribution

ADP, GG and NMB designed and analysed experiments. ADP, AR, GG, JP, NO'N, AJC, RO and SL performed experiments. ADP, GG and NMB conceived the study and wrote the manuscript. All authors discussed the results and manuscript.

Conflicts of Interest

None of the authors declare a conflict of interest.

References

Alexander SPH, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E*, et al.* (2015). The Concise Guide to PHARMACOLOGY 2015/16: Overview. *British Journal of Pharmacology* **172**(24)**:** 5729-5743.

Amin J, Weiss DS (1993). GABAA receptor needs two homologous domains of the beta-subunit for activation by GABA but not by pentobarbital. *Nature* **366**(6455)**:** 565-569.

Andersen N, Corradi J, Sine SM, Bouzat C (2013). Stoichiometry for activation of neuronal α7 nicotinic receptors. *Proceedings of the National Academy of Sciences* **110**(51)**:** 20819-20824.

Barann M, Molderings G, Brüss M, Bönisch H, Urban BW, Göthert M (2002). Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulatory site. *British Journal of Pharmacology* **137**(5)**:** 589-596.

Barnes N, Hales T, Lummis S, Peters J (2009). The 5-HT3 receptor-the relationship between structure and function. *Neuropharmacology* **56:** 273 - 284.

Bentley KR, Barnes NM (1998). 5-hydroxytryptamine3 (5-HT3) receptor-mediated depolarisation of the rat isolated vagus nerve: modulation by trichloroethanol and related alcohols. *Eur J Pharmacol* **354**(1)**:** 25-31.

Brady CA, Stanford IM, Ali I, Lin L, Williams JM, E. Dubin A*, et al.* (2001). Pharmacological comparison of human homomeric 5-HT3A receptors versus heteromeric 5-HT3A/3B receptors. *Neuropharmacology* **41**(2)**:** 282-284.

Corradi J, Gumilar F, Bouzat C (2009). Single-Channel Kinetic Analysis for Activation and Desensitization of Homomeric 5-HT3A Receptors. *Biophysical Journal* **97**(5)**:** 1335-1345.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SPA, Giembycz MA*, et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. *British Journal of Pharmacology* **172**(14)**:** 3461-3471.

daCosta Corrie JB, Baenziger John E (2013). Gating of Pentameric Ligand-Gated Ion Channels: Structural Insights and Ambiguities. *Structure* **21**(8)**:** 1271-1283.

Deiml T, Haseneder R, Zieglgänsberger W, Rammes G, Eisensamer B, Rupprecht R*, et al.* (2004). α-Thujone reduces 5-HT3 receptor activity by an effect on the agonist-induced desensitization. *Neuropharmacology* **46**(2)**:** 192-201.

Downie DL, Hope AG, Belelli D, Lambert JJ, Peters JA, Bentley KR*, et al.* (1995). The interaction of trichloroethanol with murine recombinant 5-HT3 receptors. *Br J Pharmacol* **114**(8)**:** 1641-1651.

Dubin AE, Huvar R, D'Andrea MR, Pyati J, Zhu JY, Joy KC*, et al.* (1999). The Pharmacological and Functional Characteristics of the Serotonin 5-HT3A Receptor Are Specifically Modified by a 5-HT3B Receptor Subunit. *Journal of Biological Chemistry* **274**(43)**:** 30799-30810.

Duffy NH, Lester HA, Dougherty DA (2012). Ondansetron and Granisetron Binding Orientation in the 5-HT3 Receptor Determined by Unnatural Amino Acid Mutagenesis. *ACS Chemical Biology* **7**(10)**:** 1738-1745.

Gill JK, Savolainen M, Young GT, Zwart R, Sher E, Millar NS (2011). Agonist activation of α7 nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proceedings of the National Academy of Sciences* **108**(14)**:** 5867-5872.

Hapfelmeier G, Tredt C, Haseneder R, Zieglgänsberger W, Eisensamer B, Rupprecht R*, et al.* (2003). Co-expression of the 5-HT3B Serotonin Receptor Subunit Alters the Biophysics of the 5-HT3 Receptor. *Biophysical Journal* **84**(3)**:** 1720-1733.

Hassaine G, Deluz C, Grasso L, Wyss R, Tol MB, Hovius R*, et al.* (2014). X-ray structure of the mouse serotonin 5-HT3 receptor. *Nature* **512**(7514)**:** 276-281.

Hu X-Q (2015). Auto-inhibition at a ligand-gated ion channel: a cross-talk between orthosteric and allosteric sites. *British Journal of Pharmacology* **172**(1)**:** 93-105.

Hu X-Q, Lovinger DM (2008). The L293 residue in transmembrane domain 2 of the 5-HT3A receptor is a molecular determinant of allosteric modulation by 5-hydroxyindole. *Neuropharmacology* **54**(8)**:** 1153-1165.

Jørgensen CG, Frølund B, Kehler J, Jensen AA (2011). Discovery of Benzamide Analogues as a Novel Class of 5-HT3 Receptor Agonists. *ChemMedChem* **6**(4)**:** 725-736.

Karnovsky AM, Gotow LF, McKinley DD, Piechan JL, Ruble CL, Mills CJ*, et al.* (2003). A cluster of novel serotonin receptor 3-like genes on human chromosome 3. *Gene* **319**(0)**:** 137-148.

