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RESEARCH PAPER

Auto-inhibition at a ligand-gated ion channel: a cross-talk between orthosteric and allosteric sites

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BACKGROUND AND PURPOSE

A ligand is believed to produce either positive or negative responses, or to block both of them. However, an indole compound was found to promote both positive and negative effects at the 5-HT_3AB receptor, which displays a low level of spontaneous activity. The present study attempted to delineate the mechanisms underlying this phenomenon.

EXPERIMENTAL APPROACH

The spontaneously active V291S 5-HT_3A receptor was used to explore the properties of 5-hydroxyindole (5-HoI) and 5-methoxyindole (5-MoI), structural analogues of 5-HT, either alone or in combination with orthosteric probes.

KEY RESULTS

Two types of efficacy switching were initiated by altering ligand structure and concentration. At lower concentrations, a subtle structural change at position 5 of the indole molecule resulted in opposite effects. 5-HoI apparently elicited partial allosteric inverse agonism, whereas 5-MoI induced allosteric agonism. Interestingly, at a higher concentration, these indoles produced distinct auto-inhibition, manifested as a switch from positive to negative effects. 5-HoI induced a transition from orthosteric agonism to allosteric inverse agonism, whereas 5-MoI produced a shift from allosteric agonism to orthosteric inverse agonism. The auto-inhibition appears to involve communication between orthosteric and allosteric sites of the active receptor conformation and/or between inactive and active conformations. An additive effect of orthosteric and allosteric inverse agonism and insensitivity of allosteric agonism to orthosteric antagonism were also demonstrated.

CONCLUSIONS AND IMPLICATIONS

Together, the results suggest that the moiety at position 5 of the indole structure is a critical determinant of a ligand's properties at the 5-HT_3A receptor, providing new insights into understanding ligand–receptor interactions.

Abbreviations

5-HoI, 5-hydroxyindole; 5-MoI, 5-methoxyindole; LGIC, ligand-gated ion channel; nACh receptor, nicotinic ACh receptor; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate

Tables of Links

TARGETS	
α7 nACh receptor	$GABA_A$ receptor
$5\text{-}HT_3$ receptors	Glycine receptors
$5\text{-}HT_3A$ receptor	nACh receptors
$5\text{-}HT_3AB$ receptor	

LIGANDS	
5-HT	Physostigmine
5-hydroxyindole	TMB-8
[3H]-GR65630	Tubocurarine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

A cell can sense environmental changes via receptors on its surface membrane and inside the cell. The binding of ligands to their corresponding receptors alters the functions of the cell (Cooper, 2000). The $5\text{-}HT_3$ receptor belongs to the Cys-loop ligand-gated ion channel (LGIC) superfamily that also includes nicotinic ACh (nACh), $GABA_A$ and glycine receptors (Connolly and Wafford, 2004). The activity of the $5\text{-}HT_3$ receptor is regulated by the binding of ligands to orthosteric and/or allosteric sites on the receptor. $5\text{-}HT_3$ receptors expressed in central and peripheral nervous systems mediate fast excitatory neurotransmission (Sugita *et al.*, 1992; Ferezou *et al.*, 2002) and modulate the release of various neurotransmitters (van Hooft and Vijverberg, 2000; Schicker *et al.*, 2008). They have been implicated in cognition, anxiety, nociception, emesis, substance abuse and addiction, cardiovascular regulation, and gut motility (Thompson and Lummis, 2007; Walstab *et al.*, 2010).

Classical receptor theories assume that a receptor is either on or off upon ligand binding, and ligands are thus classified into agonists, antagonists and inverse agonists based on their ability to alter receptor activity (Neubig *et al.*, 2003; Kenakin, 2004). Agonists produce positive effects; inverse agonists yield negative effects; and neutral antagonists block both positive and negative effects. However, this classification of ligands has been challenged by recent advances in receptor pharmacology (Schwartz and Holst, 2007; Smith *et al.*, 2011). We previously demonstrated that a 5-HT analogue could produce both agonism and inverse agonism collaterally at the heteromeric $5\text{-}HT_3AB$ receptor, which exhibited a low level of spontaneous (i.e. ligand-independent) activity (Hu and Peoples, 2008a). This observation is irreconcilable with classical receptor concepts. The mechanism underlying the co-occurrence of both positive and negative effects for a ligand remains unknown, and further analysis of it is hindered by the low level of constitutive activity of the $5\text{-}HT_{3AB}$ receptor. Work from Lester's laboratory demonstrated that a mutated pore-lining residue (V291S) in the $5\text{-}HT_3A$ receptor enables the channel to open spontaneously, leading to significant constitutive activity (Dang *et al.*, 2000; Bhattacharya *et al.*, 2004). Consequently, we took advantage of the spontaneous activity of the V291S receptor and revealed opposing effects; 5-hydroxyindole (5-HoI) and 5-methoxyindole (5-MoI), two structural analogues of the endogenous $5\text{-}HT_3$ receptor agonist 5-HT, were found to have distinct effects induced by their interactions with discrete ligand-binding sites on the receptor.

Methods

Cell culture, mutagenesis and transient receptor expression

HEK293 cells (ATCC, Manassas, VA, USA) were grown in minimum essential medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS and maintained in a humidified incubator at 37°C in 5% CO_2. Mouse $5\text{-}HT_3A$ isoform 1 receptor subunit was subcloned into the vector pcDNA3.1 (Life Technologies). The mouse $5\text{-}HT_3A$ receptor mRNA sequence is available through GenBank accession D49395. Point mutation (valine to serine at position 291) of the $5\text{-}HT_3A$ receptor was accomplished using a QuikChange site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA). The successful incorporation of the mutation was verified by double-strand DNA sequencing. HEK293 cells were transiently transfected with $5\text{-}HT_3$ receptor cDNAs using Lipofectamine 2000 transfection reagent (Life Technologies) or calcium-phosphate transfection reagent. Green fluorescent protein (pGreen Lantern, Life Technologies) was co-expressed with $5\text{-}HT_3A$ receptor subunits to permit optical selection of transfected cells.

Whole-cell patch-clamp recording

Whole-cell patch-clamp recording was performed as described previously (Hu and Lovinger, 2005). Cells were continuously superfused with an external solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES (pH to 7.4 with NaOH and osmolarity to ~340 mosmol with sucrose). The recording pipettes had resistances of 2–5 MΩ when filled with pipette solution containing 140 mM CsCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES (pH to 7.2 with CsOH and osmolarity to ~315 mosmol with sucrose). Membrane current was recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) at 20–22°C. Cells were held at –60 mV unless otherwise indicated. Data were acquired using pClamp9.0 software (Molecular Devices). Currents were filtered at 2 kHz and digitized at 5–10 kHz. Ligands were

applied with a stepper motor-driven apparatus (SF-77B Fast-step, Warner Instrument Co., Hamden, CT, USA) through a two-barrel theta glass tubing (TGC150, Warner Instruments) that had been pulled to a tip diameter of ~200 μm. The cell was placed in front of the stream of control solution. The stepper motor-driven apparatus was driven by voltage pulses from the pClamp9.0 software, which produced a rapid lateral displacement of the theta tubing to move the interface between two different solutions such as control and agonist solutions.

Data analysis

The present study used a randomized experimental design. Data analysis and graphing were performed with Origin 7.5 (OriginLab, Northampton, MA, USA), pClamp 9.0 (Molecular Devices) or GraphPad InStat 3.0 (GraphPad Software Inc., San Diego, CA, USA) software. A decrease in the holding current, manifested as an apparent outward current, represents a negative effect, whereas an increase in the holding current exhibited as an inward current, denotes a positive effect. To facilitate a comparison, the effects were expressed as percentage changes over holding currents: % change over holding current = $[I_{outward}$ ($_{or}$ I_{inward}) $- I_{holding}]/I_{holding} \times 100$ ($I_{holding}$: holding current; $I_{outward}$: apparent outward current; and I_{inward}: inward current). Data are presented as mean ± SEM. Statistical significance was determined with ANOVA or Student's *t* test, where appropriate. Differences were considered significant at $P < 0.05$.

