INVESTIGATION OF THE ALLOSTERIC PHARMACOLOGY OF THE 5-HT$_3$ RECEPTOR IDENTIFYING THE POTENT ALLOSTERIC MODULATOR 5-CHLOROINDOLE

by

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Abstract

The 5-HT_3 receptor is a ligand-gated ion channel that mediates for example fast synaptic neurotransmission in the CNS and PNS. 5-HT_3 receptor antagonists are established anti-emetics in the clinic, they also offer symptomatic relief for patients with irritable bowel syndrome, yet, sometimes serious side-effects limits their use in this indication. The 5-HT_3 receptor is modulated allosterically by various compounds including colchicine, alcohols and volatile anaesthetics but as yet, these modulators either lack potency or selectivity, which hinders investigation.

The present study reports a novel 5-HT_3 receptor allosteric modulator that displays relatively high potency and selectivity; 5-chloro-indole (Cl-indole). Cl-indole potentiated 5-HT_3 receptor mediated responses arising from heterologous expression of the h5-HT_3A receptor (assessed by the affinity shift of agonists to compete for the radioligand binding site and by the increase in agonist action upon the h5-HT_3A receptor-mediated increase in $[Ca^{2+}]_i$; the latter action was evident with a range of agonists with very low intrinsic activity to full agonists). Cl-indole was also able to modulate allosterically the mouse native 5-HT_3 receptor.

Additional studies provided further support for the role of the C-terminus of the h5-HT3A subunit to promote stability of the arising 5-HT_3 receptor complex and that ligand interaction with the 5-HT_3A receptor impacted cell surface expression.

In summary, the study reports the identification of Cl-indole as a positive allosteric modulator of the 5-HT_3 receptor along with extensions to our knowledge concerning a structural component of the 5-HT3A subunit that promotes stability and the trafficking of the subunit into the cell membrane. These studies increase our understanding of the 5-HT_3 receptor, which may contribute to the design of better drugs targeting this receptor for therapeutic benefit.

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“Our brains seem to be organised to make random comparisons of the contents of our memories. Daydreaming allows the process to go into free fall. Suddenly, there is a new idea, born with intense excitement. We cannot organise this process but we can distort or even defeat it. ”

- Sir James Black

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Materials and Methods

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List of Abbreviations

5-HIAA	**5-hydroxyindol acetic acid**
5-HT	**5-hydroxytryptamine (serotonin)**
5OHI	**5-hydroxyindole**
AM	**acetoxymethyl**
AMPA	**α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid**
BSA	**bovine serum albumin**
cAMP	**adenosine cyclic 3' 5'-monophosphate**
cDNA	**complementary deoxyribonucleic acid**
Cl-indole	**5-chloroindole**
E5-HT	**reversal potential for 5-HT-evoked current**
EDTA	**ethylenedinitrilo-tetraacetic acid**
FBS	**foetal bovine serum**
GABA	**γ-aminobutyric acid**
GPCR	**G-protein coupled receptor**
HEKh5-HT3A	**HEK293 cells stably expressing the human 5-HT3A subunit**
HKE293	**human embryonic Kidney cells**
kDa	**kilo Dalton**
LGIC	**ligand gated ion channel**
LGIC	**ligand-gated ion channel**
mCPBG	**mchlorophenylbiguanide**
nACh	**nicotinic acetylcholine receptor**
NMDA	**N-methyl-D-aspartic acid or N-methyl-D-aspartate**
PAMs	**positive allosteric modulators**
PBS	**phosphate buffered saline**
PCR	**polymerase chain reaction**

PKG and PKC	**phospho kinase G and C**
TAE	**tris-acetate-EDTA**
TBS	**tris-buffered saline**
TCE	**2,2,2-trichloroethanol**
TEMED	**tetramethylethylenediamine**
TM	**transmembrane**
TRP	**tryptophan**

Chapter 1: Introduction

History of 5-HT

Since 1868 scientists have been mindful of a substance in the blood that could cause vasoconstriction. However, it was another 80 years before the active compound was positively identified. In 1940, Erspamer isolated a substance that was secreted by the enterochromaffin cells in the gastric mucosa, which produced causing smooth muscle contraction. He named it enteramine due to the fact that it was found in the gut (entero) and it was monoamine (amine). However, it was not until 1948 that Rapport was first isolated and purified this substance from the blood by (Rapport 1948) and named it serotonin. The term serotonin describes the function of the molecule i.e. sero (found in serum) and tonin (causes smooth vascular muscle constriction). (Batis N., 2006, BMedSc)

In 1952, Espamer and Asero associated the two substances and discovered that serotonin and enteramine is one and the same molecule, 5-hydroxytryptamine (5-HT). Nowadays only the term serotonin is used. Within another two years Amin et al. (1954) had discovered that 5-HT was also found in the CNS. Nowadays, it is known that 5-HT has a very widespread distribution throughout the body and diverse functions including vasoconstriction (Fozard et al, 1989), constriction of smooth muscle in the gastrointestinal tract GIT (Graig and Clarke 1991), neuromodulatory actions in the central and peripheral nervous systems, as well as neurodevelopmental influences (Whitaker-Azmitia et al., 1996; Levit et al., 1997). Moreover, it is well documented that 5-HT exerts its actions through at least 14 functionally and pharmacologically distinct receptors (Barnes and Sharp, 1999, Hoyer et al., 1994).

Distribution of 5-HT

The concept that 5-HT acts as a neurotransmitter within the central nervous system (CNS) was first suggested by Twarog and Page in 1953. However, the majority of physiological 5-HT, in terms of absolute mass, resides within the gut where it acts as a neurotransmitter in the myenteric plexus and a local hormone secreted from enterochromaffin cells (Bertrand et al., 2006). 5-HT is also present, at a high concentration, in platelets from where it is released as part of the blood coagulation process. Indeed, it was this action that lead to its initial discovery (Rapport et al., 1948).

Localisation of the serotonergic cell bodies and axons within the CNS was originally documented by Dahlström and Fuxe in 1964. Nuclei were found to be concentrated along the central or midline (raphé) of the brainstem and were originally classified in terms of clusters of cells referred to as B1-B9. More extensive anatomical studies have sub-divided these into two main groups based on their position within the brainstem and their axonal projections. The rostral group, which includes the dorsal raphé nucleus (DRN), is comprised of the B7 and B8 cell clusters and represents the largest anatomical grouping of serotonergic cell bodies. The median raphé nucleus (MRN) is comprised of the B5, B8 and B9 cell clusters. Together these two raphé nuclei provide the majority of serotonergic innervation to forebrain areas including the cerebral cortex, hippocampus, limbic system and hypothalamus (Jacobs and Azmitia, 1992). The caudal group, consisting of cell clusters B1-B4 is located in the medulla and caudal pons. This group includes the following nuclei; nucleus raphé obscurus (NRO; B2), nucleus raphé pallidus (NRP; B1) and nucleus raphé magnus

(NRM; B3). The axons of these cells form the major descending pathways of the serotonergic system that innervate the spinal cord, modulating sensory, motor and autonomic processing (Törk, 1990) as well as cells that have branching ascending and descending axons (Lovick and Robinson, 1983).

The axons of the serotonergic fibres innervating forebrain regions provide a further basis for subdivision of the serotonergic system. Morphologically the axons appear as either thin, extensively branched fibres that possess small fusiform varicosities (D-fibres) or axons that appear thick and devoid of varicosities at origin, which branch in terminal areas to form thinner fibres with comparatively larger, oval varicosities (M-fibres). These two sub-populations of serotonergic fibres originate primarily from cell bodies of the dorsal raphé nucleus (D-fibres) or the median raphé nucleus (M-fibres). Further to morphological and anatomical distinctions, the D-fibres appear more vulnerable than M-fibres to neurotoxins such as the amphetamine derivatives, PCA and MDMA (Törk, 1990). Finally, it appears that D-fibre varicosities form less conventional synapses, which has led to speculations that these projections are indicative of a volume or paracrine transmission (local hormone) of the serotonergic system (Törk 1990; Hornung 2003; Hensler, 2006).

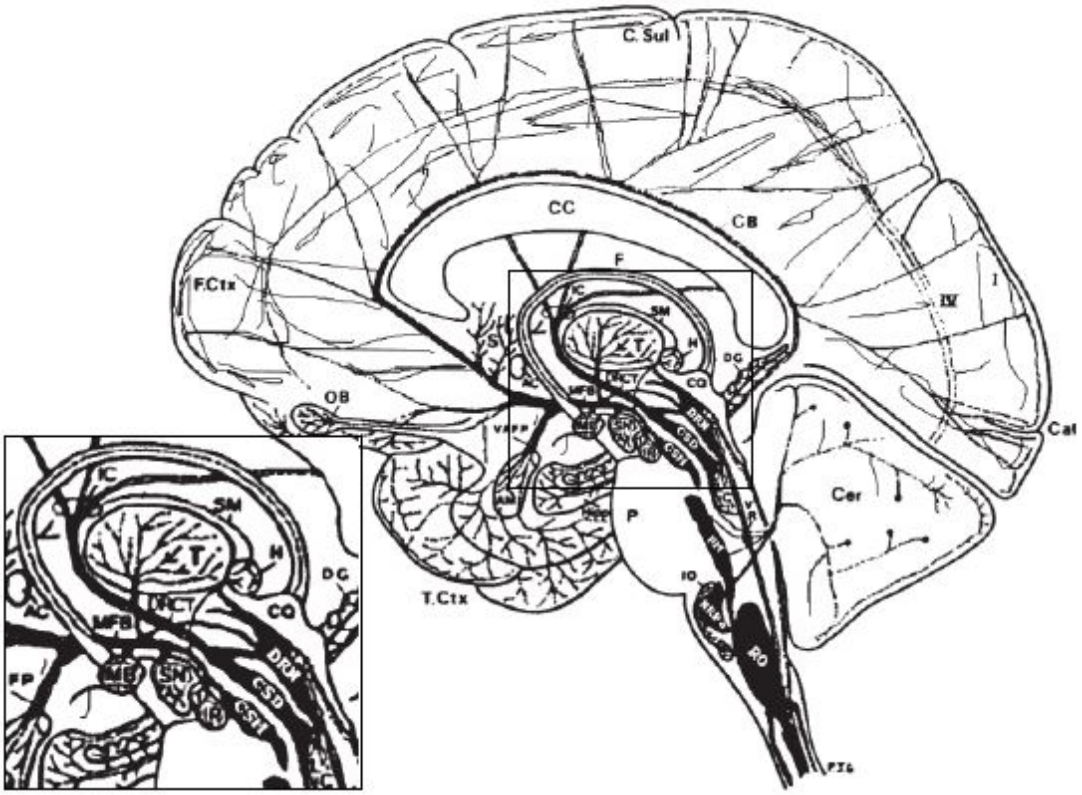


Diagram 1. Schematic of main serotonergic projections in human brain and spinal cord frpm raphe nuclei. Demonstrating extensive efferents of theis et of reticular nuclei. DRM, dorsal raphe nuclei; CSD, centralis superior nucleus, pars dorsalis; CSM, centralis superior nucleus, pars medianus; RO, nucleus raphe obscruus; NRPa, nucleus raphe pallidus; RM, nucleus raphe maglus; VR, nucleus raphe ventricularis (adapted from Azmitia and Gannon 1986)

Volume Transmission

There is indication that volume transmission of 5-HT within dorsal raphé nucleus (DRN) (Bunin & Wightman, 1998) potentially be similar to volume transmission of neuropeptides in hypothalamus (de Kock et al. 2003), due to its relatively slow action, effecting the activity of the cell it is released from via activation of metabotropic receptors. Neurones in DRN discharge substantial quantities of 5-HT locally in the nucleus from extrasynaptic sites (Bunin & Wightman, 1998), acting both on dendritic 5-HT_1A autoreceptors (Liu et al. 2005), and on 5-HT_2 receptors (Liu et al. 2000) located on presynaptic GABAergic neurones.

Further, it has been estimated the concentrations of 5-HT in the substantia nigra reticulata (SNr) from its initial stored state until it reaches its receptor site, if all molecules released per terminal were all in one vesicle could reach concentration of 90 mM (Bunin and Wightman 1998). When released into a space with the dimensions of a SNr synapse the concentration would be 6 mM (Bunin and Wightman 1998). This is very high as compared with the affinity of the 5-HT_1 receptors. When 5-HT diffuses into the extracellular compartment, its measured maximal concentration is 55 nM, a value much closer to the affinity for receptors and Km for transport. This concentration is removed from the extracellular space with a half-life of ~200 msec and allows 5-HT to diffuse >20 μm, a distance sufficient to interact with many extrasynaptic elements (Bunin and Wightman 1998).

5-HT Receptors

The classification of the 5-HT receptors was reviewed in 1994 by the Receptor Nomenclature Committee of the International Union of Pharmacology (IUPHAR) and the Serotonin Club, which decided that the structural, physiological and pharmacological properties of the receptors would determine their classification and, moreover, that newly cloned receptors would be noted in lower case appellation prior to confirming the function of the endogenous receptor (Hoyer and Martin, 1997).

The 5-HT receptors are divided in 7 classes (5-HT_{1-7}) with at least 14 subtypes and they belong to the G-protein coupled receptor (GPCR) family (seven transmembrane domains), with the exception of the 5-HT_3 receptors which are ligand gated ion channels of the cys-cys-loop superfamily (similar to nACh, $GABA_A$ and glycine receptors). (Batis N., 2006, BMedSc)

Metabotropic 5-HT Receptors

5-HT$_1$ receptors

The 5-HT$_1$ receptor family is a subdivided into 5 receptor subtypes (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D,}$ 5-HT$_{1E}$, 5-HT$_{1F}$) all of which are GPCRs negatively coupled to adenylyl cyclase and consequently they can reduce cAMP activity. 5-HT$_1$ receptors can be found either pre-synaptically (autoreceptors) or post-synaptically (Barnes & Sharp, 1999). (Batis N., 2006, BMedSc)

5-HT$_2$ receptors

The 5-HT$_2$ receptor family consists of the 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ (previously 5-HT$_{1C}$) receptors (Pritchett et al, 1988; Saltzman et al 1991; Kursar et al 1994). The gene of the receptor family has either two introns (5-HT$_2$A, 5-HT$_2$B) or three introns (5-HT$_2$C) with no evidence of splice variants contributing to the functional difference of the three subtypes. However, it has been reported that the 5-HT$_2$C receptor undergoes RNA editing, giving rise to diverse isoforms of the receptor subtype that have distribution discrepancy and a minor pharmacological distinction (Burns et al 1997). The 5-HT$_2$ receptor's amino acid sequence suggesting it is a member of the seven transmembrane receptor superfamily (GPCR). Functionally, all three receptor subtypes are positively coupled to phospholipase C and therefore are able to increase intracellular calcium levels via the inositosol pathway (Barnes and Sharp, 1999). (Batis N., 2006, BMedSc)

5-HT$_4$ receptors

The 5-HT$_4$ receptor family includes 8 isoforms of the receptor, which are all coded by a very complex gene (700Kb, 38 exons) which generates 7 carboxy-terminal variants *5-HT$_{4A}$*, (Blondel et al. 1997, Claeysel et al. 1997; *5-HT$_{4B}$* Van den Wyngaert et al. 1997; *5-HT$_{4C}$*, Blondel et al. 1998); *5-HT$_{4D}$* (Mialet et al. 2000b, Blondel et al. 1998);

5-HT$_{4E}$ (Claysen et al. 1999, Mialet et al. 2000a); *5-HT$_{4F}$*, *5-HT$_{4G}$* (Claysen et al. 1999) with their sequences varying after the amino acid 358 (leucine) of the 5-HT$_4$ receptor sequence (see Diagram 1). Additionally, a novel splice variant 5-HT$_{4H}$ or 5-HT$_{4HB}$ has been characterised, which is generated by a 14 amino acid long insertion in the 2nd extracellular loop of the 5-HT$_{4B}$ subtype (Bender et al 2000) giving rise to the 8th isoform of the receptor. All the receptor subtypes are GPCRs and appear to be positively coupled to adenylyl cyclase. The splice variants are rare among 5-HT receptors, most genes coding for the receptors have no introns. However, the only subtype that shows this variation is the 5-HT$_7$ receptor, which is also positively coupled to adenylyl cyclase (Heidmann et al., 1997; Barnes & Sharp 1999).

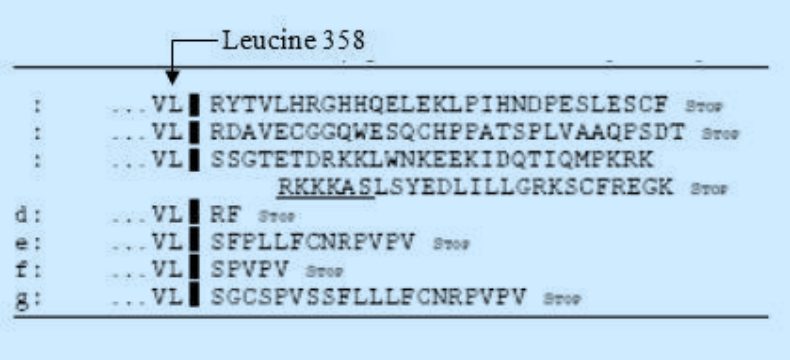


Diagram 2. 5-HT$_4$ receptor splice variants after the 358th amino acid which corresponds to Leucine (L). *Adapted from Bender et al., 2000*

Interestingly the 5-HT$_4$ receptor subtype is the only 5-HT receptor whose physiological function (adenynyl cyclase stimulation) was established prior to the demonstration of its pharmacological profile (Hungen et al 1997; Barnes & Sharp 1999) and cloning the gene. (Batis N., 2006, BMedSc)

5-HT$_5$ receptors

Two subtypes: the 5-ht$_{5A}$ and 5-ht$_{5B}$ receptor have been identified. The gene for the 5-ht$_5$A receptor has been cloned in humans, rats, and mice and it has been suggested (Francken et al 1998) that it is negatively coupled to adenylyl cyclase and thus causes cAMP levels to decrease (Carson et al 1996). Additionally it is also believed that it is G-protein coupled to an inward rectifying K^+ channel; GIRK$_1$ (Grailhe et al. 2001). In contrast, the 5-ht$_5$ receptor is not believed to form functional receptor in humans (only

in rats) as it contains a stop codon within the human receptor sequence. At present, no physiological responses mediated by this receptor family have been identified (Batis N., 2006, BMedSc)

5-HT_6 receptors

This receptor class comprises of a single gene product as no subtypes or functional splice variants (Kohen et al. 1996) have been identified so far. It has been suggested that it is positively coupled to adenylyl cyclase causing increased cAMP activity .(Batis N., 2006, BMedSc)

5-HT_7 receptors

The 5-HT_7 receptor family is comprised of 4 isoforms (5-HT_{7A}, 5-HT_{7B}, 5-HT_{7C}, 5-HT_{7D}) generated by alternate splicing of a single gene (Heidmann et al 1997). The receptor has been to shown to increase cAMP levels by the associated G-protein interacting with calmodulin-stimulated adenylyl cyclase upon receptor activation (Bard et al. 1993; Adham et al. 1998). In addition, the 5-HT_7 receptor may also be able to activate the mitogen-activated protein kinase Erk, *in vitro* (Errico et al., 2001). (Batis N., 2006, BMedSc)

Ligand gated ion channels (LGIC)

5-HT_3 receptors

The 5-HT_3 receptor is the only monoamine-gated ion channel (Derkach et al 1989). Five subunits have been identified within the 5-HT_3 receptor class; 5-HT_3A, 5-HT_3B, 5-ht_3C, 5-ht_3D, 5-HT_3E receptors (Mariq et al, 1991; Davies et 1999; Dubin et al 2002, Hanna et al., 1991; Niesler et al., 2003; Karnovsky et al., 2003). It is believed that they have a pentameric structure (Boess et al, 1995) similar to other members of the cys-cys loop LGIC superfamily such as $GABA_{A,}$ nicotinic acetylcholine and glycine receptors. All five subunits of the 5-HT_3 receptor have been demonstrated to have similar structure, with an extracellular N-terminus, possibly containing the ligand binding site (Eisele et al., 1993), four putative transmembrane domains (TM1-4), a large intracellular loop, and a short extracellular C-terminus (Reeves and Lummis, 2002). It is believed that the TM2 domain of the subunits forms the central channel pore. The homomeric 5-HT_3A receptor has been shown to mediate fast excitatory responses by depolarising the cell membrane. However, the “non-alpha”

subunits (non-5-HT_3A subunits) do not appear to form functional homomeric receptors. More likely they form heteromeric receptors co-expressing with the 5-HT_3A subunit, influencing the functional rather the pharmacological properties of the heteromeric receptor. For example, the 5-HT_3AB heteromeric receptor has similar pharmacological profile to the homomeric 5-HT_3A receptor but the homomeric receptors are inwardly rectifying as opposed to the linear relationship of the heteromeric receptor (Davies et al., 1999; Brady et al., 2001). However, the conductance of the heteromeric receptor is closer to that found in native tissue (Davies et al, 1999; Brady et al., 2001).

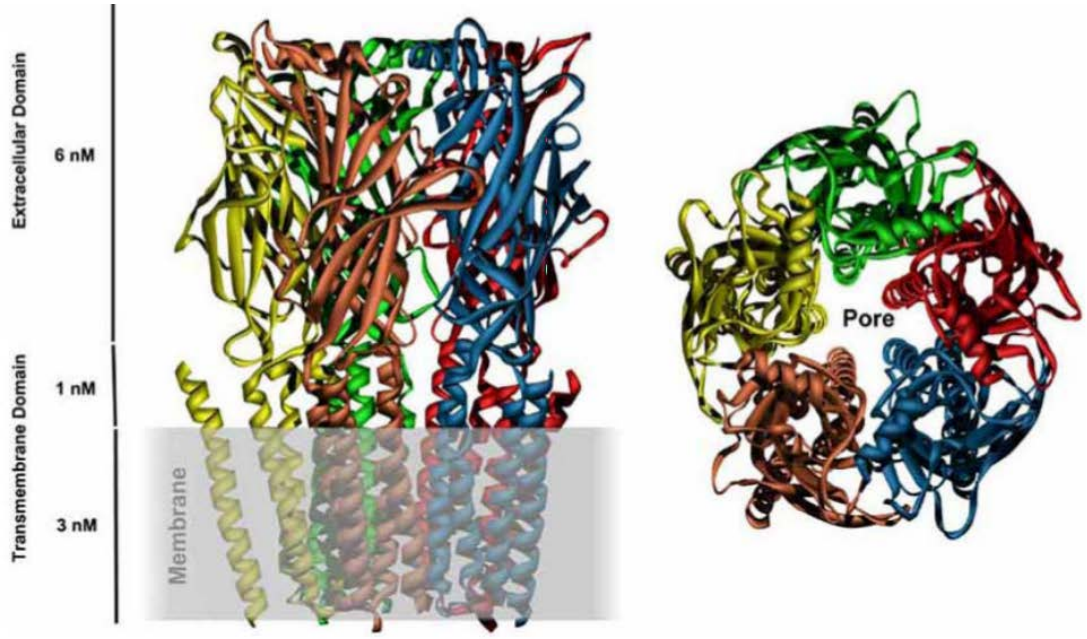


Diagram 3. Adapted from Thomson and Lummis 2006; homology model of the extracellular and transmembrane domains of the 5-HT_3 receptor. The receptor is depicted from the side and the cell membrane is highlighted as a grey box. On the right receptor is shown from above, looking down towards the membrane and through the central ion-conducting pore.

The 5-HT_3 receptor ion channel allows cations to be conducted non-selectively, and similar to the nACh receptor, it conducts monovalent ions at similar rates. The TM1-2 linker sequence contributes towards ion selectivity (Gunthorpe and Lummis, 2001) for sodium and potassium ions, as well as Ca^{2+}. However, even though the homomeric channel is non-selective for mono and bivalent cations, the heteromeric 5-HT_3AB

receptor was less permeable to Ca^{2+} than Na^{+} and K^{+} and impermeable to Mg^{2+} ions (Davies et al., 1999)

The pharmacological profile of the 5-HT_3 receptor is well established (Barnes and Sharp, 1999) and a large number of selective ligands are available, such as the selective agonists m-chlorophenylbiguanide (Kilpatrick et al., 1990) and SR57222A (Bachy et al., 1993), as well as the selective antagonists ondansetron and granisetron. These antagonists are the ones that are most widely used for clinical purposes (for review Thompson and Lummis, 2007). Similar to other members of the ligand-gated ion channel superfamily, 5-HT_3 receptors also possesses distinct allosteric binding sites via which substances such as alcohol (ethanol), general anaesthetics like ketamine and isoflurane, barbiturates and also steroids influence the function of the receptor (Parker et al., 1996).

The receptor distribution has been mapped within the central nervous system (CNS) and peripheral nervous system (PNS), as well as in the colon, intestine, lung, muscle and stomach, kidney and liver. Additionally, recent evidence has shown that the 5-HT_3 receptor is also expressed in a range of lymphocyte populations although its importance with respect to lymphocyte function remains to be determined. In fact, the mRNA coding for the 5-HT_3A receptor is present in a lot of B cell germinal centres (GC) and memory B cell populations but is decreased dramatically after the maturation of the B cells (the 11^{th} most downregulated gene; Rinaldi et al., 2010); implying an important role of the receptor in the transit from GC to mature B cells. Indeed, future investigation of the presence and function of a fast excitatory ligand-gated ion channel in B cells could reveal new insights into the function of the immune system and a possible evolutionary link between the maturation of the immune and nervous systems.

Endocytosis of the 5-HT_3 receptor

Interestingly, many members of the LGIC superfamily exhibit cell membrane receptor internalisation, via adaptin AP2 mediated endocytosis; these include the ionotropic glutamate receptors (Carroll et al., 1999) and $GABA_A$ receptors (Kittler et al., 2000;

Herring et al., 2003). Further roots of endocytosis could include lipid raft sas vehicles as well as caveolae can facilitate clathrin-independent endocytosis(Allen et al., 2007); the influence of lipid raft integrity for ligand-gated ion channel function has been shown for a variety of receptors (Allen et al., 2007).

The potential involvement of similar endocytotic mechanisms in the internalisation of the 5-HT_3 receptor has yet to be identified; however, potential ligand-dependent 5-HT_3 receptor internalisation has been reported (Ilegems et al., 2004). The adaptin AP2 complex mediates clathrin mediated endocytosis of integral membrane proteins and is comprised of four subunits: α-, β-, μ-, and σ-adaptin (Marsh and McMahon, 1999; Owen et al., 2004). The adaptin AP2 complex mediates clathrin dependent endocytosis by recruiting integral membrane proteins into clathrin coated pits (Marsh and McMahon, 1999; Owen et al., 2004). At least two intracellular recognition motifs within the recruited protein have been identified: Yxxθ where θ is a large hydrophabic amino acid, and dileucine (LL) motifs.

The 5-HT_3 receptor subunits have a large intracellular loop between the TM3 and TM4. Within this region, the 5-HT_{3A} subunit does not contain any Yxxθ motifs (known recognition sites for AP2 complex; Marsh and McMahon, 1999; Owen et al., 2004). However, it does possess three dileucine (LL) motifs (secondary recognition sites for AP2; Marsh and McMahon, 1999; Owen et al., 2004), which may indicate interaction with the adaptin AP2 complex. Interestingly, the 5-HT_{3B} subunit possesses a Yxxθ [YXEH] and two LL motifs in the large intracellular loop. This could indicate and possibly narrow the possible sites for association, if that occurs, between the receptor and the AP2-clathrin complex. This could be of particular interest as receptor endocytosis influences the cell surface expression of receptors, the regulation of which is important in controlling the receptor-mediated function, hence regulating the synaptic efficiency of the neurotransmitter. Indeed, initial studies in our laboratory using radioligand binding have demonstrated that sucrose, a known inhibitor of clathrin mediated endocytosis (Heuser and Andersen, 1989) increases membrane associated 5-HT_3 receptor expression in 5-HT_3A receptor stably expressing HEK-293 cells (Barnes et al, unpublished).

5-HT_3 Receptor ligand binding domain

The 5-HT_3 receptor ligand binding site is situated within the extracellular *N*-terminal domain of the receptor. Although initially predicted from homology with other LGIC's, this was first demonstrated convincingly by Eiselé et al. (1993), with a chimera of the acetylcholine receptor subunit *N*-terminal domain and the *C*-terminal 5-HT_3 receptor four transmembrane domains, which displayed pharmacological characteristics of the acetylcholine receptor but 5-HT_3 channel-like properties (Eiselé et al., 1993). As mentioned previously, a large fraction of the structural data available concerning the 5-HT_3 ligand binding site is extrapolated from studies on the acetylcholine binding protein (AChBP) and nAChR. The AChBP consists of 10 β-strands and 10 loops, with a predicted binding pocket formed by a series of loops on the principle subunit face (binding loops A-C) and a series of β-strands on the complementary subunit face (binding loops D-F; Brejc at al., 2001). Homology studies between the AChBP and the 5-HT_3A receptor have identified the putative corresponding loop A-F regions within the 5-HT_3A receptor and the particular residues within the binding cleft (Maksay et al., 2003, Reeves et al., 2003, Thompson et al., 2005, Joshi et al., 2006). Mutating these residues to structurally similar or dissimilar amino acids has indicated their role in ligand binding and validates much of the information arising from structural homology models. Individual residues within the loops that effect agonist and/or antagonist binding include Loop A (numbering of amino acids residues is in accordance to that of the murine 5-HT_3A receptor, on which the majority of the studies were preformed); N128, E129, F130 (Boess et al., 1997, Steward et al., 2000, Thompson et al., 2005, Sullivan et al., 2006, Yan et al., 2006), Loop B; S182,W183, H185, D189 (Spier and Lummis, 2000, Reeves et al., 2003, Joshi et al., 2006), Loop C; E225, F226, I228, D229, Y234, E236 (Mochizuki et al., 1999, Hope et al., 1999, Schreiter et al., 2003, Beene et al., 2004, Thompson et al., 2005, Suryanarayanan et al., 2005), Loop D; W90, R92, Y94, (Spier and Lummis, 2000, Yan and White, 2002, Yan et al., 2006), Loop E; Y141, Y143, Y153, G148 (Venkataramen et al., 2002, Beene et al., 2004, Price and Lummis, 2004, Joshi et al., 2006), Loop F; W195, D204 (Spier and Lummis, 2000, Thompson et al., 2006). The h5-HT_{3A} subunit amino acid sequence is identical to that of the murine receptor in binding loops A, B and D. The differences between the sequences in loops C, E and F maybe partially responsible for pharmacological inter-species differences (see below).

In addition, a number of residues have been identified within the *N*-terminal domain, which may not have direct involvement in ligand binding but are essential for subunit structure/assembly. These include; W95, W102, W121, W214 (Spier and Lummis, 2000), P56, P104, P123, P170 (Deane and Lummis, 2001), Y50, Y91, Y141, Y234 (Price and Lummis, 2004).

There are a somewhat limited number of 5-HT_3 receptor agonists available in comparison to the extensive list of antagonists, many of which are highly selective. 1-(m-Chlorophenyl)-biguanide (mCPBG) is a potent high affinity 5-HT_3 receptor agonist (Kilpatrick et al., 1990), derived from the less potent agonist, phenylbiguanide (PBG) (Hoyer and Neijt, 1987). PBG is approximately equipotent to another, but less selective, 5-HT_3 receptor agonist, 2-methyl-5-HT (Richardson et al., 1985; Hoyer and Neijt, 1987; Kilpatrick et al., 1990). However, the above agonists appear to be partial agonists in at least some systems (Boess et al., 1992). The endogenous 'full' agonist, 5-HT, is placed in the rank order of agonist affinity above PBG and 2-methyl-5-HT, but below mCPBG (Bufton et al., 1993). Agonist binding displays positive co-operativity with Hill coefficients ranging from 1.5 - 3 (Kilpatrick et al., 1990; Boess et al., 1992; Miyake et al., 1995; Steward et al., 2000; Mott et al., 2001). This suggests that at least two agonist molecules are required to fully activate the receptor ion-channel.

Identification of the original selective 5-HT_3 receptor antagonists (e.g. tropisetron, ondansetron; Richardson et al., 1985; Butler et al., 1988) greatly helped the characterisation of 5-HT_3 receptors. Modelled on the low potency antagonist cocaine, MDL 72222 (Fozard, 1984) was the first 5-HT_3 receptor selective antagonist to be identified. However, many selective, high affinity compounds soon became available including ICS 205-930 (tropisetron, Richardson et al 1985), GR38032F (ondansetron, Butler et al., 1988), BRL 43694 (granisetron; Sanger and Nelson, 1989), GR65630, and zacopride (for a review on 5-HT_3 receptor antagonist structures see, Gozlan and Langlois, 1992). A number of non-selective 5-HT_3 receptor antagonists are recognised including quizapine, which displays nanomolar affinity, the dopamine D_2 receptor antagonist, metoclopramide, and the nicotinic acetylcholine receptor

antagonist, d-tubocurarine. The appearance of radiolabelled ligands ([^{3}H]-ICS205-930; [^{3}H]-GR65630; [^{3}H]quipazine; [^{3}H]zacopride; Hoyer and Neijt 1987; Kilpatrick and Tyers, 1987; Barnes et al., 1988a; Milburn and Peroutka, 1988) allowed the direct labelling of 5-HT_3 receptors and the performance of competition studies to allow a detailed affinity profile of competing ligands (Kilpatrick et al., 1987; Barnes et al., 1988a; Milburn and Peroutka, 1988; Barnes et al., 1989a; Bufton et al., 1993; Steward et al., 1995).

The affinity of many 5-HT_3 receptor selective/non-selective ligands varies between species, resulting in characteristic individual pharmacological profiles. For example, d-tubocurarine shows approximately 1800-fold lower affinity of for the guinea pig and h5-HT_3 receptor compared to the murine orthologue (Newberry et al., 1991; Bufton et al., 1993; Hope et al., 1999, Yan et al., 2006). In addition, mCPBG is 100 times less potent at the h5-HT_3A receptor than at the rat orthologue and the partial agonist, 2-Me-5-HT acts as a full agonist at h5-HT_{3A} receptors (Miyake et al,. 1995). More significantly, PBG does not act as either an agonist or an antagonist at the guinea-pig 5-HT_3A receptor (Butler et al., 1990, Lankiewicz et al., 1998).

Despite speculation that a heteromeric 5-HT_3AB receptor would display differing pharmacology to that of the 5-HT_3A homomer, the heteromeric 5-HT_3AB receptor is relatively pharmacologically indistinguishable using 5-HT_3 selective ligands. Since pentameric is the arrangement of receptor subunit, a succession of diverse binding sites is possible in the heteromeric receptors. The atomic force microscopy (AFM) proposed a subunit arrangement of BBABA in human 5-HT_3AB receptors, presenting the possibility that ligands bind to A+B–, B+A– and/or B+B– binding sites (Barrera et al. 2005). Nevertheless, the understanding is further complicated by the fact that it has been indicated by Lummis and colleagues primarily in the murine receptor that at least one binding site is at A – A interface for receptor function in the 5-HT_3AB receptor (Lochner and Lummis 2010); however potentially there may be differences in the stoichiometries of mouse and human heteromeric receptors, or potentially data from either the atomic force microscopy or mutagenesis study may not represent the situation in vivo (Lochner and Lummis 2010; Thomson et al., 2011). Only minor

differences are apparent, with a slight reduction in potency of 5-HT and d-tubocurarine at heteromeric compared to homomeric 5-HT_3AB receptors (Davies et al., 1999; Hanna et al., 2000; Brady et al., 2001). Selectivity between 5-HT_3A and 5-HT_3AB receptors can be achieved, however, with the $GABA_A$ receptor channel blocker, picrotoxin. The IC_{50} for picrotoxin is around 30 μM for homomeric 5-HT_3A receptors compared to 3 mM for heteromeric 5-HT_3AB receptors (Das and Dillion, 2003).