Kesters D, Thompson AJ, Brams M, van Elk R, Spurny R, Geitmann M*, et al.* (2013). Structural basis of ligand recognition in 5-HT3 receptors. *EMBO Rep* **14**(1)**:** 49-56.

Kooyman AR, Van Hooft JA, Vijverberg HPM (1993). 5-Hydroxyindole slows desensitization of the 5-HT3 receptor-mediated ion current in N1E-115 neuroblastoma cells. *British Journal of Pharmacology* **108**(2)**:** 287-289.

Kozuska JL, Paulsen IM, Belfield WJ, Martin IL, Cole DJ, Holt A*, et al.* (2014). Impact of intracellular domain flexibility upon properties of activated human 5-HT3 receptors. *British Journal of Pharmacology* **171**(7)**:** 1617-1628.

Lansdell SJ, Gee VJ, Harkness PC, Doward AI, Baker ER, Gibb AJ*, et al.* (2005). RIC-3 Enhances Functional Expression of Multiple Nicotinic Acetylcholine Receptor Subtypes in Mammalian Cells. *Molecular Pharmacology* **68**(5)**:** 1431-1438.

Lansdell SJ, Sathyaprakash C, Doward A, Millar NS (2015). Activation of Human 5-Hydroxytryptamine Type 3 Receptors via an Allosteric Transmembrane Site. *Molecular Pharmacology* **87**(1)**:** 87-95.

Lochner M, Lummis SCR (2010). Agonists and Antagonists Bind to an A-A Interface in the Heteromeric 5-HT3AB Receptor. *Biophysical Journal* **98**(8)**:** 1494-1502.

Miles Timothy F, Dougherty Dennis A, Lester Henry A (2013). The 5-HT3AB Receptor Shows an A3B2 Stoichiometry at the Plasma Membrane. *Biophysical Journal* **105**(4)**:** 887-898.

Miles TF, Lester HA, Dougherty DA (2015). Allosteric activation of the 5-HT3AB receptor by mCPBG. *Neuropharmacology* **91:** 103-108.

Monk SA, Williams JM, Hope AG, Barnes NM (2004). Identification and importance of N-glycosylation of the human 5-hydroxytryptamine3A receptor subunit. *Biochem Pharmacol* **68**(9)**:** 1787-1796.

Mott DD, Erreger K, Banke TG, Traynelis SF (2001). Open probability of homomeric murine 5-HT3A serotonin receptors depends on subunit occupancy. *The Journal of Physiology* **535**(2)**:** 427-443.

Newman AS, Batis N, Grafton G, Caputo F, Brady CA, Lambert JJ*, et al.* (2013). 5-Chloroindole: a potent allosteric modulator of the 5-HT3 receptor. *British Journal of Pharmacology* **169**(6)**:** 1228-1238.

Niesler B (2011). 5-HT(3) receptors: potential of individual isoforms for personalised therapy. *Curr Opin Pharmacol* **11**(1)**:** 81-86.

Niesler B, Frank B, Kapeller J, Rappold GA (2003). Cloning, physical mapping and expression analysis of the human 5-HT3 serotonin receptor-like genes HTR3C, HTR3D and HTR3E. *Gene* **310:** 101-111.

Parker RM, Bentley KR, Barnes NM (1996). Allosteric modulation of 5-HT3 receptors: focus on alcohols and anaesthetic agents. *Trends Pharmacol Sci* **17**(3)**:** 95-99.

Rayes D, De Rosa MJ, Sine SM, Bouzat C (2009). Number and Locations of Agonist Binding Sites Required to Activate Homomeric Cys-Loop Receptors. *The Journal of Neuroscience* **29**(18)**:** 6022-6032.

Rossokhin AV, Sharonova IN, Bukanova JV, Kolbaev SN, Skrebitsky VG (2014). Block of GABAA receptor ion channel by penicillin: Electrophysiological and modeling insights toward the mechanism. *Molecular and Cellular Neuroscience* **63:** 72-82.

Schwartz TW, Holst B (2006). Ago-Allosteric Modulation and Other Types of Allostery in Dimeric 7TM Receptors. *Journal of Receptors and Signal Transduction* **26**(1-2)**:** 107-128.

Schwartz TW, Holst B (2007). Allosteric enhancers, allosteric agonists and ago-allosteric modulators: where do they bind and how do they act? *Trends in pharmacological sciences* **28**(8)**:** 366-373.

Short CA, Cao AT, Wingfield MA, Doers ME, Jobe EM, Wang N*, et al.* (2015). Subunit interfaces contribute differently to activation and allosteric modulation of neuronal nicotinic acetylcholine receptors. *Neuropharmacology* **91:** 157-168.

Smith NJ, Bennett KA, Milligan G (2011a). When simple agonism is not enough: Emerging modalities of GPCR ligands. *Molecular and Cellular Endocrinology* **331**(2)**:** 241-247.

Smith NJ, Ward RJ, Stoddart LA, Hudson BD, Kostenis E, Ulven T*, et al.* (2011b). Extracellular Loop 2 of the Free Fatty Acid Receptor 2 Mediates Allosterism of a Phenylacetamide Ago-Allosteric Modulator. *Molecular Pharmacology* **80**(1)**:** 163-173.

