The pharmacology of the 5-HT_{2A} receptor and the difficulty surrounding functional studies with single target models

A thesis presented for the degree of Master of Science by research

Stacy Steven April 2012

Treatment of many disorders can be frequently problematic due to the relatively non selective nature of many drugs available on the market. Symptoms can be complex and expansive, often leading to symptoms representing other disorders in addition to the primary reason for treatment. In particular mental health disorders fall prey to this situation. Targeting treatment can be difficult due to the implication of receptors in more than one disorder, and more than one receptor in a single disorder. In the instance of GPCRs, receptors such as the serotonin receptors (and in particular the 5-HT_{2A} for the interest of this research) belong to a large family of receptors, the GPCR Class A super family. Around 50% of the drugs now commercially available target GPCR receptors (Wise et al 2004, Katugampola & Davenport.,2003) and drugs with action at serotonin receptors are used in the treatment of many disorders, particularly psychotic disorders such as schizophrenia. Inability to target single receptors selectively means that the therapeutic values of the drugs are much lower than desired.

In this study, the 5-HT_{2A} receptor was incorporated into a stable, inducible cell line using HEK 293 cells and the Flp-in T-REx system, allowing receptor expression to be under the control of the antibiotic doxycycline and hence allow pharmacology to be explored. There is a variation when looking at the potency of agonists in relation to calcium mobilisation and IP-one accumulation, although following the same order of potency the values differ between each experiment type. The order of potency for the majority of the antagonist ligands is very different when looking at IP-one and Ca^{2+} experiments, as are the values obtained for affinity. This was surprising due to the both lying downstream of the IP3 pathway. The most closely relating results to the published IUPHAR values stem from binding experiments.

Understanding the pharmacology of the single receptor by several methods is essential when screening drugs for effectiveness at the receptor. Here the data exploring the pharmacology of the 5-HT_{2A} receptor demonstrated the difficulty surrounding functional studies using single target models.

Acknowledgements

It goes without saying the depth of my gratitude towards my friends in the Milligan lab both past and present for keeping me sane, offering me support and a great deal of advice throughout my research journey. I am also hugely grateful to Prof. Graeme Milligan for giving me a fantastic opportunity and a great deal of patience throughout my time in his lab.

Author’s declaration

The work presented in this thesis was conducted by the author and has not previously been submitted for a degree or diploma at this university of any other institution.

Contents Page

List of figures Page

List of Tables Page

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HT_{2A}	5-hydroxytryptamine 2A receptor
5-HTP	5-hydroxytryptophan
7TM	7 transmembrane
AA	arachidonic acid
AMP	adenosine monophonsphate
Arg	arginine
Asp	aspartic acid
BNDF	brain-derived neurotrophic factor
BRET	bioluminescence resonance energy transfer
Ca^{2+}	calcium
CFP	cerulean fluorescent protein
CNS	central nervous system
D1, D2 etc	dopamine receptor 1, 2 . . .
DAG	diacylglycerol
DOI	4-methoxy-2,5-di-methoxyphenylisopropylamine
DOX	doxycycline
DYN	dynamin
EC	extracellular
ELISA	enzyme linked immunosorbent assay
ENK	enkephalin
EPS	extrapyramidal symptoms
ERK	extracellular signal regulated kinases
FRET	fluorescence resonance energy transfer
GABA	gamma Amino Butyric Acid

GDP	guanosine diphosphate
Gly	glycine
Glu	glutamic acid
GPCR	g protein-coupled receptor
GRK	guanosine receptor kinase
GTP	guanosine triphosphate
^{125}I	Iodine
IC	intracellular
IP	inositol phosphate
IP1	inositol monophosphate
IP3	inositol-1,4,5-trisphosphate
IUPHAR	international union of basic and clinical pharmacology
K^+	potassium
kDa	kilodalton
Li^+	lithium
LSD	lysergic acid diethylamide
Lys	lysine
M1	muscarinic receptor
MAPK	mitogen activated protein kinase
OCD	obsessive compulsive disorder
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine