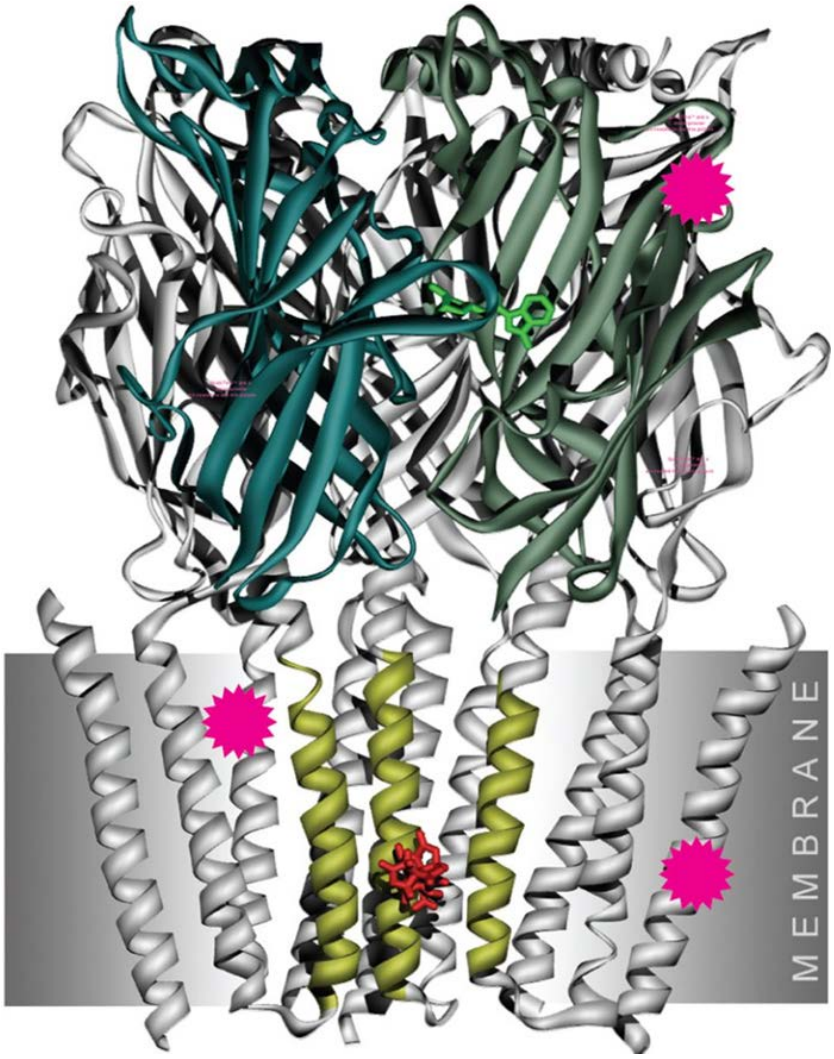


Diagram 3. 5-HT_3 receptor binding sites. Model of the 5-HT_3 receptor (with two subunits removed for clarity) showing granisetron (*green*) and picrotoxin (*red*) docked into their known binding sites in the ECD and in the pore, respectively. Other possible binding sites (*pink*), whose locations have not yet been confirmed, are an allosteric site in the Extracellular domain, an interhelical site in the TMD, and a lipid transmembrane site at the membrane-receptor boundary. Adapted from Lummis 2012

Coupling of agonist binding to channel gating

The second transmembrane domain (TM2) of each subunit forms the central ion-channel pore of the cys-cys loop ligand-gated ion-channel family (Akabas et al., 1994; Xu and Akabas., 1996; Miyazawa et al., 2003). TM2 is believed to be an α-helical structure that bends to form a central "gate" controlling the permeation of ions through the cell lipid membrane (Reeves et al., 2001; Panicker et al., 2002; Peters et al., 2005). It appears that there is at least one break in the α-helical structure, forming a kink in TM2, where the highly conserved 9' leucine and 13' valine residues form hydrophobic interactions, bringing together adjacent subunit α-helices to form a core diameter too small for ion-permeation (Miyazawa et al., 2003; Lummis, 2004).

The gate acts as both a barrier to prevent ion permeation in the closed state and a link to transduction mechanisms conveying channel opening (Panicker et al., 2002). It has long been documented that the TM2 helices undergo a conformational change upon receptor opening (Unwin, 1995). Unwin and colleagues have more recently demonstrated that an inner β sheet formation of the nAChR α subunit extracellular binding domain is rotated 15º in an activated state (Unwin et al., 2002). Structural studies of the nAChR indicated that a region within this inner β sheet, the β1- β2 loop, may have an interaction with the protruding TM2 and thereby relay the activation twist that breaks the hydrophobic interactions and causes channel opening (Miyazawa, 2003). In support of this hypothesis, an interaction between the β1-β2 loops and the extracellular end of TM2 within the 5-HT_3 receptor has been confirmed (Reeves et al., 2005). Additional channel gating studies with both the nAChR and $GABA_A$ receptor indicate the importance of residues within the cys-loop and TM2-TM3 linker (Kash et al., 2003; Bouzat et al., 2004; Sala et al., 2005). In the resting state the nAChR cys-cys loop and β1-β2 loop straddles the TM2-TM3 linker (Unwin, 2005), providing further supporting evidence for these regions being responsible for the transduction mechanism that links agonist binding and gating of the pre-transmembrane domain one region. Specifically, increased potency for both 5-HT and 2-Me-5-HT resulted after mutation of the amino acid arginine (R^{222}), to an alanine residue, with the latter converting from a partial to a full-agonist (Hu et al., 2003). More recently, the *cis-trans* isomerisation of a proline residue, conserved between the

5-HT_3 receptor and the nAChR, within the TM2-TM3 linker has been identified as a requirement for receptor activation (Lummis et al., 2005). This group has therefore suggested that upon agonist binding the cys-cys loop/β1-β2 loop "clamp" is released from the TM2-TM3 linker, allowing *cis-trans* isomerisation of the proline residue, resulting in a switch from the closed to open states. The binding of two molecules in non-adjacent binding sites, as postulated for the nAChR, may be required to create the full torsion twist required for ion-channel opening (Lummis, 2004).

Ion-channel conduction of the 5-HT_3 receptor

Early studies on 5-HT-induced responses led to the proposal that the 5-HT_3 receptor incorporated a ligand-gated ion-channel that produced rapid depolarising currents mediated by cations (Higashi and Nishi, 1982; Surprenant and Crist, 1988). Further direct evidence however, was produced by Derkach et al. (1989). Using an excised (outside-out) patch clamp recording technique from on neurons of the guinea-pig submucousal plexus, they demonstrated that channel activity similar to that produced by the nAChR, could be evoked up to 5 hours after excision of the outside-out patch. This finding indicated that neither GTP-binding proteins nor a diffusible intracellular second messenger system appeared necessary for 5-HT_3 mediated currents. Finally, 5-HT was shown to act as a fast-synaptic neurotransmitter within the mammalian brain, specifically rat amygdala neurons (Suigita et al., 1992). This was the first demonstration that an amine, rather than an amino acid, was capable of such activity.

Despite having similar electrophysiological properties to the nAChR, single-channel conductance of the 5-HT_3 receptor is considerably (see further) lower (~ 40 pS for the nAChR; Derkach et al., 1989). Moreover, single-channel conductance values varied considerably between different studies. Outside-out preparations of native neuronal tissue generally yielded single-channel conductances between 8 and 17 pS; In comparison, neuronal cell lines expressing the 5-HT_3 receptor typically had sub-pS single-channel conductances (see table below).

Anatomical area/ cell line	**Conductance (pS)**	**Reference**
Guinea pig submucosal plexus	9.2 & 14.8	Derkach et al., 1989
Rabbit nodose ganglion	16.5	Malone et al., 1991
Mouse superior cervical ganglion	8.9	Hussy et al., 1994
Rat superior cervical ganglion	11	Yang et al., 1992
Mouse and rat hippocampus	8.3 & 10.5	Jones and Surprenant 1994
N18	0.59	Yang 1990
N1E-15	0.31/ 0.63/ 5.6	Lambert et al., 1989 Hussy et al., 1994 van Hooft et al., 1994
NG108-15	9 & 13 (undifferentiated) 4.4 (differentiated)	Shao et al., 1991
Cloned receptor	0.63 (m5-HT_3A) and 0.24-0.74 (h5-HT_3A)	Hussy et al., 1994 Brown et al., 1998

Single channel conductance of 5-HT_3 receptor from various species and cell types.

When taking into consideration that the sub-pS values were determined by fluctuation analysis and not directly measured and that the findings of Yang et al. (1992), showed significantly lower conductance with fluctuation analysis compared to single-channel recording of the rat superior cervical ganglion, the authors suggested that two populations of 5-HT_3 receptors may exist within these cells. The lower conducting population would not be detectable over noise level when directly measuring single-

channel openings but would influence the fluctuation analysis, resulting in an average current of the two receptor forms.

Despite high homology with the nAChR TM2 amino acid sequence and similar receptor pore size values being estimated (Yang, 1990; Boess et al., 1992; Peters et al., 1992) the TM2 domains for both 5-HT_3 and nAChR are surrounded by rings of negatively charged amino acids, which appear to control the rate of cationic transport through the ion-channel (Imoto et al., 1988; Konno et al., 1991). Investigations into the possible mechanisms that lower 5-HT_3 receptor cationic conductance highlighted the importance of a positively charged lysine residue at position 4' of the 5-HT_3 TM2 domain. However, mutagenic studies targeted at this residue failed to provide support for this hypothesis (Gunthorpe et al., 2000).

Upon cloning of the 5-HT_3B subunit it was discovered that the heteromeric 5-HT_3AB receptor displays a larger single-channel conductance (16 pS) than the homomeric, resembling that from studies on native neuronal receptors (Davies et al., 1999). Somewhat paradoxically however, the sequence of the 5-HT_{3B} receptor subunit does not align with the pattern of negatively charged amino acids shown to promote cationic conductance (Imoto et al., 1988; Davies et al., 1999). A series of studies on chimeric 5-HT_3A and 5-HT_3B receptors, led Kelley and colleagues (2003a) to identify a region within the TM2-TM3 large cytoplasmic loop which determines single-channel conductance, the HA-stretch. More specifically, mutagenesis of three arginine residues within this region in the 5-HT_3A subunit, to their 5-HT_3B subunit equivalents, resulted in single-channel conductance levels similar to the heteromeric receptor (Kelley et al., 2003a; Peters et al., 2005). The authors proposed that the TM2-TM3 large cytoplasmic loops of adjacent subunits form a vestibule with portals framed by the residues within the HA-stretch, thus directly controlling ion-flux (Kelley et al., 2003a; Peters et al., 2004).

In addition to affecting the single-channel conductance of the receptor, the presence of h5-HT_{3B} subunits within the heteromeric 5-HT_3AB receptor also alters the current-voltage relationship (Davies et al., 1999). Although both homomeric and heteromeric receptors have a reversal potential of approximately 0 mV (Brown et al., 1998; Davies et al., 1999), the homomeric receptors are inwardly rectifying, opposed to the linear current/voltage relationship displayed by the human heteromeric receptor (Brown et al., 1998; Davies et al., 1999; Brady et al., 2001). The rodent heteromeric receptors are similar to the homomeric receptor and display inward rectification (Hanna et al., 2000).

Ion-channel selectivity

Early electrophysiological studies indicate that the 5-HT_3 receptor current consists largely of monovalent cations, with relatively low Ca^{2+} and Mg^{2+} permeabilities (Peter et al., 1988; Lummis 2012). Moreover, divalent cations have an inhibitory effect on the 5-HT_3 mediated current at higher concentrations (Yang et al., 1990; Lovinger 1991; Gill et al, 1995). Ca^{2+} blockade in these studies did not affect the current-voltage relationship, indicating that these effects are voltage-independent. However, in *Xenopus* ooyctes expressing the cloned rat 5-HT_3A receptor, Ca^{2+} mediated blockade did appear voltage-dependent, suggesting some species differences may be present (Maricq et al. 1991). Some studies have identified Ca^{2+} permeability of the 5-HT_3 receptor in both native preparations (Rondé and Nichols, 1998) and in heterologous expression systems (Hargreaves et al., 1994) with more recent ion permeation studies calculating relative permeabilities of P_{Na}/P_{Cs} and P_{Ca}/P_{Cs} to 0.9 and 1.00, respectively (Brown et al., 1998).

Upon cloning of the 5-HT_{3B} subunit, some of the aforementioned discrepancies in 5-HT_3 receptor ion-channel permeation were accounted for. Davies et al. (1999) reported that the homomeric 5-HT_3A receptor is non-selective for both monovalent and divalent cations, in agreement with that determined by Brown et al. (1998). The heteromeric 5-HT_3AB receptor however, was substantially less permeant to Ca^{2+} ions, and impermeable to Mg^{2+} ions than the homomeric recpetor (Davies et al., 1999).

In addition to controlling the conductance of the 5-HT_3 receptor, rings of negatively charged amino acids within the TM2 domain are important for ion-selectivity (see figure 2). Mutation of the negatively charged glutamate residue at position -1' to an uncharged alanine residue, resulted in a relatively non-selective 5-HT_3 receptor (P_{Na}/P_{Cl} = 0.89; Thompson and Lummis, 2003). Further mutation of 13'valine to threonine (the corresponding amino acid of the anionic glycine and $GABA_A$ receptors) and the insertion of a proline residue preceding the Glu -1'Ala residue, produced a 5-HT mediated receptor with significant chloride permeability (P_{Cl}/P_{Na} = 12.3; Gunthorpe and Lummis, 2001). Interestingly, the 5-HT_{3B} subunit sequence contains an alanine residue rather than a glutamate at position -1' of the TM2 domain, which suggests that only 2 glutamate residues at this position are required for cation selection and may contribute to the reduced Ca^{2+} permeability of the heteromeric receptor (Davies et al., 1999).

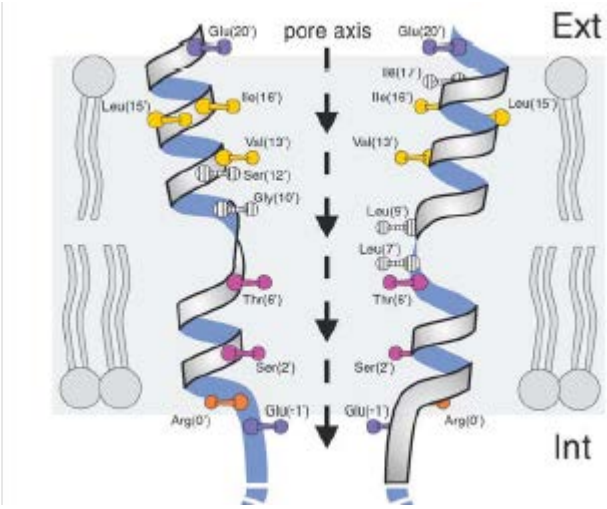


Figure 2. Representation of TM2 of the 5-HT_3A receptor. Coloured according to side chain charge of residue; basic (orange); acidic (blue); polar (purple); non-polar (yellow). Adapted from Keramidas et al. (2004).

Desensitisation kinetics

5-HT_3 receptor currents rapidly desensitize in the presence of agonist, with a decay phase that has been fitted to both monophasic (Neijt et al., 1989; Yang, 1990; Hu and Lovinger, 2005) and biphasic (Yakel et al., 1991; Boddeke et al., 1996) exponentials. Superfusing concentrations of 5-HT which do not activate the ion-channel also reduce

the inward current in response to pressure-applied 5-HT, with receptors fully recovering within 2 minute rest periods (Bartrup and Newberry, 1996). The kinetics of 5-HT_3 receptor desensitization can be modified by a number factors including; [Ca^{2+}] (Yakel et al., 1993; Lobitz et al., 2001), voltage (Yakel, 1996), tetraethylammonium ions (Kooyman et al., 1993a), phosphatases e.g. calcineurin (Boddeke et al., 1996) and alcohols e.g. 5-hydroxyindole (5OHI) and trichloroethanol (TCE; the active metabolite of the anaesthetic chloral hydrate; Kooyman et al., 1993b; Bentley and Barnes, 1998; Zhou et al., 1998). Specific amino acid substitutions at the following residues also alter desensitization kinetics; S248 within TM1 (Lobitz et al., 2001), 4' lysine and 9' leucine within TM2 (Yakel et al., 1993; Gunthorpe et al., 2000), D298 within the TM2-TM3 linker (Hu and Lovinger, 2005) and R427 within the large intracellular loop (Hu et al., 2006). Furthermore, Arginine residue A427 exhibits critical importance for receptor desensitisation through potential interaction with adjoining amino acids (Hu et al., 2006). Finally, 5-HT_3 receptor subunit composition also appears important. Co-expression of a truncated h5-HT_3A subunit and the wild-type h5-HT_3A subunit, within a heterologous expression system, forms a slower desensitizing receptor (Brüss et al., 2000b). Heteromeric 5-HT_3AB receptors recover from desensitization considerably faster than the homomeric 5-HT_3A receptor and kinetic modelling studies indicate that the mechanism of desensitisation is altered by the 5-HT_3B subunit (Hapfelmeier et al., 2003).

Allosteric modulation

An array of different compounds, modulate the 5-HT_3 receptor mediated response. Assuming a similar action to other ligand-gated ion-channels, the interactions possibly occur at sites distinct from the agonist binding domain. Potentiation of the 5-HT_3 mediated current occurs by alcohols such as, ethanol, butanol, isopentanol and trichloroethanol (Machu and Harris, 1994; Lovinger and Zhou., 1994; Downie et al., 1995; Bentley and Barnes, 1998). Furthermore, allosteric modulation of 5-HT_3 receptor function has been shown within neuronal cells of transgenic mice over-expressing 5-HT_3 receptors in frontal cortex neurons, which may indicate a function for 5-HT_3 receptors in mediating the behavioural effects of alcohol (Sung et al., 2000). In contrast, some functional studies and experiments with the guinea-pig 5-HT_{3A} subunit orthologue have shown that ethanol inhibits 5-HT_3 receptor mediated

responses (for review see, Parker et al., 1996a). These effects do not seem to occur via direct phosphorylation of the putative phosphorylation sites within the 5-HT_3 receptor (Machu et al., 1999). Further, via post translational modification by protein kinases the kinetics of the receptor can change. The large cytoplasmic loop contains a cluster of putative protein phosphorylation sites for PKA which substantially accelerate desensitisation (Lankiewicz et al., 2000)

Anaesthetic agents also appear to modulate the 5-HT_3 receptor current. Both local and general anaesthetics were shown to inhibit the 5-HT-induced influx of [^{14}C]-guanidinium via a mechanism not correlating with anaesthetic lipophilicity (Barann et al., 1993). In addition, the local anaesthetics, bupivacaine, tetracaine, cocaine and procaine were shown to inhibit 5-HT_3 receptor-mediated currents in rat nodose ganglion (Fan and Weight, 1994). Barbiturate anaesthetics, including thiopentone and pentobarbital, also inhibit the responses to 5-HT (Jenkins et al,. 1996; Barann et al., 1997). In contrast, the volatile anaesthetics, which include halothane and isoflurane, potentiate the 5-HT_3 receptor-mediated current, an effect that is additive to the potentiation by ethanol, which therefore indicates that these compounds exert their effects at different molecular locations (Parker et al., 1996a; Jenkins et al., 1996).

Other compounds that have an allosteric effect on the 5-HT_3 receptor include; 5-hydroxyindole analogues that delay agonist dissociation by increasing channel opening (van Hooft et al., 1997), gonadol steroids, for example, 17β-estradiol and progesterone (Wetzel et al., 1998), imidazolines; ifenprodil and ketamine (Peters et al., 1991; Molderings et al., 1996), bisindolymaleimide (Coultrap et al., 1999), L-type Ca^{2+} channel blockers; verapamil, diltiazem, and nimodipine (Hargreaves et al., 1996) and divalent cations, for example, Cu^{2+} and Cd^{2+}, Zn^{2+} (Lovinger, 1991, Hubbard and Lummis, 2000), Mg^{2+} (Maricq et al., 1991) and Ca^{2+} (Maricq et al., 1991; Nieymer and Lummis, 2001; van Hooft and Wadman, 2003), which appear to inhibit 5-HT_3-mediated currents at high concentrations (Lovinger, 1991),

The effect of some of the previously mentioned 5-HT_3 receptor allosteric modulators is altered by the presence of the 5-HT_3B subunit. For example, the heteromeric 5-HT_3AB receptor seems less prone to modulation by ethanol and also the effects of

trichloroethanol are much less apparent compared to the homomeric 5-HT_3A receptor (Hayrepetyan et al., 2005). Similarly, the ability of the volatile anaesthetics to potentiate the 5-HT_3 receptor mediated responses was reduced, or even inverted to an inhibition in the case of isoflurane, by the co-expression of the 5-HT_3B subunit with the 5-HT_3A subunit (Stevens et al., 2005).

5-HT and digestive function

Serotonin is predominately (~95%) synthesised and stored within the gastrointestinal (GI) tract in the enterocromaffin cells (EC) in the enteric mucosa. Smaller stores are present in serotonergic neurons of the enteric nervous system (ENS), where 5-HT mediates both slow and fast neurotransmission (Gershon, 2004,2005: Gershon and Tack, 2007) by acting via multiple receptor subtypes (5-$HT_{1,2,3,4,7,\ 1P}$). Animal studies indicated that serotonin can activate submucosal intrinsic primary afferent neurons (IPANs) via 5-HT_{1P} receptor to initiate a peristaltic reflex (Pan and Gershon, 200; Wade et al., 1994). Further, myenteric IPANs by 5-HT_3 receptors (Bertrand et al., 1997) are able to initiate giant migrating contractions (Karaus and Sarna, 1987; Nagakura et al., 2002: Sarna, 2007). Additionally, stimulation of the 5-HT_4 receptor can increase secretion of acetylcholine and calcitonin gene-related peptide (CGRP; Grinder, 2003) facilitating neurotransmission in prokinetic pathways (Liu et al., 2005; Pan and Callgan, 1994; Ren et al., 2008). 5-HT_3 receptors also play a role in the regulation of GI motility, visceral sensation and nociception and GI secretion processes as well as the modulation of brain-gut axis influencing the GI tract and brain crosstalk. (Gershon 2004; 2005).

5-HT_3 receptor expression has been shown in the myenteric and submucosal plexus in the somata of neuronal cells in human gut. Additionally, they have been identified in enterocytes within the mucosal layer and in immune cells within the lamina propria (Sakurai-Yamashita et al 2000; Kapeller et al., 2011). Moreover 5-HT_3 receptors have been shown to be present on cholinergic myenteric neurons modulating the release of acetylcholine (Kapeller et al 2011; Gershon 1999; Zhou et al., 1999), as well

as-submucosal and myenteric ganglia involved in control of secretion and gut motility (Galligan 1996; Mawe et al., 1986; Tack et al., 1992; Wade et al 1994). Additionally, 5-HT_3 receptors appear on intrinsic afferents and the vagus nerve and are directly linked with crosstalk between GI tract and the brain, known as the brain-gut axis (Gershon 2004, 2005). Finally, 5-HT_3 receptors are expressed in immune cells and activation leads to T cell activation and secretion of cytokines and prostaglandins (Khan and Hichami 1999; Fiebich et al., 2004).

5-HT_3 receptor and emesis

Vomiting can be initiated by the release of serotonin from enterochromaffin cells of the intestinal mucosa, resulting in the stimulation of peripheral 5-HT_3 receptors in the adjacent vagal afferent neurons (Minami et al., 2003). This effect is concurrent with the local release of serotonin in the area postrema, located on the dorsal medulla elongata. The co-action of 5-HT at both locations triggers the vomiting reflex, in which co-ordinated contractions of abdominal muscles diaphragm and the muscles used in respiratory inspiration causing intense pressure to formed in the stomach which released when the upper oesophageal sphincter relaxes leading to rapid expulsion of the stomach content through the mouth.

Whilst vomiting can be considered a protective reflex to rapidly clear potentially poisonous substances from the GI tract, it can also be triggered by emotional events e.g. fear, disgust and is a common after effect of general anaesthetics. Vomiting is also a distressing side effect of cancer chemotherapy and radiotherapy.

At present the 5-HT_3 receptor antagonists tropisetron, ondansetron, granisetron, dolasetron and palonosetron are used for controlling chemotherapy and radiotherapy induced nausea and vomiting as well as in postoperative nausea and vomiting. Furthermore, there is clinical evidence that 5-HT_3 receptor antagonists could be useful for the alleviation of nausea and vomiting during pregnancy and following caesarean section (Fujii et al., 1998; Einarson et al., 2004). The therapeutic effect of 5-HT_3 receptor antagonists derives via the inhibition on the vomiting reflex. It has been suggested that the 5-HT_3B receptor subunit could play an important contribution to the effectiveness of these compounds. Further, Tremblay et al. in 2003 in a study

on polymorphisms have demonstrated a positive link between mutation in the promoter region of the 5-HT_3B gene and the frequency of vomiting

5-HT_3 receptor involvement in eating disorders

There is evidence that 5-HT receptor antagonists may be helpful in the treatment of eating disorders such as anorexia and bulimia, which often co-exist with depressive disorder. Increased vagal afferent nerve activity has been demonstrated in association with the binge eating and vomiting associated with bulimia. The 5-HT_3 receptor antagonist ondansetron has been used to suppress the effect (Faris et al.,2000, 2006). Additionally, the symptoms of depression were also reduced in those subjects (Faris et al., 2006). A number of 5-HT_3 receptor antagonists, such as tropisetron, ondansetron and bemesetron, are able to improve anorexia (Hammer et al., 1990; Jiang and Gietzen, 1994; Washburn et al., 1994). Another 5-HT_3 receptor antagonist m-chlorophenylpiperazine has also been reported to produce a similar improvement in patient mood and self-perception of body image, although it has been suggested that these affects could be facilitated by actions at other 5-HT receptor subtypes (Frank et al., 2001).

5-HT_3 receptor in pain and inflammation

Peripheral and central 5-HT_3 receptors are thought to be involved in nociception and inflammation (Zeitz et al., 2002; Fiebich et al., 2004; De la Vega et al., 2005; Khan and Hichame 1999). In particular, the expression of 5-HT_3 receptors has been reported on primary afferents (Hamon et at., 1989) that transmit sensory and nociceptive signals from periphery to the brain. Clinically, 5-HT_3 receptors are targeted in treatments for migraine, postoperative pain (Schowrer and Ramadori 1993; Schowrer et al., 1995; Kyriakides et al., 1999). Furthermore, following tissue injury a complex process of inflammation and nociception is initiated. Painful symptoms have been attributed to the serotonin-mediated release of various neuropeptides in particular substance P from damaged/ affected tissue. Indeed, 5-HT_3 receptor antagonists appear to have analgesic effects (Saria et al 1990), particularly, tropisetron, in fibromyalgia, rheumatoid arthritis, tendonitis and lower back pain

(Faerber et al., 2001; Stratz and Muller 2000; Stratz et al., 2001; Samborski et al 2004; Stratz and Muller 2004a, 2004b). Interestingly, co-administration of 5-HT_3 receptor antagonists with some NSAIDs have interesting outcomes on their ability in alleviating pain. The pain relieving properties of paracetamol when in co-administration with granisetron or tropisetron are blocked. However, the analgesic effect of aspirin when co-administered with ondansetron is unaffected. (Sandrini et al., 2002, 2003; Pickering et al., 2006).

5-HT_3 receptors and Irritable bowel syndrome (IBS)

Irritable bowel syndrome (IBS) is a chronic dysfunctional condition of the gastrointestinal (GI) tract that affects between 5-20% of the general adult population (Agreus et al., 2000; Hillila et al., 2004; Hungin et la., 2003). The incidence among women is twice as high as men, and onset after the age of 50 is unusual (Mertz HR 2003). Symptoms ranging from constipation (IBS-C) to diarrhoea (IBS-D) predominate, or a combination/succession of the two (alternating IBS), often accompanied with severe abdominal pain and discomfort (Longtreth et al., 2006). The direct annual medical costs in the US were estimated to be close to $8 billion, the greater proportion of this cost (94%) not attributed to medication but implying unmet medical need for IBS therapy. The cost has been estimated as direct medical cost and as indirect medical cost approximating productivity loss and sick leave (Hulisz D., 2004). The exact pathogenesis of IBS is currently unknown; however, the typical symptoms of pain, altered GI motility and secretion disturbance suggest a potential dysfunction in neural pathways involved in sensory and motor function. Moreover, the interaction and relationship between causal and secondary alterations are unclear. Subpopulations of sufferers present changes in gut flora (e.g. post infectious), and there is an increased incidence of psychological co-morbidities (depression, anxiety, stress) in IBS sufferers. (clinical practice guide-Andresen et al., 2011)

Since the initial description of 5-HT_3 receptor presence in the GI tract, various groups have speculated on the role of the diversity of 5-HT_3 receptor subtypes (Fletcher and Barnes 1998; Gaddum and Picarelli 1957). It has been hypothesised that sequence

variants of HTR3 genes may contribute to the aetiology of functional GI disorders (Humphrey et al., 1999; Jones and Blackburn, 2002). It has been highlighted by the Niesler group that variants of HTR3A and HTR3E are associated with IBS-D patients, leading to a significant up-regulation of the expression of the receptor subunit in vitro, indicating an increased susceptibility in those individuals (Kapeller et al., 2008).

Given the evidence implicating the involvement of 5-HT_3 receptor dysfunction in IBS, drugs acting at 5-HT_3 receptors have been targeted as potential treatments for the condition. Visceral hypersensitivity, as often experienced in IBS, is positively influenced by 5-HT_3 receptor antagonists (Mayer et al., 2001). Alosetron, ondanseron and cilasetron have been shown to display beneficial effects on visceral sensation, gut motility and secretion in clinical trials of IBS patients (De Ponti, 2004; Gershon and Tack, 2007; Ford et al., 2009). In particular, alosetron appears effective in alleviating the symptoms of IBS-D by decreasing gut transit time, increasing fluid absorption and reducing pain (De Ponti, 2004; Ford et al., 2009). Unfortunately, the broad utilisation of 5-HT_3 receptor antagonists (alosetron, ramosetron, palonosetron) has been hampered due to incidences, although rare, of severe constipation and ischaemic colitis associated with alosetron (Clayton et al 1999; Ford et al 2009) the first of the setron class to be introduced. However, ramoretron, was launched in Japan in 2008 with no reports of the same serious adverse effects indicating that safer modulators of 5-HT_3 receptor can be achieved (Matsueda et al., 2008).

Future directions; ligands

Partial activation of ligand gated ion channels is an established drug discovery strategy that could potentially improve side effect profiles of classical ligands (Hogg and Bertrand 2007) and could potentially be utilised in the treatment of IBS. Based on the intrinsic activity that would possess could be used in the treatment either of IBS-C or IBS-D. A high affinity 5-HT_3 receptor low intrinsic activity partial agonist would be predicted to attenuate 5-HT_3 receptor function in the presence of excessive endogenous serotonin, but still maintain a basal level of receptor function. Such compounds could reduce risk of constipation and other unwanted side effects associated with classical antagonists currently used in receptor inhibition in IBS-D. One such compound is AMR-SER-67 (Usyatinsky et al., 2008) along with other

potential compounds by AMRI (Manning et al., 2010). Furthermore, compounds with a high intrinsic activity should alleviate IBS-C symptoms. Indeed, one of the leading candidates DDP733 with high intrinsic activity has passed Phase IIb trial for IBS-C (Evangelista 2007). However, in some patients the compound caused emesis, predictable outcome for a high intrinsic activity 5-HT_3 receptor partial agonist.

Although 5-HT_3 agonists show some promise, the incidence of side effects is a hindrance to further development. In addition to the orthosteric 5-HT site the 5-HT_3 receptor complex possesses a topologically distinct allosteric modulatory site, with the potential of presenting therapeutic target. The advantage of positive allosteric modulators (PAMs) as a target for therapeutic pharmaceutical purposes would be the ability to modulate receptor function whilst retaining spatial and temporal control (Williams et al., 2011). In particular, a positive allosteric modulator may be able to offer relief from reduced gastrointestinal motility disorders such as IBS-C. Of particular interest is 5-chloroindole (Cl-indole), a steel corrosion inhibitor (Moretti et al., 1996; see Figure 1), which we demonstrated (Newman et al., 2013) to be a relatively potent Type II PAM of the 5-HT_3A receptor (using nomenclature coined for PAMs of the nicotinic Ach receptors (Gronlien et al., 2007; Williams et al., 2011). An intriguing possibility is that an endogenous ligand could associate with the Cl-indole recognition site expressed in the 5-HT_3 receptor analogous to the b-carbolines at the benzodiazepine site on the $GABA_A$ receptor (Peña et al., 1986; Evans and Lowry, 2007). This could offer further pharmacological rationale for drug design and more avenues to manipulate selectively the function of 5-HT_3 receptor for therapeutic purposes in the treatment of IBS. As an added advantage, such compound (negative allosteric modulators) could prove a useful pharmacological tool in identifying and characterise low intrinsic activity partial agonists. This could be of particular assistance in the development of partial agonists in targeting the therapy of all forms of IBS.

Aims

In an attempt to increase our understanding of the 5-HT_3 receptor pharmacology and in an effort to aid in the development of drugs that could provide better targeting of the receptor with perspective therapeutic applications we aimed to investigate the Cl-indole and the effect that has on 5-HT_3 receptor pharmacology utilising an array of experiments and techniques.

I would test the hypothesis that Cl-indole is a positive allosteric modulator of the 5-HT_3 receptor. I would attempt to characterise and identify the potential response in the presence of Cl-indole. Further I would attempt to classify the compound as potent (or not) Type I or II PAM of the 5-HT_3 receptor, in the human and murine receptor subtype. I would then be able to further assess if there is any species selectivity of the drug, which would potential allows to identify mechanistically how it produces its effects compared to other PAM (for instance TCE and 5-Hydroxyindole). I would then proceed to, using data for the murine receptor subtype from Changeux group already published accordingly to the h-5HT3A subunit employing single amino acid mutation in attempted to knock-out the modulatory effect of Cl-indole, thus allowing us to map the binding pocket of the allosteric compound.

Furthermore, I would try to characterise the pharmacology of Cl-indole using a range of full and partial agonists of the 5-HT_3 receptor (mCPBG, 2-methyl-5HT, DDP733, quipazine, dopamine and 5-HT) in order to understand the potential allosteric site, as well as gaining knowledge in distinguishing low intrinsic activity partial agonist from antagonists to the 5-HT_3 receptor

In order to fully characterise the response that Cl-indole is able to facilitate, I would use Cl-indole to assess if it is able to reactivate desensitised 5-HT_3 receptors once an agonist has stimulated them.

Additionally, I would test the hypothesis that the presence of 5-chloroindole and other allosteric modulator, like progesterone (an endogenous allosteric modulator), are able to modulate the receptor internalisation, as well as the activity of agonists and/or antagonists, which would provide us with further understanding of the mechanism of allosteric modulation (positive and negative). Moreover, we would gain further understanding of the function of the homomeric 5-HT_3A receptor

Moreover the stability of the h5-HT_3A receptor and the importance of the c-terminus are of interest as the understanding of the receptor pharmacology and allosterism maybe be linked to the stability of the receptor and hence I would examine in the presence of chaotropic factor the importance of the c-terminus of the subunit.