Solt K, Ruesch D, Forman SA, Davies PA, Raines DE (2007). Differential Effects of Serotonin and Dopamine on Human 5-HT3A Receptor Kinetics: Interpretation within an Allosteric Kinetic Model. *The Journal of Neuroscience* **27**(48)**:** 13151-13160.

Stevens R, Rüsch D, Solt K, Raines DE, Davies PA (2005). Modulation of Human 5-Hydroxytryptamine Type 3AB Receptors by Volatile Anesthetics and n-Alcohols. *Journal of Pharmacology and Experimental Therapeutics* **314**(1)**:** 338-345.

Thompson AJ, Price KL, Lummis SCR (2011). Cysteine modification reveals which subunits form the ligand binding site in human heteromeric 5-HT3AB receptors. *The Journal of Physiology* **589**(17)**:** 4243-4257.

Trattnig SM, Harpsøe K, Thygesen SB, Rahr LM, Ahring PK, Balle T*, et al.* (2012). Discovery of a Novel Allosteric Modulator of 5-HT3 Receptors. *Journal of Biological Chemistry* **287**(30)**:** 25241-25254.

Van Hooft JA, Van Der Haar E, Vijverberg HPM (1997). Allosteric Potentiation of the 5-HT3 Receptor-mediated Ion Current in N1E-115 Neuroblastoma Cells by 5-Hydroxyindole and Analogues. *Neuropharmacology* **36**(4–5)**:** 649-653.

Wang J, Kuryatov A, Sriram A, Jin Z, Kamenecka TM, Kenny PJ*, et al.* (2015). An Accessory Agonist Binding Site Promotes Activation of α4β2* Nicotinic Acetylcholine Receptors. *Journal of Biological Chemistry* **290**(22)**:** 13907-13918.

Yamazaki K, Terauchi H, Iida D, Fukumoto H, Suzuki S, Kagaya T*, et al.* (2012). Ago-allosteric modulators of human glucagon-like peptide 2 receptor. *Bioorganic & Medicinal Chemistry Letters* **22**(19)**:** 6126-6135.

Zhou Q, Lovinger DM (1996). Pharmacologic characteristics of potentiation of 5-HT3 receptors by alcohols and diethyl ether in NCB-20 neuroblastoma cells. *J Pharmacol Exp Ther* **278**(2)**:** 732-740.

Ziemba PM, Schreiner BSP, Flegel C, Herbrechter R, Stark TD, Hofmann T*, et al.* (2015). Activation and modulation of recombinantly expressed serotonin receptor type 3A by terpenes and pungent substances. *Biochemical and Biophysical Research Communications* **467**(4)**:** 1090-1096.

[5-HT] nM	IC_{50} (μM)	
	Cl-indole	PBG
0	>100	15±2
10	77±12	14±1
30	49±12	14±1
100	27±6	15±3
200	10±1	10±2

Table 1. Ability of Cl-indole and PBG to compete for [^{3}H]-granisetron binding to the orthosteric site of the 5-HT$_3$A receptor. Data represent IC_{50} values for the competing drugs in the absence or presence of 5-HT (mean±SEM, n=4-8).

[5-HT] (nM)	Cl-indole IC_{50} (μM)
0	144±41
10	306±63
30	198±42
100	222±40
200	255±83
300	210±40

Table 2. Ability of Cl-indole to compete for [^{3}H]-granisetron binding to the orthosteric site of the 5-HT$_3$AB receptor. Data represent IC_{50} values for the competing drugs in the absence or presence of 5-HT (mean±SEM, n=5).