Results

Spontaneous activity of the V291S 5-HT$_3$A receptor

After establishing the whole-cell recording configuration, HEK293 cells expressing the wild-type 5-HT$_3$A receptor displayed a negligible holding current at a holding potential of −60 mV, whereas cells expressing the V291S receptor exhibited a large holding current (Figure 1A). The averaged amplitude of holding currents was −11.7 ± 1.5 pA for the wild-type receptor and −107.7 ± 11.5 pA for the V291S receptor ($n = 30$, $P < 0.05$). Functional expression of wild-type and V291S receptors were demonstrated by the generation of inward currents in response to 5-HT (Figure 1B). The concentration-response curve for 5-HT was significantly shifted to the left by the V291S mutation. Fitting data with the Hill equation revealed EC_{50} values of 1.92 ± 0.05 μM and 0.02 ± 0.005 μM for wild-type and V291S receptors respectively. Holding currents in cells expressing the V291S receptor, but not the wild-type receptor, bore similar biophysical properties to that of 5-HT currents (Supporting Information Fig. S1, data for the wild-type not shown). The 5-HT$_3$ receptor open channel blocker 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8, 100 μM) reversibly reduced holding currents in cells expressing the V291S receptor by 78.0 ± 3.2%, manifested as an apparent outward current ($n = 17$, Figure 1C). This finding is comparable to the observation from a previous study in which the V291S receptor was expressed in *Xenopus* oocytes (Bhattacharya *et al.*, 2004). In addition, holding currents in cells expressing the V291S receptor could also be reversibly inhibited by MDL 72222 and tubocurarine (Figure 1D), which are conventionally classified as competitive 5-HT$_3$ receptor antagonists. MDL 72222 and tubocurarine at 1 μM, a concentration corresponding to several hundred-fold of their individual IC_{50}s (Downie *et al.*, 1994; Gill *et al.*, 1995), decreased holding currents in cells expressing the V291S receptor by 24.6 ± 1.8% ($n = 11$) and 28.3 ± 1.5% ($n = 16$) respectively. In contrast, TMB-8, MDL 72222 and tubocurarine failed to alter holding currents in cells expressing the wild-type receptor (data not shown).

Distinct effects induced by 5-HoI and 5-MoI at the V291S receptor

5-HoI and 5-MoI are structural analogues of the endogenous 5-HT$_3$ receptor ligand 5-HT (Supporting Information Fig. S2A). Consistent with previous findings (Hu and Lovinger, 2008b; Hu and Peoples, 2008a), 5-HoI was a positive allosteric modulator, whereas 5-MoI was a partial agonist at the wild-type 5-HT$_3$A receptor (Supporting Information Fig. S2B). However, 5-HoI was found to produce apparent partial inverse agonism, and 5-MoI yielded mixed partial agonism and inverse agonism at the spontaneously active 5-HT$_3$AB receptor (Hu and Peoples, 2008a). To examine the behaviours of 5-HoI and 5-MoI at the V291S receptor, currents in response to various concentrations (100 μM to 1 mM) of these indole compounds were measured. Remarkably, 5-HoI and 5-MoI induced distinct effects at the V291S receptor (Figure 2). 5-HoI produced a concentration-dependent negative effect by suppressing spontaneous activity, manifested as an inhibition of holding currents (Figure 2A and C). In contrast, 5-MoI had a positive effect, inducing concentration-dependent activation of the receptor (Figure 2B and C).

Different responses induced by high concentrations of 5-HoI and 5-MoI

Responses induced by a high concentration (5 mM) of these indole compounds along with 5-HT (30 μM) were also examined in cells expressing the V291S receptor. An application of 5-HT rapidly increased the inward current over the holding current, and this 5-HT current desensitized negligibly (Figure 3A). Amazingly, both 5-HoI and 5-MoI produced a biphasic response: an inward current (i.e. positive effect) followed by an apparent outward current (i.e. negative effect) over the holding current (Figure 3A). This is similar to the observation reported previously for 5-MoI at the 5-HT$_3$AB receptor (Hu and Peoples, 2008a). Positive effects elicited by 5-HT, 5-HoI and 5-MoI and negative effects produced by 5-HoI and 5-MoI are summarized in Figure 3B. In contrast, both 4-hydroxyindole (4-HoI) and 4-methoxyindole (4-MoI) only elicited an inward current over the holding current in cells expressing the V291S receptor (Supporting Information Fig. S3). Although the co-application of 5-HT with open channel blockers TMB-8 or Zn^{2+} also produced biphasic responses at the V291S receptor, the actions of 5-HoI and 5-MoI did not appear to involve an open channel blockade mechanism (Supporting Information Fig. S4).