Chapter 2: Materials and Methods

2.1.1 Quantitation of DNA samples

The quantity of DNA within a sample was calculated using a spectrophotometer (Thermo Spectronic Biomate 3). At a wavelength of 260 nm (1 cm path length) the absorbance, or A_{260}, reflects the quantity of DNA or RNA. An Optical Density (OD) reading of one corresponds approximately to, 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and RNA, and 33 µg/ml for single-stranded oligonucleotides. Comparing the ratio of absorption at, OD_{260}:OD_{280}, gives an indication of the purity of the sample, where protein absorbance is measured at 280 nm. Typically an OD_{260}: OD_{280} > 1.8 is considered relatively pure and the concentration of the DNA accurate (Sambrook and Russell, 2001).

2.1.2 Restriction endonuclease digest

One unit of restriction enzyme will completely digest 1 µg of DNA in a 50 µl reaction in 60 minutes. Restriction endonucleases are supplied with a 10X buffer, which all contain magnesium chloride. Some restriction enzymes require bovine serum albumin (BSA) at a final concentration of 100 µg/ml, however, the presence of BSA is not inhibitory to enzymes that do not require it. Table 3 represents a typical 50 µl single digest used in this study; most enzymes require incubation for 1-2 hours at 37ºC. If a double digest was required for cleavage at two distinct regions within a DNA molecule, this could be completed simultaneously with a buffer compatible to both enzymes, or sequentially either after heat inactivation (at 65ºC/80ºC) of the initial digesting enzyme or purification via agarose gel electrophoresis (see section 2.3.3). If less than 1 µg of DNA was to be digested a smaller scale reaction was used, with a

total volume of 20 µl, reducing the volume of constituents, proportionally. Digests using specific enzymes were altered to meet the conditions described in the manufacturer's instructions (New England Biolabs (NEB), UK).

Restriction Digest	Volume (µl)
DNA (1-5µg)	x
100x Bovine Serum Albumin (BSA)	0.5
10x Buffer	5.0
Restriction Endonuclease (20 U/µl)	1.5
ddH_2O	50- sum of above

Table 3. Constituents within a typical restriction enzyme digest.

2.1.3 Agarose gel electrophoresis

Separation of DNA through a solid agarose matrix, can be used to purify products from a PCR reaction, fragments from a restriction digest or characterise the restriction map of cDNA clones. The net negative charge of DNA allows it to migrate towards the anode of an electrophoresis tank, at a rate directly proportional to the size of the fragment.

TAE 50x	
TRIS	2 M
Glacial acetic acid	50 mM
EDTA pH 8	5% (v/v)

Table 4. Agarose gel electrophoresis buffer (stored at room Temperature).

Agarose 1.5% in 1X TAE buffer (Table 4) was heated in a microwave until fully dissolved. After brief cooling, a 10 mg/ml ethidium bromide stock was added giving a final concentration of 0.05 µg/ml, the latter binds to the DNA and allows visualisation under ultraviolet (UV) illumination. Gels were cast with an appropriate comb and allowed to set at room temperature.

6x DNA Loading Buffer	
Bromomphenol Blue	0.25% w/v
Xylene cyanol FF	0.25% w/v
Ficoll (Type 400)	15%

Table 5. Agarose gel sample buffer (stored at room temperature).

Samples were mixed with a 6X DNA loading buffer (Table 5) and ran alongside DNA molecular weight markers (Hyperladder I, Bioline). Typically, the gel was immersed in 1X TAE in an electrophoresis tank (Thistle Scientific) and ran for approximately 1 hour at 100 V. Gels were then viewed under UV illumination using a UV transilluminator (Ultraviolet Products (UVP)).

2.1.4 Agarose gel extraction and purification of DNA

Extraction of DNA from an agarose matrix was achieved using the GenElute^{TM} Gel Extraction Kit (Sigma, NA1111). DNA bands, visualised under ultraviolet illumination, were excised from the gel, weighed and incubated with three gel volumes (w/v) of gel solubilisation solution at 50-60ºC for 10 minutes. Spin columns

were prepared with 500 µl of column preparation solution and centrifuged at 13,000 x g for 1 min at room temperature. When the gel had completely dissolved, DNA was precipitated with one gel volume of molecular biology grade isopropanol. Gel solution was then loaded onto the column, in 700 µl aliquots, centrifuged at 13,000 x g for 1 min, discarding the flow-through. The column was then washed with 700 µl of Wash solution (Wash Solution Concentrate G supplied, diluted with 95-100% ethanol, giving a final concentration of approximately 75% ethanol) centrifuged at 13,000 x g, for 1 minute, discarding the flow-through and repeating the spin to remove residual ethanol. The column was then transferred to a fresh collection tube and 50 µl of ddH_20 was added to the column and incubated for 1 min. The column was then centrifuged at 13,000 x g for 1 min, and the eluted DNA solution stored at -20ºC.

2.1.5 DNA ligation

DNA ligases catalyse the formation of phosphodiester bond between the 5'-phosphate and 3'-hydroxyl termini of DNA or RNA, of both cohesive ended or blunt ended fragments. The majority of enzymes used in molecular cloning are isolated from bacteria or bacteriophage. T4 DNA ligase, from bacteriophage T4, was used predominantly to ligate cDNA into plasmid vectors (NEB, UK). Reactions to ligate cohesive ends were incubated at 4ºC overnight with the buffer supplied containing the cofactor and activators, Mg^{2+}, ATP and dithiothreitol (Promega Incorporation, USA). Typically, at least two insert vector molar ratios were assembled (1:1 or 3:1) , with 1U (Weiss Units) of T4 DNA ligase (Weiss et al., 1968). Table 6 below represents a standard 20 µl reaction with a 0.5 kb insert, a 3kb plasmid vector and a molar ratio of 1:1. Control reactions omitting either the cDNA insert to assess the incidence of self-

ligating vector or T4 DNA ligase to observe the presence of any undigested vector were also prepared.

T4 DNA Ligation	
Vector DNA	200 ng
Insert DNA	34 ng
10x Ligase Buffer	2 µl
T4 DNA Ligase (Weiss Units)	1U
ddH_20	Final vol. of 20 µl

Table 6. Components of a typical DNA ligation reaction.

2.1.6 Bacterial Culture

Growth of bacteria was performed using Luria-Bertani Broth (LB) 25g/l (Sigma). Sterility was achieved via autoclaving, and liquid media supplemented with the appropriate antibiotic where necessary. Liquid culture was incubated at 37ºC shaking at 225-250rpm. For solid phase growth of bacterial colonies, agar was added to the LB at 2% w/v and autoclaved. When required, it was re-melted in a microwave before allowing cooling to 55ºc for supplementing with antibiotic. Plates poured and set at room temperature were stored at 4ºc for up to 2 weeks.

2.1.6A Generation of chemically 'super competent' bacterial cells

The use of bacteria to propagate plasmid vectors has become wide standard laboratory practice. Bacterial cells for example, *E.coli*, may be electroporated or heat shocked to

induce the uptake of DNA. Bacteria cells required for DNA propagation were treated chemically with cations resulting in a 'competent state'. The protocol below is based on that first described by Cohen et al. (1972).

5 µl of XL10-Gold® supercompetent cells (Stratagene, USA) frozen at -80ºC, were used to inoculate a 5 ml LB culture, supplemented with tetracycline 12.5 µg/ml and grown for approximately 12 hours at 37ºC with agitation. The culture was then used to inoculate a 250-500 ml expansion culture containing tetracycline 12.5 µg/ml and $MgCl_2$ 20 mM. Growth of the bacteria was monitored until the culture had an OD_{600} of 0.4-0.8 (exponential growth phase). The cells were centrifuged at 4,000 x g at 4ºC and resuspended in 0.4 culture volumes of TB1 (Table 5) and incubated on ice for 5 minutes. Cells were recovered by centrifugation, the supernatant removed and the pellet resuspended in 0.04 volumes of TB2 (Table 5) and incubated on ice for 45-60mins. Aliquots of 200 µl were frozen in liquid nitrogen before storage at -80ºC. Both TB1 and TB2, were filter sterilised prior to use (Fisher Brand, UK).

[A] TB 1 pH 5.8 (Acetic acid)	
$KC_2H_3O_2$	30 mM
$CaCl_2$	10 mM
$MnCl_2$	50 mM
RbCl	100 mM
Glycerol	15%

[B] TB 2 pH 6.6 (KOH)	
MOPS	10 mM
$CaCl_2$	75 mM
RbCl	10 mM
Glycerol	10%

Table 7. [A]-TB1 [B]-TB2 Buffer constituents for generation of "supercompetent" bacterial cells (stored at room temperature).

2.2 Transformation of competent bacteria

Approximately 10µl of diluted maxiprep in ddH2O (1:10) was incubated with 200µl of super-competent bacterial cells on ice for 20 minutes. A heat shock of 42ºC was applied for 1 minute, followed by incubation on ice for 5 minutes. Furthermore, 500µl of LB was added and the bacteria were incubated for 2 hours at 37ºc with agitation. Then they were plated on to agar LB containing ampicillin10µg/ml and grown overnight for approximately 16 hours at 37ºc.

Single colonies from LB agar plates were chosen and they were grown over 6 hours in a 5ml ampicillin supplemented (10µg/ml; Sigma) LB culture. Subsequently, 1ml of this culture was added into 250ml of LB containing 10µg/ml ampicillin (Sigma) and grown overnight at 37ºc with agitation. The cells were then harvested by centrifugation for 5 minutes at 10,000 x g.

A high purity plasmid purification commercial kit (Marligen Biosciences, Inc) was followed as per manufacturer's instructions. The DNA pellets were resuspended in 300µl of TE buffer, followed by purity and concentration determination using spectrophotometry.

2.3 DNA extraction and purification from bacterial hosts

Section 2.3A Small scale

Preparation of plasmid DNA from *E.Coli* was achieved using alkaline lysis with sodium dodecyl sulphate (SDS), a method first outlined by Birnboim and Doly in 1979. The use of an anionic detergent at high pH, disrupts the cell wall, denatures proteins and chromosomal DNA. However, the alkali is unable to destroy base pairing in a tightly coiled closed circular plasmid (Sambrook and Russell, 2001). Small scale purification of plasmids was primarily required for subsequent automated sequencing

of cDNA clones. In which case, a commercially available kit (Wizard® Plus SV Minipreps DNA Purification System – Promega) using a column to bind, isolate and elute DNA was followed as to manufacturer's instructions. Where such a pure preparation was unnecessary, excess reagent buffers supplied in the kits or produced following manufacturer's instructions were used with an organic solvent i.e. chloroform, added to increase purity without the columns. Buffer constituents are listed below (Table 8), and the procedure for more crude preparations outlined.

Single colonies from LB agar plates were picked and grown overnight in a 5 ml LB culture supplemented with the appropriate antibiotic, typically ampicillin 100 µg/ml (Sigma). 1-5 mls of the culture were harvested by centrifugation, for 5 minutes at 10,000 x g. Supernatant was removed and the pellet completely resuspended in 250 µl of Cell Resuspension Solution. Cells were lysed with 250 µl of Cell Lysis Solution, mixing gently by inverting the tube and incubated for 5 minutes at room temperature. Upon partial clearing, samples were neutralised with 350 µl of neutralisation solution. Precipitated cell debris and chromosomal DNA were pelleted by centrifugation, at 14,000 x g for 10 minutes at room temperature. An equal volume of chloroform was added, mixing with gentle inversion and samples spun at 14,000 x g for 2 minutes. The aqueous layer was transferred to a fresh tube and an equal volume of isopropanol added to precipitate DNA, followed by centrifugation at 14,000 x g for 30 minutes at 4ºC. The resulting pellet was washed in approximately 70% ice cold ethanol, spun for 3 minutes at 14,000 x g, and left to air dry for approximately 10 minutes. Pellets were resuspended in 30-50 µl ddH_20.

Cell Resuspension Solution	
Tris-HCl pH7.5	0.125 M
EDTA	10 mM
RNase A	100 µg/ml

[A]: Stored at 4ºC

Cell Lysis Solution	
NaOH	0.2 M
SDS	1%

[B]:Stored at room temperature

Neutralization Solution	
Guanidine hydrochloride	4.09 M
Potassium acetate	0.759 M
Glacial acetic acid	2.12 M

[C]: Stored at room temperature

Table 8. Buffers supplied by manufactures for small DNA extraction from bacterial hosts. **[A]** Cell resuspenion buffer; **[B]** Cell lysis solution; **[C]** Neutralization solution.

2.3B Large scale

Small scale preparations of plasmid vectors does not yield the quantity of cDNA required for experimental procedures such as mammalian cell transfection. Large scale extraction was achieved using the same Alkaline – SDS method described in section 2.1.7.1. A high purity plasmid purification commercial kit (Marligen Biosciences, Inc) was followed to manufacturer's instructions. DNA pellets were resuspended in 300 µl of ddH_20, purity and concentration determined by spectrophotometry (see section 2.1.1)

2.4 Polymerase chain reaction

The polymerase chain reaction (PCR) utilises thermostable DNA polymerases to replicate specific regions of DNA, *in vitro.* Two synthetic oligonucleotides are required to prime DNA synthesis by the polymerase. Additional requirements include the four deoxynucleoside triphosphates (dNTPS; Bioline), monovalent and divalent

cations, typically Mg^{2+}, a buffer to maintain pH and the template DNA. For faithful copying of the cDNA template a proofreading polymerase, *Pfu* (Stratagene), was preferred due to the greater fidelity achieved compared with more common polymerase's such as *Taq*. The cycles of PCR are divided in to three segments; 1) denaturation, 2) annealing and 3) extension. The denaturing temperature is determined by the G/C content of the double stranded DNA template and the polymerase stability. Higher G/C content, results in a higher denaturing temperature; typical maximum temperatures are 94-95ºC. An initial denaturing step may be employed prior to cycling to ensure complete separation of the template DNA, especially those that are large and/or closed circular.

The annealing temperature is affected largely by the melting temperature (T_m) of the oligonucleotides. This can be estimated using a number of equations (see Sambook and Russell, 2001) and the annealing temperature is typically set at 3-5ºC lower than the oligonuleotide T_M, however, optimisation is often required. Extension of the annealed primer by the DNA polymerase occurs at the optimum temperature for each particular enzyme, for example *Pfu*'s optimum condition is 72-78ºC. Polymerases vary in time required to extend a set number of nucleotides/minute, however, extension is often carried out for approximately 1-2 minutes per 1000 base pairs of template DNA. A final extension step after cycling has been completed may help to fully extend products. Typically, 30 cycles were programmed and carried out in an automated thermal cycler (TC-312 Techne®). An example reaction used in this study and the appropriate cycling parameters are shown in Table 7

PCR reaction	
DNA polymerase buffer x10	5 µl
Each dNTP	200 µM
Template DNA	~ 10 ng
Forward Primer	1 µM
Reverse Primer	1 µM
DNA polymerase	1- 5U
ddH_20	Final Vol. of 50 µl

Initial Denature		2 min at 95ºC
30 Cycles		1 min at 95ºC 1 min at 55ºC 2 min at 72ºC
Final Extension		10 min at 72ºC

Table 9. Components within a typical PCR reaction with example cycling parameters.

2.5 Mutagenesis

2.5A Site-directed mutagenesis- QuickChange®

The QuikChange® site-directed mutagenesis method, (Stratagene) may be used to delete, insert or switch nucleotides of a particular cDNA clone within a plasmid vector.

Manufacturer's instructions for the design of oligonucleotide primers were followed meticulously. Forward and reverse complementary oligonucleotides 25-45 base pairs in length, contained the desired mutation and had a melting temperature ≥ 78ºC. Typically, PCR parameters were as followed; an initial denaturing step of 95ºC for 30 seconds followed by 12-18 cycles of denaturing for 30 seconds at 95ºC, annealing for 1 minute at 55ºC and extending for 2 minutes/kb of template at 68ºC.

QuikChange® Reaction	
10X *pfu* reaction Buffer	5 µl
dsDNA template	5-50 ng
Forward Primer	150 ng
Reverse Primer	150 ng
dNTP mix (10mM of each)	1 µl
ddH_20	Final vol. of 50 µl
Pfu Turbo	1 µl

Table 10. Components of a typical QuikChange® reaction.

2.5B Automated DNA sequencing

Verification of clone sequence and the success of mutagenesis were achieved by automated DNA sequencing. Samples prepared using small scale purification kits (section 2.1.7.1) were sent to Lark Technologies LTD (Hope End, Takeley, Essex) and sequenced using fluorescent dye terminator sequencing, a method derived from the original Sanger di-deoxy chain termination method (Sanger et al., 1977).

2.6 Cell cultures

2.6A Maintenance of HEK-293 cells

Cells were maintained at 37^{o}C in an atmosphere of 95% air, 5% CO_2, 100% relative humidity (LEEC cell culture incubator). Grown in T75 (75cm^2) tissue culture flask containing Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin (Sigma). To maintain healthy proliferation the cells were routinely passaged. At 100% confluency, media was aspirated off the cell monolayer and washed in 5 ml Dulbecco's Phosphate-Buffered Saline (D-PBS; Sigma). Cells were then dissociated from the flask with 2 mls of trypsin/EDTA (Sigma) wash followed by incubation with trypsin/EDTA for

5mins. The reaction was terminated with the addition of 8mls of serum containing DMEM that dilutes the trypsin and contains trypsin inhibitor α-1-antitrypsin. The cell suspension was then aliquoted 1:5 passage, thus 2mls of cell suspension in a T75 flask containing 15mls of complete media.

All cell culture was completed in a microbiological safety cabinet, preventing contamination with bacteria, fungi and viruses.

Section 2.6B Maintenance of COS-7 cells

Cell lines isolated from numerous species and tissues have become readily accessible commercially. These allow the expression of particular proteins in controlled environments, where the effect of mutated proteins can be determined in the absence of wild-type protein. COS-7 cells, an 'established' cell line from African green monkey kidney fibroblast cells, were used in this study, obtained originally by the European Collection of Cell cultures (ECACC; 87021302). They have no detectable endogenous 5-HT_3 receptor protein and are capable of expressing high levels of heterogeneously expressed protein.

Cells were maintained at 37^{o}C in an atmosphere of 95% air, 5% CO_2, 100% relative humidity (LEEC cell culture incubator). For experiments where temperature effects on protein expression were investigated, cells were incubated in identical atmospheric conditions, at reduced temperatures. Cultures were grown in T75 (75 cm^2) tissue culture flasks containing Dulbecco's Modified Eagles Medium (DMEM; Sigma), supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin

(Sigma). To maintain healthy proliferation the cells were routinely passaged. At approximately 100% confluency, media was aspirated off the cell monolayer and the cells washed in 5 mls of Dulbecco's Phosphate-Buffered Saline (D-PBS; Sigma). Cells were then dissociated from the flask with 2 mls of trypsin/EDTA (Sigma) for 2-5 mins. The reaction was quenched with serum containing DMEM, which in addition to diluting the trypsin, contains a trypsin inhibitor, α-1-antitrypsin. Routinely, 8 mls of DMEM quenched the reaction forming a 10 ml suspension culture. As an example, for a 1:5 passage, 2 mls of this suspension was used to seed a T75 flask containing 15 mls of media. Dividing this suspension culture between 10 or 2 flasks manipulated the speed of proliferation. All cell culture was completed in a microbiological safety cabinet, preventing contamination with bacteria, fungi and viruses.

2.6C Maintenance DG75 Lymphocytes

Cells were maintained at 37^{o}C in an atmosphere of 95% air, 5% CO_2, 100% relative humidity (LEEC cell culture incubator). Grown in T75 (75cm^2) tissue culture flask containing RPMI 1640 media (P.A.A) supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin (Sigma) (complete media). At 100% confluency, 10mls of cell suspension was added to 20mls complete media.

2.6D Long term storage of cell lines over liquid nitrogen and recovery

Cells were grown to 100% confluency, the adhere cell lines were dissociated from the flask with trypsin/EDTA. Media was added to quench the reaction and all cell lines were pelleted from the suspension by centrifugation at 1200 RCF for 5 minutes. The pellet was resuspended in 1ml of freezing media, 50% foetal calf serum, 40% DMEM

or RPMI 1640 as required and 10% Dimethylsulphoxide (DMSO; Sigma). To protect cells from 'snap freezing' 1ml aliquots in cryovials were frozen overnight at -80^{o}C in Mr Frosty™ (Wessington Cryogenics), containing room temperature isopropanol which very slowly allowed cells to reach –80^{o}C. Subsequently, the vials were stored in liquid nitrogen cell bank.

When required, cryovials were defrosted at 37^{o}C and added immediately to 7mls of supplemented media in a T25 (25cm^{2}) flask. Media was changed the following day and when cells reached confluency they were transferred to T75 flasks for culture, as described above as required for each cell line.

2.7 Transient Transfection of Mammalian cell lines by electroporation

The capacitance, voltage, time of pulse and the size of the cuvette were optimized previously for both HEK-293, COS-7 cell lines and the following protocol was used for both.

Pulsed electrical fields were used to insert plasmid cDNA into COS-7/HEK 293 cells. Although the exact mechanism underlying this highly effective technique is not fully understood, it is believed that hydrophilic pores within the cell membrane form with greater ease, as the transmembrane voltage increases (Weaver, 1993). During this transient pore opening period DNA may enter the cytoplasm. The effectiveness of electroporation relates to the pulse field length and strength. The capacitance, voltage, time of pulse and the size of the cuvette were optimized previously for the COS-7 cell line and the following applied.

Cells were grown to 100% confluency, dissociated using trypsin-EDTA and quenched with 5mls DMEM/MEM. The cells were pelleted by centrifugation at 1200 rcf and

resuspended in 1ml of media per flask of cells. The 1ml of cell suspension was added to the electroporation cuvette, 4 mm path length, (GeneFlow), containing the appropriate cDNA, total amount of 30 µg. The cell suspension containing the cDNA was incubated for 15 minutes before electroporating with an Easyject Plus (Equibio) at 400 V, 3 msec pulse with capacitance of 150µF.

Cells were immediately transferred to a T75 flask containing 40 mls of media which was subsequently divided in two. When transfected cells were required for immunocytochemistry techniques, 200 µl of transfection mixture was taken from the 40mls and added to appropriate wells of a 24 well plate that contained 500 µl of media and an autoclaved glass coverslip (13 mm diameter; VWR International). Cells were harvested 48-72 hours post transfection. Media was aspirated, and the cellular monolayer washed with 5 mls of ice-cold PBS. Cells were dissociated with 5 mls of ice-cold PBS using a cell scraper; the flask was subsequently rinsed with an additional 5 mls of ice-cold PBS to remove all remaining cells. The suspension was centrifuged, at 3700 x g for 6 minutes, PBS aspirated, and the pellet 'snap' frozen in liquid nitrogen and stored at -80 ^{o}C until required.

2.7.1 DG75 Cells Transfection

In order to transfect the DG75 B lymphocytes two transfection techniques were used, electroporation and P.E.I. The following protocol was used after intense optimisation.

2.7.1A Electroporation

When the cells reached 100% confluency, they were split 1:2 24 hours prior to transfection. The cells were, then, pelleted by centrifugation at 1200 rcf for 5mins and

resuspended in fresh HEPES buffered RPMI 1640 medium (5µl 1M HEPES buffer/ml) at concentration ~10^7 cells in 1 ml. The cell suspension was cooled on ice for 5mins. Additionally, 80µg of plasmid DNA was mixed in with 30µl of TE buffer into the electroporation cuvette. Then the 1 ml of cold cell suspension was added to the cuvette and it was gently mixed. The cell suspension was then electroporated using an Easyject Plus (Equibio) at 310 V, with capacitance of 900µF. Subsequently, the cell suspension was mixed well in the cuvette and incubated at room temperature for 10mins. The cell suspension was then added to 20mls of warm RPMI 1640 medium supplemented with 10% FCS and incubated for 24-36 hours at 37^{o}C in an atmosphere of 95% air, 5% CO_2, 100% relative humidity.

2.7.1B Polyethelyne (PEI)

The cells were pelleted by centrifugation at 1200 rcf for 5mins and resuspended in fresh RPMI 1640 medium. Moreover, 0.2-10mM PEI was mixed into 10-40µg of plasmid DNA in a ratio 1:1 and final volume of 500µl (the plasmid DNA was diluted in 5% Glucose in PBS). The mixture was incubated for 5-20mins at room temperature. Furthermore, 1ml of the cell suspension was added to the PEI-DNA mixture and incubated for 20mins at room temperature. Then the mixture was added into 40mls of warm RPMI 1640 media supplemented with 10% FCS and incubated for 24-36 hours at 37^{o}C in an atmosphere of 95% air, 5% CO_2, 100% relative humidity.

2.7.2 Stably expressing the human 5-HT3A subunit

HEK293 cells stably expressing the human 5-HT3A subunit (HEKh5-HT3A cells; Brady et al., 2001) 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 penicillin and 10 mg/ml streptomycin) and G418 (500 µg/ml) and maintained at 37°C, 5% CO2, 95% relative humidity. Approximately 24 hrs prior to [Ca2+]i assays, cells were seeded directly into poly-D-lysine coated, black-walled, clear bottomed, 96-well plates (Costar) with ~1 x 105 cells per well and similar for ELISA.

2.8 Preparation of Dialysis Tubing

We used dialysis tubing to dialyse FBS for ELISA experiments. Cut the tubing into pieces of convenient length (10-20cm). The tubes were then boiled for 10 minutes in 0.5L of tubing preparation solution 1 ((2% (w/v) sodium bicarbonate 1 mM EDTA (pH 8.0)). They were then rinsed twice in distilled water followed by boiling for 10 minutes in 0.5L of tubing preparation solution 2 (1 mM EDTA (pH 8.0)). Tubes were allowed to cool down and stored them in tubing preparation solution II at 4°C. Before used tubing was rinsed twice inside and out with distilled water.

Foetal Bovine serum was dialysed by ultrafiltration in dialysis tubing with cut off 10-14 KDa against 0.15 M NaCl over 48 hrs and the NaCl was renewed 3 times over that period. The dialysis reduces the concentration of "free" low molecular weight components such as nucleotides and amino acids. We were able to determine by HPLC that tryptophan and kynurenine reduced over 100 fold.

2.9 Preparation of protein samples

Whole cell homogenates

Whole cell lysates were prepared for radioligand binding (section 2.3.4) and Western blotting (section 2.3.4). Cell pellets harvested from transfections and frozen in pairs, were resuspended

in 5 mls of ice-cold Tris; 50 mM, pH 7.5. The suspension was homogenised, using a Polytron (Polytron; Kinematica EmbH Kriens, Switzerland) at maximum speed for 10 seconds ensuring the sample was kept on ice. When a high protein concentration was required for Western blots, pellets were homogenised by hand in a glass homogeniser; 500 µl of ice-cold Tris; 50 mM, pH 7.5 (Fisher Brand, UK).

Isolation of cell membranes

To obtain a purer sample of cell membranes, pellets homogenised in 5 mls Tris, as described in 2.2.4.1, were centrifuged, for 12 minutes at 20,000 x g, 4 ^{o}C. The pellet was then resuspended in 20 mls of ice-cold Tris; 50 mM pH 7.5, homogenised for 5 seconds with a polytron (max setting) and centrifugation repeated. The pellet, containing the membrane fraction of the cellular debris, was washed in 20 mls of Tris a further 3-8 times. The pellet was finally resuspended in 1-2 mls of Tris; 50 mM pH 7.5.

2.10 Characterisation of proteins

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins according to molecular weight via electrophoresis.

Gel preparation and electrophoresis

The gels were cast using glass plates (10.5 x 9.8 cm, with 1mm spacers) from a Minigel-Twin tank electrophoresis system (Biometra).

A 1:29 molar ratio of bisacrylamide:acrylamide (Severn Biotech Ltd), in a 30% stock, was used to prepare gel solutions. Table 2 indicates the components of the resolving and stacking gels (as well as buffers used for the assay).

Table 2	**RESOLVING GEL (10%)**	**STACKING GEL (3.5%)**
30% (w/v) Acrylamide stock (ml)	5	0.93
4x Lower Buffer (ml)	3.75	0
4x Upper Buffer (ml)	0	2
Water (ml)	6.12	4.95
10% (w/v) APS (µl)	100	100
TEMED (µl)	30	20
Total volume (ml)	15	8

Lower buffer: 1.5M Tris, 0.4% (w/v) SDS; pH 8.8

Upper buffer: 0.5M Tris, 0.4% (w/v) SDS; pH 6.8

SDS-PAGE reservoir buffer: 25mM Tris, 200mM glycine, 0.1% (w/v) SDS

2x SDS-PAGE loading buffer: 15% (v/v) glycerol, 125mM Tris, 5mM EDTA, 2% (w/v) SDS, 0.1%(w/v) bromophenol blue, 1% (v/v) 2-mercaptoethanol; pH 6.8

Resolving gel was poured into plates, 1 cm below the bottom of the required comb wells. To ensure a clean levelled line between stacking and resolving gels, butanol was added on top of the resolving gel until the resolving gel was set at room temperature. Butanol was subsequently removed and the plates were rinsed with water to remove any excess unpolymerised resolving gel components. The stacking gel was then added and either a 10 well comb was then inserted to cast the wells. The gel was then left to set at room temperature.

The tank was filled with SDS-PAGE running buffer ensuring the wells were fully submerged. Samples prepared in equal volume of 2 x SDS-PAGE sample buffer, boiled for approximately 10 minutes and centrifuged briefly, they were loaded into wells 20µl. Prestained protein molecular weight markers were also loaded (10µl) to

visualise migration and identify molecular weight of protein bands (New England Biolabs®, Inc). Electrophoresis was performed at 20mA per gel.

Western Blotting

Following gel electrophoresis the proteins were transferred via electrophoresis to an immunblotting membrane Polyvinylidene difluoride (PVDF; Immobilon P, Millipore). Transfer of the protein was achieved using a semi-dry system (Biometra), with Whatman 3MM paper saturated in transfer buffer acting as a reservoir. Presoaked Whatman papers were stacked ensuring no air bubbles were formed. Four papers were layered, before the PVDF membrane, soaked briefly in methanol and then in transfer buffer, was placed on the stack, followed by the polyacrylamide gel and four additional Whatman papers. Proteins required 0.8mA/cm^2 current, in order to be transferred onto the PVDF membrane due to their movement towards the anode, for approximately 1 hour.

Prior to the incubation with primary antibody, the PVDF was placed in blocking buffer (5% milk in TBS-T) for 1 hour at room temperature to block non-specific binding. The primary antibody (mouse anti-*myc* monoclonal antibody;) was diluted to 1:1000 concentration in 10mls of blocking buffer and incubated overnight at 4°c with gentle rocking (50 rpm Mini Orbital Shaker SO5; Stuart Scientific). Washing was performed for 1hour with multiple changes of TBS-T washing buffer. The secondary antibody was then diluted to 1:1000 in 10mls blocking buffer and incubated for 2 hours at room temperature on orbital shaker; secondary antibody coupled to the enzyme horseradish peroxidise (rabbit anti-mouse HRP). PVDF was then washed for 1 hour with multiple changes of TBS-T washing buffer. The chemiluminescent

substrate, SuperSignal® West Pico (Pierce) was mixed as described in manufactures instruction and incubated with the PVDF membrane for 5 minutes. Subsequent exposure of the blot to Hyperfilm™ ECL™ (Amersham Biosciences) and developing using Kodak chemicals, revealed any immunoreactive bands.

Amidoblack Staining

Amido black powder was dissolved (0.1% w/v) in a 50:10:40 methanol:acetic acid:water and used to highlight proteins on the PVDF membrane, before or after immunoblotting. Staining appeared after incubation for as little as 10 minutes and any background was removed with washing in buffer omitting the dye.

2.11 Radioligand Binding

5-HT_3 binding assay were performed using the labelled antagonist [^{3}H] Granisetron (63.5 Ci/mmol, final assay concentration 1nm, PerkinElmer). Transfected cells were homogenised by sonication. Calculation of specific binding capacity per mg of cellular protein was achieved using 300µl total volume assays. Assays, carried out in triplicates, contained 100µl of radioligand, 100µl of whole cell homogenate and 100µl of binding buffer (Ice-cold 50mM Tris pH 7.5). To determine the level of non-specific binding, additional assays replacing binding buffer with 100µl of "cold" competing ligand, typically 10µM Ondansetron, were also measured in triplicate. Assays were incubated for 1 hour at room temperature on a shaker and terminated with rapid filtration using a Brandel Cell Harvester (Model M-25RP). Ligand-receptor complexes were captured with Whatman GF/B glass fibre filters (Semat Technical (UK) Ltd), pre-soaked for 1-3 hours in Tris, Polyethyleneimine 3% (v/v). Filters were rinsed 2 times, with 3 mls of ice-cold binding buffer and placed into vials with scintillation fluid (Optiphase HiSafe 2; Perkin Elmer) for counting the radioactivity content, in a liquid scintillation counter (Tri-carb 1500 TR Liquid Scintillation

Analyser; Packard). Mean of triplicates was calculated and the specific binding (Total-Nonspecific) of [^{3}H] Granisetron expressed per mg of whole cell homogenate protein.

Pharmacological profile

Studies to determine the range of ligand concentration required to inhibit specific binding of [^{3}H]granisetron were performed as described above. Competition curves were fitted to a one-site competition model with an iterative curve fitting computer programme (KaleidaGraph™, Abelbeck Software).

$$b = (B_{max}[L]^n/([L]^n + (K)^n)$$

Where b = bound ligand, B_{max} = the maximal specific binding, [L] =concentration of competing ligand, K = IC_{50} of the competing ligand (the concentration at which 50% of the binding sites are occupied by the competing ligand), n = the Hill coefficient.

2.12 High Progesterone Tratment

NG108-15 mouse neuroblastomas x rat glioma hybrid cells express functional 5-HT3 receptor (Yakel and Jackson, 1988; Hoyer and Neijt, 1987) were cultured in T75 flasks. DMEM was supplemented with 10% fetal bovine serum, 1% penicillin – streptomycin and HAT; 0.1 mM hypoxanthine, 400 nM aminopterin, 0.016 mM thymidine. We utilised this cell line gonadal steroid progesterone interacting with ligand gated ion channels (Review Rupprecht et al., 2001). To investigate the ability of progesterone to effect membrane bound 5-HT_3 receptor expression after prolonged exposure NG108-15 cells were culture for 3 days in culture media in the presence of either vehicle (chloroform) or 1 µM progesterone (HP) or 1 µM progesterone that was withdrawn for the final 6hrs of the incubation (PWD). Cells were then counted and

undergone a membrane preparation in 50mM Tris buffer, as described previously. Membrane preparations were then used utilising radioligand binding protocol as previously were ~1nM [^{3}H]granisetron defined specific binding when competed by specific antagonist ondansetron (10 µM) and expressed in fmol bound per million cells.

2.13 Protein Assay Quantification Bradford standard

Protein concentration of samples was determined using Bio-rad reagent (Bio-rad) based on an assay first described by Bradford in 1976. Bio-rad reagent was diluted 1:5 with ddH_20 and filtered with chromatography paper to remove particulate. Bovine serum albumin (BSA) was diluted to 0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml with the appropriate sample buffer, for use as protein standards. 1ml of filtered Bio-rad reagent and 20µl of each sample was added to disposable polystyrene cuvettes (Biorad). Cuvettes were inverted, for mixing, and the reaction incubated for 10 minutes. Absorbance was measured at 595nm in a spectrophotometer (Thermo Spectronic Biomate 3) and BSA standards used to calculate a protein concentration/ absorbance curve. Measuring each sample, the spectrophotometer programme gave a protein concentration reading for each sample calculated from absorbance at 595nm and the BSA standard curve

2.14 Immunoreactivity

2.14.1 Immunocytochemistry

To investigate the expression and cellular localisation of specific proteins appropriate antibodies were used.