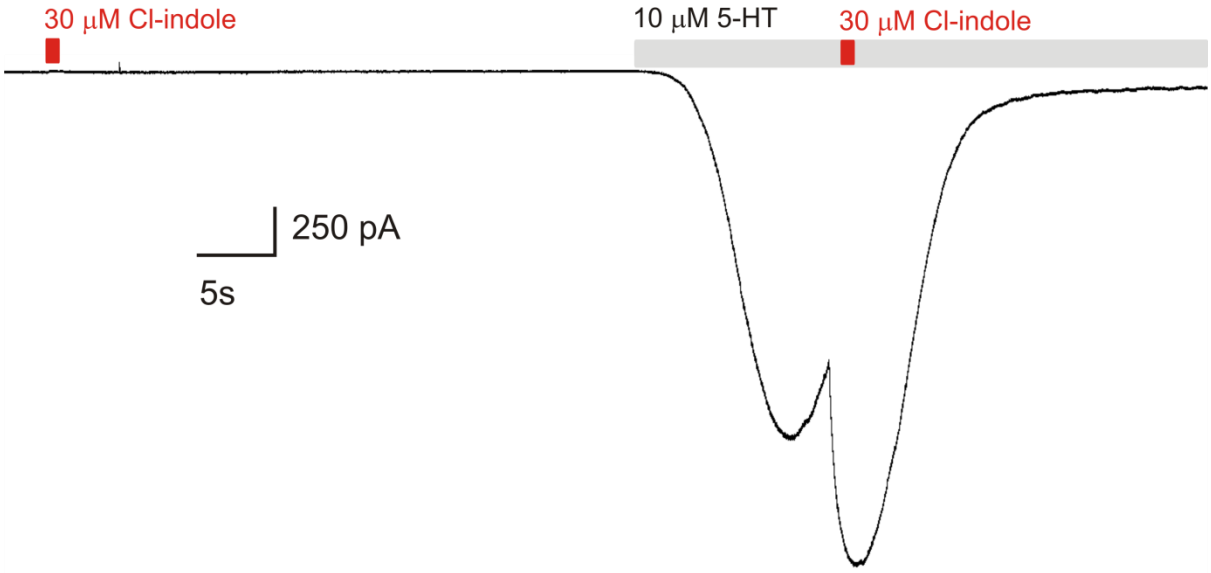

Figure 1. Cl-indole is not a 5-HT$_3$A receptor agonist

Cl-indole (30 μM 100 ms) did not evoke a response. However in the presence of 5-HT (10 μM, bath application), Cl-indole (30 μM 100 ms) provoked an inward current. Representative trace, n=7.

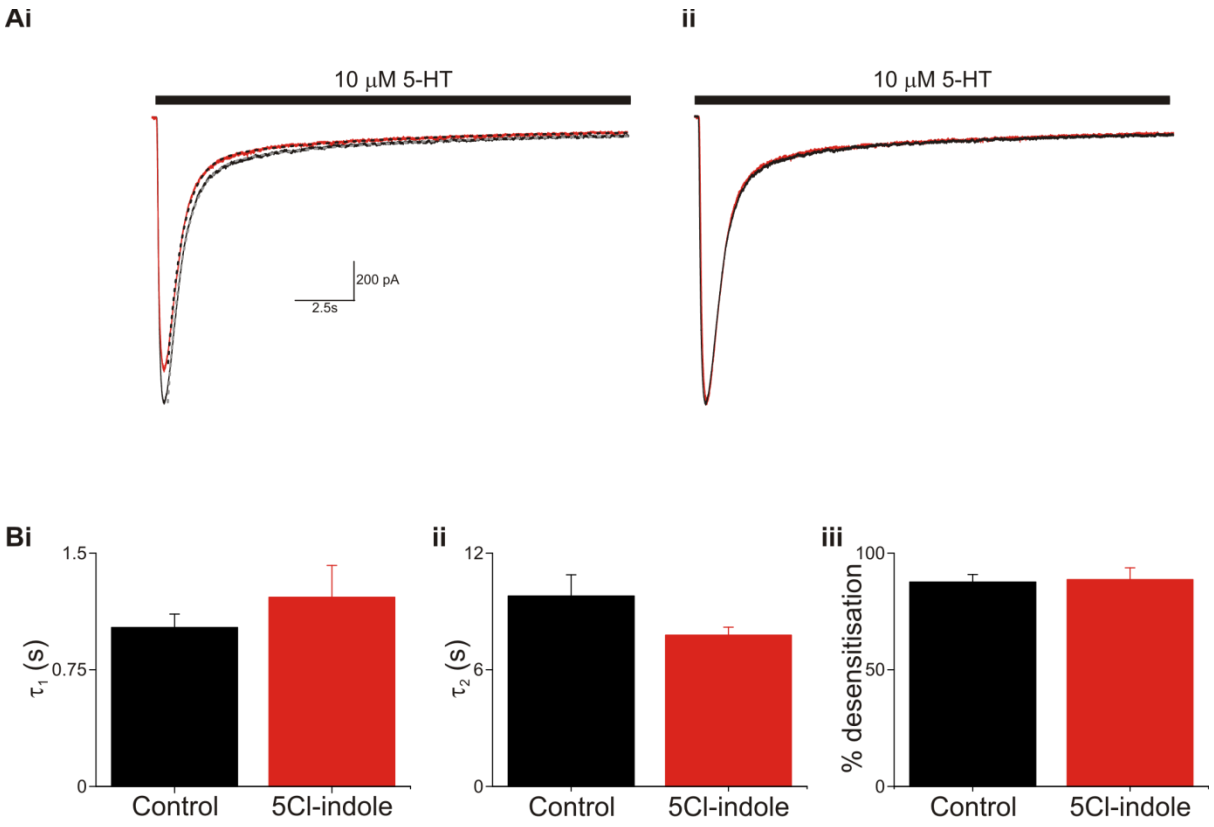

Figure 2. Cl-indole does not modulate 5-HT$_3$A receptor desensitisation (**Ai**); Prolonged application of 5-HT (10 μM, 20 seconds) activated a large 5-HT$_3$A receptor current that displayed pronounced desensitisation under control conditions (black trace); single recording representative of 11 recordings.The magnitude and kinetics of desensitisation of the current evoked by 10 μM 5-HT were unaffected by concomitant bath application of Cl-indole (10 μM) (red trace).(**Aii**) Normalising 5-HT (10 μM) induced current to the peak response highlighted the lack of effect of Cl-indole on receptor desensitisation. (**Bi & ii**) The time constants of the current decay in the absence (black bar) or presence (red bar) of Cl-indole (10 μM). (Biii) Degree of receptor desensitisation in the absence (black bar) or presence (red bar) of Cl-indole (10 μM). Data represent the mean ± SEM, n=11).