Thr	threonine
TM	transmembrane
Tyr	tyrosine
YFP	yellow fluorescent protein

1. Introduction

Overview

Mental disorders as we know them, span wide ranging categories, including anxiety, mood and psychotic disorders, and exhibit an extensive list of symptoms. Sufferers of such disorders often suffer co-morbid conditions on top of their main condition as a result of many over lapping symptoms. Therapeutics based on ligand biased activity target cardinal symptoms, activating only specific pathways associated with therapeutic benefits. In the instance of GPCR however, for families such as the serotonin receptors which belong to the GPCR Class A super family, this is often difficult to achieve , due to many ligands being generally non selective for one receptor sub type specifically over another. The drug market today has now shifted its focus slightly towards multi target therapy, in order to treat a variety of symptoms, lessening the impact of such disorders. The notion of polypharmacology of antipsychotic drugs focuses on the treatment of psychotic disorders, with drugs targeting multiple receptors, in order to produce drugs with greater therapeutic value. Several serotonin receptors are known already to be involved in cognitive functions, one key player being 5-HT_{2A}.

1.1 Cellular communication

Cellular communication is undoubtedly an integral process in the existence of life forms. This communication occurs via a system of receptors and messengers, which elicit many different types of cellular signals and effects to modulate all body systems. 4 main receptor classes are:

1) Ligand-gated ion channels
2) G protein-coupled receptors
3) Kinase-linked receptors
4) Nuclear receptors

These receptors contribute to virtually all physiological processes and hence dysregulation can result in a multitude of disease states.

1.2 G protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest and most versatile group of cell surface receptors, and detect a diverse array of chemical signals in a highly selective way (Hill, 2006).

GPCRs constitute a super family, with each and every GPCR containing the characteristic 7 transmembrane (7TM) spanning hydrophobic domain.

Some 865 genes in man encode GPCRs (Milligan and Kostensis, 2006).

1.21 Classification of GPCRs

There are 2 main classification systems of GPCR families. The A-F classification system, proposed by Kolakowski in 1994, in which Class A: Rhodopsin-like, with over 80% of all GPCRs in humans; Class B: Secretin-like; Class C: Metabotropic glutamate receptors; Class D: Pheromone receptors; Class E: cAMP receptors; and the smallest Class F: Frizzled/smoothened family. The second classification system is the GRAFS classification system, proposed by Fredricksson et al.,2003. The system is based on common ancestry, taking into account things such as chromosomal positioning.

1.22 GPCR families in more detail

There are 5 main families of GPCR.

Class A- Rhodopsin like: This is the largest family of GPCRs and is known to contain around 670 human proteins. The rhodopsin family of GPCRs is highly heterogenous when both primary structures and ligand preference are considered (Langerstrom and Schioth, 2008). This group of receptors are most targeted by drugs of a clinical nature, containing at least 18 important drug targets: The histamine receptors 1 and 2, the dopamine receptors

1 and 2, the serotonin receptors 1A, 1D and 2A, the adrenoceptors 1A, 2A, B1 and B2, the muscinarinic receptor 3, the prostanoid receptors TP,EP,EP3,IPI and FP, and the cannabinoid receptor 1 (Langerstrom and Schioth,2008).

Class B- Secretin like receptor: All receptors in this class have extracellular hormone binding domains and bind peptide hormones. Examples of these are the corticotrophin releasing hormone receptors (CRHR1 and CRHR2), the glucagon receptor and the secretin receptor. Hormone treatments targeting these receptors are for clinical conditions such as diabetes and osteoporosis.

Class C- glutamate like receptor: a family of 22 proteins, including glutamate receptors and sweet and umani taste receptors.