Culture media was aspirated from the wells and the coverslips washed 3 times with ice-cold PBS. Fixation with 2% (v/v) paraformaldehyde, lower than standard PFA to minimise permeabilisation, was achieved at room temperature for 20mins, with subsequent washing with PBS for 30mins. We used lower than the standard 4% to

minimise cell membrane damage and to allow more reliable results of our cell surface assay.

Cells were incubated for 1 hour at room temperature with blocking buffer (PBS, 10% foetal bovine serum; FCS). Moreover, primary antibody (monoclonal anti-myc clone 9B11, Cell Signalling) was diluted 1:1000 with blocking buffer and incubated overnight at 4°c. The cells were then washed in PBS prior to incubation for 2 hours at room temperature with secondary antibody, horse anti mouse IgG2A AlexaFluor 488 (Molecular Probes), diluted 1:300 in blocking buffer. After extensive washing the cells were then permeabilised with PBS/TX100 0.3% for 1 hour at room temperature followed subsequently by hour incubation with blocking buffer TX100 0.3%. A second incubation with primary antibody (anti-myc) was preformed overnight at 4°c, with antibody diluted 1:1000 in blocking buffer TX100 0.3%. Cells were washed repeatedly and incubated for 2 hours at room temperature with secondary antibody, 1:300 horse anti mouse IgG2A AlexaFluor 568. Cells were then washed extensively with PBS. The slides were mounted with VectorShield Hardset mounting medium (Vector). Images were captured using a confocal microscope (Carl Zeiss Laser Scanning System LSM 510).) or using a digital camera with IPLab (Nikon) software.

2.14.2 Surface expression of receptor using ELISA

Cell surface ELISA was performed under nonpermeant conditions as described previously (Kniazeffet al., 2004; Cheng et al., 2005). HEK 293 cells stably expressing myc-tagged h5-HT3A receptor or vector control HEK293 cells seeded at 105 cells per well in a 96 well tissue culture plate and culture in dialysed or “normal” non- dialysed FCS enriched media. 24hrs later, the cells were incubated with 100µg/ml cycloheximide or 10µg/ml Brefeldin A and the appropriate drug treatment (vehicle, 5-HT, ondansetron, DDP733, Cl-Indole) for 5hrs.

The media was then aspired and plates were incubated on ice with 2 ml of ice cold PBS; subsequently they incubated PBS containing 5% skimmed milk powder (Blocking buffer) for 30 min. Cells were then incubated on ice with the anti-myc monoclonal antibody 9E10 / 9B11 in blocking buffer for 2 hrs. The cells were aspirated and washed with ice cold PBS, following by incubation with 4% paraformaldehyde for 10 minutes and then washing with PBS. Cells were then incubated with secondary antibody coupled to the enzyme horseradish peroxidise (rabbit anti-mouse HRP) for 1 h. The cells were aspirated and washed three times with PBS, three times with PBS, and the reaction product was developed with 3,3',5,5'-tetramethylbenzidine (TMB) and the reaction was terminated with the addition of 2 M H_2SO_4 after ~15 min. Optical density was measured at 450nm.

2.14.3 Free floating cell Immunocytochemistry

Typically 10^7 cells were washed with cold PBS followed by centrifugation. Cells were then fixed for 10 min with 4% paraformaldehyde, resuspended in cold PBS and then spun at 1200rcf for 5 min. The cells were resuspended in blocking buffer (10% FCS/PBS) for 30mins at room temperature (RT) followed by resuspension in anti-human IgM Alexafluor 488 (1:1000; in PBS), 2hrs at RT. The cells were subsequently wash in cold PBS and 0.3% Triton-X100/PBS was added to cells to permeabilise cell membranes, incubated for 10 mins at RT then centrifuged for 5 min. Mouse anti-myc monoclonal antibody was diluted in 0.3% Triton-X100/PBS buffer (1:1000) was incubated with cells for 1 hr at RT. The secondary, goat anti-mouse Alexa Fluor 568 (1:1000), was added after washing with blocking buffer and incubated for 1hr at RT. Cells were then washed with excess PBS to allow membranes to reseal, the final pellets were resuspended in 500µl of PBS and an aliquot of cells spotted in duplicate onto multiwell slides and left to air-dry. To visualise the nuclei Vectorshield was

added to each cell spot, slides were kept in the dark at 4^{o}C. Staining was analysed using a Nikon Eclipse E600 epifluorescence microscope.

2.15 Functional assay

2.15.1 Calcium influx single Cuvette assay for DG75 cells

The activity of the expressed h5-HT$_3$A receptors was assayed by measuring elevations in the intracellular calcium concentration induced by application of 5-HT or other agonists. Two methods were used; either a Fura-2 two-wavelength fluorescence ratio measurement or Fluo-4AM. Fura-2 acetoxymethyl ester (Molecular Probes) diluted from a 1 mM stock solution in dimethylsulfoxide, was used at a final concentration of 2 µM in Hank's balanced salt solution (HBSS). The cells were loaded with Fura-2 or Fluo-4AM for 60 min at 37^{o}C temperature before the solution was replaced with fresh HBSS.

Furthermore, into quartz cuvette 2mls cell suspension was added. Through a hole in the holder, chemicals such as receptor agonists could be injected during data acquisition to measure acute responses. A magnetic stir crossbar on the bottom of the cuvette ensured rapid mixing of the solutions. The experiments were done at 37°C. Any changes in intracellular calcium ion concentration were measured by determining the ratio of 510 nm light emitted by alternate stimulation with 340 and 380 nm. Ionomycin, a known ionophore with preference to Ca^{2+}, (1µM concentration) was used to determine the maximum value of response, giving also a positive control for the experimental set up, and EGTA/HBSS to chelate Ca^{2+} from the extracellular solution in order to identify minimum response.

Fluorescence measurements were performed with a Hitachi F-2000 (Tokyo, Japan) fluorescence spectrophotometer. The background autofluorescence of the unloaded cells was measured and subtracted from the raw signal before calculation of the ratio.

2.15.2 HEK293 cell Flex Station Quantification of $[Ca^{2+}]_i$

Cells were washed with 1 x HBSS (Hank's balanced salt solution; Invitrogen) and incubated with Fluo-4 acetoxymethyl (AM) ester (2.5 µM; Molecular Probes) for 60 min at room temperature. Cells were then washed in HBSS and incubated for a further 30 min (room temperature) prior to assay. Alteration in $[Ca^{2+}]$i was measured using a FlexStation (Molecular Devices) with fluorescence levels assessed every 3 seconds. Buffer or Cl-indole was added after 20 seconds, agonist/partial agonist (or antagonist tested for agonist activity) was added after 80 seconds and recordings continued with the subsequent addition of carbachol (1 mM final concentration; a muscarinic receptor agonist used as positive control of calcium influx on HEK293 cells) at 320 seconds. In antagonism studies, antagonists were preincubated for half an hour prior to analysis (Newman et al., 2013).

For the desensitisation studies the above assay was modified; we were able to instead of carbachol to add to the following stimulation by agonist/ partial agonist the cells were exposed to vehicle, Cl-indole, trichloroethanol (TCE), 5-HT or quipazine.

Concentration response data were analysed by computer-assisted iterative curve fitting according to the three parameter logistic equation (Barnes et al., 1992a).

2.15.3 Single cell electrophysiology

Macroscopic currents were recorded in the whole-cell recording mode of the patch-clamp technique from HEKh5-HT3A cells cultured on coverslips using infrared DIC (inverted Olympus FV1000 confocal microscope; Olympus Keymed, Southend on Sea, UK). 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 potentials and currents were recorded using an NPI SEC-10L amplifier (Scientifica, Harpenden, UK), low-pass Bessel filtered at 1 kHz (NL-125, Digitimer Ltd, Welwyn Garden City, UK) and digitized at 10 kHz by a Power 1401 (CED Ltd, Cambridge, UK). Experiments were performed at room temperature with the cells voltage- clamped at -60 mV. Stimulation and data acquisition were controlled using Signal software (version 3; CED) (Newman et al., 2013).

2.15.4 Neuronal contraction of mouse bladder

Male BALB/c mice, aged 8-12 weeks, were killed with a rising CO_2 concentration followed by cervical fracture. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and European Communities Council Directive 86/09/EEC. The urinary bladder was removed and placed in physiological saline (in mM; NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3, glucose 11.1 pH and [O_2] were regulated by continuously bubbling the

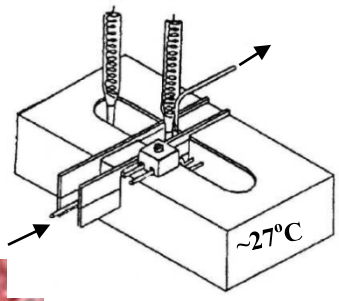
solution reservoir with 95% O_2 and 5% CO_2). Other organs were harvested for experimental use in other projects. Connective tissue surrounding the bladder was first removed before the ventral wall of the bladder was opened longitudinally from the bladder neck to the apex of the dome with the urothelium left intact. Tissue strips (6-8 mm long, 1-2 mm wide) were cut along the craniocaudal axis of the dorsal surface for organ bath studies. Each bladder strip was mounted in a 500 µl organ bath and connected to an isometric transducer (HLT050/D ADinstrument, Chalgrove, UK) under an initial tension of 9.8 mN and allowed to equilibrate (i.e. to accommodate under tension) for at least 60 min. Electrical field stimulation was delivered every minute by a Grass S48 stimulator (Grass Instruments, Quincy, MA) with a 0.1 ms pulse width (PW), at 90 V with a 500 ms train duration (TD) at a train frequency of 10 Hz. Contraction data were digitized using a Powerlab/4SP data acquisition system using Chart v.4.2.3 software (ADInstruments, Chalgrove, UK). (Newman, Batis et al., 2013)

2.15.5 Vagus nerve grease gap electrophysiological assay

Tissue preparation

Female Wistar rats 150–250 g were killed by cervical dislocation and segments of cervical vagus nerve 15–20 mm, without the associated nodose ganglion, were gently dissected away from the attached carotid artery and placed in gassed 95% O_2/ 5% CO_2 chilled Krebs buffer mM: $NaCl_2$ 120, KCl 4.75, KH_2 PO 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11). The connective tissue sheath was removed from the nerve and the desheathed nerve e was transferred to an inclined lightly greased Vaseline heated Perspex block 27^{o}C. The wick of a silver–silver chloride electrode, mounted in a Pasteur pipette, was placed on one end of the isolated nerve. The grease barrier was formed by applying grease over the wick of the electrode thereby

electrically isolating one end of the nerve. The wick of a second silver–silver chloride electrode was placed on to record the potential difference between the two portions of the vagus nerve either side of the grease barrier which was monitored After placing of the recording electrodes, Krebs buffer 4–5 ml/min; 27^{o}C, constantly gassed with 95% O_2, 5% CO_2 over the portion of vagus nerve in the continuous perfusion chamber. The whole procedure was usually completed within 15 min of dissection.



Vagus nerve marked V and trachea marked t as was used for our experemental preparation.

Agonists / Partial agonist assay

Noncumulative concentration response curves to agonists were obtained by including the agonist in the perfusing Krebs buffer for appropriate time followed by a washout period between exposures to each agonist concentration.

Chapter 3: Results

3.1 Allosteric modulation

3.1.1 5-Chloroindole

3.1.1A Impact of Cl-indole upon ligand affinity for the h5-HT$_3$A receptor

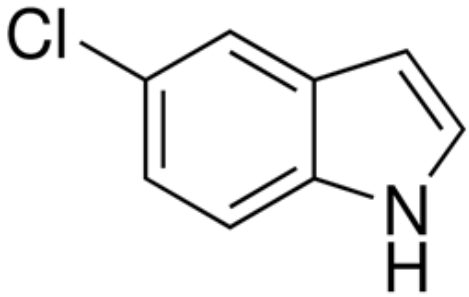


Figure 1 Structure of Cl-indole.

Cl-indole (10 µM; Figure 1) failed to impact radioligand binding saturation data arising from [^{3}H]granisetron (0.1-10 nM) binding the h5-HT$_3$A receptor expressed by HEKh5-HT3A cells (Bmax = 1130 ± 146 and 1027 ± 66 fmol/mg protein, pKd = 8.85 ± 0.20 and 8.77 ± 0.16 calculated in the absence and presence of Cl-indole, respectively [mean ± SEM, n = 3], P > 0.05; non-specific binding defined by ondansetron, 10 µM; non-specific binding defined by ondansetron, 10 µM; experiments performed previously in the lab, curves not shown). In competition studies with 5-HT and the antagonist, tropisetron, Cl-indole (10 µM) increased the affinity of 5-HT for the [^{3}H]granisetron specific binding site in HEKh5-HT3A cell homogenates but did not modify the affinity of tropisetron (Figure 2; Table 2; Newman et al., 2013) Indicating potentially agonist dependency..

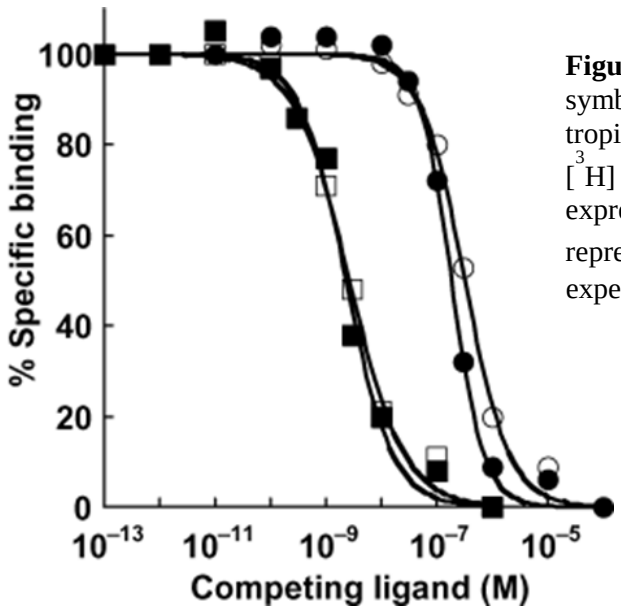


Figure 2. Impact of Cl-indole (10 µM; filled symbols) on the ability of 5-HT (circles) and tropisetron (squares) to compete for specific [^{3}H] granisetron binding to HEK293 cells expressing the h5-HT$_3$A receptor. Data represent mean from 4 independent experiments (Newman et al., 2013).

Table 2. Impact of Cl-indole (10 µM) upon the affinity (pK_i) of 5-HT and tropisetron (ICS 205–930; (1***R***,5***S***)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl 1***methyl***-indole-3-carboxylate) and Hill coefficient for the h5-HT_3A receptor radiolabelled with [^{3}H]granisetron (Newman et al., 2013)

	pKi	Hill coefficient
5-HT	6.31 ± 0.03	1.59 ± 0.21
5-HT + Cl-indole	6.57 ± 0.03*	1.44 ± 0.13
Tropisetron	8.50 ± 0.10	1.00 ± 0.03
Tropisetron + Cl-indole	8.39 ± 0.06	0.99 ± 0.04

Data represents mean ± SEM from four independent experiments.
Effect of Cl-indole *$P < 0.05$ paired two tailed-t-tset.

3.1.1B 5-HT_3 receptor agonists increase $[Ca^{2+}]_i$ in HEKh5-HT3A cells

In order to further investigate the function of the receptor we have used a calcium influx assay using FlexStation platform. The endogenous full agonist, 5-HT (10 nM – 10 µM) or a range of structurally diverse partial 5-HT_3 receptor agonists ((S)-zacopride, DDP733, RR210, quipazine, dopamine, 2-methyl-5-HT, SR57227A, mCPBG) evoked concentration-dependent increases in $[Ca^{2+}]_i$ in HEKh5-HT3A cells (Figure 3; Figure 5; Figure 6). Maximal responses evoked by the partial agonists ranged from inconsistent responses barely above baseline (3% (S)-zacopride) to 89% (mCPBG) of the maximal response to 5-HT (Figure 3; Figure 5; Figure 6; Newman et al., 2013). It was notable that in the continued presence of 5-HT or partial agonists with relatively high intrinsic activity, particularly at the higher concentrations investigated, there was an associated tachyphylaxis in the $[Ca^{2+}]_i$ response (e.g. Figure 3; Newman et al., 2013)indicating potential receptor desensitisation.

HEK293 cells express muscarinic receptors, which are able via G-protein coupling to mobilise calcium. The transient rise in calcium has been attributed to the accompanied inositol assembly and is anticipated to be generated by release from intracellular stores in endoplasmic reticulum and the sustained calcium increased of the plateau

phase has been attributed to extracellular calcium influx (Edelman et al., 1994). Therefore, in our experimental set up we use muscarinic acetylcholine receptor agonist carbachol, as a positive control in our plates.

Neither 5-HT (10 µM) nor DDP733 (1 µM) increased $[Ca^{2+}]_i$ in untransfected HEK293 cells, although these cells responded to the muscarinic acetylcholine receptor agonist, carbachol (1 mM; data not shown).

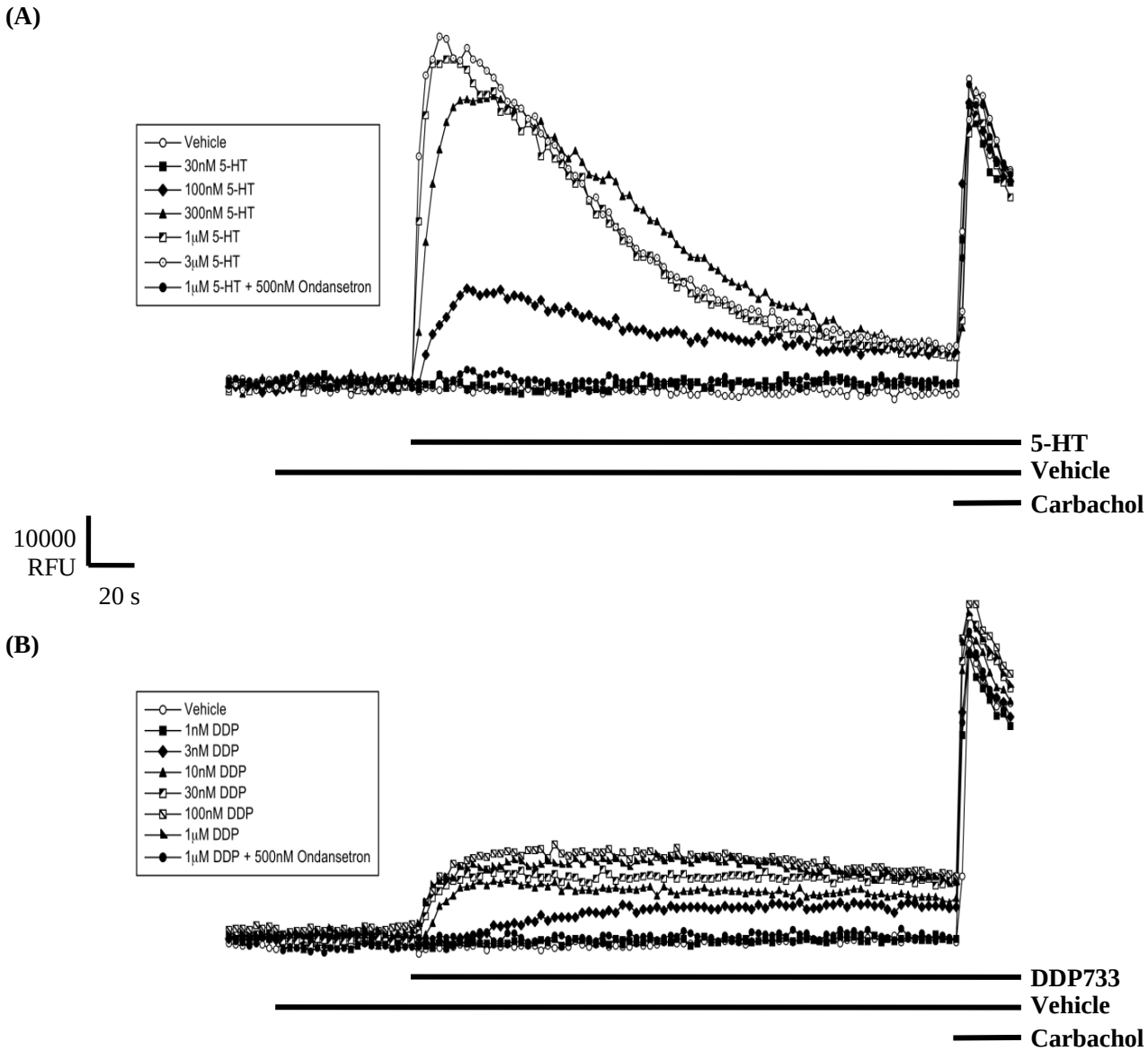


Figure 3. Representative experiment of concentration-dependent ability of 5-HT (A) and DDP733 (B) to increase $[Ca^{2+}]_i$ in HEK293 cells expressing the h5-HT_3A receptor and blockade of the response by the prior incubation with the selective 5-HT_3 receptor antagonist, ondansetron (500 nM). The muscarinic receptor agonist, carbachol (1 mM) added where indicated. Data representative from at least 5 independent experiments (Newman et al., 2013; Poster, Batis et al., SFN 2010).

3.1.1C Potentiation by Cl-indole of the h5-HT$_3$A receptor-mediated increase in $[Ca^{2+}]_i$ in HEKh5-HT3A cells

Application of Cl-indole (1-100 µM) generally failed to evoke $[Ca^{2+}]_i$ response in HEKh5-HT3A cells (occasionally a small response was noted with 100 µM Cl-indole) yet potentiated in a concentration-dependent manner the impact of a subsequent application of 5-HT (Figure 4; Table 3). Whilst Cl-indole induced a relatively small percentage increase in the response to a maximally effective concentration of 5-HT (3 µM), Cl-indole reduced the tachyphylaxis associated with the continued presence of relatively high concentrations of 5-HT (Figure 4). The Cl-indole-induced potentiation in the maximal 5-HT response was greater at lower sub-maximal concentrations of 5-HT (Figure 4; Newman et al., 2013).

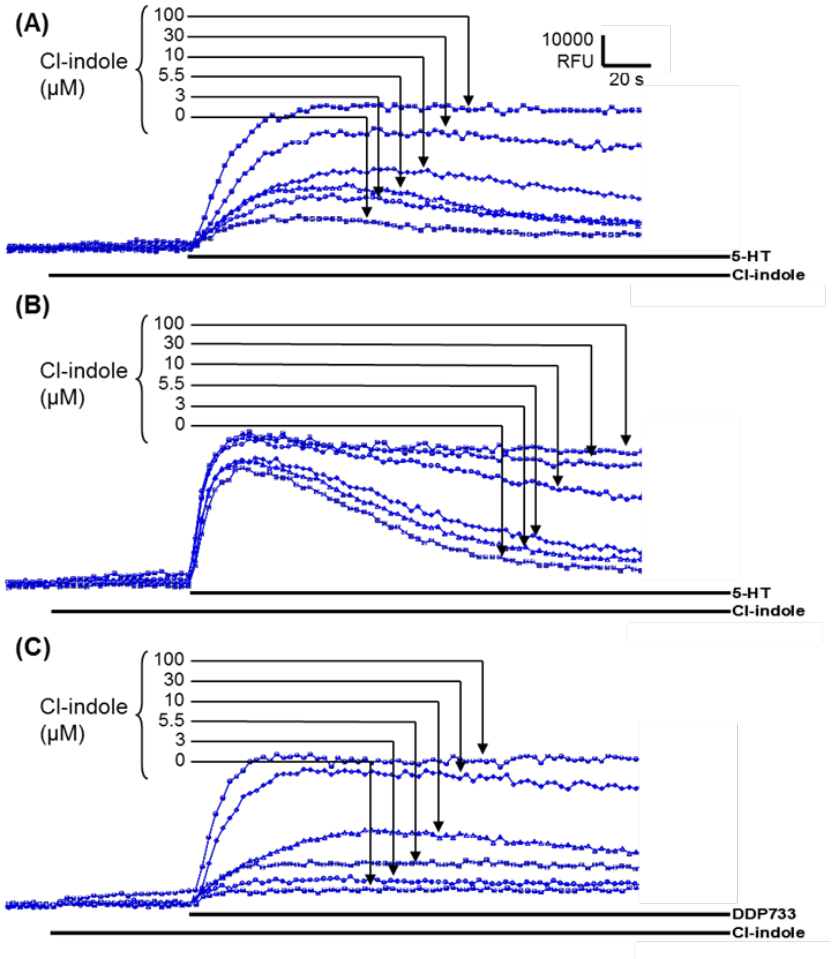


Figure 4. Concentration-dependent ability of Cl-indole to potentiate responses to 5-HT (A; 0.3 µM, B; 3.0 µM) and DDP733 (C; 100 nM) to increase $[Ca^{2+}]_i$ in HEK293 cells expressing the h5-HT$_3$A receptor (data representative from 3-6 experiments. In A-C, drugs were added where indicated by the horizontal bar (Newman et al., 2013; Poster, Batis et al, SFN 2010) see next page Figure 4D.

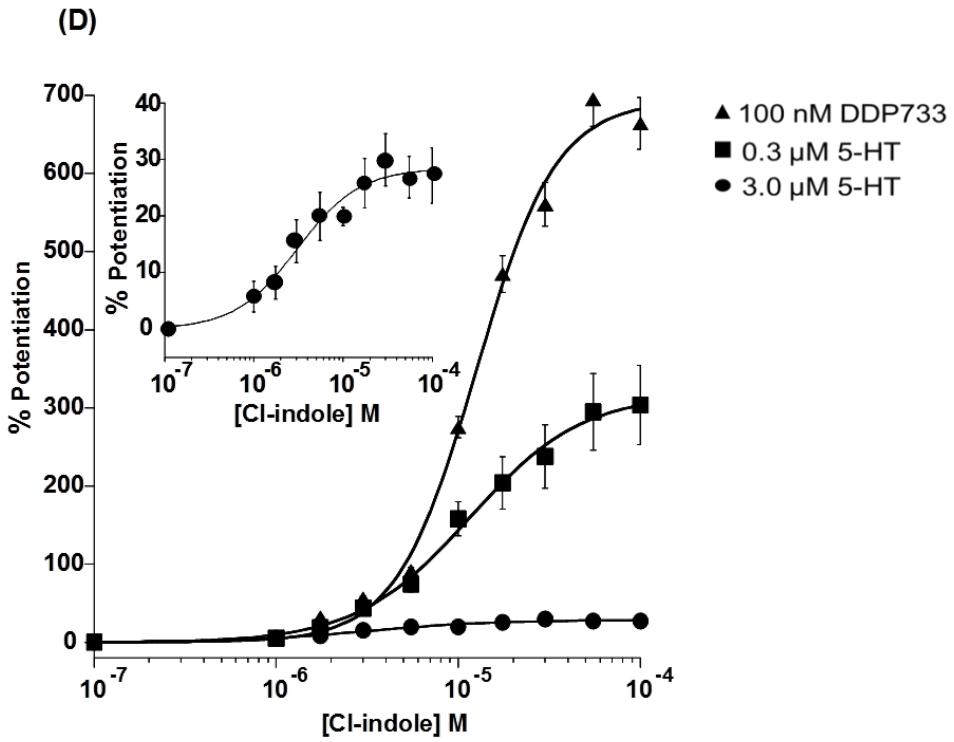


Figure 4. D. Concentration-dependent ability of Cl-indole to potentiate responses to 5-HT (0.3 µM, 3.0 µM) and DDP733 (100 nM) to increase $[Ca^{2+}]_i$ in HEK293 cells expressing the h5-HT_3A receptor (data representative from 3-6 experiments; Cl-indole concentration-responses fitted to a three-parameter logistic equation (see Table 2 for data arising from the analysis). (Newman et al., 2013; Poster, Batis et al, SFN 2010).

Table 3. Efficacy (Emax), potency (pEC_{50}) and Hill coefficient concerning the concentration-dependent ability of Cl-indole to potentiate the response of 5-HT (0.3 and 3.0 µM) and DDP733 (100 nM) to increase $[Ca^{2+}]_i$ in HEK293 cells expressing the h5-HT_3A receptor. Data represent mean±SEM, n=3-6. (Poster, Batis et al., SFN 2010)

5-HT3 receptor agonist	Cl-indole		
	Emax (% increase)	pEC_{50}	Hill coefficient
5-HT (3.0 µM)	29±4	5.49±0.05	1.62±0.31
5-HT (0.3 µM)	318±52	4.95±0.05	1.30±0.06
DDP733 (100 nM)	730±96	4.87±0.07	1.83±0.32

Cl-indole also induced a concentration-dependent increase in the maximal responses evoked by a range of 5-HT_3 receptor partial agonists (Figure 4C; Figure 5; Figure 6A; Table 4; (Newman et al., 2013)).

Table 4. Efficacy (Emax), potency (pEC_{50}) and Hill coefficient concerning the ability of various 5-HT_3 receptor agonists, in the absence and presence of Cl-indole (10 µM), to increase $[Ca^{2+}]_i$ in HEK293 cells expressing the h5-HT_3A receptor. Data represent mean±SEM, n=4-9. Effect of Cl-indole *P<0.05, **P<0.01, ***P<0.001 paired two tailed t test (Newman et al., 2013; Poster, Batis et al., SFN 2010)

[1]% Maximum response compared to the maximum response of 5-HT in the absence of Cl-indole.

Agonist ± Cl-indole	**Emax[1]**	**pEC_{50}**	**Hill coefficient**
5-HT	100	6.70±0.06	5.20±1.95
5-HT + Cl-indole	117±5**	6.85±0.03*	2.84±0.26
2-Methyl-5-HT	72±8	5.67±0.06	2.04±0.25
2-Methyl-5-HT + Cl-indole	121±18*	5.83±0.08	4.74±2.51
mCPBG	89±2	6.41±0.07	2.14±0.35
mCPBG + Cl-indole	106±10	5.73±0.07**	3.18±0.22*
Quipazine	46±9	7.85±0.17	4.34±1.49
Quipazine + Cl-indole	115±14**	8.11±0.17	2.70±0.25
DDP733	23±5	8.34±0.10	5.66±2.03
DDP733 + Cl-indole	71±11**	8.06±0.05*	1.29±0.10
Dopamine	53±5	4.08±0.04	1.19±0.12
Dopamine + Cl-indole	94±11*	4.29±0.05*	2.44±0.59
SR57227A	82±6	6.43±0.02	2.89±0.76
SR57227A + Cl-indole	137±18*	7.00±0.00**	1.78±0.10
RR210	30±3	7.10±0.06	0.89±0.03
RR210 + Cl-indole	92±8***	7.45±0.05**	2.52±1.16
(S)-Zacopride	3±3	ND	ND
(S)-Zacopride + Cl-indole	25±5**	7.65±0.55	3.50±1.40

It is important to note that an orthosteric ligand dependency (probe-dependence) is apparent were depending on the agonist / partial agonist used to evoke a response the co-operativity, indicators of efficacy (Emax) and affinity (pEC50) of the orthosteric ligand is different in the presence of the Cl-indole, which in turn is different from one to the next partial agonist/ agonist. The affinity of the endogenous agonist 5-HT is not affected by the PAM but the efficacy (Emax) is increased (Table 4, Figure 5). On the other hand partial agonists like SR57227A exhibit a shift of the concentration response curve to the left (increase in affinity; fo pEC50 consult table 4) accompanied an upward shift of the Emax plateau (increase in efficacy; Figure 5; for Emax consult table 4) characteristics of a positive allosteric modulator. Furthermore in Table 4 (derived from Figure 5) it is shown that Cl-indole affecting almost all drug (both full and partial agonist; with the exception of mCPBG; $P>0.05$) Emax statistically significantly, in varying degrees, by potentiating the calcium influx respond.

Additionally, almost all the drugs tested had an increase in their affinity for the receptor, which was statistically significant. Interestingly from our data (Figure 5) we are able to conclude that there is an apparent ligand dependency on the modulatory effect transduced by Cl-indole establishing an agonist dependency in the efficacy of Cl-indole for the receptor. Thus at maximally effective concentrations of agonists Cl-indole promoted only small percentage increase in the maximal effect of full agonist, like 5-HT, however, responses by agonists with lower intrinsic activity for the receptor were potentiated much in a greater manner.