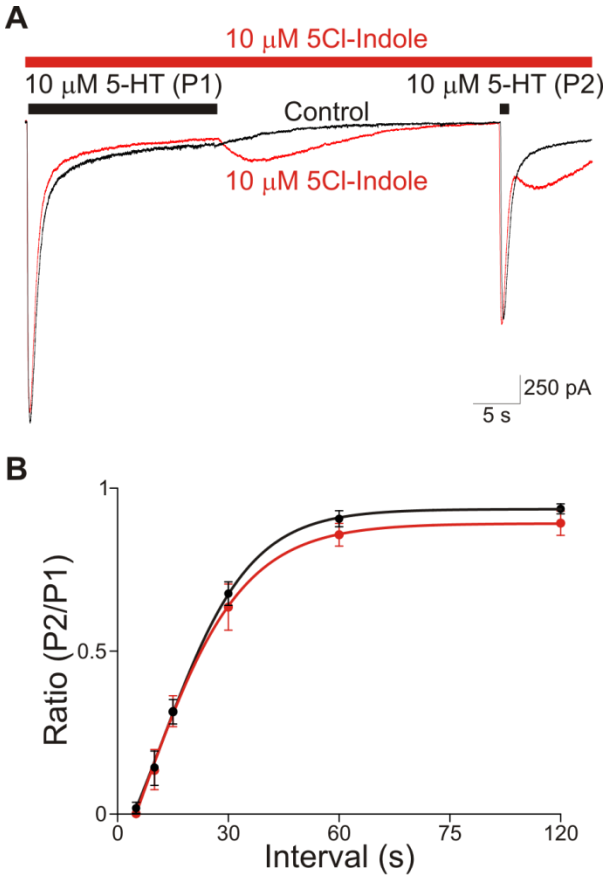

Figure 3. Cl-indole does not modulate 5-HT$_3$A receptor recovery from desensitisation. (A) A single prolonged application (P1) of 5-HT (10 μM, 20 s) induced similar receptor desensitisation in absence (black trace) or the presence of Cl-indole (10 μM, red trace). After removal of the 5-HT, a second test pulse (P2) of 5-HT (10 μM, 1 s) with a delay between 5 s and 2 minutes tested recovery from desensitisation; a single representative trace from 5 recordings. Traces show an example recording with a 30 s interpulse interval. (**B**) Fractional recovery of the current amplitude elicited by the second 5-HT pulse (P2/P1) was plotted against the inter-pulse interval in the absence (black symbols) or presence of Cl-indole (10 μM, red symbols); data represent the mean±SEM, n=5.