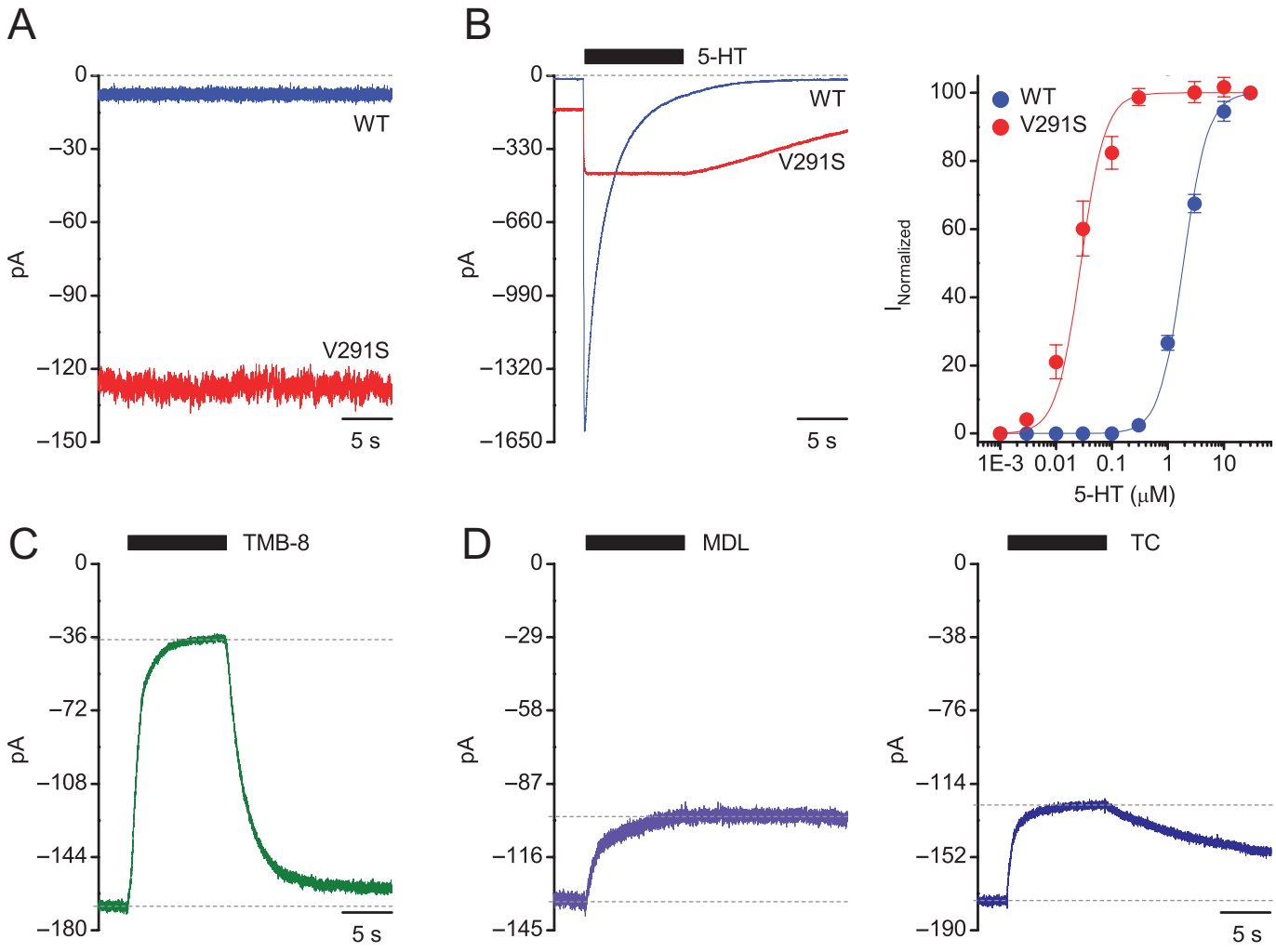

Figure 1

Spontaneous activity of the V291S 5-HT_3A receptor. (A) Tracings show representative holding currents obtained at –60 mV in cells expressing wild-type (WT) and V291S receptors. (B) Tracings of whole-cell currents activated by 30 μM 5-HT and concentration-response curves induced by 5-HT in cells expressing WT and V291S receptors. Each data point represents mean ± SEM from 6–10 cells. (C) A representative tracing shows the response to an application of 100 μM TMB-8 in cells expressing the V291S receptor. (D) Tracings show responses to applications of 1 μM MDL 72222 and 1 μM tubocurarine in cells expressing the V291S receptor. Note that inhibition of holding currents by TMB-8, MDL 72222 and tubocurarine in cells expressing the V291S receptor was manifested as apparent outward currents.

Divergent allosterism conferred by 5-HoI and 5-MoI at the V291S receptor

To probe the potential binding sites on the V291S receptor that mediate the opposite responses to low concentrations of 5-HoI and 5-MoI, the effect of saturating the orthosteric site with MDL 72222 (1 μM; Downie *et al.*, 1994; Gill *et al.*, 1995) on responses induced by 500 μM 5-HoI or 5-MoI was subsequently investigated using co-application and sequential application protocols (Figure 4A and B). 5-HoI and MDL 72222, when applied alone, suppressed the holding currents, and a combination of both compounds resulted in an additive inhibition regardless of whether the co-application or sequential application protocols were used (Figure 4C). When applied separately, 5-MoI and MDL 72222 produced positive and negative effects, respectively, resulting in an increase and a decrease over holding currents. 5-MoI still induced positive effects when co-applied or sequentially applied with MDL 72222, which were not different from that produced by 5-MoI alone ($P > 0.05$, Figure 4D).

Distinct switching of effects induced by high concentrations of 5-HoI and 5-MoI at the V291S receptor

A sequential application protocol was then used to probe potential binding sites concurrently mediating the contrasting effects induced by high concentrations of 5-HoI or 5-MoI. In this protocol, the orthosteric binding site was initially saturated with 1 μM MDL 72222 or tubocurarine (Downie *et al.*, 1994; Gill *et al.*, 1995); and 5-HoI and 5-MoI (5 mM) were subsequently applied approximately at the maximal

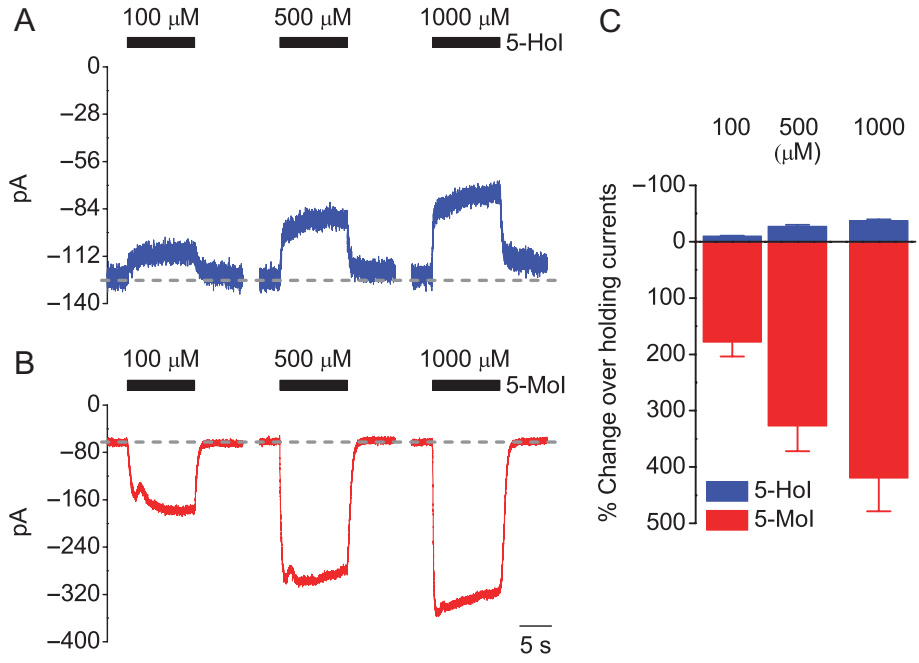

Figure 2

Distinct effects induced by lower concentrations of 5-HoI and 5-MoI at the V291S receptor. Tracings show responses to lower concentrations (100 to 1000 μM) of 5-HoI (A) and 5-MoI (B) in the same cells. (C) Bar graph summarizes negative effects induced by 5-HoI and positive effects induced by 5-MoI. Each bar represents mean ± SEM from 7 to 11 cells. Dashed lines illustrate levels of holding currents.

response produced by MDL 72222 or tubocurarine. As shown in Figure 5A and B, an application of MDL 72222 caused a ~25% inhibition of holding currents. The sequential application of 5-HoI in the presence of MDL 72222 produced a cumulative inhibitory effect, resulting in inhibition of holding currents. Nevertheless, 5-MoI still activated the receptor in the presence of MDL 72222 and elicited an inward current corresponding to an increase over holding currents. In contrast to the biphasic responses produced by both indole compounds illustrated in Figure 3, neither the positive effect induced by 5-HoI nor the negative effect induced by 5-MoI was observed in the presence of MDL 72222. Similar findings were obtained with a sequential application of tubocurarine and 5-HoI/5-MoI (Figure 5C and D).

Interactions of 5-HoI and 5-MoI at the V291S receptor

Possible interactions between 5-HoI (5 mM) and 5-MoI (5 mM) were also investigated. As aforementioned, an application of 5-HoI or 5-MoI alone produced an initial receptor activation followed by receptor inactivation (Figure 6A and B). Despite the auto-inhibition produced by 5-HoI, an application of 5-MoI in the presence of 5-HoI was still able to produce receptor activation, manifested as an inward current with reduced amplitude and inactivation accompanied by an outward current. However, the auto-inhibition produced by an initial application of 5-MoI prevented the agonist effect, but not the inverse agonist effect induced by a successive application of 5-HoI (Figure 6C and D).

Discussion

A key finding arising from this study is that a subtle modification of the indole structure (Supporting Information Fig. S2A) dramatically changed the behaviour of the ligand at the spontaneously active V291S 5-HT$_{3A}$ receptor. The substitution of the hydroxyl group at position 5 with a methoxy group in the indole molecule converted apparent inverse agonism into agonism, suggesting that the moiety at this position of the indole is a critical determinant of ligand efficacy at the 5-HT$_3$A receptors. The Monod-Wyman-Changeux model predicts that a ligand has its preference towards a given conformation of the receptor (Monod *et al.*, 1965). An agonist prefers to bind to the active R* conformation, whereas an inverse agonist favours an interaction with the inactive R conformation. The results from the present study suggest that 5-HoI and 5-MoI at low concentrations favour R and R*conformations, respectively, producing opposite responses.

Saturation of the orthosteric binding site with MDL 72222 would preclude access of the other molecule to this site on the V291S receptor. The inability of MDL 72222 to alter responses produced by low concentrations of both 5-HoI and 5-MoI suggests that these two indoles predominantly bind to the allosteric site of different receptor conformations to elicit apparent partial inverse agonism and agonism respectively (Figure 7; Supporting Information Figs S6 and S7). Efficacy alterations due to ligand structure modifications is common for allosteric ligands (Wenthur *et al.*, 2014).