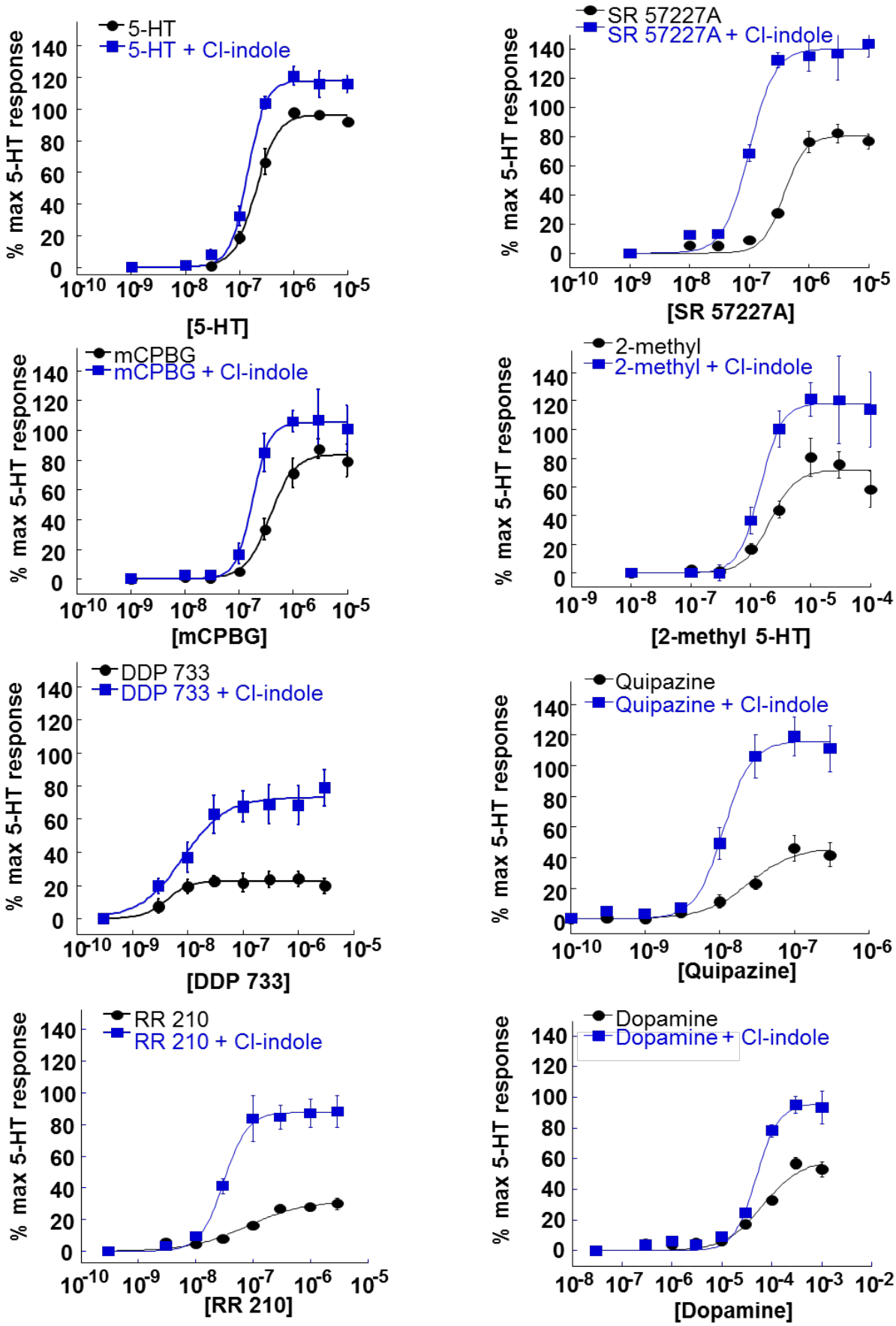


Figure 5 Concentration-dependent ability of various 5-HT_3 receptor agonists to induce increases in intracellular calcium in HEK293 cells expressing the h5-HT_3A receptor in the absence and presence of Cl-indole (10 μM). Data represent mean ± SEM, n = 3–6. (Newman et al., 2013; Poster, Batis et al., SFN 2010)

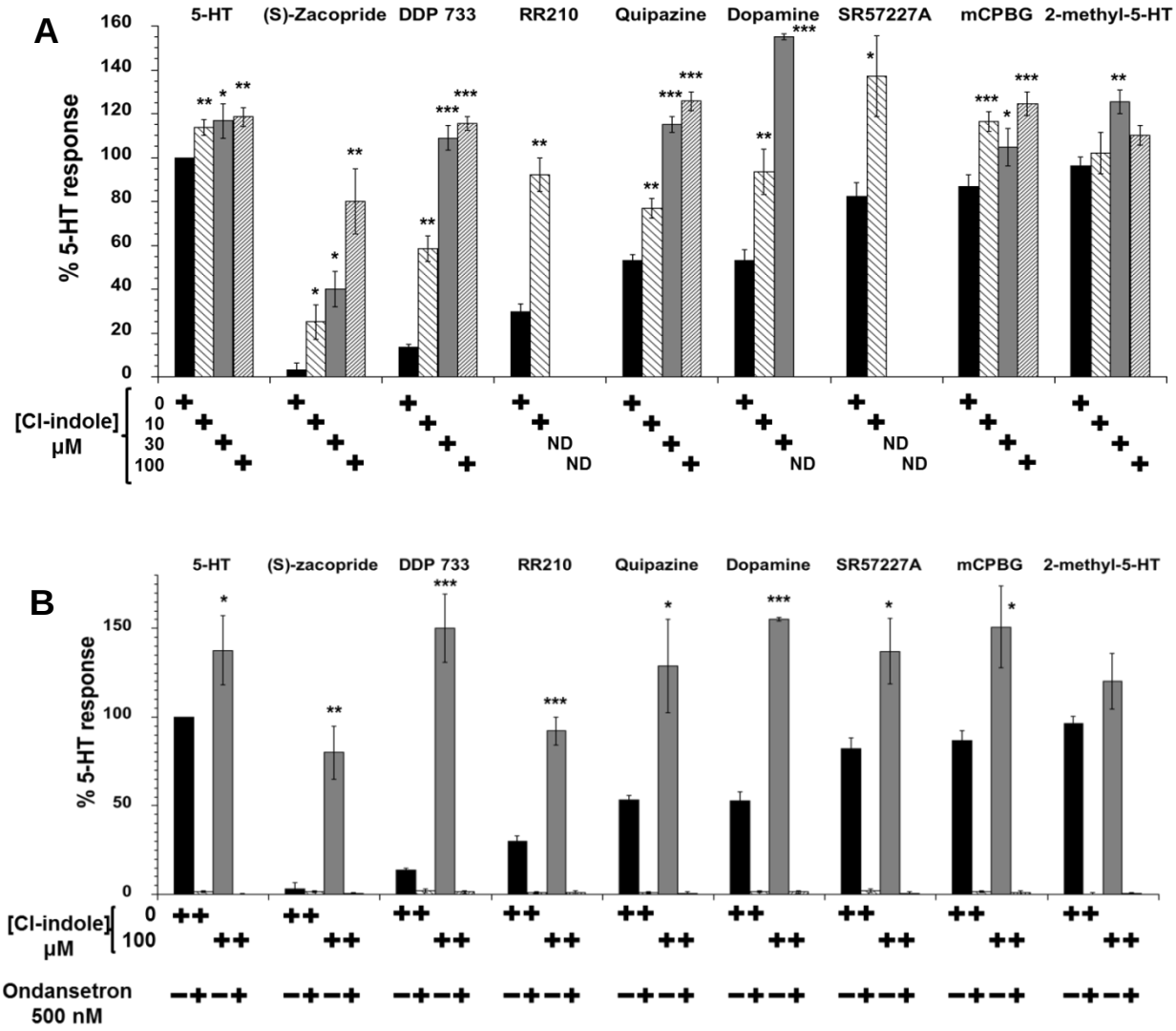


Figure 6 Ability of Cl-indole (10, 30 and 100 µM) to potentiate the action of various 5-HT_3 receptor agonists (**A**) to increase intracellular calcium in HEK293 cells expressing the h5-HT_3A receptor and the blockade of the response by the selective 5-HT_3 receptor antagonist, ondansetron (500 nM) (**B**) 5-HT (3.0 µM), (S)-zacopride (1.0 µM), DDP 733 (1.0 µM), RR210 (1.0 µM), quipazine (30 nM), dopamine (300 µM), SR57227A (1.0 µM), mCPBG (1.0 µM) and 2-methyl-5-HT (10 µM). Data represent mean % response compared with 5-HT ± SEM (n = 3–15). Effect of Cl-indole *P < 0.05, **P < 0.01, ***P < 0.001; antagonism by ondansetron P < 0.01 in all instances except compared with (S)-zacopride alone. (Newman et al., 2013; Poster, Batis et al., SFN 2010)

$[Ca^{2+}]_i$ responses in HEKh5-HT3A cells evoked by 5-HT and the partial agonists in the absence and presence of Cl-indole (10-100 µM) were fully antagonised by prior application of the selective antagonists, ondansetron (500 nM; Figure 6B data for 10 µM Cl-indole not shown) and granisetron (500 nM; data not shown). In contrast to the 5-HT_3 receptor agonists, the selective 5-HT_3 receptor antagonists; alosetron, BRL46470, ondansetron, ramosetron, and palonosetron (all used at 3 µM) failed to evoke $[Ca^{2+}]_i$ responses in HEKh5-HT3A cells in either the absence or presence of Cl-indole (10-100 µM; Figure 7; (Newman,et al., 2013)). Proving that the receptor requires an agonist or partial agonist binding to the orthosteric site for the channel to become permeable providing us with a potential tool for distinguishing antagonists form very low intrinsic acticity partial agonists like S-Zacopride.

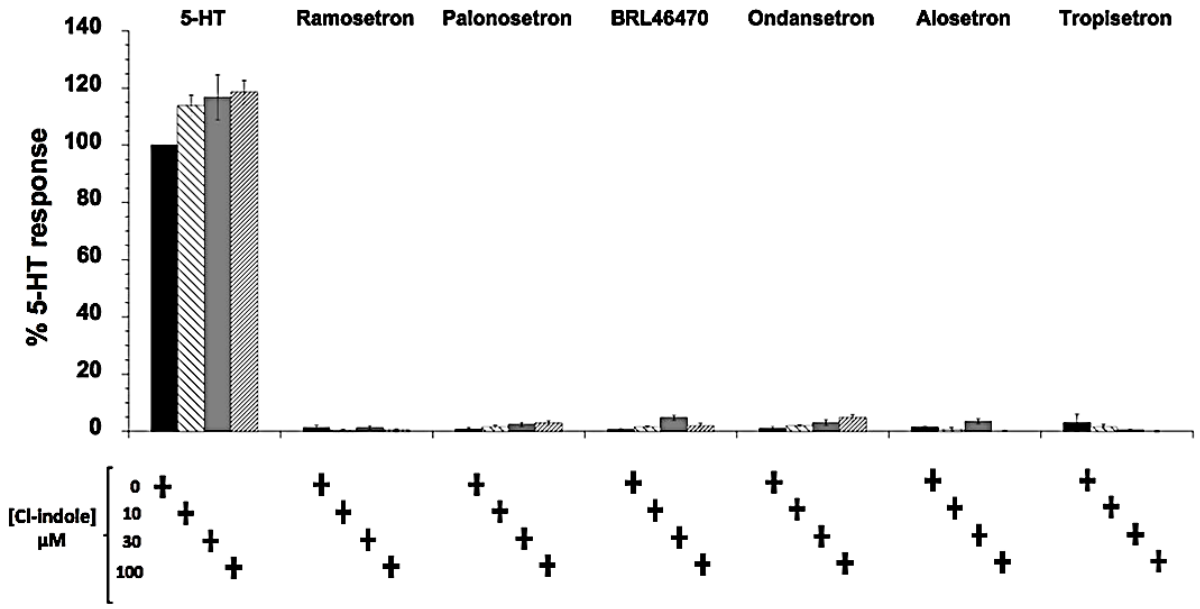


Figure 7 Failure of Cl-indole (10, 30 and 100 µM) to induce responses from various 5-HT_3 receptor antagonists assessed by intracellular calcium in HEK293 cells expressing the h5-HT_3A receptor. 5-HT, full agonist for comparison, and the antagonists; ramosetron, palonosetron, BRL46470, ondansetron, alosetron and tropisetron (all 3 µM). Data represent mean % response compared with 5-HT ± SEM (*n* = 3–8). (Newman et al., 2013; Poster, Batis et al., SFN 2010)

3.1.1D Potentiation by Cl-indole of agonist-evoked currents recorded under voltage-clamp from HEKh5-HT3A cells

In order to further investigate the positive allosteric modulation effects that we were able to identify using cell populations to monitor intracellular calcium influx we also employed the use of electrophysiological experimental assay in the form of voltage-clamp, which performed by Drs Gillian Grafton and Andrew Powell and was published recently (Newman et al., 2013)

In detail, at a holding potential of −60 mV, a brief application of 5-HT (10 μM; 100 ms) to HEKh5-HT3A cells elicited a transient inward current (560 ± 130 pA, n = 6; Figure 8A). The rising phase was best fitted by a single exponential function with a mean time constant of 0.9 ± 0.1 s and the current decayed slowly back to baseline (t_{50} 2.1 ± 0.4 s). Repeated applications (5 min interval) of 5-HT significantly reduced the amplitude of the inward current, such that by the third application the peak amplitude was approximately one-third of the initial response (Figure 8A). In contrast, 5-HT_3 receptor currents evoked by a 10 ms application of 5-HT (10 μM) at the same frequency were not reduced by repeated stimulation (Figure 8A) and hence this protocol was employed for further investigations (Newman, Batis et al., 2013).

Superfusion of Cl-indole (10 μM) did not alter the holding current of 5-HT_3A receptor expressing HEK 293 cells, nor did the compound alter the amplitude (control 411 ± 107 pA, Cl-indole 399 ± 106 pA; n = 6; P = 0.86) or rise time (control 76 ± 16 ms, Cl-indole 110 ± 40 ms; n = 6;P = 0.31) of 5-HT (10 μM)-induced currents (Figure 8B). However, Cl-indole significantly increased the decay time for 5-HT_3 receptor-mediated currents (control t_{50} = 2.8 ± 0.7 s, Cl-indole t_{50} = 12.2 ± 2.5 s; n = 6; P = 0.005); the effect of Cl-indole on decay rate was partially reversible after washout (t_{50} = 6.3 ± 1.0 s; P = 0.04; Figure 8B; (Newman et al., 2013)).

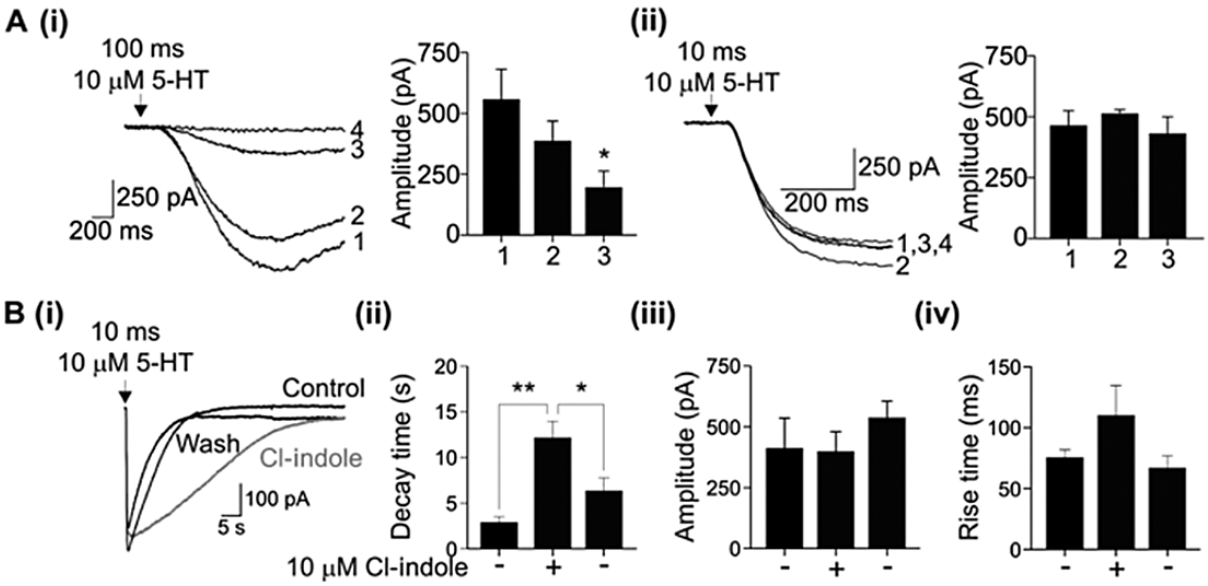


Figure 8. A: ability of repeated application of 5-HT (10 μM) for 100 ms (i) and 10 ms (ii) to evoke responses from voltage-clamped HEK293 cells expressing the h5-HT_3A receptor. Traces are representative of at least three independent experiments, numbers represent the traces corresponding to the application number of the 5-HT. Data in histograms represent mean ± SEM, n = 5–6. B: ability of 5-HT (10 μM applied for 10 ms) in the absence (control) and presence of Cl-indole (10 μM, grey trace) to evoke responses from voltage-clamped HEK293 cells expressing the h5-HT3A receptor (i) with histograms displaying impact upon decay time (ii), amplitude (iii) and rise time (iv); the right hand column in each of these istograms represents results after wash-out of Cl-Indole. *P < 0.05, **P < 0.01. Experiments performed by Dr Gillian Grafton and Dr Andrew Powel. For details see Newman et al., 2013

The selective 5-HT_3 receptor antagonist, ondansetron (100 nM), completely blocked the activation of 5-HT_3 receptors by 5-HT (10 μM) in both the absence and presence of Cl-indole (10 μM). The effect of ondansetron was reversible upon washout with subsequent application of 5-HT evoking inward current, which was modulated by Cl-indole (Figure 9A; (Newman et al., 2013)).

To assess whether Cl-indole affected the ionic permeability of the h5-HT_3A receptor, the I–V relationship of the macroscopic current response to 5-HT was constructed by a voltage ramp (−100 to +60 mV) recorded at the peak of the current response to bath-applied 5-HT (1.0 μM; 15 s). The reversal potential ($E_{5\text{-HT}}$) in the presence and absence of Cl-indole (10 μM) was −2.2 ± 3.1 and −0.5 ± 2.9 mV, respectively n = 6, P = 0.74) (Figure 9B), suggesting that Cl-indole does not grossly affect the ionic permeability of h5-HT_3A receptors. The peak 5-HT current recorded at −60 mV was significantly increased by Cl-indole (Control 265.8 ± 127.8 pA; Cl-indole 1430.8 ± 362.9 pA; n = 5, P < 0.05; (Newman et al., 2013))

Application of the 5-HT_3 receptor partial agonist, DDP733 (100 nM; 100 ms), evoked a relatively small inward current (149 ± 58 pA, n = 6; Figure 9C) which had a slow rise time (10.2 ± 3.2 s) and slow decay time (46.5 ± 10.5 s). Superfusion of Cl-indole (10 μM), significantly increased the amplitude of DDP733-induced currents (912 ± 272 pA; $P < 0.05$; Figure 9C) and slowed the rise time (18.6 ± 3.9 s; $P < 0.005$). Cl-indole also prolonged the decay rate of DDP733-induced currents (137 ± 35 s; $P < 0.01$; Figure 9C). The effect of Cl-indole upon DDP733-evoked amplitude, rise time and decay rate was fully reversed by washout of Cl-indole (33.1 ± 6.5 pA, 11.6 ± 3.9 s, 52 ± 20 s, respectively). (For details see Newman et al., 2013)

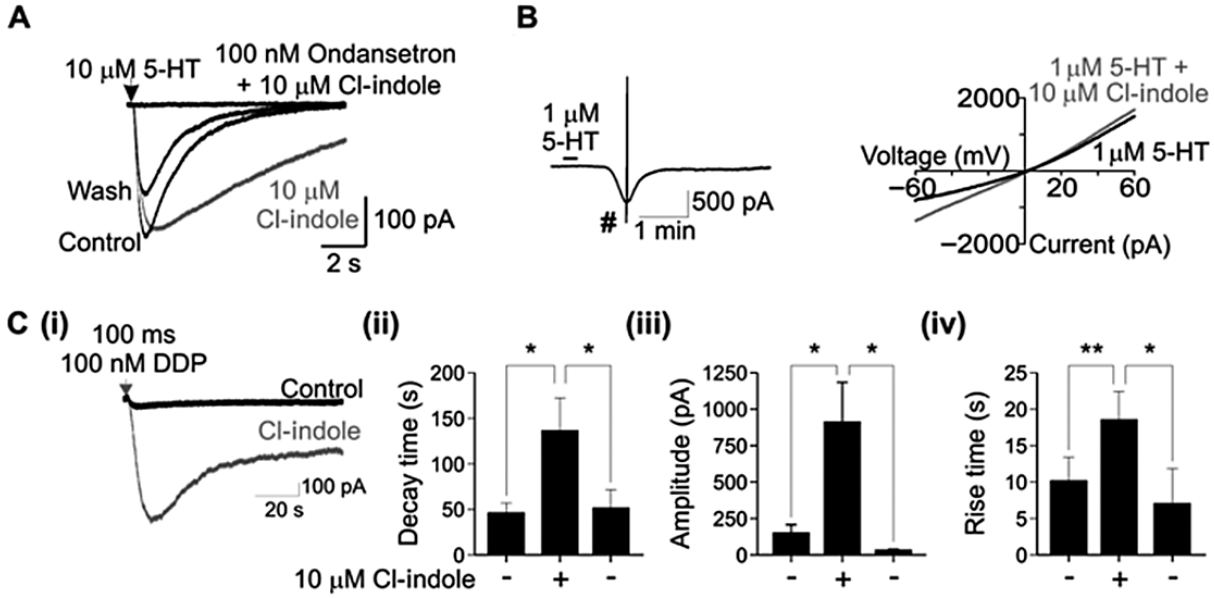


Figure 9. Ability of Cl-indole (10 μM) to potentiate 5-HT_3 receptor agonist responses. A: The presence of the selective 5-HT_3 receptor antagonist, ondansetron (100 nM), completely prevented a response to 5-HT (10 μM applied for 10 ms) in the presence of Cl-indole (10 μM). The effect of ondansetron was partly reversible by wash-out [subsequent response to 5-HT (10 μM applied for 10 ms; wash)]. Traces are representative of at least three independent experiments. B: I–V plots arising in the presence of 5-HT (1.0 μM) or 5-HT (1.0 μM) plus Cl-indole (10 μM) determined around the peak response (indicated #). Drugs were bath applied for 15 s. Traces are representative of at least three independent experiments. C: DDP733 (100 nM) in the absence and presence of Cl-indole (10 μM); recordings from voltage-clamped HEK293 cells expressing the h5-HT_3A receptor (i) with histograms displaying impact upon decay time (ii), amplitude (iii) and rise time (iv); the right-hand column in each of these histograms represents results after wash-out of Cl-indole. Traces are representative of at least three independent experiments. Data in histograms represent mean ± SEM, n = 5–6. *$P < 0.05$, **$P < 0.01$. Experiments performed in collaboration with Drs Gillian Grafton and Andrew Powell; for details see Newman et al., 2013

3.1.1E Native 5-HT3 receptor responses

Modulation of neuronally mediated contraction of mouse bladder

Furthermore in order to investigate the ability of Cl-indole to modulate native 5-HT_3 receptors, and in a whole tissue assay, the 5-HT_3 receptor-mediated increase in bladder smooth muscle 'twitch' was investigated (Chetty et al., 2007) in collaboration with Francesca Caputo and Dr Keith Brain (see details Newman et al., 2013). Under control conditions, electrical field stimulation induced a tetrodotoxin (100 nM)-sensitive contraction of the muscle strips. Using a sub-maximal stimulus protocol (six pulses at 10 Hz), consistent contraction amplitudes obtained when the stimuli were delivered at 1 min intervals. The selective 5-HT_3 receptor agonist, mCPBG (3.0 μM), transiently increased the amplitude of contraction, reaching a peak increase of 20 ± 4% (n = 4 strips; P < 0.05), which subsequently declined in the continued presence of mCPBG (30 μM; Figure 10). Cl-indole (30 μM) alone had no effect on the amplitude of contraction (6 ± 3%; n = 4; P = 0.46; Figure 10), but subsequent addition of mCPBG (3.0 μM) greatly increased the peak amplitude by 54 ± 3%, an effect that was maintained in the continued presence of mCPBG (30 μM; Figure 10). (Newman et al., 2013)

In the presence of the 5-HT_3 receptor antagonist ondansetron (500 nM) the subsequent application of Cl-indole (30 μM), mCPBG (3.0 μM) did not significantly increase the amplitude of contraction (8 ± 7%; n = 6; P = 0.30; Figure 10Cii, open bars): in the matching set of bladder strips, Cl-indole (30 μM) + mCPBG (3.0 μM) in the absence of ondansetron increased the peak amplitude by 31 ± 10% (n = 5; P < 0.05; Figure 10Cii - filled bars). The experimental protocols were performed by Francesca Caputto and Dr Keith Brain (Newman et al., 2013).

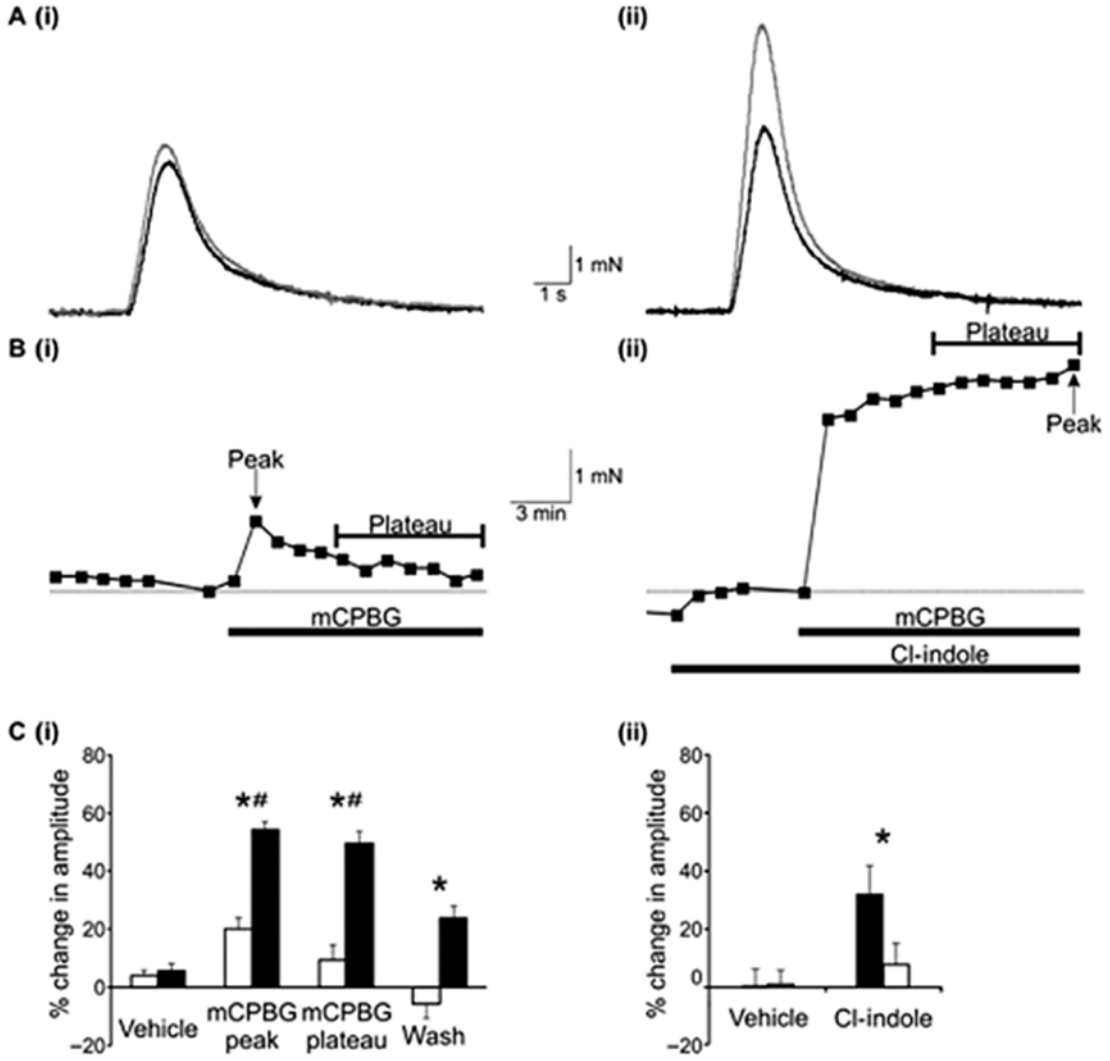


Figure 10. Cl-indole augments the mCPBG-induced potentiation of the neurogenic contractile response in mouse bladder strips. (A) Sample traces from different bladder strips showing that Cl-indole (30 μM) augments the mCPBG-induced potentiation of contractile response in mouse bladder strips. (Ai) mCPBG (3.0 μM) alone only subtly increases the amplitude of contractile force generated following field stimulation. (Aii) In the presence of Cl-indole (30 μM), mCPBG increases to a greater relative extent the contractile force. For each panel, the black line indicates the neurogenic contraction in the absence of mCPBG, and the grey line indicates the subsequent response in the presence of mCPBG in the same bladder strip. (Ai) and (Aii) show the response of different bladder strips from the same mouse and measured in parallel; the mean amplitudes did not vary significantly amongst the two test groups, although different muscle strips (even from the same animals) showed a range of typical contractile forces. (Bi) Field stimulation of mouse bladder strips (open bars) is subtly augmented by mCPBG (3.0 μM), an effect that shows tachyphylaxis (i.e. the plateau contractile amplitude in the continued presence of mCPBG is lower than the peak response). This effect is reversible upon removing mCPBG (washout). (Bii, Ci) In the presence of Cl-indole (30 μM), the peak response to mCPBG (3.0 μM) was potentiated and the tachyphylaxis was prevented (closed bars). The effect of Cl-indole was only partially reversed on wash. Cl-indole alone had no significant effect on contraction. (Cii) Responses to mCPBG (3.0 μM) in the presence of Cl-indole were blocked by the 5-HT_3 receptor antagonist ondansetron (open bar). *P < 0.05 compared with vehicle, $^{\#}P$ < 0.05 compared with mCPBG response in the absence of Cl-indole.

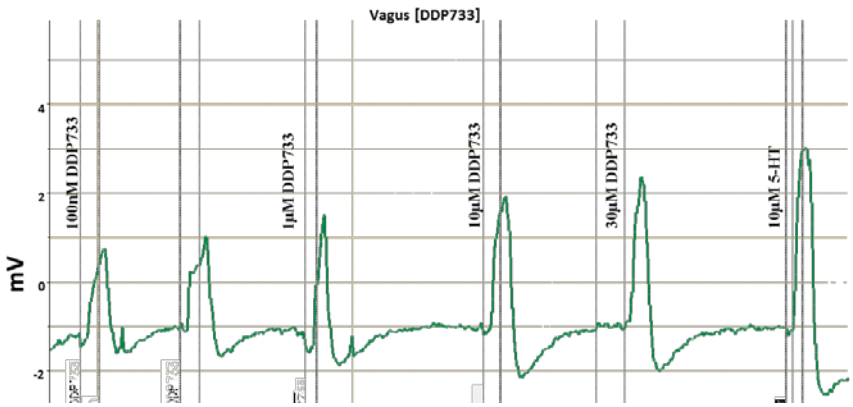
Experiments performed by Dr Keith Brain and Francesca Caputo. (For details of protocol, see Newman et al., 2013)

Rat Vagus nerve electrophysiology – preliminary data

In a further attempt to establish a model of native 5-HT_3 receptor the vagus nerve of the rat was used in a grease gap electrophysiological experiment was investigated. Under control condition noncumulative concentration response curves to agonists were obtained by including the agonist in the perfusing Krebs buffer for appropriate time followed by a washout period between exposures to each agonist concentration.

I was able to demonstrate in preliminary experiments of at least 3 vagus nerves from 3 individual rats donated port-mortem by Dr Lovick, that the partial DDP733 is able to induce a concentration dependent response via depolarisation of the perfused side compared to the control side of rat vagus nerve in the grease gap electrophysiological experiment comparable to the endogenous ligand 5-HT response.

A



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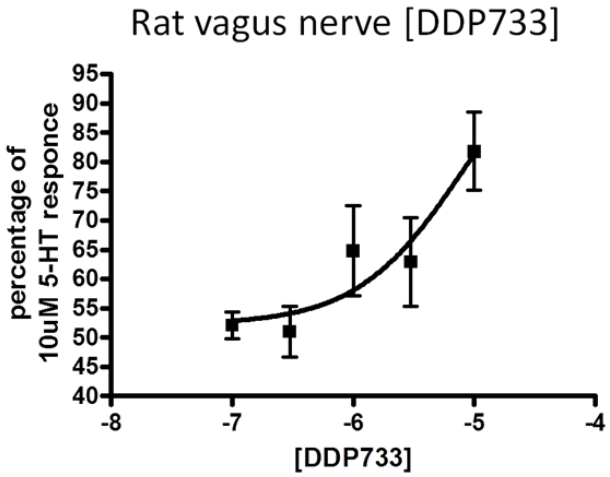


Preliminary Figure. Concentration-dependent ability of increasing concentration of DDP733 a 5-HT_3 receptor partial agonist to increases in depolarisation (mV)of the perfused side compared to the control side of rat vagus nerve in a grease gap electrophysiological experiment. A. Typical trace of non-cumulative response to DDP733 (100, 300 nM, 1, 10, 30µM) and 10µM 5-HT. B. data representative from 3 experiments DDP733 concentration-responses fitted to a three-parameter logistic equation. Data represent mean ± SEM, n = 3.

This allows the demonstration in native tissue the potential of the vagus nerve as a tool for screening and assessing potential compounds with activity in 5-HT_3 receptor identified in cell lines as well as potential use for testing protocol for preclinical screening of drugs for emetogenic and other vagally facilitated effects. We were, due to time contains, not able to complete the study intended initially; were we would have assessed the ability of Cl-indole to modulate the effect of agonist and partial agonist have on native 5-HT_3 receptor.

3.1.1F Potentiation by Cl-indole and reversal of the apparent desensitisation of the h5-HT_3A receptor-mediated increase in $[Ca^{2+}]_i$ in HEKh5-HT3A cells

Similar to our initial hypothesis full agonists, like 5-HT, caused near complete desensitisation of the response; the response did not fully recover; with only a small percentage of the initial response, when Cl-indole (100μM) was added subsequently (Figure 11A). However, after response to a partial agonist (DDP733; Figure 11B), the addition of Cl-indole increased $[Ca^{2+}]_i$ to levels similar to those observed by full agonist (Figure 11B and 11C). These findings suggest that while the apparent full desensitisation induced by full agonists is almost irreversible in our population model, the partial desensitisation, or the partial population of cells, induced by partial agonists can be reversed, by Cl-indole. The addition of vehicle control post stimulation by either full agonist or partial agonist had no effect on the $[Ca^{2+}]_i$ (Figure 11A, 11B and Table 5) in HEK293 cells stably expressing h5-HT_3A receptor. Similar effect with the use of Cl-indole was observed with the known positive allosteric modulator TCE used at the higher concentration (5mM) on $[Ca^{2+}]_i$ following HEKh5-HT3A cells exposure to either the maximal and submaximal concentrations of 5-HT, but not when the lower concentrations of TCE were introduced (1 – 3 mM; Table 5). When TCE was used following partial agonist (DDP733 and quipazine) effect on $[Ca^{2+}]_i$ the response levels increased to comparable levels to those observed from full agonist alone was used to initiate responses above the base line and at least partially to those observed following 5-HT addition (Table 5).

Furthermore the subsequent introduction of maximal 5-HT (100 μM) was unable to overcome the apparent desensitisation caused by 5-HT either at submaximal or

maximal concentration. However, following initial responses to partial agonist DDP733 both at maximal and submaximal concentration 5-HT appeared to be able to overcome the apparent receptor desensitisation, at least partially (Table 5).

Notably the addition of maximal quipazine (10μM) following a calcium response initialised by either full agonist 5-HT (3 or 100 μM) or partial agonists DDP733 (0.1 or 1 μM) and quipazine (10 μM) appeared to cause a decrease in the observed $[Ca^{2+}]_i$ compared to the proceeding base line of response.