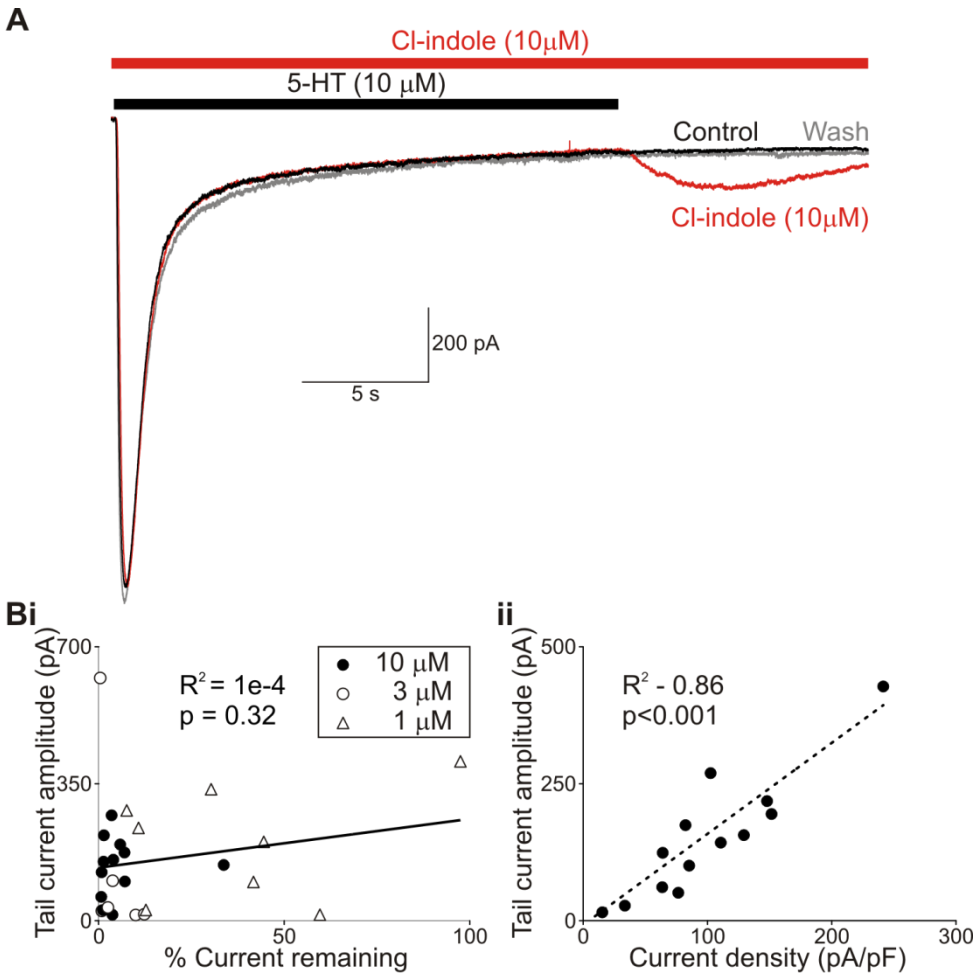

Figure 4. Cl-indole evokes tail currents (**A**) In control conditions, the current decayed back to baseline levels after removal of 5-HT (black trace). In contrast, removal of the agonist in the presence of Cl-indole (red trace) induced a tail current with slow activation and deactivation kinetics. The presence of the tail current was completely reversible upon removal of Cl-indole (grey trace); trace representative of 13 recordings. **(B)** Peak tail current amplitude did not correlate with the degree of receptor desensitation (**Bi**) resulting from a 20s 5-HT application (● - 10 μM, ○ - 3 μM, △ - 1 μM), but correlated with 5-HT-induced current density (**Bii**). Data represent individual recordings.

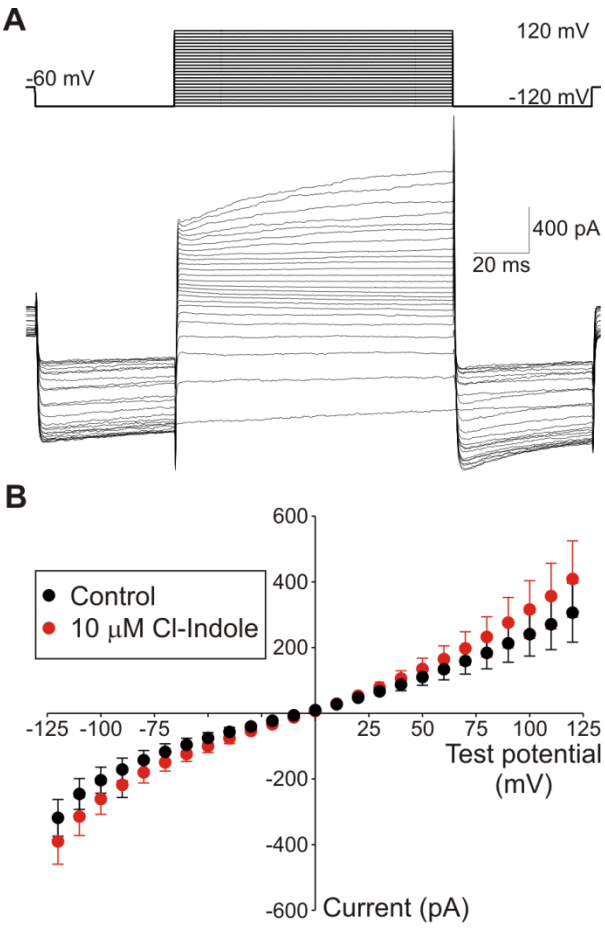

Figure 5. Cl-indole does not modulate the current voltage relationship of the 5-HT$_3$A receptor current. (**A**) IV curves (-120 mV – 120 mV) were constructed at the peak of the Cl-indole-induced tail current. (**B**) The current voltage relationship was unaltered by Cl-indole (10 μM). Data represent the mean±SEM, n=6.

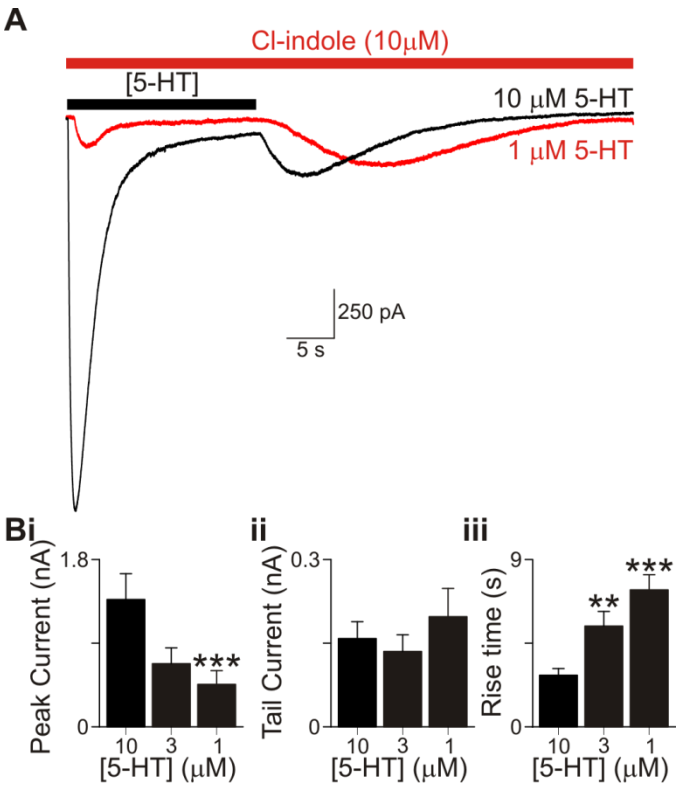

Figure 6. **Cl-indole induced tail current amplitude is independent of 5-HT concentration.**(**A** and **Bi**) In the presence of Cl-indole (10 μM), application of 5-HT evoked a concentration dependent inward current (10 μM – black trace, 1 μM – red trace; trace representative of 9 recordings). (**A** and **Bii**) A similar amplitude tail current was observed at all agonist concentrations. (**Biii**) The rise time of the tail currents was dependent on 5-HT concentration. Bar graphs represent mean+SEM, n=9.