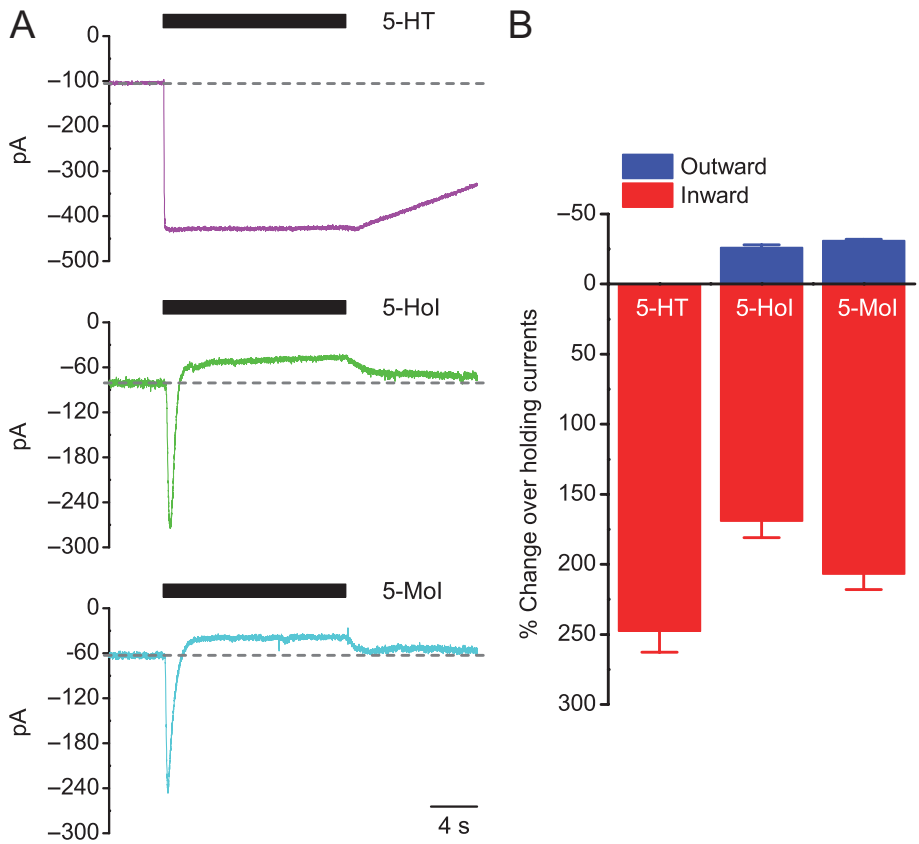

Figure 3

Efficacy switching induced by a high concentration of 5-HT analogues at the V291S receptor. (A) Tracings show negligibly desensitizing current activated by 30 μM 5-HT (upper panel) and biphasic responses, manifested as a transient inward current followed by an apparent outward current over holding currents, elicited by 5 mM 5-HoI (middle panel) and 5 mM 5-MoI (lower panel). Dashed lines illustrate levels of holding currents. (B) Averaged data summarize percentage changes of inward and apparent outward currents over holding currents induced by 5-HT, 5-HoI and 5-MoI. Each bar represents mean ± SEM from 23 to 29 cells.

Moiety substitutions to a ligand was found to convert a positive allosteric modulator to a negative allosteric modulator at the mGlu receptor (Wood *et al.*, 2011) and to transform an allosteric agonist to a positive allosteric modulator at the α7 nACh receptor (Gill *et al.*, 2012). Hence, a subtle modification of a given moiety in a ligand could function as an 'efficacy switch'. Understanding the molecular basis of this phenomenon might shed light on ligand-based design of novel therapeutics.

Allosteric agonism has been previously demonstrated in various members of the Cys-loop LGIC superfamily (Schrattenholz *et al.*, 1993; Akk and Steinbach, 2005; Campo-Soria *et al.*, 2006; Militante *et al.*, 2008; Gill *et al.*, 2011; Jorgensen *et al.*, 2011). Responses produced by allosteric agonists may be resistant to conventional orthosteric antagonists (Kenakin, 2010). Consistent with this notion, allosteric agonism induced by 5-MoI at the 5-HT_3A receptor (Figures 4 and 5, Supporting Information Fig. S7B) and by physostigmine at the nACh receptor (Schrattenholz *et al.*, 1993) was insensitive to classical competitive antagonists. Furthermore, allosteric activation of the nACh receptor caused little desensitization (Palczynska *et al.*, 2012) and was not affected by receptor desensitization induced by binding of ACh to the orthosteric site (Schrattenholz *et al.*, 1993). Therefore, allosteric agonists may offer great potential to increase therapeutic ligands for disorders associated with Cys-loop LGICs.

Inverse agonism could originate from binding of a ligand to either orthosteric or allosteric sites. Little is known about the co-occurrence of both orthosteric and allosteric inverse agonism. Since orthosteric and allosteric inverse agonism occurred as a results of ligands interacting with different binding sites, they could be additive if the spontaneous activity of a receptor is only moderately inhibited by each mechanism. Co-application of MDL 72222/tubocurarine/5-MoI and 5-HoI demonstrated that partial orthosteric and allosteric inverse agonism could indeed be additive (Figures 4–6, Supporting Information Fig. S7A), providing evidence to support this view.

Strikingly, 5-HoI and 5-MoI at higher concentrations initially activated and subsequently inactivated the spontaneously active V291S receptor, suggesting the occurrence of auto-inhibition. Auto-inhibition is common in biology including enzymology and pharmacology (Kuhl, 1994; Bindslev, 2004). Open channel blockade may account for the auto-inhibition induced by high concentrations of ligands at