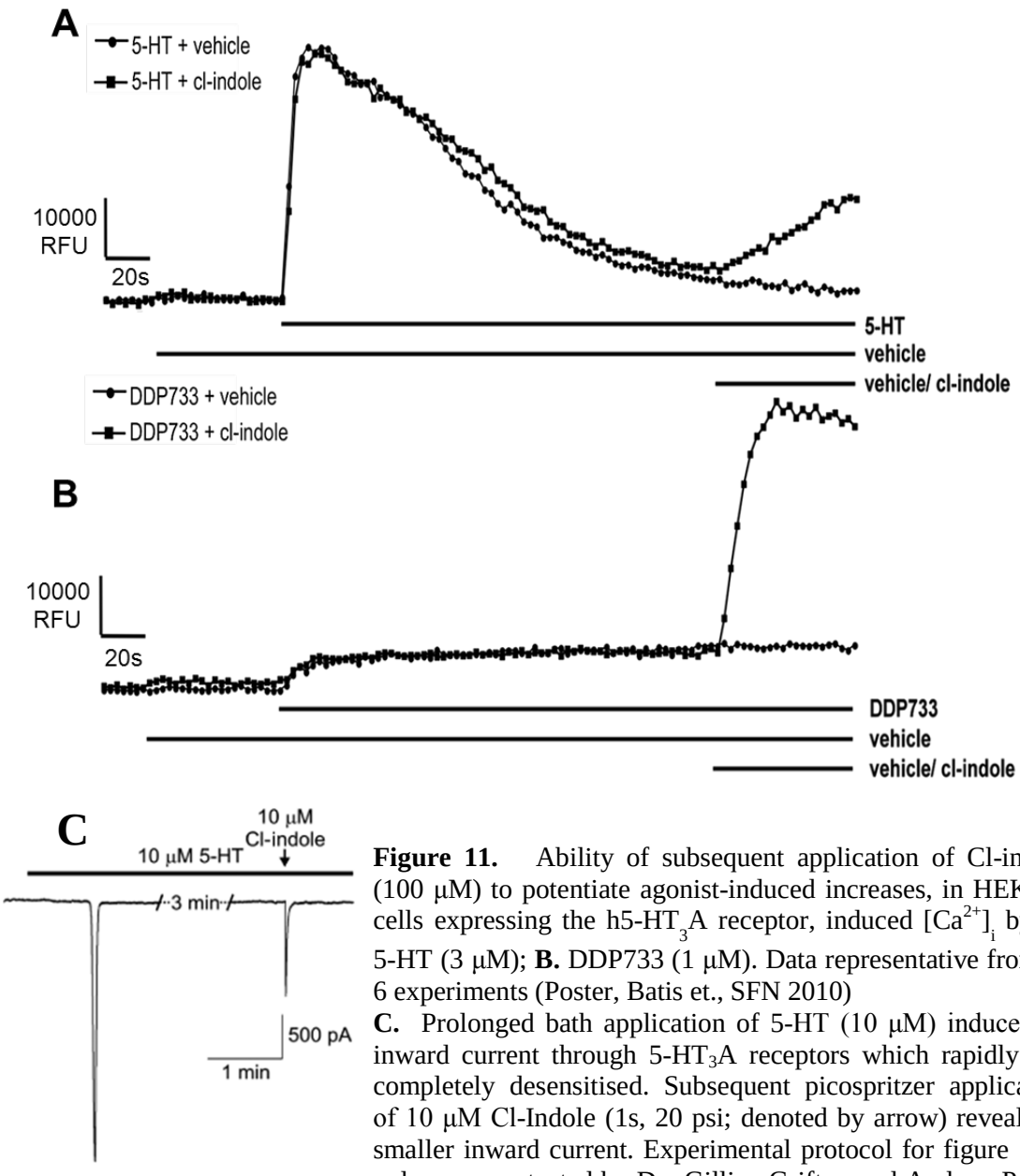


Figure 11. Ability of subsequent application of Cl-indole (100 μM) to potentiate agonist-induced increases, in HEK293 cells expressing the h5-HT_3A receptor, induced $[Ca^{2+}]_i$ by **A.** 5-HT (3 μM); **B.** DDP733 (1 μM). Data representative from 3-6 experiments (Poster, Batis et., SFN 2010)
C. Prolonged bath application of 5-HT (10 μM) induced an inward current through 5-HT_3A receptors which rapidly and completely desensitised. Subsequent picospritzer application of 10 μM Cl-Indole (1s, 20 psi; denoted by arrow) revealed a smaller inward current. Experimental protocol for figure 11C, only, was contacted by Drs Gillian Grifton and Andrew Powel (Newman et al., 2013)

280s Drug Added / 80s Drug Added	Vehicle	100µM Cl-indole	1mM TCE	2mM TCE	5mM TCE	100µM 5-HT	10µM Quipazine
3µM 5-HT	0	+	0	0	+	0	–
100µM 5-HT	0	+	0	0	++	0	
100nM DDP733	0	+++++	++++	+++	+++	+	– –
1µM DDP733	0	+++++	++++	++++	++++	+	–
10µM Quipazine	0	+++	0	++	++	–	– – –

Table 5 representing post stimulation additions of drugs and the effect they have on intracellular [Ca^{2+}] in HEK293 cells stably expressing h5-HT_3A receptor. Increase (+) compared to control in calcium or decrease (-) or no effect (0); (the greater the number of individual symbols the greater the deviation from the control)

To further characterise the effect of Cl-indole, we also employed an electrophysiological protocol in collaboration with Drs Gillian Grifton and Andrew Powel. In more detail, we examined, once more, whether the compound could re-activate desensitized 5-HT_3 receptors. Prolonged bath application of 5-HT (10 µM) produced a large transient current (2.52 ± 0.75 nA; *n* = 4) that decayed rapidly to 8.9 ± 4.0% of the peak response. In the continued presence of 5-HT, pressure ejection of Cl-indole (10 µM) elicited an inward current (0.97 ± 0.54 pA; Figure 11C; Newman et al., 2013)

3.1.2 Potentiation by other allosteric modulators of the h5-HT$_3$A receptor-mediated increase in [Ca^{2+}]$_i$ in HEKh5-HT3A cells

3.1.2A TCE allosteric modulators of the h5-HT$_3$A receptor-mediated increase in [Ca^{2+}]$_i$ in HEKh5-HT3A cells

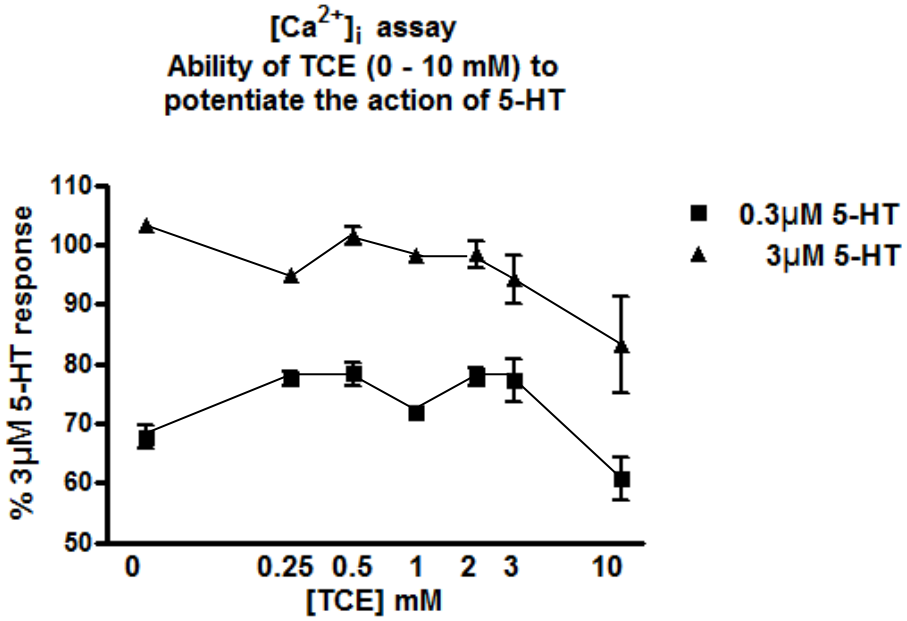


Figure 12 Concentration-dependent ability of increasing concentration of trichloroethanol (TCE) when administered prior to 5-HT either 0.3 µM or 3 µM to induce increases in intracellular calcium in HEK293 cells expressing the human 5-HT$_3$A receptor; representing the percentage of response compared to the response identified when in the presence of vehicle 3µM of 5-HT was used. Data represent mean ± SEM, n > 3.

Further to investigate if other potential PAMs were able to induce changed to the receptor pharmacology we introduced in our assay the application of 2,2,2-trichloroethanol (TCE) (0.25 - 10 mM), a known 5-HT$_3$ receptor modulator (Bemtley and Barnes, 1998; Lovinger et al., 2000), that generally failed to evoke [Ca^{2+}]$_i$ response in HEKh5-HT3A cells yet potentiated in a concentration-dependent manner the impact of a subsequent application of 5-HT (Figure 10). Whilst TCE induced relatively no change in the response to a maximally effective concentration of 5-HT (3 µM); there was an apparent decrease in the percentage response obtained at the higher concentrations of TCE (3 and 10 mM). The TCE-induced potentiation in the maximal response was greater at lower sub-maximal concentrations of 5-HT (0.3 µM; ~10%; Figure 12); however, once more there was an apparent decrease in the percentage response obtained at the higher concentrations of TCE (3 and 10 mM).

$[Ca^{2+}]_i$ responses in HEKh5-HT3A cells evoked by 5-HT in the absence and presence of TCE were fully antagonised by prior application of the selective antagonists, ondansetron (500 nM; not shown).

3.1.2B 5OHI allosteric modulators of the h5-HT_3A receptor-mediated increase in $[Ca^{2+}]_i$ in HEKh5-HT3A cells

The well-characterized positive allosteric modulator (PAM), 5-hydroxyindole (5OHI), has diverse effects on 5-HT_3 receptors. It has been noted that a mix of allosteric and orthosteric effects (Kooyman et al., 1994; Hu and Lovinger, 2008) is demonstrated by 5OHI. Indeed, one study failed to show any effect, suggesting that 5-hydroxyindole is a PAM at rodent receptors, with no activity at their human counterpart (Grønlien et al., 2010).

Application of 5-hydroxyindole (5OHI) (0.01 - 3 mM) generally failed to evoke $[Ca^{2+}]$i response in HEKh5-HT3A cells; yet potentiated in a concentration-dependent manner the impact of a subsequent application of quipazine (Figure 13). Whilst 5OHI induced a relatively negligible percentage change in the response to a low concentration of quipazine (10 nM); there was an apparent decrease in the percentage response obtained at the higher concentrations of 5OHI used (3 mM). The 5OHI-induced potentiation in the maximal 5-HT response was greater at higher concentrations of quipazine (30 nM; Figure 13) however once more there was an apparent decrease in the percentage response obtained at the higher concentrations of 5OHI (3 mM); resembling more of a “bell” shape response curve.

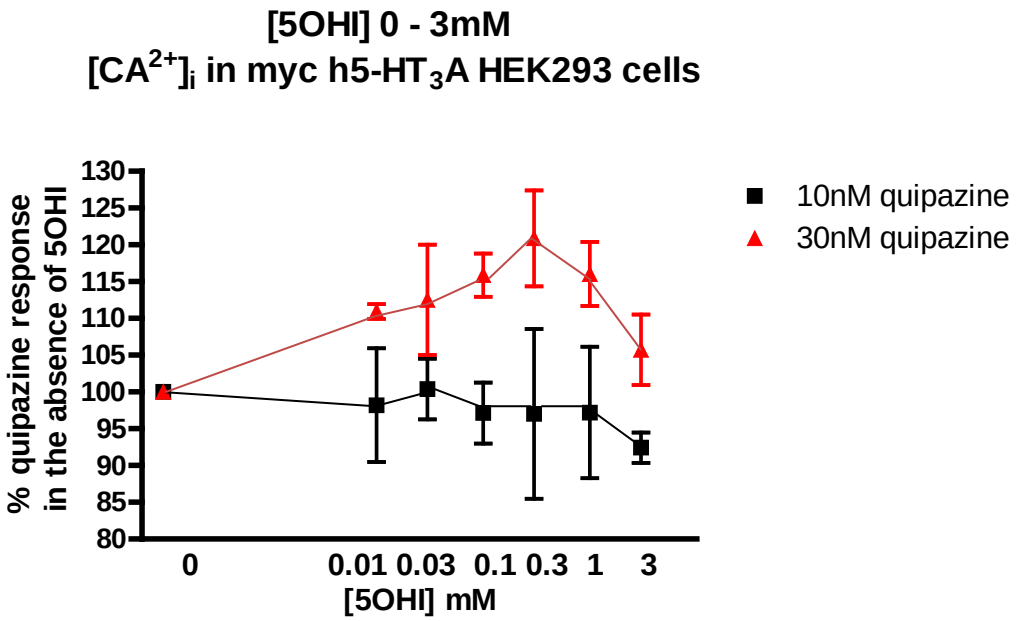


Figure 13. Concentration-dependent ability of increasing concentration of 5-hydroxyidole (5OHI) when administered prior to either 10nM or 30nM of quipazine, a 5-HT_3 receptor partial agonist, to induce increases in intracellular calcium in HEK293 cells expressing the human 5-HT_3A receptor; representing the percentage of response compared to the response identified when in the presence of vehicle appropriate quipazine concentration was used. Data represent mean ± SEM, *n* >3.

$[Ca^{2+}]_i$ responses in HEKh5-HT3A cells evoked by quipazine in the absence and presence of 5OHI were fully antagonised by prior application of the selective antagonists, ondansetron (500 nM; data not shown).

3.1.2C Potentiation by 5OHI of the h5-HT3A compared to the mouse receptor-mediated increase in [Ca2+]i in HEK293 cells transiently expressing either the murine or the human myc tagged 5-HT3A subunit

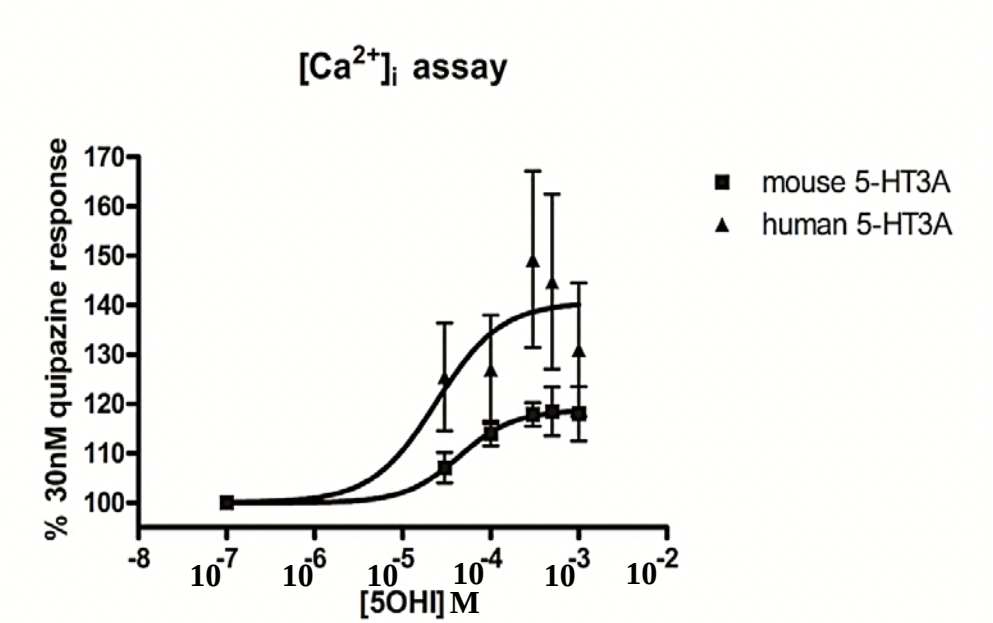


Figure 14. Concentration-dependent ability of increasing concentration of 5-hydroxyindole (5OHI) when administered prior to quipazine (30nM) a 5-HT_3 receptor partial agonist to induce increases in intracellular calcium in HEK293 cells expressing either the human or the mouse 5-HT_3A receptor. Data represent mean ± SEM, n = 3–4.

In a further attempt to characterise the allosteric modulation Cl-indole is able to produce we were interested in potentially comparing if the murine and the human subtype of the receptor would behave differently in the presence of Cl-indole allowing us to use preciously identified amino acids important for the activity of other PAMs identified in the murine receptor. Application of 5-hydroxyindole (5OHI; PAM) (0.03 - 1 mM) appear to potentiate the impact of a subsequent application of quipazine (30nM; Figure 14) to [Ca2+]i response in HEK293 cells expressing either the human or the mouse 5-HT3A subunit, in a concentration-dependent manner,. Whilst 5OHI induced a significant percentage change (to 118% from the 100% of the control) in the response to quipazine (30 nM) in the mouse receptor subunit; there was an apparent plateau in the percentage response obtained at the higher concentrations of 5OHI used (0.3 - 1 mM). The 5OHI-induced potentiation in the maximal quipazine response was greater (138% relative to control) at the HEK293 cells expressing the human 5-HT3A subunit (Figure 14); however, there was an apparent decrease in the

percentage response obtained at the higher concentrations of 5OHI (0.5 - 1 mM); introducing an apparent in increased affinity of 5OHI for the human receptor compared to the mouse 5-HT3A subunit (Figure 14; apparent EC_{50} of 44 µM for the murine compare to 24.1 µM for the human 5-HT_3A receptor). Indicating the human subtype to be more sensitive to Cl-indole, which would not limit us from using.

3.1.3 L288S subunit Myc-tagged h-5HT3A single amino acid mutation by quickchange attempted to knock-out the modulatory effect of 5OHI

Hu et al (2006) have previously identified in the murine 5-HT_3 receptor mutation to serine of Leucine 293 (L288 in human) mutation to alter gating and alcohol modulatory actions. We subsequently wanted to test if this was true in our model.

Application of 5-hydroxyindole (5OHI) (0.1 µM - 1 mM) appear to potentiate the impact of a subsequent application of quipazine (30nM; Figure 15) to $[Ca^{2+}]_i$ response in HEK293 cells expressing either the myc tagged (h)5-HT_3A or L288S mutated 5-HT_3A receptor, in a concentration-dependent manner, with response reaching a plateau at 10µM 5OHI (Figure 15). No significant difference was observed in the pharmacodynamics of 5OHI in the presence of the Leu288Ser mutation compared to the wild – type receptor (response range between 100 and ~ 112 %; Figure 15).

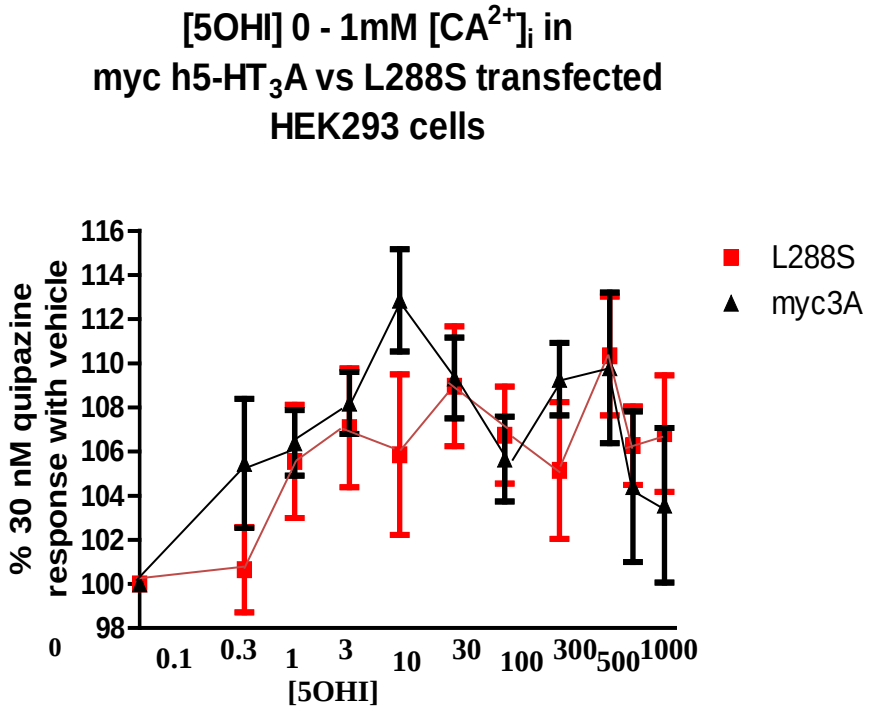


Figure 15 Concentration-dependent ability of increasing concentration of 5-hydroxyindole (5OHI) when administered prior to quipazine (30nM) a 5-HT_3 receptor partial agonist to induce increases in intracellular calcium in HEK293 cells expressing either the myc tagged h5-HT_3A receptor or the mutated L288S receptor. Data represent mean ± SEM, *n* = 3–6.

Application of quipazine (0.3nM - 1 µM) was introduced to evoke $[Ca^{2+}]_i$ response in HEK293 cells expressing either the myc tagged (h)5-HT_3A receptor or the mutated L288S receptor in a concentration-dependent which was subsequently compared to responses arising from 5-HT (Figure 16). Furthermore, the prior presence or absence of 10 µM 5OHI was used to assess if changes in the binding site of 5OHI, as described in the murine 5-HT_3A receptor which is located at Leu293 of the M2 domain in the mouse and rat (Hu and Lovinger) which is equivalent to the L288 in the human subunit changed to Serine, was able alleviate the induced increase in the response to quipazine. No apparent difference was established when $[Ca^{2+}]$i flexstation assay was utilised (Figure 16) and the pharmacological profile of quipazine in the presence or absence of 5OHI appeared almost identical within the particular set of cells expressing either L288S or "wild-type" 5-HT3A subunit.

However, when we compare the two receptors between them we see a different apparent affinity of quipazine (EC50 2.39e-8 and 1.96 e-8 M for vehicle and 5OHI L288S compared to EC50 1.30e-8 and 1.29e-8 M for vehicle and 5OHI wild type 5-HT3A subunit).

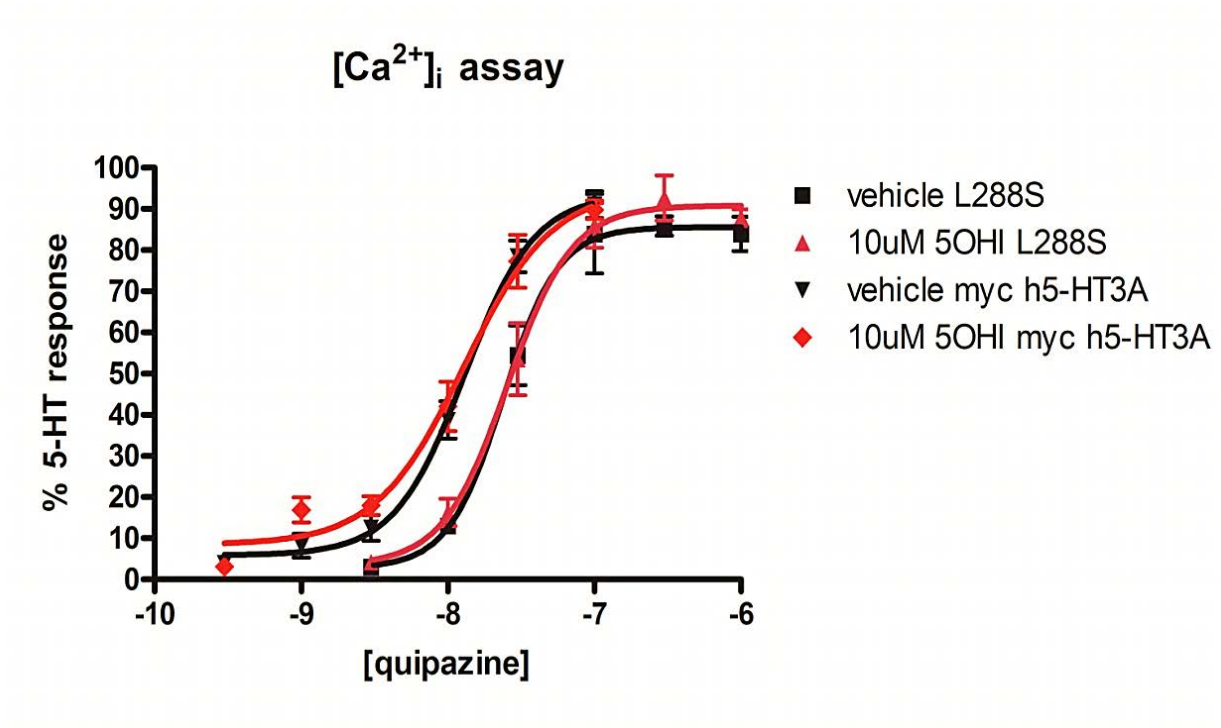


Figure 16 Concentration-dependent ability of increasing concentration of quipazine (Molar) a 5-HT_3 receptor partial agonist to induce in the absence or presence of 10µM 5OHI increases in intracellular calcium in HEK293 cells expressing either the myc tagged h5-HT3A receptor or the L288S mutated receptor. Data represent mean ± SEM, n = 3–6.

Cl-indole also induced a concentration-dependent increase in the maximal responses evoked by a range of 5-HT_3 receptor partial agonists (Figure 4C; Figure 5; Figure 6A; Table 4).

3.1.3 L288S mutant compared to h-5HT3A to potentially knock-out the modulatory effect of Cl-indole in HEK293 cells

We further wanted to characterise the potential effect the mutant would have on the Cl-indole modulation. Application of Cl-indole (30 µM) appear to potentiate the impact of a subsequent application of endogenous full agonist 5-HT (3 µM; Figure 17) to $[Ca^{2+}]_i$ response in HEK293 cells expressing either the myc tagged (h)5-HT_3A or L288S mutated h5-HT_3A receptor, as well as partial agonist DDP733 (0.3µM; Figure 17). The effect of Cl-indole potentiation of the response appear to be blocked by the substitution of leusine 288 to a serine for when challenged with 5-HT however the statistics were blunted when a t-test was done for the paired observation (P= 0.0504).

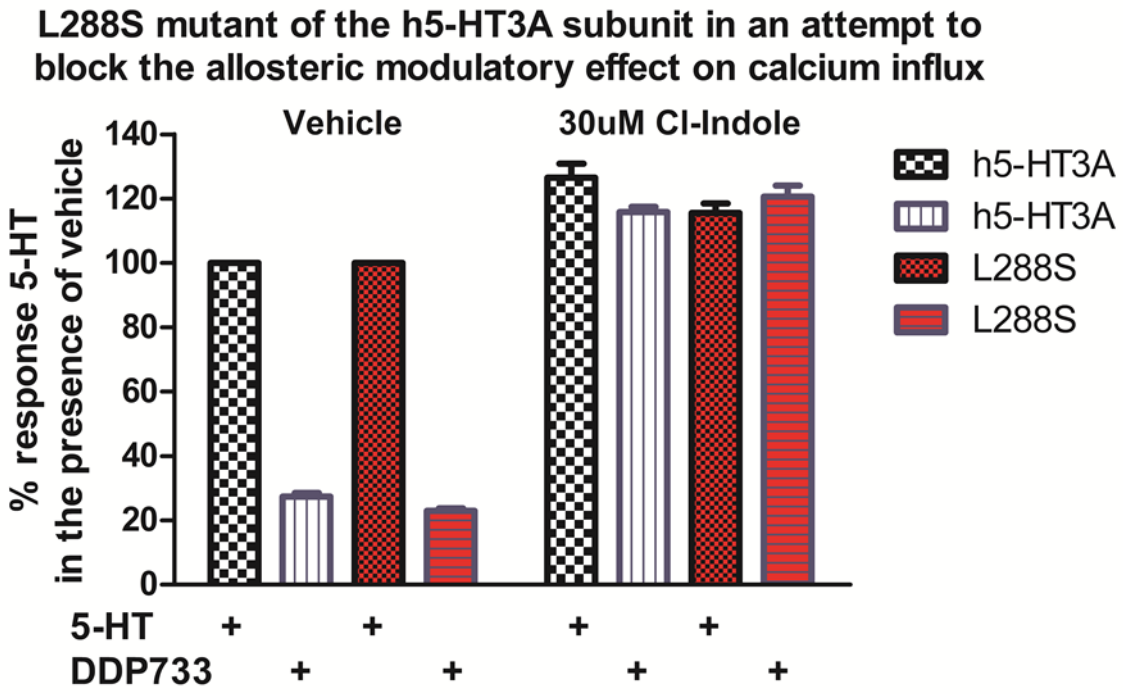
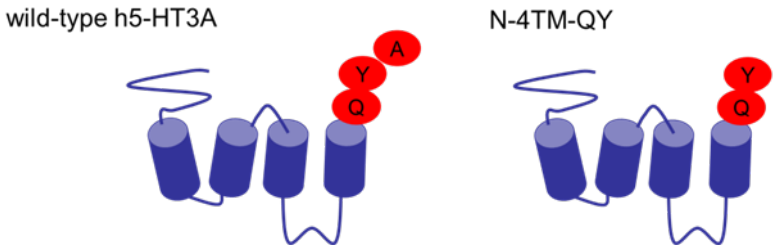


Figure 17 Ability of Cl-indole (30 µM) to potentiate the ability of 5-HT_3 receptor full agonist 5-HT (3 µM) and partial agonist DDP733 (0.3 µM) to increase intracellular calcium in HEK293 cells expressing either the h5-HT_3A receptor or the single amino acid L288S mutant of the h5-HT_3A Data represent mean ± SEM, *n* = 4 - 8.

3.2 Utilising Recombinant receptors

3.2.1 The stability of the h5-HT$_3$A receptor and the importance of the c-terminus

In order to explain potential role different regions have in the receptor stability and potential play a role to receptor function and pharmacology we have been also interested in previously reported regions of the receptor. Following previously published observation that the C-terminus of the human (h) 5-HT3A subunit is important for the expression of the homomeric 5-HT$_3$A receptor within the cell membrane, suggesting a role for the C-terminus in the promotion of receptor stability (Butler et al., 2008). We further assessed the stability of the h5-HT$_3$A receptor in comparison with that arising when the C-terminal alanine is truncated (ΔAla455 [N-4TM-QY]; see Butler et al., 2008 for construct terminology; see Template 1).



Template 1. Graphical representation of the wild-type h5-HT3A subunit and truncation mutant of the h5-HT3A subunit N-4TM-QY (ΔAla455) (Poster, Batis et al., BPS 2008)

Expression of the h5-HT3A subunit and mutants/truncations by COS-7 cells and radioligand binding assays were performed as described previously (Butler et al., 2008). Briefly, COS-7 cells were transiently transfected with the appropriate cDNA via electroporation and harvested 48 h post-transfection to generate binding homogenates to assess the impact of urea upon specific ~ 1 nM [^{3}H]granisetron binding. [^{3}H]Granisetron specific binding (defined by ondansetron, 10 µM) arising from expression of the truncation mutant of the h5-HT3A subunit, N-4TM-QY (ΔAla455), was more susceptible to disruption by increasing concentrations of urea relative to the specific binding arising from expression of the wild-type h5-HT3A subunit (Figure 18; $P<0.05$, Mann Whitney U test). In contrast, there was no significant difference in the urea sensitivity of [^{3}H]granisetron specific binding arising from expression of the and N-4TM-AAA relative to the wild-type h5-HT3A subunit (Gln453Ala and Tyr454Ala; data not shown).

Additionally, the pharmacology of the wild-type h5-HT_3A receptor and the N-4TM-QY were assessed when challenged with cold 5-HT, ondansetron and quipazine assessed [^{3}H]granisetron (~1nM; Figure 19) and no difference in the pharmacology was identified (table 6) with pKi values and Hill slope numbers being almost identical for endogenous agonist 5-HT, specific competitive antagonist ondansetron and partial agonist quipazine.

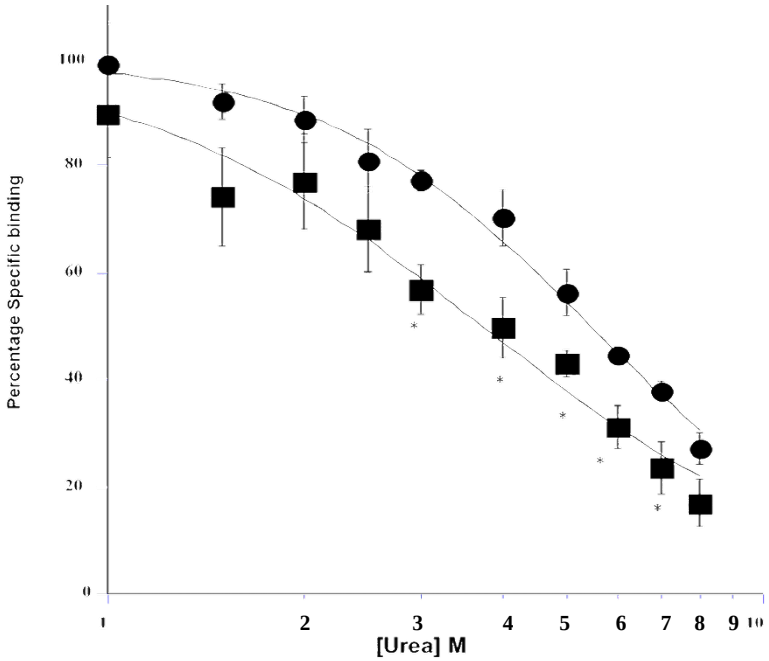


Figure 18 Specific [^{3}H]granisetron (~1 nM) binding levels arising from expression of the wild-type h5-HT3A subunit (●) and the truncation mutant N-4TM-QY (■) by COS-7 cells. Data represent mean ± SEM, n= 4. Comparison between the urea-induced inhibition of specific binding between the two constructs; *P<0.05, Mann Whitney U test (Poster, Batis et al., BPS 2008).

Table 6. pK_i values and Hillslope number of various 5-HT_3 receptor ligands, to compete with [^{3}H]granisetron (~1 nM) at either wild-type h5-HT3A subunit and the truncation mutant N-4TM-QY. Data represents mean ± SEM, (n≥3) (Poster, Batis et al., BPS 2008).

pKi			
Ligand / Subunit	5-HT	Ondansetron	Quipazine
wild-type h5-HT3A	6.59 ±0.18	8.46 ±0.19	8.89 ±0.12
N-4TM-QY	6.74 ±0.29	8.46 ±0.22	8.94 ±0.12

Hill number			
Ligand / Subunit	5-HT	Ondansetron	Quipazine
wild-type h5-HT3A	1.41 ±0.13	0.85 ±0.08	1.11 ±0.04
N-4TM-QY	1.48 ±0.44	0.82 ±0.09	1.40 ±0.30

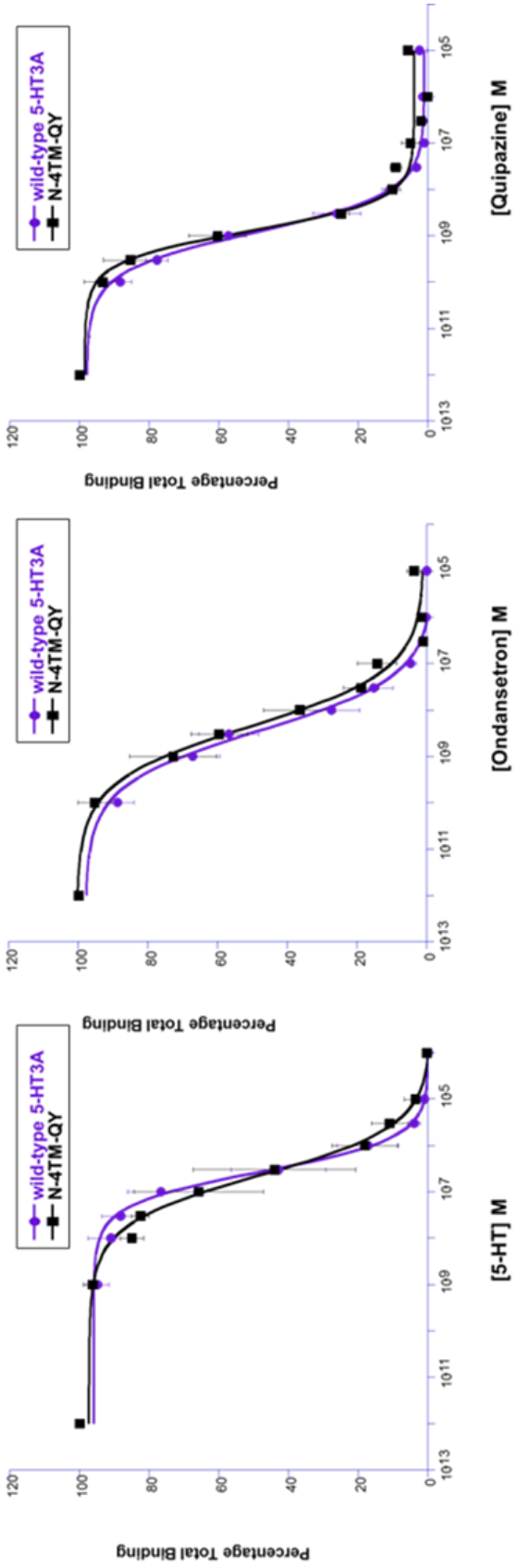


Figure 19. Comparison of wild-type h5-HT3A subunit and the truncation mutant N-4TM-QY by competition binding of [^{3}H]granisetron (~1 nM) with 5-HT, the 5-HT$_3$ receptor agonist quipazine and 5-HT$_3$ receptor antagonist ondansetron (n≥3, (Poster Batis et al., BPS 2008))

3.3 Receptor internalisation / up-regulation

The potential ability of agonist, partial agonist, PAM and antagonist to affect the receptor internalisation and recycling would be investigated further by employing an ELISA assay.