The adhesion receptors : This is the 2nd biggest super family, consisting of 33 members and divided into 8 sub-groups. De orphanised adhesion GPCRs have recently been shown to suppress melanoma metastasis and tumour growth. The majority of adhesion GPCRs are still orphans meaning that their endogenous ligands remain unknown. Owing to the present limitation of ligands to these receptor proteins, no drugs are targeted against these GPCRs (Langerstrom and Schioth,2008).

Frizzled/taste2 receptors: Frizzled and smoothened receptors are grouped together, and contain 10 frizzled and 1 smoothened receptor. These play key roles in development and they are thought to be potential anti cancer targets, although as yet no clinical drugs are available. Taste 2 receptors are present in humans as 25 functional genes, and these receptors allow the detection of bitter tastes.

All GPCRs in the class A family share homology with Rhodopsin,comprising of a single serpentine polypeptide chain with 7 transmembrane helices linked by 3 extracellular and 3 intracellular loops and with an amino terminal which is extracellular, and a carboxyterminal which is intracellular (Eglen,2005).

1.23 GPCR structure

Each of the 7 TM domain is composed of 20-27 amino acids. The N terminal and the C terminal vary substantially in size in different GPCRs. N terminal segment is between 7-595 amino acids long whilst the C terminus ranges between 12-359 amino acids. There are also variation in particular in the intracellular loops (5-230 amino acids), these variations are an indication of their diverse structures and functions (Ji et al., 1998).

7 TM structures characteristically have N and C terminals which span opposite membranes (figure 1.1), allowing for 1 extra cellular and 1 intracellular. 7 TM's provide a sufficient size and versatility to offer a prodigious number of specificities, regulatory mechanisms and contact sites for G proteins and other signalling molecules (Ji et al., 1998).

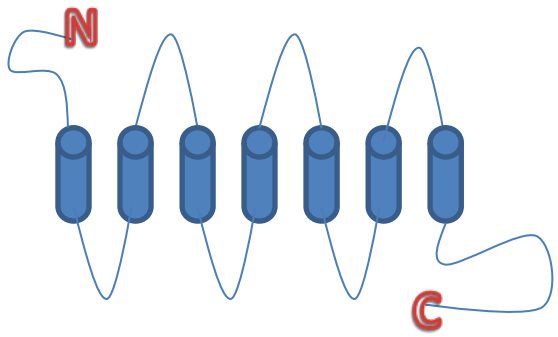


Figure 1.1 Representation of the typical GPCR structure, containing 7 transmembrane spanning domains ,with the N terminus in the extracellular domain and the C terminus spanning the intracellular domain.

Arrangement of the 7 transmembrane helices is a closed loop system, which follows a counter clockwise direction from transmembrane domain1 to transmembrane helix 7. The

orientation of TMs imposes a stereo- and geometric specificity on ligands entry into, and binding to the TM core (Ji et al., 1998).

1.24 Rhodopsin

Rhodopsin is a GPCR which has been studied in thorough detail, and is used as an example when describing the 7TM structure of all GPCRs. Bovine rhodopsin was the first GPCR for which an atomic level crystal structure was obtained (Palczewski et al., 2000). This breakthrough allowed insight into the location of its integral ligand retinal. However the covalent link of retinal is distant from other GPCR ligands and made designing ligands based on this model alone inadequate (Mustafi and Palczewski 2008).