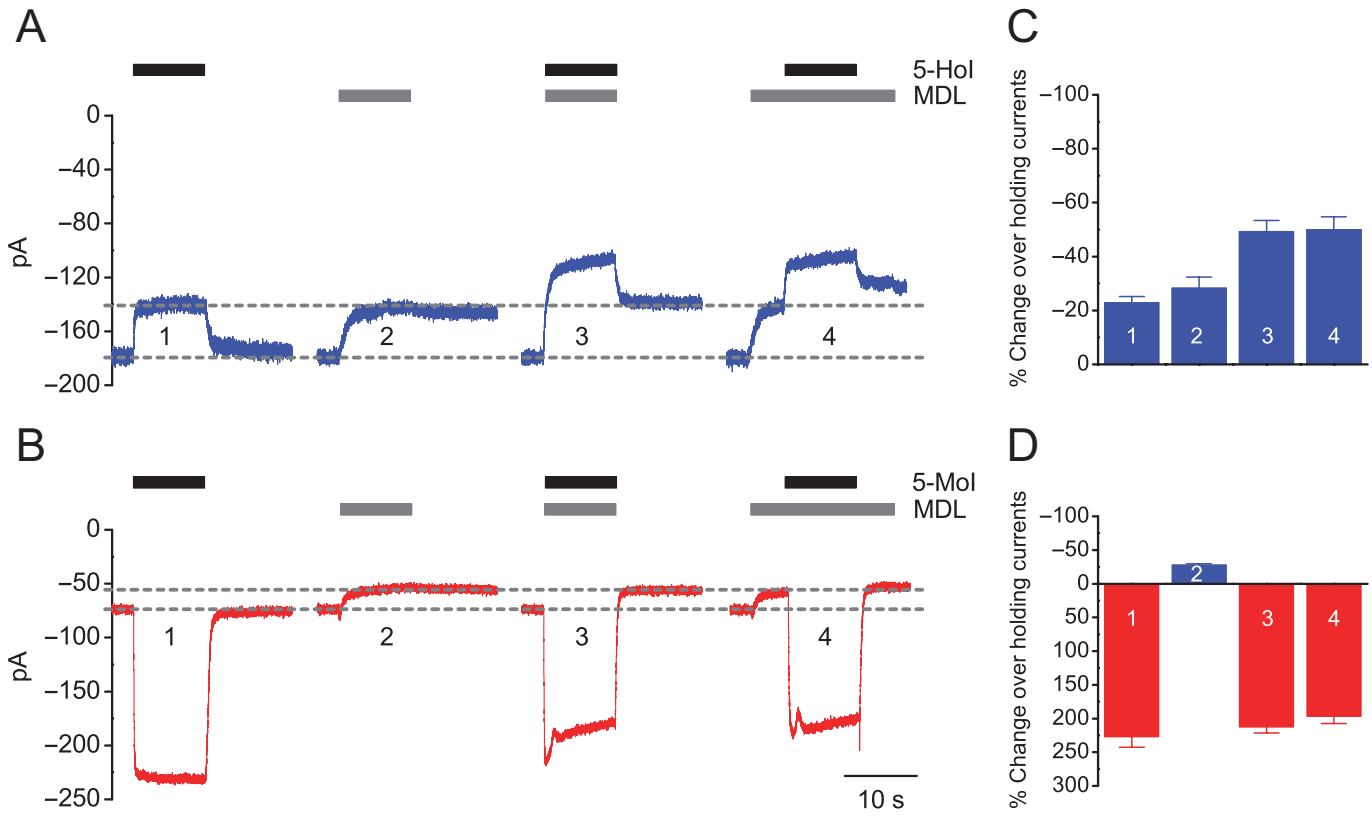

Figure 4

Effects of MDL 72222 on responses induced by a low concentration of 5-HoI and 5-MoI. (A and B) Tracings show responses produced by 500 μM 5-HoI (upper panel)/5-MoI (lower panel) and/or 1 μM MDL 72222 using various application protocols: 1, 5-HoI/5-MoI alone; 2, MDL 72222 alone; 3, co-application of 5-HoI/5-MoI and MDL 72222; and 4, sequential application of MDL 72222 and 5-HoI/5-MoI. Dashed lines illustrate levels of holding currents and the inhibition of holding currents by MDL 72222 respectively. (C and D) Bar graph summarizes responses to applications of 5-HoI/5-MoI and/or MDL 72222. Each bar represents mean ± SEM from 5 to 6 cells.

LGICs (Arias, 1996; Mercik *et al.*, 2002). However, the observations that the 5-HT-induced current was largely unaffected by 5-HoI and 5-MoI (Supporting Information Fig. S4D) and that these indoles produced concentration-dependent enhancement and inhibition of the spontaneous activity rules out this possibility. Desensitization of LGICs is a process in which the ion channel enters a non-conducting state despite the presence of a ligand on the receptor. High to saturating concentrations of agonist usually increase the likelihood of the channel entering this state. The auto-inhibition induced by 5-HoI and 5-MoI appears to be a combination of desensitization and inverse agonism. However, the V291S mutation created a virtually non-desensitizing receptor; and negligible desensitization occurred even at super-saturating concentrations of 5-HT (Supporting Information Fig. S5). Therefore, receptor desensitization is unlikely to contribute to the auto-inhibition induced by these indoles.

GPCRs adopt multiple conformations linked to distinct signalling pathways, and ligand efficacy is signalling pathway-dependent. For example, propranolol functions as an inverse agonist towards G protein-dependent pathways, whereas it acts as a positive agonist for β-arrestin-mediated ERK1/2 phosphorylation (Galandrin and Bouvier, 2006). This phenomenon has been commonly termed 'functional selectivity' (Kenakin and Miller, 2010; Kenakin, 2011; Valant *et al.*, 2012). However, an integrated ion channel is the solitary effector coupled to LGICs. Ligand binding either promotes or blocks channel opening. Remarkably, 5-HoI and 5-MoI were able to produce both positive and negative effects at the V291S receptor by initially opening and subsequently closing the very same channel pore. Furthermore, functional selectivity involves singular binding of a ligand to distinct receptor conformations, whereas the auto-inhibition induced by these indoles seems to require dual binding of orthosteric and allosteric sites of the same or different conformations. Thus, the variable effect conferred by high concentrations of 5-HoI and 5-MoI is dissimilar from functional selectivity observed in GPCRs.

Positive and negative efficacies are *a priori* opposites. It is hard to imagine that a single ligand could produce agonism and inverse agonism concurrently via binding to a single site of the receptor. Receptors possess an orthosteric binding site for their respective endogenous ligand(s) and other active sites for allosteric ligands. The coexistence of orthosteric and allosteric sites on a receptor allows possible co-occupancy of both sites by the same ligand simultaneously or sequentially, resulting in additional ligand-receptor interactions and functional outcomes. 5-HoI displaced the orthosteric antagonist

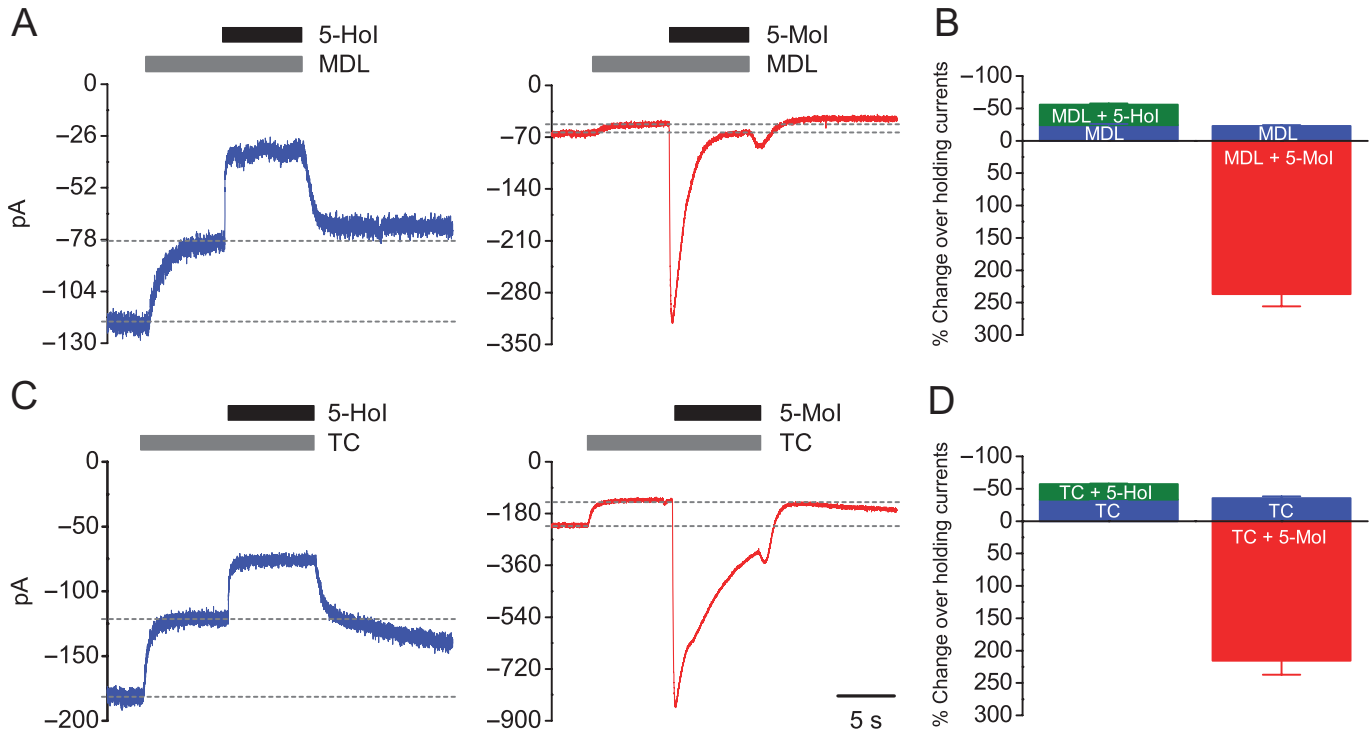

Figure 5

Distinct effects induced by a high concentration of 5-HoI and 5-MoI after saturating the orthosteric binding site with 5-HT_3 receptor competitive antagonists. (A) Tracings show responses produced by a sequential application protocol of MDL 72222 (MDL, 1 μM) and 5-HoI (5 mM) or 5-MoI (5 mM). (B) Averaged data summarize percentage changes of responses over holding currents produced by 5-HoI and 5-MoI in the presence of MDL 72222. Each bar represents mean ± SEM from 22 to 29 cells. (C) Tracings show responses produced by a sequential application protocol of tubocurarine (TC, 1 μM) and 5-HoI (5 mM) or 5-MoI (5 mM). (D) Averaged data summarize percentage changes of responses over holding currents produced by 5-HoI and 5-MoI in the presence of tubocurarine. Each bar represents mean ± SEM from 9 to 18 cells. Dashed lines illustrate levels of holding currents and the inhibition of holding currents by MDL 72222/tubocurarine respectively.