3.3.1 Cycloheximide utilised in whole cell ELISA assay to determine Surface expression of myc - tagged h5-HT_3A receptor expressed in HEK293 cells

To assess if the interaction of h5-HT_3A receptor with ligands has an effect on the cell surface we utilised an ELISA assay. HEK293 cells expressing myc – tagged h5-HT3A receptor were cultured on 96 well plates. In order to identify the receptor internalisation we introduced to the cells cycloheximide (100 µg / ml) a known inhibitor of protein biosynthesis regularly used in assays to determine protein half – life (Zhou P. 2004) and trafficking (Ilegems et al.,2004). In order to establish the appropriate incubation length of the cells with cycloheximide, without disrupting the cell surface expression of the 5-HT_3A receptor, we used 100 µg/ml (~355.42 µM) cycloheximide to incubate HEK293 cells expressing myc- tagged h5-HT_3A receptor. 24 hours following the transfer onto 96 well plates, for 19, 9, 6, 3 and 0 hours prior to fixing them. Cells were then fixed using 2% paraformaldehyde and cell surface detection was performed in the absence of detergent, and total expression levels were determined following Triton X-100 treatment. Receptor expression was determined using an HRP-conjugated secondary antibody and assayed using 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) as the substrate, with detection at 450 nm after 30 min following the addition of 0.5 M H_2SO_4.

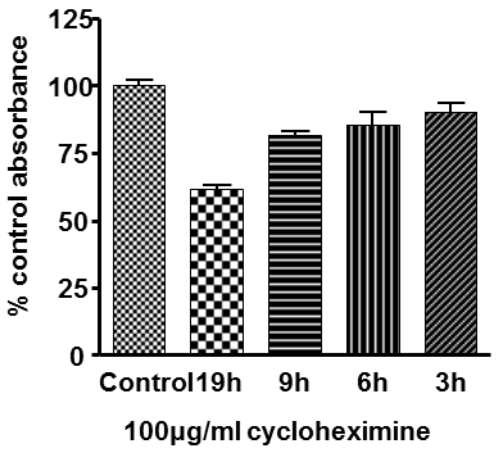


Figure 20. ELISA in HEK293 cells stably expressing myc-tagged h5-HT3A subunit. Incubated with 100µg/ml cycloheximide inhibitor of protein biosynthesis at various time points (0, 19, 9, 6 3 hrs) at the final period prior to fixing the cells

We were able to determine that following 6 or more hours of incubation with cycloheximide were sufficient to start observing increases greater than ~10% in receptor down regulation (Figure 20) and therefore we decided that as previously described assay (Ilegems et al., 2004) we would use 5 hours incubation with cycloheximine (Wettstein, et la., 1964).

3.3.2 Ability of various ligands to influence surface receptor expression of myc - tagged h5-HT_3A receptor expressed in HEK293 cells

We investigated whether using HEK293 cells expressing myc-tagged h5-HT_3A receptor and following incubation with different types of ligands we can influence the cell surface expression of the myc – tagged h5-HT_3A receptor. Moreover since foetal bovine serum contains 5-HT (~0.3 μM; Mothersill et al., 2010) we were interested to further investigate the potential difference the normal serum compared to the dialysed serum could potentially have on cell surface receptor expression.

HEK293 cells expressing the myc –tagged h5-HT_3A receptor were plated onto 96 well plates (in hexaplets wells) and following 24hrs of growth cells were incubated with either normal serum media or dialysed serum media for an additonal 24 hours of which the final 5 hours cycloheximide (100 μg/ml) was also added. Moreover, in addition to the cycloheximide that all cells were treated a proportion had 5-HT (10 μM) introduced or ondansetron (30 μM) or 30 μM ondansetron with 10 μM 5-HT or Cl-indole (30μM) or 30μM Cl-indole with 10μM 5-HT (Figure 21). Following whole cell ELISA assay we identified surface receptor immunoreactivity and expressed it as a percentage relative to control. The results when analysed statistically and they were grouped to be compared to the type of ligand used into categories agonist, antagonist and allosteric modulator if two drugs were combined the antagonist or allosteric modulator was introduced in the incubation 1 minute prior to the agonist.

The cells that exposed to non-dialysed serum for the period of our investigation when incubated with the various treatments (5 hrs) exhibited no significant changes in the receptor levels expressed in the cell surface relative to the control (5 hrs cycloheximide).

However, the cells growing in dialysed serum exhibited differences compare to control in surface receptor expression. Specifically we were able to identify apparent up – regulation of surface myc – tagged h5-HT_3A receptor (~21.7 %; figure 19) induced by the specific antagonist ondansetron (30 μM; Figure 21); further when the antagonist ondansetron was co-incubated with 5-HT induced an apparent receptor up-regulation (~ 31.7 %; figure 21). However, neither of the changes were statistically significant.

Interestingly when the positive allosteric modulator 5-chloroinole (30 μM) was used on its own in HEK293 cells expressing myc – tagged h5-HT_3A receptor grown in dialysed serum, we identified no significant change to the surface receptor expression however when co-incubated with 5-HT (10 μM) we identified an apparent up-regulation in surface myc – tagged 5-HT_3A receptor expression. Total level of signal did not change, used to standardise the experiments through the plates to establish consistent receptor expression.

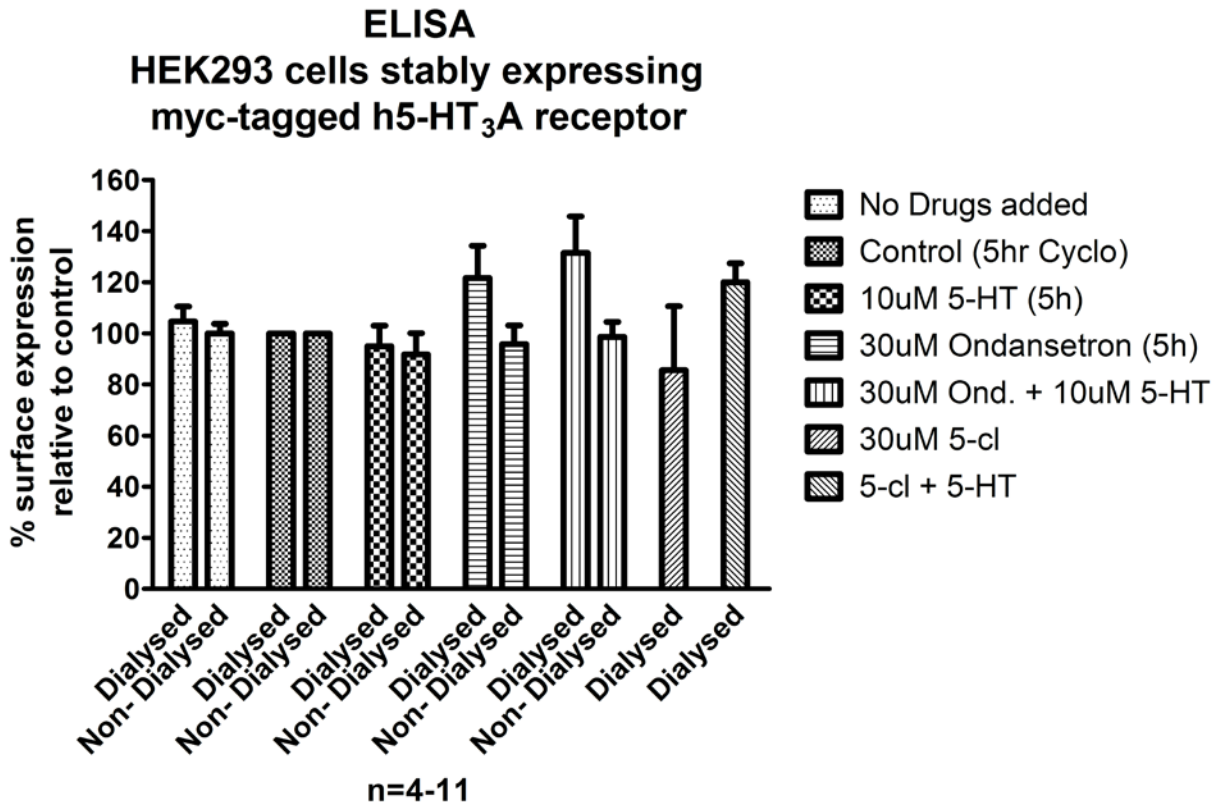


Figure 21. HEK293 cells stably expressing myc-tagged h5-HT_3A receptor subunit were utilised in a live cell ELISA to test the ability of agonist like 5-HT and antagonist ondansetron to induce internalisation of the receptor (in the presence of cycloheximide), as well as the allosteric modulator 5-Chloroindole when incubated either with dialysed or non-dialysed FCS in the culture media.

Further, there is an apparent inhibition of ondansetron response between dialysed and non-dialysed serum used indicating potential 5-HT (0.3 μM estimated in FBS) left non-broken down or a pool of compounds that can anchor the receptor in non-dialysed serum.

3.3.3 The ability of Progesterone treatment to influence 5-HT$_3$ receptor trafficking in NG108-15 cells

NG108-15 mouse neuroblastoma x rat glioma hybrid cells express functional 5-HT$_3$ receptor (Yakel and Jackson, 1988; Hoyer and Neijt, 1987). We utilised this cell line in order to investigate the potential effect the gonadal steroid progesterone which appear to interact allosterically with ligand gated ion channels (Review Rupprecht et al., 2001) and potentially have an effect on behavioural changes and various disorders. To investigate the ability of progesterone, a non-competitive inhibitor of 5-HT$_3$ receptor, to effect membrane bound 5-HT$_3$ receptor expression after prolonged exposure NG108-15 cells were culture for 3 days in culture media in the presence of either vehicle (chloroform) or 1 µM progesterone (HP) or 1 µM progesterone that was withdrawn for the final 6 hrs of the incubation (PWD). Cells were then counted and undergone a membrane preparation in 50 mM Tris buffer. Membrane preparations were then used utilising radioligand binding protocol were ~1nM ^{3}H]granisetron defined specific binding when competed by specific antagonist ondansetron (10 µM) and expressed in fmol bound per million cells (Figure 22). Statistically significant increase in membrane bound 5-HT$_3$ receptor was identified for both high progesterone and progesterone withdrawn incubation compared to the vehicle treated cells (Figure 22)

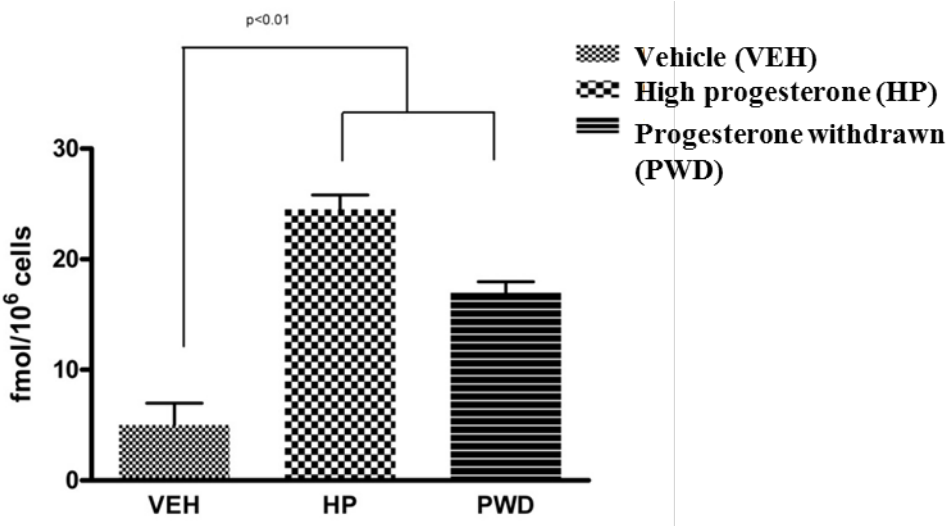


Figure 22. High progesterone treatment to identify 5-HT$_3$ receptor radioligand binding in NG108-15 cells. They were incubated in the presence of vehicle (VEH), high progesterone (1 µM; HP) and also high progesterone (1 µM) that was subsequently was withdrawn the final 6hrs of incubation (PWD). Then competition binding was performed on the membrane preparations. n>3

3.4 Native response investigation in cell system

3.4.1 Identification of expression of mRNA of various 5-HT3 subunits in DG75 cells.

As model of a Burkitt's lymphoma disease and a tool of testing a peripheral 5-HT3 subunits, we utilised the DG75 cell line which could potentially also is a good vehicle for transfection. We wanted initially to establish if the cells were expressing native receptor. DG75 cells, a lymphoblastoid cell line, were used for mRNA extraction and the construction of cDNA in order for RT-PCR screening assay to determine the detectable levels of the five individual human HT3A-E subunit coding. We were able to identify expression of mRNA coding for the 5-HT3A, 5-HT3B and 5-HT3E subunit but lacked detectable levels of 5-HT3C and 5-HT3D subunit mRNA in separate experiments with n= 3 -6 (table 7) and representative results were always compared with HEK293 cells overexpressing the different subunits as positive control (Figure 23).

Table 7; Screening for the identification in DG75 cells the presence of the different subunits of h5-HT_3 receptor. Demonstration of separate experiments were cDNA was used to identify presence positively (+) or not (-) of mRNA expression in DG75 cells.
(Symbols represent individual experiments)

Receptor subunit	DG75 cells expression
HT3A	+ + + +
HT3B	+ + + + +
HT3C	- - - - - -
HT3D	- - -
HT3E	+ + +

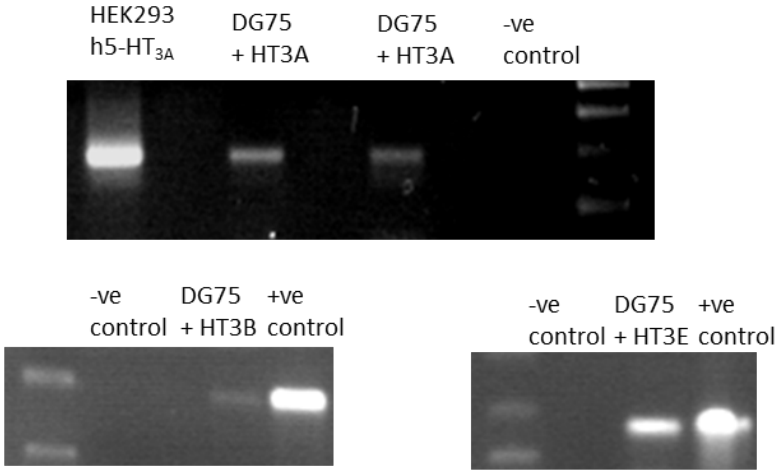


Figure 23. Typical example of h5-HT3A (HT3A), h5-HT3B (HT3B), h5-HT3E (HT3E) subunit trace in DG75 cells compared to positive control and negative control

3.4.2 Ability of 5-HT to evoke responses investigated in DG75 cell by calcium influx assay

Using calcium influx assay using a single using a cuvette fluorometer to measure sequential chances in absorbance representing calcium influx bound to Fluo-4 AM we have further assayed the 5-HT (0 – 1mM) interference with fluorescence, prior to testing any compounds, in lysed DG75 cells that were previously loaded with Fluo-4 AM (Figure 24). We have found that as 5-HT additive concentration increases the percentage absorbance relative to 0 mM 5-HT is decreasing to almost complete abolition indicating that the autofluorescent properties of the 5-HT are interfering with the absorbance recorded due to calcium influx into the cells.

Utilising DG75 cells, concentration response curve were obtained to 5-HT (0.1 – 3 μM; Figure 25), however, the specificity of the response to 5-HT had not been possible to be attributed to a specific 5-HT sensitive receptors. We attempted to identify which 5-HT sensitive receptor was responsible for the observed response by utilising the following antagonists; 0.5 and 1 μM ondansetron, 0.5 and 1 μM ritanserin, 0.5 and 1 μM spiperone, 1 and 10 μM paroxetine1 and 10 μM clozapine, as well as all possible combinations of the previous drugs, and 1 μM GR113808, 1 μM methiothepin and 10 μM renzapride (individual traces not shown; Table 8 for Ki for receptors).

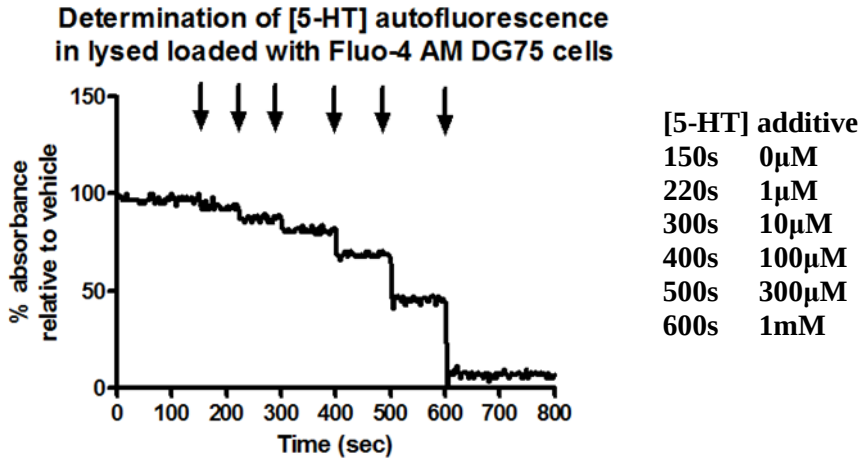
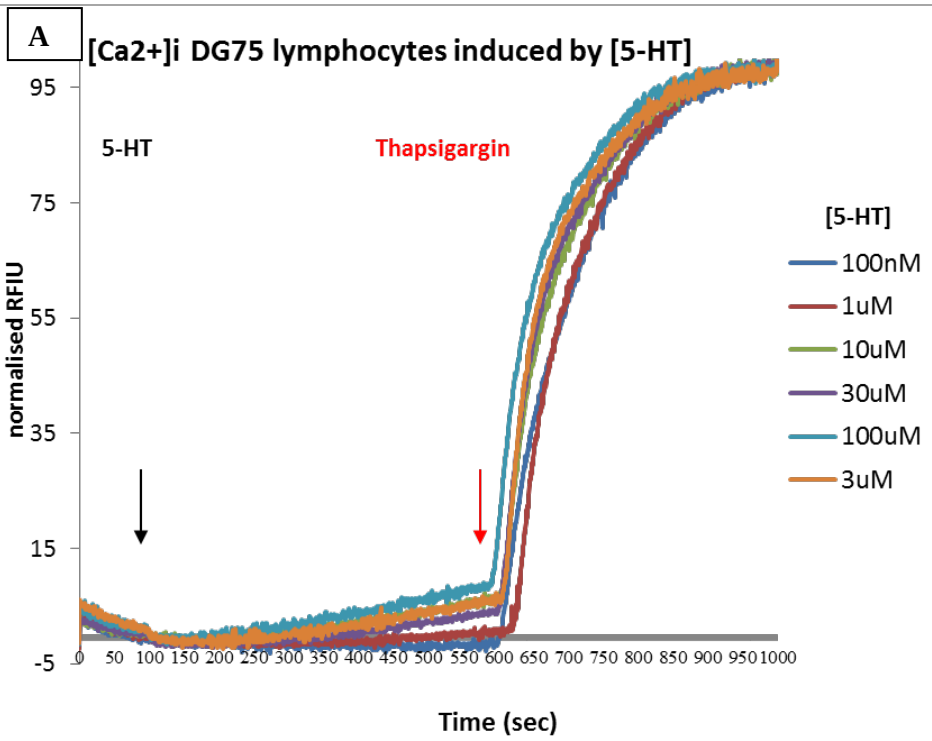


Figure 24. Concentration dependent ability of additive concentrations of 5-HT (0, 1, 10, 100, 300 μM; 1mM) to interfere with absorbance relative to vehicle when added to lysed DG75 cells previously loaded with 2 μM Fluo-4 AM



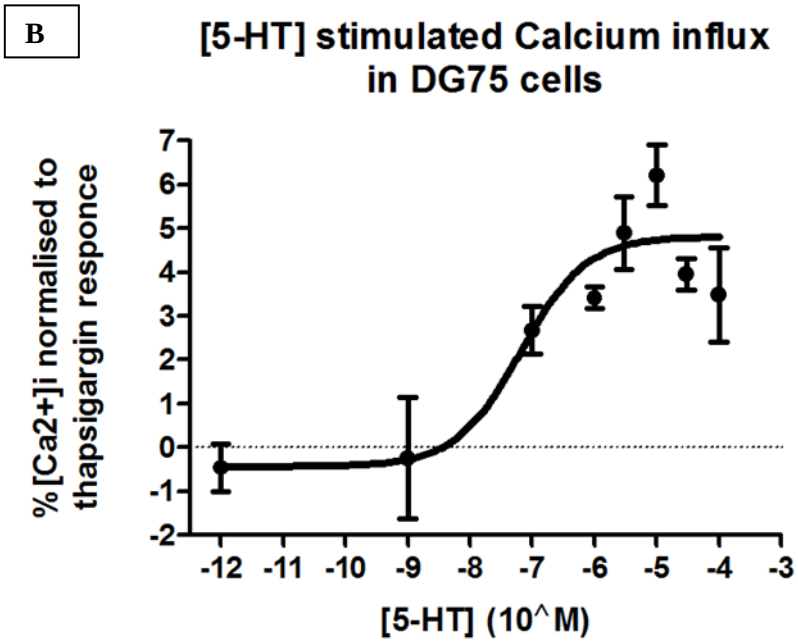


Figure 25. Concentration-dependent ability of 5-HT to increase $[Ca^{2+}]_i$ DG75 cells and the muscarinic receptor agonist, thapsigargin (non-competitive inhibitor of sarco / endoplasmic reticulum Ca^{2+} ATPase) added where indicated. (A) is representative of typical trace from data of at least 5 independent experiments. (B) 5-HT concentration-responses fitted to a three-parameter logistic equation. Data represent mean ± SEM, n > 4.

	Receptor [Ki(nM)]														
Drug	5-HT1A	5-HT1B	5-HT1D	5-HT1E	5-HT1F	5-HT2	5-HT2A	5-HT2B	5-HT2C	5-HT3	5-HT4	5-HT5	5-HT6	5-HT7	SERT
methysergide	16.98	2.5	69	237	34	6.66	3.25	4.3	1.27	> 10,000		63.09	116	83.09	> 10,000
ritavserin	2919	289	72				0.45	3.28	6.60693	> 10,000			16	44.83	
spiperone	194.6	10000	2396.76	5051	> 10,000		1.17	1114.18	602.56	> 10,000		2511.9		109.82	
clozapine	123.7	519	2132	698	130		15.81	8.37	13.4896	241	> 10,000	3857	13.49	48	1624
paroxetine															0.58
ondansetron										7.33					
methiothipin	41.34	12.05	5.78	179.53	648.83		3.1623	1.81	4.46684	38.9		87.17	0.39	3.71	
GR113808								870.96		1000	0.25				

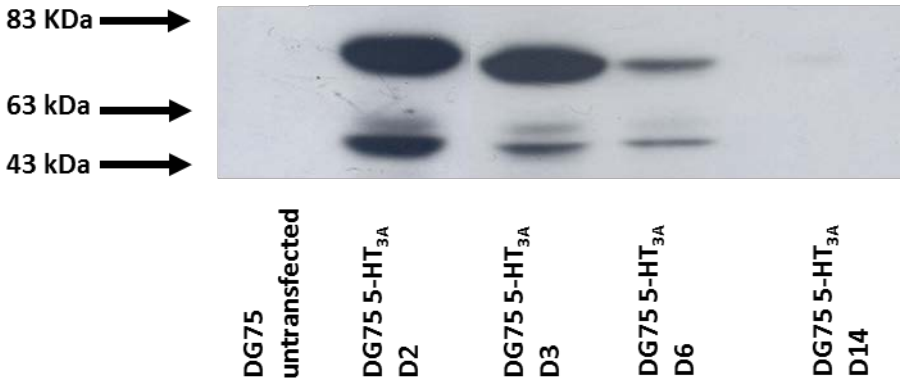
	Receptor [Ki(nM)]												
Drug	adrenergic Alpha1A	adrenergic Alpha2A	adrenergic Alpha2C	dopamine D1	dopamine D2	dopamine D3	Dopamine D4	histamine H1	muscarinic M1	muscarinic M2	muscarinic M3	muscarinic M4	NERT
methysergide	> 10,000	106	88.3										
ritavserin				933	84	24	30						
spiperone	74.47			398.5	0.125	0.295		272					
clozapine	1.6	7	142	266.25	53.5			1.63	16.2	204	58	16.5	
paroxetine									72	340			56.67
ondansetron													
methiothipin													
GR113808													

Table 8. Ki for various receptors of drugs used to identify 5-HT sensitive response

3.5 Transiently transfected DG75 cells with myc –tagged h5-HT3A cDNA

3.5.1 Investigation of immunoreactivity of myc –tagged h5-HT3A receptor expression in DG75 cells

Protein expression was verified by SDS-PAGE followed by Western blotting. 20 µg of protein from whole cell homogenates were loaded per well on 10% polyacrylamide gels. Western blotting was achieved using mouse anti-myc monoclonal (9E10) primary antibody, with an anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP).



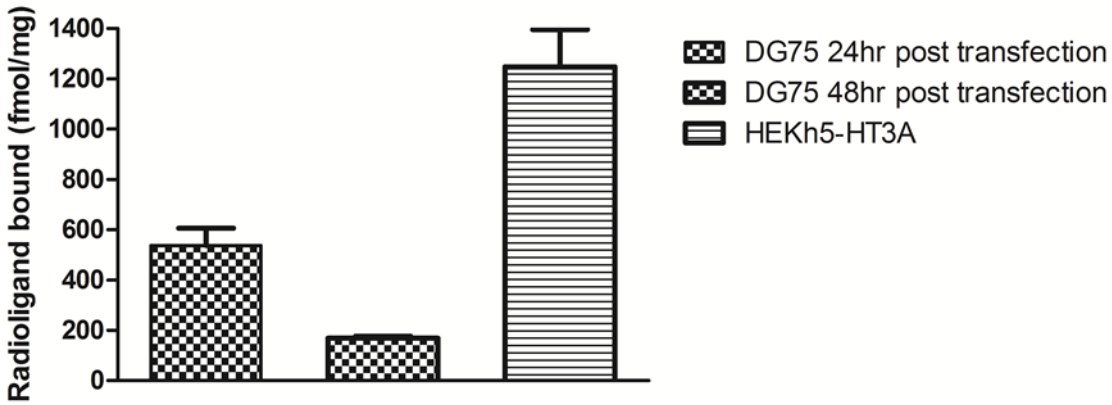


Figure 26 (A) Myc-tagged-5-HT_3A subunit immunoreactivity expressed in DG75 lymphocytes transiently transfected with myc-h5-HT_{3A} subunit cDNA and harvested at 48 hours (D2), 3 days (D3) and 6 days (D6). Untransfected DG75 cells were used as control. Immunoreactivity was identified with monoclonal mouse anti-myc primary antibody 9E10 (2:100); primary antibody was detected using a HRP-conjugated goat anti-mouse (1:1000). (B) Radioligand Binding performed on the same cells were competition binding of [^{3}H]granisetron (~1 nM) with and 5-HT_3 receptor antagonist ondansetron to identify specific binding.

Figure 26, confirms the expression and is representative from at least 3 experimental replicates. Prestained protein markers were run alongside all samples to determine the approximate molecular weight of immunoreactive protein bands. Protein appears faithfully expressed in DG75 cells transfected with the cDNA. Receptor produced multiple immunoreactive bands between the molecular weights of 43 kDa and 63 kDa. This is consistent with previous studies on the 5-HT_3A receptor subunit (Hovius et al., 1998, Monk et al., 2004). Predicted molecular weight of the back bone peptide (53 kDa, Belelli et al., 1995) appear to be an underestimate, with the differences in values reported from molecular size determined by SDS-PAGE attributed to the addition of oligosaccharides (N-glycosylation). Further, cells were harvested at different time points post transfection (2, 3, 6, and 14 days) to identify the optimum time point for using the cells with the strongest signal identified as being between 48 and 72 hours post transfection (Figure 26A).

3.5.2 Radioligand binding assay to identify formation of myc – tagged h5-HT_3A receptor complex expression in DG75 cells

To establish whether the DG75 cells were able to form receptors exhibiting expected radioligand binding levels, competition binding studies were performed for the two strongest time points identified in the Western Blot assay (48 and 72 hours post transfection; Figure 26A) and alongside whole cell homogenates of HEK293 cells stably expressing h5-HT_3A receptor was used. Whole cell homogenates were assayed with ~1 nM [^{3}H] granisetron and competing ligand, the selective 5-HT_3 receptor antagonist ondansetron (Figure 26B). Similar pattern of receptor expression with the one observed with the Western Blot assay was also observed with the radioligand birding competition assay. Both of the observations gave us the confidence that DG75 cells were a good enough expression system for the receptor for us to pursue at approximately 48 hours post transfection.

3.6 The ability of endogenous agonist 5-HT to evoke increase in calcium influx in DG75 cells

Transiently transfected DG75 cells expressing the myc – tagged h5-HT_3A receptor were used to determine receptor function. Furthermore, using a cuvette fluorometer to measure sequential chances in absorbance representing calcium influx bound to Fluo-4 AM (Figure 27) when cells were challenged with endogenous agonist 5-HT. Experiments were repeated at least on three separate occasions following separate transfections of DG75 cells with the receptor cDNA. Additionally, prior incubation of the cells with specific competitive antagonist ondansetron (100nM) inhibited the prior observed 5-HT response. The response was re-established when using the same pool of DG75 cells expressing myc – tagged h5-HT_3A receptor the addition of 5-HT produced comparable response to the one initially observed. We therefore can conclude that we were able to have a functional h5-HT_3A receptor response in a model of a B lymphocyte that we could use further in an investigation and exploitation of 5-HT3 receptor in a potential treatment targeting immune cells.

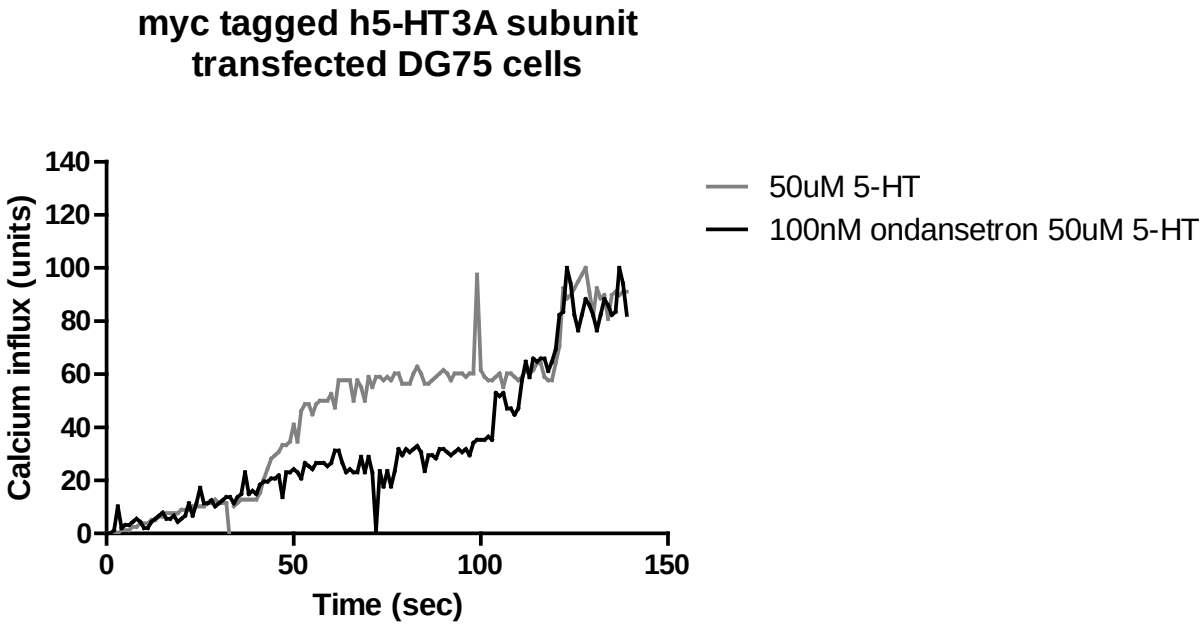


Figure 27. Representative trace of the ability of 5-HT added at 30sec to increase $[Ca^{2+}]_i$ in DG75 cells expressing the myc tagged h5-HT_3A receptor and blockade of the response by the prior incubation with the selective 5-HT_3 receptor antagonist, ondansetron (100 nM). Data representative from at least 3 independent experiments were a 5-HT response was reestablished following a wash out period of the ondansetron.

Chapter 4: Discussion

POSITIVE ALLOSTERIC MODULATOR FOR 5-HT$_3$ RECEPTOR

In the present study we have demonstrate that Cl-indole is a relatively potent positive allosteric modulator of the h5-HT$_3$A receptor and also the native and recombinant receptor expressed in other species; like the mouse native 5-HT$_3$ receptor. In order to demonstrate this, primarily we utilised the rapid 5-HT$_3$ receptor mediated increase in $[Ca^{2+}]_i$, assessed using a Ca^{2+}-sensitive chromophore and quantified using a FlexStation; as has been previously established (Price and Lummis, 2005). Thus, we demonstrated that HEKh5-HT3A cells formed a functional h5-HT$_3$A receptor, based on confirming receptor pharmacology by utilising a range of selective and non-selective 5-HT$_3$ receptor ligands. Additionally, the pharmacological profile we assembled comprised differential maximal responses by a diversity of partial agonists, with the majority of which with recognised sub-maximal intrinsic activities.

Further evidence supporting a selective modulation of $[Ca^{2+}]_i$ via the 5-HT$_3$A receptor in our protocol arise from studies performed in parallel where 5-HT$_3$ receptor agonists failed to evoke responses in native HEK293 cells, indicating that our recombinant receptor had to be involved in the response. Furthermore, when we used 5-HT$_3$ receptor selective concentrations of the specific competitive antagonist ondansetron prior to the regular protocol the response was completely abolished.

Cl-indole when applied alone failed to evoke increase in $[Ca^{2+}]_i$; with the exception of an occasional minor increase, only detected when used at the highest concentration of Cl-indole tested (100 μM).

Notably, Cl-indole potentiated the response to a range of agonists of the 5-HT_3 receptor. It is noticeable that the efficacy of Cl-indole was agonist-dependent. Therefore, at maximally effective concentrations of the agonists, Cl-indole promoted only a comparatively small percentage increase in the maximal effect of the full agonist 5-HT, yet responses by agonists with lower intrinsic activity (efficacy) for the 5-HT_3 receptor, were potentiated to a rather greater degree. Consequently, in the presence of Cl-indole, all of the partial 5-HT_3 receptor agonists tested in the present study ((S)-zacopride, DDP733, dopamine, mCPBG, 2-methyl-5-HT, quipazine, RR 210, SR57727A; Richardson et al., 1985; Neijt et al., 1986; Kilpatrick et al., 1990; Bachy et al., 1993; Downie et al., 1995; Yamazaki et a., 1996; Evangelista, 2007), which displayed intrinsic activities ranging from 3-89%, evoked responses comparable to the responses elicited by maximal (saturating) concentrations of the endogenous full agonist, 5-HT. This observation was also accompanied the apparent shift in an agonist dependent manner of the efficacy and/or affinity of the orthosteric ligand used to evoke the calcium response. This agonist dependence has major implications for drug discovery, and even possible therapeutic use, because the identification and consequent classification of an allosteric ligand will depend on the orthosteric ligand that is used to identify the response.