Rhodopsin itself is a light photo receptor protein, present in the rod cells. When activated by light, this initiates the signalling pathway that leads to vision. The 7TM portion of Rhodopsin is known as opsin (Palczweski et al., 2000). Opsin is linked covalently to the ligand11-cis-retinal through Lys 296. When 11-cis-retinal absorbs a photon this causes isomerisation to all-trans, leading to a conformational change of the protein moiety from the inactive to active (R*) state (Mustafi and Palczewski 2008). A cascade of reactions is then initiated. Absorption of a single photon results in the activation of hundreds of G protein molecules (Fung et al., 1980). Crystalisation confirmed the presence of an 8^{th} helic running parallel to the plasma membrane and terminated by a pair of palmitoylated cysteine residues, Other identified key micro domains include the DRY motif, Helix III, the NPXXY domain connecting transmembrane helix VII and cytoplasmic helix VIII. The NPXXY domain has been seen to play an essential role in the switching of 5-HT_{2C} receptor between active and inactive conformational states (Weinstein et al, 2002). The Ballesteros group put in place a numbering system :The *Ballesteros*-Weinstein *numbering system* which would allow easy location of amino acid residues. The numbering system uses a 50 decimal point to signify the location of the most conserved residue (Ballesteros and Weinstein,1995). The reference residue amongst the most conserved residues will always

be numbered 50, eg in tm 4 there is a tryptophan which is most conserved and so this is referenced 4.50. There is a serine residue 5 amino acids after this tryptophan, and so its location is 4.55 (Ballesteros and Weinstein,1995). This is much easier and quicker to use than counting in ascending order the entire length of the receptor to find a particular residue, eg the proline in TM6 is 267 amino acids along. Several residues are conserved among Class A GPCRs, The conserved residues are in helix I (Gly and Asn), helix II (Leu and Asp), helix III (Cys and AspArgTyr), helix IV (Trp and Pro), helix V (Pro and Tyr), helix VI (Phe, Trp, and Pro), and helix VII (Asn, Pro, and Tyr of the NPXXY motif) (Mirzadegan et al.,2003). The tyrosine residue tyr 5.58 on tm 5 is 80% conserved amongst class A GPCRs (Ballesteros and Weinstein,1995).

The molecular size of rhodopsin is intermediate in comparison to other members of the family, so therefore contains all the essential functionally important components.
This groundwork in x-ray crystallography studies paved the way for the emergence of other high resolution crystal structures for various GPCRs, including the human β2 adrenergic receptor (Cherezov et al.,2007), β1 adrenergic receptor (Warne et al.,2008), and the human A_{2A} adenosine receptor (Jaokda et al.,2008). A 4^{th} structure materialised in 2008, that of the squid rhodopsin (Murakami and Kouyama 2008). Recognition of G protein Gαq appears to occur as a result of extended helices V and VI into the cytoplasmic medium, along with 2 cytoplasmic helices. The early crystallised structures were captured in the inactive form, having been crystallised in the presence of inverse agonists or antagonists. However recently All contain 7TM helices and an 8^{th} parallel to intracellular membrane. These emerging structures highlight structural differences between GPCRs of the class A receptor family, which result in differences in various processes such as ligand recognition and activation, including ability to alternate between activation states.

1.25 The D(E)RY Motif

The only structural common feature of GPCRs is the 7TM spanning domains, with 3IC and 3EC loops, an amino acid terminus to the extracellular side and a carboxyterminus on the intracellular side. However, major subfamily the rhodopsin contain a highly conserved sequence homology for the Asp/Glu-Arg-Tyr D(E)RY motif , between TM2 and intracellular loop C2. It functions as a regulatory body in receptor conformational states, and consequently the activation of associated G proteins because it provides an ionic lock which stabilises the inactive form of the receptor (Rovati et al.,2007, Mirzadegan et al.,2003, Mustafi and Palczewski 2008). This domain can influence ligand binding characteristics, depending on whether residues which follow are of an acidic or basic nature. Basic residue (34% Lys, 19 % Arg) results in peptide ligand binding, Acidic residue (Asp, Glu) will allow binding of biogenic amine ligands (Mirzadegan et al.,2003). The big picture derived from rhodopsin crystalisation studies indicate arginine 3.50 interacts with aspartic acid or glutamic acid, which are the amino acids at position 3.49, and a Glu residue on helix 6 position 6.30, forming salt bridges (Ballesteros et al., 1998, Palczewski et al., 2000). Disruption of these bridges (Asp/Glu 3.49, Arg 3.50 and Glu 6.30) is thought to induce activation of receptor as they are stabilizing structures forcing the receptor to remain in the inactive state, as predicted in various mutagenesis experiments (Ballesteros et al.,2001, Rovati.,2007), with arginine (a basic residue) in particular playing an important role in receptor stabilisation (Flanagan et al.,2005) 2005). Mutational analysis of the highly conserved D(E)RY motif of the thromboxane A2 receptor α (TP2α) highlight an alternate role in GPCR, implying that arginine is essential to the process of G protein coupling (Capra et al.,2004). Highlighted in β2 adrenergic rececptor studies is the importance of a rotamer “toggle switch” located on helix 6. It is thought to be important, alongside the ionic lock for agonist binding and promotion of receptor activation (Shi et al.,2002).