[^{3}H]-GR65630 bound at the 5-HT_3 receptor with a pKi of 1.96 (i.e. ~10 mM; Kooyman *et al.*, 1994). Specifically, [^{3}H]-GR65630 was negligibly displaced by 1 mM 5-HoI, which potentiated 5-HT responses, but competitively displaced by 10 mM 5-HoI which blocked 5-HT responses, suggesting that 5-HoI could bind to orthosteric and/or allosteric sites on the 5-HT_3 receptor. Consistent with this finding, a site-directed mutagenesis study suggested that direct activation and allosteric modulation of the 5-HT_{3A} receptor by 5-HoI were mediated by the interaction of this ligand with orthosteric and allosteric sites respectively (Hu and Lovinger, 2008b). Not surprisingly, the present study was able to demonstrate that 5-HoI and 5-MoI at higher concentrations could bind to orthosteric and allosteric sites collaterally to produce both positive and negative effects.

Although the allosteric binding site is topographically distinct from the orthosteric binding site, they are conformationally linked (May *et al.*, 2007). The collateral binding of indole compounds to both orthosteric and allosteric sites would initiate an energy transfer between these two sites (Supporting Information Fig. S10). Binding of 5-HoI or 5-MoI to each site engenders distinct effects; and the net outcome depends on the interaction between those two sites. Biphasic responses elicited by high concentrations of 5-HoI and 5-MoI represent two types of auto-inhibition: a conversion from orthosteric agonism to allosteric inverse agonism for 5-HoI (Figure 7; Supporting Information Fig. S8A) and a transition from allosteric agonism to orthosteric inverse agonism for 5-MoI (Figure 7; Supporting Information Fig. S9A). At a high concentration, binding of 5-HoI to the orthosteric site or 5-MoI to the allosteric site of the unliganded R* conformation yields agonism, whereas conformational changes induced by concomitant/subsequent binding of 5-HoI to the allosteric site (Figure 7; Supporting Information Fig. S8B) or 5-MoI to the orthosteric site (Figure 7; Supporting Information Fig. S9B) of the liganded R* conformation would promote negative cooperativity and/or reduce efficacy generated by ligand binding to the other site, leading to receptor inactivation. 5-HT_3 receptor agonists 5-HT, 1-(m-chlorophenyl)-biguanide (mCPBG) and tryptamine at high concentrations produced an auto-inhibition at the wild-type receptor, resulting in bell-shaped concentration-response curves (Lankiewicz *et al.*, 1998; Hapfelmeier *et al.*, 2003; Meiboom *et al.*, 2013). This auto-inhibition may represent a type of non-competitive, homotropic antagonism, possibly arising from a cross-talk between orthosteric and allosteric sites upon simultaneous binding by the same ligand.

Singly occupied receptors (i.e. only one of the orthosteric and allosteric sites was occupied) appeared to be most abundant at low concentrations of 5-HoI and 5-MoI, and these

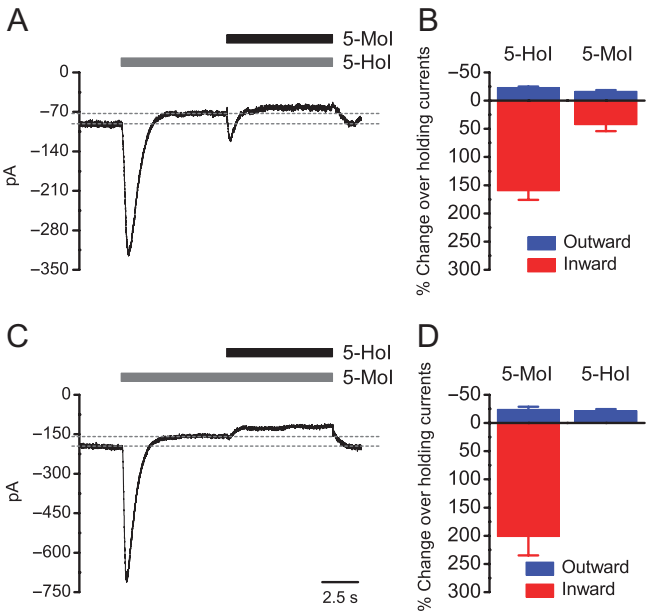

Figure 6

Interactions between 5-HoI and 5-MoI at the V291S receptor. A sequential protocol was used to investigate the interactions between high concentrations of 5-HoI (5 mM) and 5-MoI (5 mM) at the V291S receptor. Inward and apparent outward currents are expressed as percentage changes over holding currents. Note that an initial application of 5-HoI produced an auto-inhibition, which reduced the receptor activation but not inactivation induced by the subsequent application of 5-MoI (A and B). Also note that the auto-inhibition produced by an initial application of 5-MoI only inhibited the positive effect induced by 5-HoI, leaving the negative effect induced by 5-HoI intact (C and D). Each bar represents mean ± SEM from 7 to 10 cells.

ligands predominantly interacted with the allosteric site on the V291S receptor. However, doubly occupied receptors prevailed at higher ligand concentrations (Figure 7). An increase in ligand concentration from 1 mM to 5 mM, albeit a narrow increment, allowed the ligand to bind to the orthosteric site as well and provided enough binding energy to overcome (for 5-HoI) or augment (for 5-MoI) the energy barrier between inactive and active conformations (Supporting Information Fig. S10). Conformational changes leading to channel opening induced by binding of one indole molecule to one site probably exposed the other site to the same ligand; and subsequent binding of this ligand to the later site resulted in additional conformational changes that closed the channel pore. These findings are largely consistent with the theoretical homotropic two-state model proposed to explain the bell-shaped concentration–response relationship (Bindslev, 2004). According to this model, a ligand at low to middle levels of concentration could only bind to either orthosteric or allosteric sites to elicit a given response, whereas the ligand at high concentrations could bind to both orthosteric and allosteric sites resulting in auto-inhibition.

Alternatively, the efficacy switch occurring at high concentrations of 5-HoI and 5-MoI may be the result of 'population competition antagonism' (Figure 7). The binding of 5-HoI or 5-MoI to orthosteric and allosteric sites of different