In addition to Cl-indole potentiating the maximal 5-HT_3 receptor response to agonists, the tachyphylaxis associated with the response, which was particularly evident with saturating concentrations of the higher efficacy agonists, was less apparent in the presence of Cl-indole. Thus, the additional ability of Cl-indole to slow the apparent desensitisation of the receptor indicates that Cl-indole is a Type II PAM of the h5-HT_3A receptor (using the nomenclature recently devised for PAMs of the nicotinic acetylcholine receptors; Grønlien et al., 2007 and was further used Johnstone et al., 2011).

It has been suggested that all ligand-gated ion channels are both activated and desensitised by agonists in a concentration-dependent manner, and can become desensitised at lower agonist concentrations than those required to substantially activate the receptor (Keramidas and Lynch, 2013; Quick and Lester, 2002; Yakel et al., 1991). Thus, under conditions of continuous exposure, such as those that may be approached with chronic drug treatment, agonists may have a discreetly narrow effective concentration range restricted to the section of overlap in which the receptors are activated but not fully desensitised (Hogg and Bertrand, 2004). We were further interested to investigate the hypothesis that Cl-indole could convert h5-HT_3A receptors that were already desensitized by an agonist back to a conducting state. Notably, it appears that Cl-indole can also induce a potential reversal of the apparent desensitisation, assessed by $[Ca^{2+}]_i$ via the flexstation, induced by low and high intrinsic activity agonists of the h5-HT_3A receptor. When the cells were challenged with endogenous full agonist 5-HT at submaximal concentration (3 μM) and at saturating concentration (100 μM) full response was established followed by an

apparent desensitisation which was partially rescued by the addition, subsequently, of Cl-indole and by the maximal concentration of TCE; inducing a sustained response that developed slowly. These results suggest that Cl-indole potentially activated receptors that were presumably agonist-bound but desensitised. Furthermore, when the partial agonist DDP733 at submaximal and at saturating concentration was used evoked a response and produced a sustained calcium influx; when Cl-indole was subsequently added a potential full reversal, similar to the response one observes when using a full agonist, of the probable desensitisation was determined. This is in line with the reported activity of α7 nACh receptor PAM PNU-120596 resulted in a large and sustained inward current when was applied to cultured rat hippocampal neurons that were desensitised by saturating concentration nicotine (Hurst et al., 2005). Notably, a smaller rescue response was observed when TCE was used; potentially indicating that TCE can reach in a lesser extent the allosteric site of the receptor in order to overcome the apparent desensitisation.

Further, when saturating concentration of 5-HT was used instead of Cl-indole, as a subsequent addition drug, only a small response was recovered less than 10% of that of 5-HT would induce on its own, indicating probably that the DDP733 with its high affinity low intrinsic activity out competes the full agonist 5-HT and potentially induces ligand-bound desensitisation.

Moreover, it is now appreciated that 5-HT_3 receptor antagonists can have contrasting characteristics. For example, palonosetron binds with positive co-operativity (Rojas at

el., 2008), as do 5-HT_3 receptor agonists including most with low intrinsic activity (e.g. Barnes et al., 1992). In addition, palonosetron evokes receptor internalisation (Rojas et al., 2010). Interestingly, it has been demonstrated by the Connolly lab (Hothersall et al., 2013) actual surface receptor inhibition rather than internalisation could be responsible for prolonged inhibition in their assays. Another atypical 5-HT_3 receptor antagonist, BRL46470, has an enhanced binding capacity to recombinant 5-HT_3A receptors (Steward et al., 1995). However, in the present study, both palonosetron and BRL46470 behaved as other 'classical' 5-HT_3 receptor antagonists (alosetron, ondansetron, ramosetron) in that they failed to evoke 5-HT_3 receptor mediated responses even in the presence of a maximal concentration of Cl-indole. This directly contrasts the ability of Cl-indole to elicit robust 5-HT_3 receptor mediated responses from the low intrinsic activity partial agonists investigated in the present study. Such findings suggest that the atypical characteristics of the 5-HT_3 receptor ligands, palonosetron and BRL46470, are not explained by a low level of intrinsic activity associated with these molecules.

An additional 'atypical' 5-HT_3 receptor ligand (S)-zacopride; previously designated as an antagonist (Smith et al., 1988). When radiolabelled, (S)-zacopride is one of the most suitable radioligands to effectively label the 5-HT_3 receptor (e.g. Barnes et al., 1992a), yet 5-HT_3 receptor agonists and partial agonists fail to compete with positive co-operativity, unlike their competition for other 5-HT_3 receptor radioligands (e.g. Barnes et al., 1992a). In addition, (S)-zacopride does not display the same ability, as a range of 5-HT_3 receptor antagonists, to modulate animal behaviour in various tests suggestive of therapeutic potential (Barnes et al., 1990; Young and Johnson, 1991;

Barnes et al., 1992b; 1992c; File and Andrews, 1993 but see Bill et al., 1995). Furthermore, unlike other 5-HT_3 receptor antagonists, (S)-zacopride can induce emesis (King, 1990; although see Smith et al., 1989 and Costall et al., 1990) and evoke the Bezold-Jarisch reflex in the ferret (Middlefell and Price, 1991); both responses involve the vagus nerve (Brown, 1966; King, 1990), which expresses high levels of functional 5-HT_3 receptors (e.g. Ireland and Tyers, 1987; Kilpatrick et al., 1989). To interpret these findings it was subsequently hypothesised that (S)-zacopride may display agonist actions at the 5-HT_3 receptor, besides the identified 5-HT_4 receptor agonism of (S)-zacopride (e.g. Eglen et al., 1990; Baxter et al., 1991) - this receptor also activates the vagus nerve (Bley et al., 1994; Coleman and Rhodes, 1995) - complicates interpretation of animal data from in vivo models. The failure of (S)-zacopride to activate native 5-HT_3 receptors in in vitro assays, including the isolated vagus nerve (rabbit and rat vagus nerve; Barnes et al., 1990; Bentley and Barnes, 1998) clearly does not correlate with the agonist hypothesis. However, the 5-HT_3 receptor desensitises rapidly in the continued presence of agonist, which can make identification of relatively small responses difficult. Hence in the present study we utilised the ability of Cl-indole to manifest robust 5-HT_3 receptor mediated responses from 5-HT_3 receptor ligands with low intrinsic activity to make a direct in vitro assessment of the nature of (S)-zacopride's interaction with the h5-HT_3A receptor. Consistent with (S)-zacopride's atypical characteristics when compared to 'silent' 5-HT_3 receptor antagonists, (S)-zacopride displayed an inconsistent at best very low level intrinsic activity for the h5-HT_3A receptor (3% compared to 5-HT) that was converted into robust responses by the presence of Cl-indole. Hence the present finding offers direct evidence that (S)-zacopride is a very low intrinsic activity partial

agonist at least for the h5-HT_3A receptor, which distinguishes this ligand from a range of classical 5-HT_3 receptor antagonists.

We have further utilised the rat vagus nerve in a series of grease gap electrophysiological experiments in order to investigate the impact the low intrinsic activity partial agonist DDP733 would have. We were able to demonstrate the concentration-dependent ability of DDP733 to increase the depolarisation of the rat vagus nerve.

We were unable however to perform conclusive experiments on the vagus in the presence of Cl-indole due to limited tissue availability, costs and time constrains of this study.

Additionally with collaboration with Prof. Kumar we have conducted preliminary experiments utilising the rat carotid body, which is expressing 5-HT_3 receptors (Yoshioka et al., 1991; Wang et al., 2002). We have identified that potentially DDP733 shows no activity in the rat, but used in the presence of Cl-indole induces activity of the carotid body, response which was returned to normal upon wash out of the compounds. These preliminary results together with the in vitro data that we have presented in this study can potentially further support our hypothesis that Cl-indole is a PAM to the 5-HT_3 receptor and can play an important role in deciphering native integrative 5-HT_3 mediated responses.

In terms of the molecular mechanism of Cl-indole, the greater tendency to impact the maximal response to partial agonists associate a modulation of early transitions within

the conformational wave elicited by agonists, that would result in receptor function (Lape et al., 2008). The early transitions arise in the region of the orthosteric binding loops in the N-terminus, particularly the degree of capping of the agonist ligand (potentially by loops B and C; Miller and Smart, 2009). If the degree of capping of the agonist correlates with the degree of intrinsic activity of a partial agonist and by association, the stability of the pre-activated receptor 'flip-state' (Lape et al., 2008) then this molecular region of the subunit may be the target of Cl-indole within the 5-HT_3 receptor complex.

Uncompleted work by other members of the lab is investigating this hypothesis, which may also elucidate whether the Cl-indole recognition site that alters the maximum response to agonist is also responsible for the reduced apparent desensitisation rate in the presence of Cl-indole. The identification and mapping of the allosteric binding pocket and its proximity to the orthosteric binding site could potentially allow for an investigation of the some potentially bitopic (binding at both ortho- and allo- steric site) ligands which could potentially endogenously exist (maybe a break down product of monoamines or indoles). We could also speculate that such potential ligand can also give rise to an understanding of biological complexity (receptor reserves could be interpreted both ways if such theory existed); potentially providing us with pharmacological tools to discriminate between the different homomeric and heteromeric 5-HT_3 receptor and also the opportunity to develop novel drug discovery methodologies.

Patch clamp recordings using HEKh5-HT3A cells demonstrated that Cl-indole potentiated h5-HT_3A receptor mediated responses, particularly the maximal responses evoked by the partial agonist DDP733 in comparison to 5-HT. This effect of Cl-indole was not associated with change in the driving force for the 5-HT_3 receptor mediated current as the reversal potential was unchanged in the presence of Cl-indole (see further Newman et al., 2013).

5-Substituted indoles, such as 5-hydroxyindole (5OHI), have been recognized as PAMs of 5-HT_3 receptors. However, reports indicate that 5OHI has both orthosteric and allosteric binding activities (Kooyman et al**.,** 1994**;** Hu and Lovinger, 2008), signifying at least two binding sites, one of which could potentially overlap the orthosteric site. Furthermore, 5OHI has allosteric effects on the nicotinic α7 receptor (Bertrand and Gopalakrishnan, 2007) and one report suggests that 5OHI is a PAM at rodent but not human receptors (Grønlien et al., 2010). These studies, consistent also with our results, cast considerable doubt on 5-hydroxyindole's selectivity and its mixed mode of action renders its use as a pharmacological tool debatable.

Further, It has been identified in the murine 5-HT3A subunit that the substitution of the TM2 leucine 298 (human equivalent is Leu288) with serine is detrimental to the positive allosteric effects of 5OHI and TCE, on the murine receptor (Hu and Lovinger 2008). Additionally, this site (L288) has been highlighted as having a significant effect on the activity of the negative allosteric modulator (NAM) PU-02, the most potent and selective NAM for the 5-HT_3 receptor characterised, appears to be acting

through a transmembrane intersubunit site comprised of the upper three helical turns of TM2 and TM3 in one subunit and TM1 and TM2 in the adjacent subunit (Trattnig et al., 2012). In the present study, we attempted to demonstrate if the equivalent mutation would have the same effect in human 5-HT_3A receptor when exposed to 5OHI and TCE and potentially abolish the positive allosteric effect established by Cl-indole utilising the Flexstation calcium influx assay.

Initially we attempted to demonstrate that the L288S receptor would have a detrimental influence in the positive modulation of 5OHI and TCE utilising the calcium influx assay when the cells were challenged with the partial agonist quipazine. We were unable to identify any difference in the pharmacology of quipazine (Hill number and EC50, EMax) in the dose response curve obtained in the presence or absence of 5OHI with the dose response curves being almost identical.

We were not able to statistically show a reduction in the potentiation effect that Cl-indole has when co-administered with 5-HT (P=0.0504 t test; the P value is verging to significance; however, as the variation in the different experiments was minute providing an over confidence in the changes; “Statistics Notes: Interaction 2: compare effect sizes not P values” http://www.bmj.com/content/313/7060/808) and there was no effect in the potentiation of calcium influx when the cells in the presence of Cl-indole were challenged with the partial agonist DDP733.

Excitingly, however, we were able to identify a small difference in quipazine pharmacology in the L288S (pEC50 = 7.62 ± 0.062) compare to normal myc-tagged

h5-HT_3A receptor (pEC50 = 7.885 ± 0.045) but statistically significant (P=0.026; student t-test). We therefore were not able to identify the L298 to have the same effect in the human subunit compared to the reported influence that has in the murine; even though that 5OHI appears to have a greater effect in the human subunit compare to the mouse 5-HT_3A receptor in our model as our data suggest when were stimulated with quipazine (figure 14), contraindicating reports from Grønlien (Grønlien et al., 2010).

Additionally, we demonstrated in our lab, that unlike 5-hydroxyindole, 5-chloroindole is not an allosteric modulator of nicotinic α7 receptors highlighting the selectivity of Cl-indole over 5-hydroxyindole. Although closely related structures, Cl-indole exhibits a higher potency and selectivity than 5-hydroxyindole, making it a more attractive pharmacological tool for the exploration of the allosteric modulation of the 5-HT_3 receptor (see further Newman et al., 2013).

It is of interest that Cl-indole concentration-response studies identified Hill coefficients greater than unity. This suggests that multiple Cl-indole molecules bind to a given h5-HT_3A receptor in a cooperative manner. Given the pentameric molecular symmetry of the homomeric h5-HT3A receptors investigated in the present study, five identical Cl-indole recognition sites within the single receptor complex are conceivable. Studies investigating the concentration-response pharmacology of Cl-indole at heteromeric and mutant 5-HT_3 receptors may provide insights into the molecular target and mechanism of Cl-indole's interaction.

In order to identify whether the action of Cl-indole can potentially be translated from recombinant to native 5-HT_3 receptors, we investigated the effect of the compound upon the previously described 5-HT_3 receptor mediated increase in the neurogenic contraction of the mouse urinary bladder (Chetty et al., 2007). It has been identified that expression of 5-HT receptors additional to 5-HT_3 receptor subtype exists in this preparation (Chetty et al.,2007; Newman et al., 2013); thus, a just-maximal concentration of the selective 5-HT_3 receptor agonist, mCPBG, which behaves as a full agonist in this preparation, was employed (Chetty et al., 2007). While mCPBG alone caused a small increase in the amplitude of nerve-stimulation evoked contraction, this effect showed marked tachyphylaxis consistent with 5-HT_3 receptor desensitisation. This is consistent with results obtained with recombinant 5-HT_3 receptors; Cl-indole potentiated mCPBG increases in the neurogenic contraction of the mouse urinary bladder and prevented the tachyphylaxis. Responses to mCPBG in the absence or presence of Cl-indole were prevented by the inclusion of ondansetron (500 nM). Together with the data from HEKh5-HT3A cells, these observations indicating that Cl-indole acts similarly on native and heterologously expressed 5-HT_3 receptors. Thus, Cl-indole evoked equivalent responses in both species.

CONCLUSION

Whilst the ability of various alcohols and general anaesthetic agents to modulate multiple LGIC family members supports the common ancestry of these proteins, this lack of receptor selectivity may hinder therapeutic utility of pharmacologically targeting these sites. Hence, an important progression of the present work would be to investigate the selectivity of action of Cl-indole across other members of the LGIC superfamily members (e.g. preliminary data indicates Cl-indole is also Type II PAM of the 5-HT_3AB receptor isoform - potentiates agonist-evoked responses and reduces rate of desensitisation; Newman et al., unpublished observation). An allosteric recognition site selective to the 5-HT_3 receptor would offer intriguing therapeutic potential. For instance PAMs may offer relief from reduced gastrointestinal and bladder motility disorders (e.g. constipation-dominant irritable bowel syndrome and atonic bladder that commonly associated with the autonomic failure of diabetes mellitus). It also remains an intriguing possibility that an endogenous ligand impacts the Cl-indole recognition site expressed by the 5-HT_3 receptor in a way that rational drug design to prevent this interaction, or the identification of inverse allosteric receptor ligands, analogous to the action of ß-carbolines at the benzodiazepine receptor site on the $GABA_A$ receptor (e.g. Peña et al., 1986; for review see Evans and Lowry, 2007), may offer further pharmacological avenues to manipulate selectively the function of the 5-HT_3 receptor for therapeutic benefit.

In summary, the present study has identified Cl-indole to be a potent Type II PAM of the 5-HT_3 receptor.

5-HT_3 RECEPTOR STABILITY

It has previously shown that the C-terminus of the human 5-HT3A subunit is important for the expression of the homomeric 5-HT_3A receptor within the cell membrane, and suggested a role for the C-terminus in the promotion of receptor stability (Butler et al., 2008). In the present study, we further assessed the stability of the h5-HT_3A receptor in comparison with that arising when the C-terminal alanine is truncated (ΔAla^{455} [N-4TM-QY]; see Butler et al., 2008).

Expression of the h5-HT3A subunit and mutants/truncations by COS-7 cells and radioligand binding assays were performed as described previously (Butler et al., 2008)

There was no significant difference in the pharmacological profile of the wild-type h5-HT3A subunit and truncation mutant of the h5-HT3A subunit N-4TM-QY.

Specific binding arising from expression of the truncation mutant of the h5-HT3A subunit, N-4TM-QY (ΔAla^{455}), was more susceptible to disruption by increasing concentrations of urea relative to the specific binding arising from expression of the wild-type h5-HT3A subunit ($P<0.05$, Mann Whitney U test). In contrast, there was no significant difference in the urea sensitivity of [^{3}H]granisetron specific binding arising from expression of the wild-type h5-HT3A subunit and N-4TM-AAA (Gln^{453}Ala and Tyr^{454}Ala).

There was no significant difference in the pharmacological profile of the wild-type h5-HT3A subunit and truncation mutant of the h5-HT3A subunit N-4TM-QY.

The current study further supports the role of the C-terminus of the h5-HT3A subunit to promote stability of the arising 5-HT_3 receptor complex.

The present study in combination with the work published by Butler et al., (2008) demonstrates an important role for the C-terminus in the formation of the functional h5-HT_3A receptor. Butler et al. were able to demonstrate the partial restoration of 5-HT_3 receptor binding and cell membrane expression when cells expressing C-terminal mutant 5-HT3A subunits were grown at a lower temperature (27 ^{o}C) suggests that the C-terminus stabilises the 5-HT_3 receptor allowing subunit folding and subsequent maturation but were not able to exclude the 'docking' (a recognition site of this region of the subunit) role for the C-terminus as well as the stability to receptor complex; as we were able to, using a chaotropic agent, to see the difference a single amino acid can have in a mature receptor complex.

With our present study we further support the hypothesis that the C-terminus is important in the stability of the receptor complex.

5-HT_3A RECEPTOR SURFACE EXPRESSION

Receptor immunolabelling allowed monitoring of the plasma membrane targeting as well as the ligand-induced endocytosis/ up-regulation of the h5-HT_3A receptor in living cells. Reports that the receptor appears on the plasma membrane approximately 5hrs after transfection (Ilegems et al., 2004) and that maximum receptor formation occurs 36-50 hrs post-transfection was reconfirmed in this study when we were establishing our protocol.

In the current study, we have attempted to demonstrate that the cell surface expression of recombinant 5-HT_3A receptors is effected by the prolonged presence of agonist and antagonist. We were unable to detect any changes in immunoreactivity to 5-HT_3A receptor in HEK293 cell surface when all treatment protocols were contacted in medium supplemented with non-dialysed foetal bovine serum (which potentially contains 5-HT ~0.3µM; Mothersill et al., 2010). In contrast, changes in expression were indicated when dialysed serum was used. Furthermore, it has been indicated by our data that competitive antagonists (ondansetron) potentially induces an apparent up-regulation of 5-HT_3A receptor in the surface.

Potentially these findings indicate that up-regulation of 5-HT_3A receptor is independent of receptor activation as indicated by the inability of a competitive antagonist (ondansetron; to which Cl-indole failed to induce any response resulting in calcium influx) to block the 5-HT induced apparent up-regulation, and the finding that even antagonist treatment alone could potentially induce an apparent up-regulation.

Antagonist-induced up-regulation has also been shown for $GABA_A$ receptors with a competitive antagonist (Eshaq et al., 2010). Mutations of the agonist-binding site of $GABA_A$ receptors completely blocked the agonist-induced up-regulation (Eshaq et al., 2010). Competitive antagonists for nACh receptors (α4β2 subtype) are unable to induce up-regulation, however, non-competitive antagonists significantly up-regulate nACh receptors (Peng et al., 1994, Kuryatov et al., 2005). This could potentially allow the hypothesis that the non-competitive antagonists for nACh receptors are more effective at promoting subunit assembly in the endoplasmic reticulum.

Further we have also demonstrated that non-competitive allosteric antagonist (progesterone; highest reported concentration of progesterone in rat plasma is about 20 μM in late pregnancy; Ichikawa et al., 1974) was able to induce significant up-regulation in cell surface 5-HT_3A receptor in neuroblastoma cell line and that withdrawal of the progesterone reduced the surface 5-HT_3A receptor; however, the effect of previous prolonged exposure was still sufficient to present receptor up-regulation compare to control in both treatment protocols with progesterone. An intriguing possibility is that such funding could have a key role in potentially understanding why specific conditions are gender predominant (like IBS) as well as being correlated to the changes arising from the menstrual cycle. It could potentially explain also changes in mood, anxiety and depression that are potentially following a pattern depending on the gonadal steroid concentration circulating.

However, we were unable to detect any change in the surface expression of 5-HT_3A receptor when exposed to agonist (5-HT) or to PAM (Cl-indole). This is despite the fact that previous studies have demonstrated that prolonged exposure to agonists have

been shown to increase the surface expression of other members of the Cys-loop LGIC family. Nicotine, an agonist for nACh receptors, has been well documented to increase the surface expression of nACh receptors in cultured cells (Bencherif et al., 1995), rodents (Schwartz and Kellar, 1985, Wonnacott, 1990), and human smokers (Benwell et al., 1988, Perry et al., 1999, Govind et al., 2009). Additionally, Eshaq and colleagues (Eshaq et al., 2010) showed that the $GABA_A$ receptors expressed in HEK293 cells are also up-regulated in the presence of GABA. Reports also indicated that exposure to 5-HT (for 24 hours) leads to approximately a threefold increase in total binding to cells stably expressing 5-HT_3 receptors (Sanghvi et al., 2009). Furthermore, it has been demonstrated (Eshaq et al., 2010) that 5-HT increases surface levels of 5-HT_3A and GABA was unable to up-regulate 5-HT_3 receptors, as well as, the observation that 5-HT was unable to up-regulate $GABA_A$ receptors, suggesting that this phenomenon requires a ligand that works at the appropriate orthosteric site. Interestingly though, there have been reports indicating that agonist induced 5-HT_3 receptor endocytosis initialised after just 5 mins of incubation, with specific agonist, with the plasma membrane associated receptor numbers to decrease by a factor of three (Ilegems et al., 2004), bearing in mind that potentially on their study they have only established that the observation is true in the presence of ligand and not necessarily induced by ligand as further controls would have been required. Furthermore, regarding the internalisation of 5-HT_3A receptors in recombinant systems (Ilegems et al., 2004; Morton et al., 2011) as well as the expression of brain-derived neurotrophic factor reduces the 5-HT_3 receptor (Huang and Morozov, 2011), the mechanisms however remains elusive. Nottingly it has been reported that regarding trafficking to the surface of 5-HT_3A receptor the assembly and trafficking signals (Boyd et al., 2002; 2003) and post-translational modification (Sun et al., 2003;

Monk et al., 2004; Massoura et al., 2011) control recruitment to the membrane. We could speculate that ligand induced regulation of 5-HT_3 receptors may promote changes in receptor function that are isolated from acute pharmacology. 5-HT_3 receptor internalisation has been reported in the gastrointestinal system following excess 5-HT (Freeman et al., 2006) and in recombinant systems, following exposure to palonosetron (Rojas et al., 2010). Associating clinical efficacy of drugs for the receptor and for pathological explanations of high concentrations of 5-HT.

This is also true for other receptor of the cys-loop super family such as $GABA_A$ (Hearing et al., 2003; Barnes, 2000) and nicotinic acetylcholine (St John and Gordon, 2001) receptors, as well as other ligand-gated channel such as $P2X_1$ receptor (Dutton et al., 2000).

All of which are indicating that ligand induced changes in cell surface expression of ligand gated ion channel receptors could be either pro- endocytosis (down regulation) or pro- up-regulation, exhibiting possibly temporal dependency in preference of one state to the other, as well as possibly the type of ligand that is occupying the binding site either the orthosteric or even the allosteric in terms of its efficacy.

However, it has been indicated by our data that the presence of Cl-indole with a full agonist potentially has an up-regulatory effect on the 5-HT_3A receptor when co-administered with 5-HT.

The up-regulation of nACh and $GABA_A$ receptors require saturating concentrations of agonist (Peng et al., 1994, Wang et al., 1998, Kuryatov et al., 2005, Vallejo et al., 2005, Eshaq et al., 2010). Up-regulation of nACh receptors by nicotine requires the

presence of nicotine for at least six to eight hours (Wang et al., 1998, Kuryatov et al., 2005, Vallejo et al., 2005), similar to the time course we utilised for 5-HT_3A receptors. It remains unclear if the up-regulation of $GABA_A$ receptors is dependent upon agonist concentration. However, $GABA_A$ receptors were up-regulated with one hour of GABA exposure followed by 5 hours in the absence of agonist (Eshaq et al., 2010). This appears different for the nACh and potentially 5-HT_3A receptors, which require the presence of agonist for several hours of exposure. This discrepancy could be due to the proposed transport or synthesis of GABA intracellularly. Observation which could lead to the potential speculation that such ligands are providing potentially some kind of chaperone assistance to the maturation and assembly of the receptor complex and to the transportation and clustering of the receptor to the surface.

It has been well established that agonist-induced up-regulation of both nACh receptors and $GABA_A$ receptors is independent of receptor translation (Peng et al., 1994, Wang et al., 1998, Kuryatov et al., 2005, Lester et al., 2009, Eshaq et al., 2010). Multiple hypothesis for the mechanism of the translation-independent up-regulation of nACh receptors have been proposed, including alterations in surface turnover (Peng et al., 1994), and the enhancement of subunit assembly by nicotine (chaperone effect) in the endoplasmic reticulum (Kuryatov et al., 2005, Lester et al., 2009). Furthermore, $GABA_A$ receptors are also up-regulated by a chaperone effect (Eshaq et al., 2010). This hypothesis could potentially be exploited further in the investigation of 5-HT_3 receptor providing us with a comparable account of the possible existence of this phenomenon in the 5-HT_3 receptor.

Further; it has been hypothesised that clathrin mediated receptor endocytosis (Heuser and Andersen, 1989), indicated as a possible mechanism of the endocytosis of the 5-HT_3A receptor the AP2 adaptin-clathin mediated endocytosis. This is a rational possibility as other members of the ligand-gated ion channel super family potentially undergo endocytosis via this mechanism, such as the ionotropic glutamate receptors (Carroll et al., 1999) and $GABA_A$ receptors (Kittler et al., 2000; Herring et al., 2003); however, the potential involvement of similar endocytotic mechanisms in the internalisation of the 5-HT_3 receptor has yet to be reported.

Moreover, structural studies with the acetylcholine binding protein have shown that there are slight structural differences arising to the receptor complex around between agonist bound and competitive antagonist bound proteins (Shahsavar et al., 2012). Furthermore, there are distinct conformational changes on the extracellular domain of glycine receptors between activation and desensitisation (Wang and Lynch, 2011). It is possible that conformational changes in the 5-HT_3A receptors could lead to changes in the rate of internalisation and thus change the receptor expressed on the cell surface. It remains unclear how many of these extracellular changes would affect the intracellular domains of the receptor leading to changes in internalisation. Further studies are required to appreciate the structural similarities between the antagonist blocked and the desensitised receptors and if such structural changes could modify receptor internalisation.

Conclusion

Our study allowed the direct observation of ligand induced changes in surface expression of the 5-HT_3A receptor in response to exposure to various agonist, antagonist and allosteric modulators. Ligand up-regulation/ down regulation could have implications for patients using serotonin selective re-uptake inhibitors for depression, 5-HT_3 receptor antagonists like ondansetron for post-operative or chemotherapy induced nausea and emesis, or palonosetron for IBS. Potentially prolonged exposure to drugs that are directly or indirectly interacting with 5-HT_3 receptors in different areas of the body where there is receptor clustering such as the GI tract or chemoreceptor trigger zone or lymphocytes could affect treatment outcomes as well as potentially induce treatment resistance or adaptive response to the treatment. Additionally some treatments maybe could be predisposing to IBS or depression or even be silently protective against certain conditions or leukaemia's. However, further studies are needed to confirm the potential up-regulation or down regulation in tissue and whole organisms and the role they have in normal and disease function.

B lymphocytes

"Wild type" DG75 lymphocytes appear to have the capacity, determined by their expression of mRNA, to express 5-HT3A, 5-HT3B and 5-HT3E subunits of the receptor but not 5-HT3C and 5-HT3D. When investigated further, DG75 lymphocytes display relatively small amounts (~16 fmol/mg) of 5-HT_3 receptor specific radioligand binding, as determined by specific antagonist ondansetron, and did not demonstrated any detectable immunoreactivity on SDS-PAGE/Western Blotting and immunocytochemistry. However, when we investigated if a functional receptor exists in B lymphocytes (DG75 as a model) by using a calcium influx assay we determined that there is a 5-HT concentration dependent response relatively low, reflecting possibly low receptor density also identified by the low radioligand binding. In further determining which 5-HT sensitive receptor was responsible for the calcium influx, we used a number of specific antagonists (ondansetron, ritanserin, spiperone, paroxetine, clozapine, as well as all possible combinations of the previous drugs, and GR113808, methiothepin and renzapride; for receptor affinities see Table 8), but we were unsuccessful in blocking the 5-HT response; and therefore we were unable to allocate the response to a specific 5-HT sensitive receptor that could mediate a calcium influx, resulting in the arrest of further development of the investigation.

However, upon transient transfection of DG75 cells with myc-tagged h5-HT3A subunit, specific radioligand binding was identified (170 - 580 fmol/mg 24 – 48 hrs post-transfection). Additionally, when the same preparations were used for SDS/Page Western blotting protocol the expression of the myc-tagged h5-HT3A subunit was verified and the appropriate immunoreactivity band was identified at molecular

weight ~55 kDa which is in accordance with previous reports (Monk et al., 2004); representing, possibly, N-glycosylated form of the 5-HT_3A receptor.

When we used the same protocol as previously to determine the functionality of the receptor we transfected, we were able to obtain 5-HT response, induced calcium influx, that have shown to be 5-HT_3 receptor specific; as determined by the blockage of the response by specific antagonist ondansetron.

Agonist/antagonist up-regulation or down-regulation or direct stimulation or inhibition of 5-HT_3 receptors as a side effect of a main treatment could have implications for patients using serotonin selective re-uptake inhibitors for depression, ondansetron for post-operative nausea and emesis, or chronic pain patients using morphine or palonosetron for IBS management. If as the recent literature suggests and our study potentially highlights the selective expression of 5-HT_3 receptors in immune cells changing possibly their metabolic fate or the maturation pathway or even being to be proven important target for blood cancer therapies there is great interest in defining their involvement in health and disease.

General Conclusion

In summary, the present study has identified Cl-Indole as a relatively potent and selective type II PAM of the h5-HT_3A receptor and also the native and recombinant receptor expressed in other species. Further, we have demonstrated the ability of Cl-indole to slow desensitisation kinetics, which was particularly evident with saturating concentrations of the higher efficacy agonists, and was less apparent in the presence of Cl-indole; and could even reverse apparent tachyphylaxis. Compared to the previously characterised 5-hydroxy-indole, Cl-indole has a greater affinity and efficacy at the 5-HT_3 receptor and appears to be selective to this receptor over the human nicotinic alpha-7 receptor, the 5-HT_3 receptors closest family member. Availability of compounds such as Cl-indole will aid investigation of the molecular basis for allosteric modulation of the 5-HT_3 receptor, which may help in the discovery of novel therapeutic drugs targeting this receptor (Newman et al., 2013).

Our study also allowed the observation of 5-HT_3A receptor ligand-induced changes in surface expression of the 5-HT_3A receptor in response to exposure to various agonist, antagonist and allosteric modulators. These findings could have implications for patients receiving medications that impact the 5-HT_3 receptor.

In the present study, we have further reinforced the understanding that 5-HT_3 receptor stability is affected by the three amino acid C-terminus of the 5-HT_3A receptor subunit.

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“When you look at yourself from a universal standpoint, something inside always reminds or informs you that there are bigger and better things to worry about. ”

- Albert Einstein

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RESEARCH PAPER

5-Chloroindole: a potent allosteric modulator of the 5-HT$_3$ receptor

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BACKGROUND AND PURPOSE

The 5-HT$_3$ receptor is a ligand-gated ion channel that is modulated allosterically by various compounds including colchicine, alcohols and volatile anaesthetics. However the positive allosteric modulators (PAMs) identified to date have low affinity, which hinders investigation because of non-selective effects at pharmacologically active concentrations. The present study identifies 5-chloroindole (Cl-indole) as a potent PAM of the 5-HT$_3$ receptor.

EXPERIMENTAL APPROACH

5-HT$_3$ receptor function was assessed by the increase in intracellular calcium and single-cell electrophysiological recordings in HEK293 cells stably expressing the h5-HT$_3$A receptor and also the mouse native 5-HT$_3$ receptor that increases neuronal contraction of bladder smooth muscle.

KEY RESULTS

Cl-indole (1–100 μM) potentiated agonist (5-HT) and particularly partial agonist [(S)-zacopride, DDP733, RR210, quipazine, dopamine, 2-methyl-5-HT, SR57227A, *meta* chlorophenyl biguanide] induced h5-HT$_3$A receptor-mediated responses. This effect of Cl-indole was also apparent at the mouse native 5-HT$_3$ receptor. Radioligand-binding studies identified that Cl-indole induced a small (~twofold) increase in the apparent affinity of 5-HT for the h5-HT$_3$A receptor, whereas there was no effect upon the affinity of the antagonist, tropisetron. Cl-indole was able to reactivate desensitized 5-HT$_3$ receptors. In contrast to its effect on the 5-HT$_3$ receptor, Cl-indole did not alter human nicotinic α7 receptor responses.

CONCLUSIONS AND IMPLICATIONS

The present study identifies Cl-indole as a relatively potent and selective PAM of the 5-HT$_3$ receptor; such compounds will aid investigation of the molecular basis for allosteric modulation of the 5-HT$_3$ receptor and may assist the discovery of novel therapeutic drugs targeting this receptor.

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Abbreviations

AM, acetoxymethyl; Cl-indole, 5-chloroindole; $E_{5\text{-HT}}$, reversal potential for 5-HT-evoked current; HEKh5-HT3A, HEK293 cells stably expressing the human 5-HT$_3$A subunit; LGIC, ligand-gated ion channel; mCPBG, *meta* chlorophenyl biguanide; PAMs, positive allosteric modulators