1.3 Receptor activation and the ternary complex

GPCR activation and signalling was originally described by the 2 state model. This model suggests that the receptor can switch between a resting state *(R)* and an active state *(R*)* (Rang ,2006). Even in the absence of a ligand a conformational equilibrium exists between R and R* resulting in a degree of constituitive activity. Agonists have a higher affinity, preferential for R* over R.

A more appropriate and commonly used model for describing the activation of GPCRs is the ternary complex model. Analysis of radioligand binding studies was used in order to develop this theory. The ternary complex model has 3 constituents : 1) the receptor *(R)* , 2) agonist *(A)* and 3) the G protein *(G).*

Receptor activation depends on its ability to bind to and form a complex with a G protein. This receptor-G protein complex is an active conformation. Agonists can bind with high affinity in the active state, however, when the receptor is uncoupled the conformation is inactive, and there is low affinity for the agonist.

An agonist *(A)* can bind to either free receptor *(R)* or to a receptor coupled to a G protein *(RG).* The G protein binds to the free receptor or receptor coupled to an agonist. A ligand, in the ternary complex model, causes a shift in equilibrium, to favour the state for which it has greater affinity (Park et al.,2008).

This means that agonists promote activation of a receptor (active state being of higher affinity). Full agonists will exert a greater effect of shifting the equilibrium in favour of the active state than a partial agonist.

1.4 G proteins

GPCRs function along side G proteins. These proteins derive their names from their behaviours, interacting with guanine nucleotides of GTP + GDP, and hence are known as 'G' proteins. G proteins are derived from 35 genes, 16 encoding α subunits, 5 β subunits and 14 γ subunits. Each of the subunits function as guanine nucleotide exchange on /off switches, and are mechanistically similar to other proteins that are enzymatic GTPases (Milligan and Kostensis, 2006). The total number of GPCRs far exceeds the number of G proteins in humans, and thus each member of the Gα subfamilies must be able to interact with many GPCRs (Kostensis et al ., 2005).

G proteins are classified in accordance to amino acid similarities of the α subunit. 4 classes/sub families have been identified: Gαs, Gαi/o, Gαq/11 and Gα12/13. Activation of the Gαs sub family leads to stimulation of members of the membrane associated adenylyl cyclase enzyme , which in turn stimulates the production of cyclic AMP . This subsequently activates protein kinase A, generating a host of down stream processes. 9 isoforms of mammalian adenylyl cyclase have been identified and cloned to date (Hur and Kim, 2002). It is known that all 9 isoforms are susceptible to stimulation by the Gαs subfamily.

Activation of the Gαi subfamily causes the opposite to occur, and results in the inactivation of adenylyl cyclase and therefore an inhibition of cyclic AMP production. With regards to Gαi/o , only certain isoforms of the adenylyl cyclase are sensitive to inhibition, and it has also been established that each of the different isotypes are regulated by different Gβ/γ subunits. The Gαi subfamily also regulate a number of other effectors. Of great relevance is central nervous system function are a group of Ca^{2+} and K^{+} channels which control neuronal excitability.