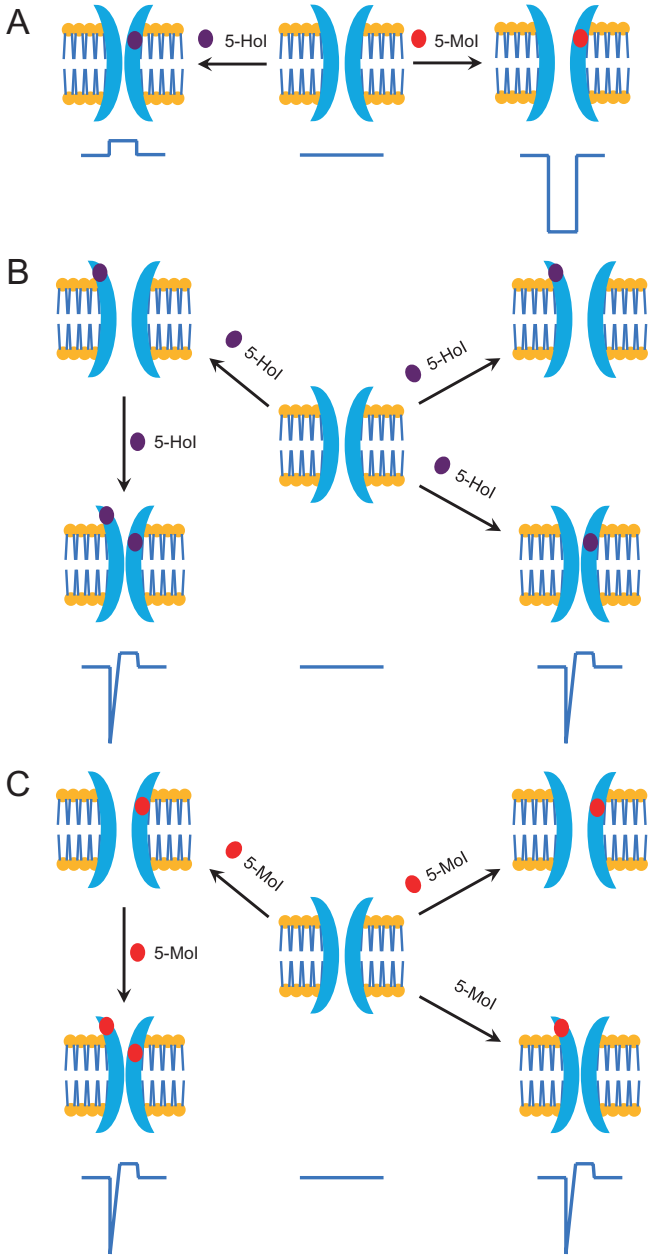

Figure 7

Schemes illustrate responses potentially induced by low and high concentrations of 5-HoI and 5-MoI at the V291S receptor. (A) Both 5-HoI and 5-MoI at low concentrations preferably bind to the allosteric site of V291S receptor. Whereas 5-HoI elicits an apparent inverse allosteric agonism, 5-MoI promotes allosteric agonism. (B) At a high concentration, these indoles may initially bind to the orthosteric site (5-HoI) or the allosteric site (5-MoI) to open the channel pore; and subsequently, they could bind to the allosteric site (5-HoI) or the orthosteric site (5-MoI) to close the channel pore. In addition, 5-HoI or 5-MoI could bind to both orthosteric and allosteric sites simultaneously to produce two populations of receptor conformations: an active conformation due to binding of 5-HoI to the orthosteric site or 5-MoI to the allosteric site and an inactive conformation due to binding of 5-HoI to the allosteric site or 5-MoI to the orthosteric site. As a result, competition for the receptor population occurs. Ultimately, the inactive conformation outweighs the active conformation, leading to auto-inhibition.

receptor conformations could cause competition for the receptor population instead of binding sites (Supporting Information Figs S8C and S9C). The binding of 5-HoI to the orthosteric site or 5-MoI to the allosteric site of the R* conformation would lower the energy barrier and/or reduce the energy of R* to R to trigger a switch to the R* conformation. On the other hand, the binding of 5-HoI to the allosteric site or 5-MoI to the orthosteric site of the R conformation would increase the energy barrier and/or reduce the energy of R relative to R* to induce a transfer to the R conformation. Therefore, the observed biphasic response is likely to represent a summation of population shift of receptor conformations upon binding of the same ligand to different conformations (Figure 7; Supporting Information Fig. S10).

A new type of ligands termed 'bitopic ligands' has been introduced recently (Valant *et al.*, 2012). These ligands, containing two distinct pharmacophores covalently linked by a flexible linker region, simultaneously target orthosteric and allosteric sites of a single receptor. Concomitant association of bitopic ligands with receptors could potentially improve affinity, efficacy, and functional selectivity (Valant *et al.*, 2012). Although 5-HoI and 5-MoI could also concomitantly bind to both orthosteric and allosteric sites, these indoles differ from bitopic ligands in that their binding to the V291S receptor appears to involve the interactions of two identical molecules with orthosteric and allosteric sites respectively.

Collectively, the present study demonstrated an unanticipated complexity of ligand efficacy using a spontaneously active 5-HT_3A receptor and two analogues of the endogenous agonist 5-HT. Subtle modifications of the moiety in a molecule may substantially alter ligand behaviour. In addition, the functional outcome induced by the binding of a ligand to either orthosteric or allosteric sites can be altered by the binding of the same ligand to the other site, which provides new insights into the pharmacology of co-occupancy of orthosteric and allosteric sites on receptors.

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Author contributions

X. Q. H. conceived, designed and performed all experiments and data analysis. X. Q. H. wrote the paper.

Conflict of interest

The author declares no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12896

Figure S1 Biophysical property of holding currents in cells expressing wild-type (WT) and V291S receptors. (A) Representative tracings show currents obtained with a voltage-step protocol in HEK 293 cells that were not transfected (HEK cell) or transfected with the WT receptor cDNAs. The membrane potential was held at –60 mV, and a voltage-step protocol from –80 mV to 20 mV with a 20-mV increment was applied. Note that the voltage-step protocol produced insignificant currents in both untransfected cells and cells expressing the WT receptor. (B) Current-voltage (I-V) relationships for holding currents in untransfected cells and cells expressing the WT receptor. Data are normalized to the current amplitude obtained at –80 mV. Holding currents in untransfected cells and cells expressing the WT receptor displayed linear current–voltage (I-V) relationships with reversal potentials of –10.4 ± 1.1 mV (n = 4) and –9.8 ± 0.6 mV (n = 6). (C) Representative tracings show currents obtained with the voltage-step protocol in cells expressing the V291S receptor in the absence and presence of 30 μM 5-HT. The voltage-step protocol generated a much larger current in cells expressing the V291S receptor than that in cells expressing the WT receptor, and exposure to 5-HT dramatically increased the current produced by the voltage-step protocol. (D) I-V relationships for holding and 5-HT currents in the same cell expressing the

V291S receptor. Data are normalized to the current amplitude obtained at –80 mV. Currents in cells expressing the V291S receptor in the absence or presence of 5-HT exhibited inward rectification at potentials negative than –40 mV and reversed at 0.03 ± 0.41 mV (-5-HT) and 0.02 ± 0.48 mV (+5-HT) (n = 5), which are comparable with the value observed for that in cells expressed the wild-type receptor (Hu and Lovinger, 2005). Note the overlap of I-V relationships for holding and 5-HT currents.

Figure S2 Pharmacological behaviour of 5-HoI and 5-MoI at the wild-type (WT) 5-HT_3A receptor. (A) Chemical structures of 5-HT, 5-HoI and 5-MoI. (B) Representative tracings show responses produced by applications of 5-HT (30 μM) alone, 5-HoI (5 mM) alone, 5-HT (30 μM) plus 5-HoI (5 mM) and 5-MoI (5 mM) alone. Note that 5-HoI itself did not elicit any response, but functioned as a positive allosteric modulator to potentiate 5-HT response. The average peak current amplitude activated by 5-HT in the presence of 5-HoI was 124.9 ± 8.4% of that in the absence of 5-HoI (n = 5). In addition, desensitization of the 5-HT current was significantly decreased by 5-HoI (desensitization time constants: 3.2 ± 0.2 s in the absence of 5-HoI versus 8.2 ± 0.7 s in the presence of 5-HoI, P < 0.05). On the other hand, 5-MoI acted as an agonist. The average peak current amplitude activated by 5-MoI was 30.2 ± 4.1% of that elicited by 5-HT (n = 6). Those observations are consistent with previous findings (Hu and Lovinger, 2008b; Hu and Peoples, 2008a).

Figure S3 Agonism elicited by 4-hydroxyindole (4-HoI) and 4-methoxyindole (4-MoI) at the V291S receptor. (A) Chemical structures of 4-HoI and 4-MoI. (B) Tracings show inward currents produced by 5 mM 4-HoI and 5 mM 4-MoI. (C) Averaged data summarize percentage changes of inward currents induced by 4-HoI and 4-MoI over holding currents. Each bar represents mean ± SEM from 6 to 10 cells.

Figure S4 5-HoI and 5-MoI are not open channel blockers at the V291S receptor. Tracings show responses induced by application of 30 μM 5-HT and co-application of 30 μM 5-HT with 100 μM TMB-8 (A) or 300 μM Zn^{2+} (B). (C) Tracings show effects of applications of 100 μM TMB-8 or 300 μM Zn^{2+} at the peak currents induced by 30 μM 5-HT respectively. (D) Tracings show effects of applications of 5 mM 5-HoI or 5 mM 5-MoI at the peak currents induced by 30 μM 5-HT respectively. Similar results have been observed in at least five cells. Dashed lines illustrate levels of holding currents.