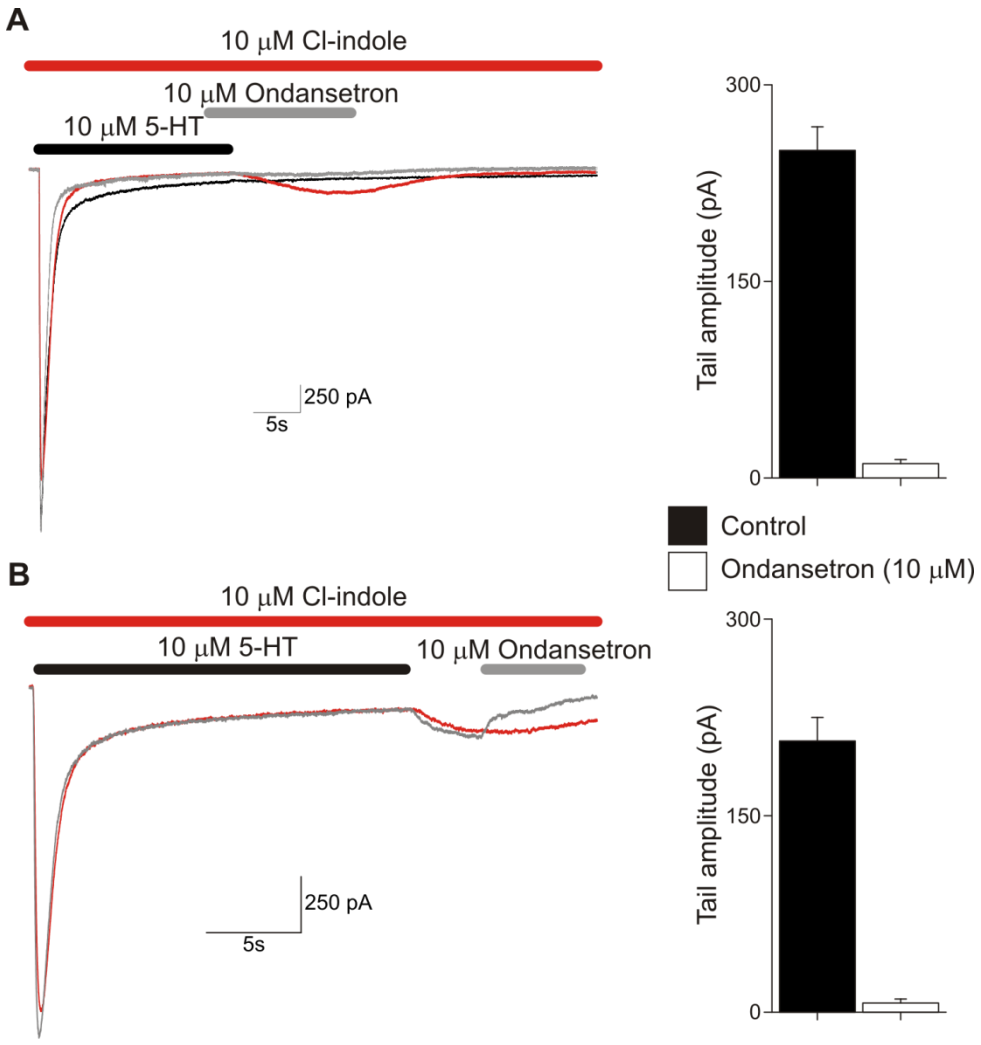

Figure 7. 5-Cl-indole mediated tail currents are blocked by ondansetron. (**A**) Selective orthosteric antagonist, ondansetron (10 μM, 15 s), applied one second prior to removal of 5-HT (10 μM, 20 s), completely blocked tail currents (grey trace), which are usually seen in the presence of 5-Cl-indole (red trace). (**B**) Ondansetron (10 μM, 10 s) applied at the peak of the tail current, completely blocked the tail current (grey trace). Traces representative of 5 recordings. Bar graphs represent mean+SEM, n=5.