Figure S5 Desensitization of 5-HT currents in cells expressing wild-type (WT) and V291S receptors. Cells transfected with 5-HT_3A receptors were exposed to 30 μM or 2 mM 5-HT for 10 s; and the extent of desensitization was determined by normalizing the current amplitude at the end of ligand application to the peak amplitude. Prolonged application of high to saturating concentrations of 5-HT reduced the current by 84.8 ± 1.3% (30 μM, n = 9) and 95.8 ± 1.3% (2 mM, n = 7) in cells expressing the WT receptor (A and B). However, the same protocol only caused 1.8 ± 0.6% (30 μM, n = 9) and 2.6 ± 0.5% (2 mM, n = 7) deacy of the 5-HT current in cells expressing the V291S receptor (C and D).

Figure S6 Models to show actions produced by low concentrations of 5-HoI and 5-MoI at the V291S receptor. The V291S receptor, which contains orthosteric and allosteric binding sites, exists in inactive (R) and active (R*) conformations in the resting condition. 5-HoI and 5-MoI at low concentrations prefer to select distinct conformations to elicit their responses. Since responses mediated by these indoles were not altered by the orthosteric probe MDL 72222, it is speculated that 5-HoI preferably binds to the allosteric site of the inactive R conformation to produce allosteric inverse agonism (RL) (A). In contrast, 5-MoI preferably binds to the allosteric site of the active R* conformation to elicit allosteric agonism (R*L) (B).

Figure S7 Models elaborate interactions between a low concentration of 5-HoI/5-MoI and a saturating concentration of the orthosteric probe MDL 72222 at the V291S receptor. (A) A reaction scheme illustrates interactions between 5-HoI and MDL 72222. When used alone, the binding of MDL 72222 (I_1) to the orthosteric site and 5-HoI (I_2) to the allosteric site of the inactive R conformation produces (orthosteric) inverse agonism (I_1R) and allosteric inverse agonism (RI_2) respectively. When co-applied, the generation of the inactive I_1RI_2 conformation due to the binding of MDL 72222 to the orthosteric site and 5-HoI to the allosteric site of the R conformation (i.e. $R \rightarrow I_1R \rightarrow I_1RI_2$ and $R \rightarrow RI_2 \rightarrow I_1RI_2$) leads to additive inverse agonism. When MDL 72222 and 5-HoI are sequentially applied, the binding of MDL 72222 (I_1) to the orthosteric site of the R conformation results in generation of the inactive I_1R conformation, and 5-HoI (I_2) subsequently binds to the allosteric site of the I_1R conformation causes accumulation of the inactive I_1RI_2 conformation ($R \rightarrow I_1R \rightarrow I_1RI_2$), producing additive inverse agonism. (B) A reaction scheme for interactions between 5-MoI and MDL 72222. When used alone, the binding of MDL 72222 (I_1) to the orthosteric site of the R conformation and 5-MoI (I_2) to the allosteric site of the active R* conformation produces orthosteric inverse agonism (I_1R) and allosteric agonism (R^*I_2) respectively. When co-applied, three possible reactions may occur (i.e. $R \rightarrow I_1R$, $R^* \rightarrow R^*I_2 \rightarrow I_1R^*I_2$ and $R^* \rightarrow I_1R^* \rightarrow I_1R^*I_2$), and the overall response is allosteric agonism due to binding of 5-MoI to the allosteric site of the R* conformation. When MDL 72222 and 5-MoI are sequentially applied, the binding of MDL 72222 (I_1) to the orthosteric site of the R conformation results in generation of the inactive I_1R conformation ($R \rightarrow I_1R$), and 5-HoI (I_2) could bind to the allosteric site of the active I_1R^* conformation to causes accumulation of the active $I_1R^*I_2$ conformation ($R^* \rightarrow I_1R^* \rightarrow I_1R^*I_2$), producing allosteric agonism. Note that in this scheme, the binding of orthosteric probe MDL 72222 fails to prevent allosteric agonism conferred by 5-MoI.

Figure S8 Models elaborate potential reactions in the autoinhibition produced by high concentrations of 5-HoI at the V291S receptor. The V291S receptor, regardless of inactive and active conformations, contains both orthosteric and allosteric binding sites. (A) A cubic model explicates possible reactions leading to co-occurrence of both agonism and inverse agonism induced by a high concentration of 5-HoI. (B) A scheme illustrates a potential cross-talk between binding of 5-HoI to both orthosteric and allosteric sites. Binding of 5-HoI to the orthosteric site of the active R* conformation to promotes agonism ($R^* \rightarrow LR^*$), and concurrent binding of 5-HoI to the allosteric site of the active LR* conformation produces an inactive LR*L conformation leading to receptor inactivation. Conformation changes induced by the binding of 5-HoI to the allosteric site could reduce affinity of 5-HoI at the orthosteric site and/or efficacy stimulated by

binding of 5-HoI to the orthosteric site. (C) A scheme describes alternate reactions that could cause auto-inhibition by 5-HoI. 5-HoI could simultaneous binds to the orthosteric site of the R* conformation (LR*) to produce agonism and allosteric site of the R conformation (RL) to generate inverse agonism. The formation of the inactive RL conformation would increase energy barrier and cause a shift of equilibrium towards inactive conformations at the expense of active conformations. As a result, a competition for the receptor population occurred, and the ultimate outcome of this competition is negative efficacy. Therefore, the binding of 5-HoI to orthosteric and allosteric sites simultaneously caused a competition for the receptor population instead of binding sites.

Figure S9 Models elaborate potential reactions in the auto-inhibition produced by high concentrations of 5-MoI at the V291S receptor. The V291S receptor, regardless of inactive and active conformations, contains both orthosteric and allosteric binding sites. (A) A cubic model explicates possible reactions leading to co-occurrence of both agonism and inverse agonism induced by a high concentration of 5-MoI. (B) A scheme illustrates a potential cross-talk between binding of 5-MoI to both orthosteric and allosteric sites. Binding of 5-MoI to the allosteric site of the active R* conformation to promotes agonism (R* $\rightarrow$ R*L), and concurrent binding of 5-MoI to the orthosteric site of the active R*L conformation produces an inactive LR*L conformation leading to receptor inactivation. Conformational change induced by the binding of 5-MoI to the orthosteric site could reduce affinity of 5-HoI at the allosteric site and/or efficacy stimulated by binding of 5-HoI to the allosteric site via allosterism. (C) A scheme describes alternate reactions that could cause auto-inhibition by 5-MoI. 5-MoI could simultaneous binds to the allosteric site of the R* conformation (R*L) to produce agonism and orthosteric site of the R conformation (LR) to generate inverse agonism. The formation of the inactive LR conformation would increase energy barrier and cause a shift of equilibrium towards inactive conformations at the expense of active conformations. As a result, a competition for the receptor population occurred, and the ultimate outcome of this competition is negative efficacy. Therefore, the binding of 5-MoI to orthosteric and allosteric sites simultaneously caused a competition for the receptor population instead of binding sites.

Figure S10 The V291S receptor can be viewed as a population of two conformations: inactive R and active R*. These two conformations are separated by a surmountable energy barrier; and the inactive R conformation is dominant at resting. Binding of a ligand to the first site on the receptor provides energy to overcome this barrier, leading to a shift of the receptor population in favor of the active R* conformation (activation). However, collateral binding of the same ligand to the second site on the receptor increases the energy barrier, triggering a switch to the inactive R conformation (inactivation) and consequent auto-inhibition. This auto-inhibition could occur in following scenarios: (i) concomitant binding of the ligand to both orthosteric and allosteric sites on the R* conformation, (ii) sequential binding of the ligand to both orthosteric and allosteric sites on the R* conformation (i.e. 5-HoI to the orthosteric site followed by to the allosteric site and 5-MoI to the allosteric site followed by to the orthosteric site) and (iii) concomitant binding to the orthosteric site on the R* conformation and to the allosteric site on the R conformation (for 5-HoI) or to the allosteric site on the R* conformation and to the orthosteric site on the R conformation (for 5-MoI).