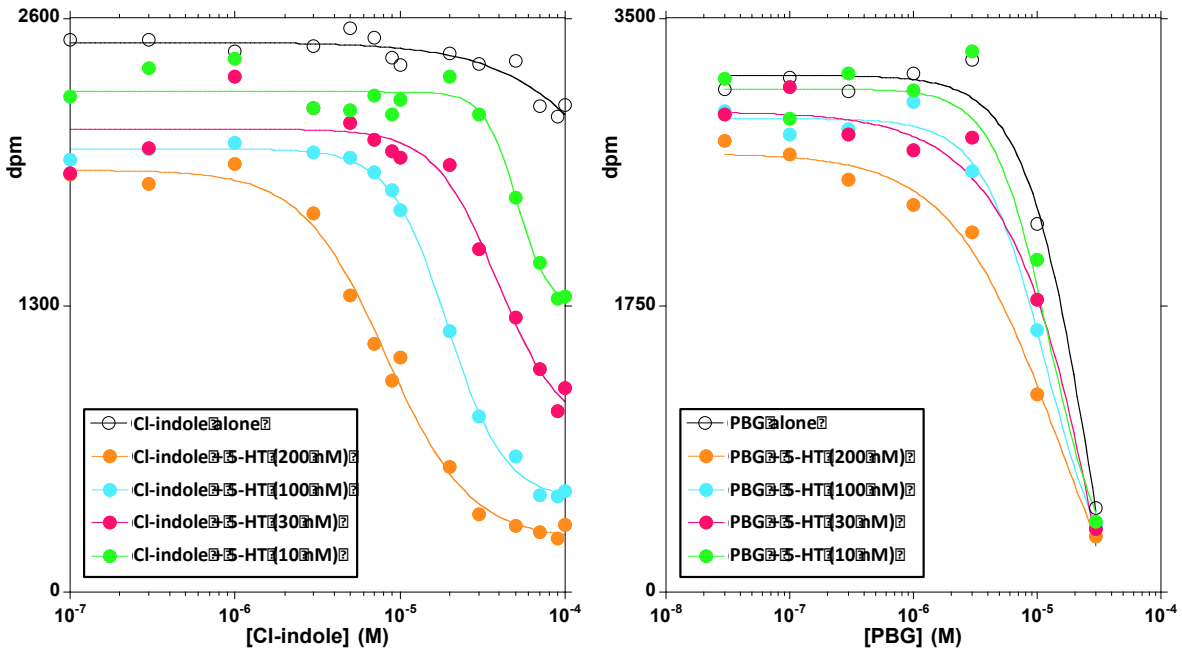

Figure 8. The presence of 5-HT allows Cl-indole to compete for [^{3}H]-granisetron binding to the orthosteric site of the 5-HT$_3$A receptor. Left graph: Cl-indole (up to 100 µM) essentially does not compete for [^{3}H]-granisetron in the absence of 5-HT, but in the presence of 5-HT (10 – 300 nM), there is a concentration-dependent increase in the affinity of Cl-indole for the [^{3}H]-granisetron-labelled orthosteric site. Right graph: the same concentrations of 5-HT fail to alter the affinity of the orthosteric agonist, PBG. Data from a single experiment that was representative of 4-8 independent experiments (see Table 1 for summary of arising data).

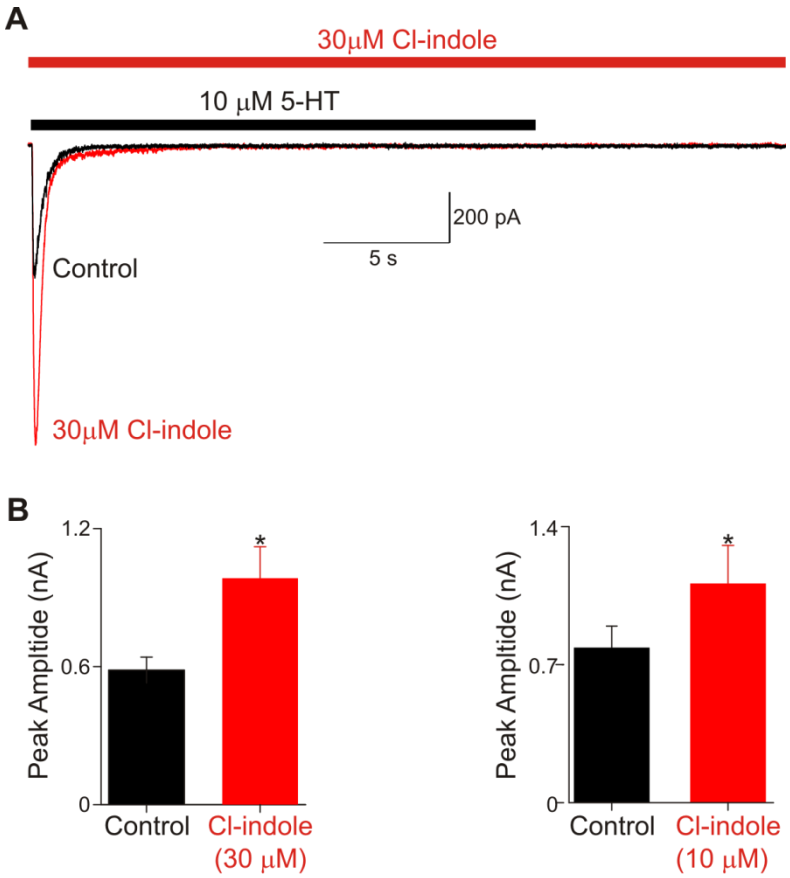

Figure 9. Cl-indole does not induce tail currents in heteromeric 5-HT3AB receptors. (**A**) Pressure ejection of 5-HT (10 μM, 20 s, 20 psi) evoked a rapidly desensitising current in both control (black trace) and in the presence of 30 μM Cl-indole (red trace). Removal of 5-HT did not induce a tail current in either condition. (**B**) Cl-indole (30 μM, left panel, n=5; 10 μM right panel, n=6) potentiated the peak 5-HT current amplitude. Bar graphs represent mean+SEM.