Gαq/11: both regulate the efflux of calcium from intracellular stores, Gαq once stimulated by a ligand+GPCR complex, functions to stimulate phospholipase Cβ and generate the second messengers IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol), controlling protein kinase c stimulation.

Gα12/13 is involved in Rho GTPase signalling cell migration, blood vessel development and metastasis, with particular emphasis in the early stages of breast cancer (Kerry et al.,2006).

It is now well established that β/γ complexes mediate at least as many functions as the α subunits (Milligan and Kostensis, 2006). Once activated, the β/γ subunit is free to bind to, and exert effect on a variety of effector molecules such as adenylyl cyclase, voltage sensitive ion channels and proteins of MAPK pathway.
Many G proteins may be lipidated in order to be able to associate with inner leaflet of the plasma membrane. G α subunits are modified by combinations of fatty acid whilst γ subunits become post-translationally modified by the addition of isoprenil groups.

1.41 The activation of a G protein

GPCRs are sensitive to activation by a variety of ligands including hormones, peptide and non peptide neurotransmitters and growth factors, as illustrated in Figure 1.2.

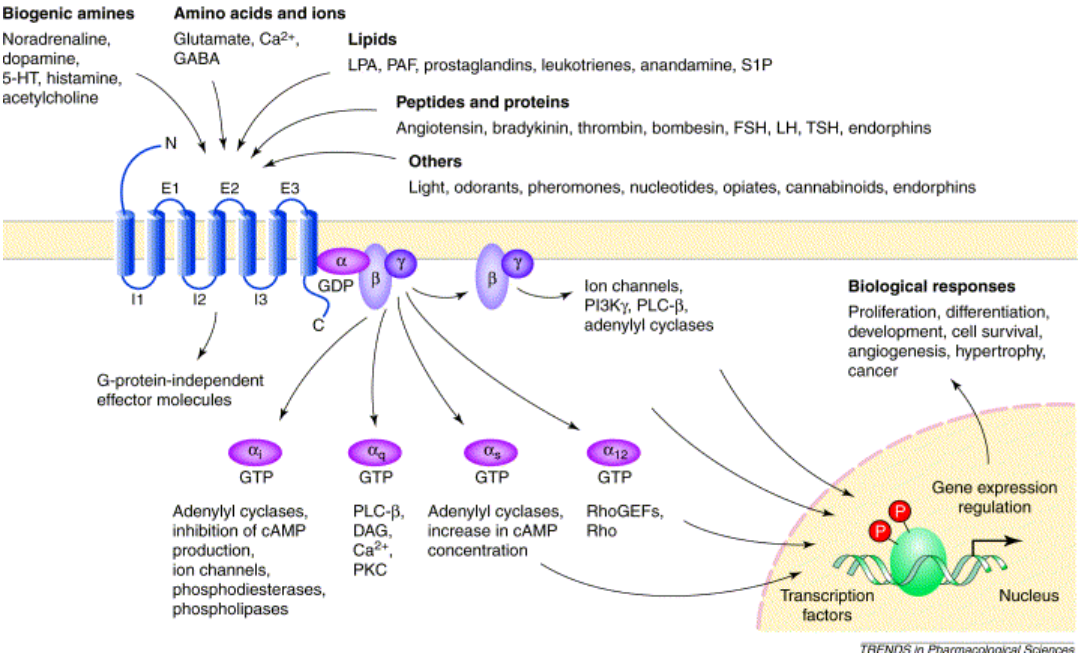


Figure1.2 Represents the diverse range of ligands which bind to GPCR'S and subsequent signalling pathways activated and second messengers generated as a result of ligand binding (Marinissen and Gutkind.,2001).

When a GPCR couples to a G protein generation of intracellular signals occurs, stimulated by a ligand/receptor interaction which occurs at cell surface level.

The GPCR and the G protein trimer, until the GPCR becomes ligand bound, are found separately on the membrane. The α subunit of this G protein is GDP bound, and once the GPCR becomes occupied by a ligand (agonist), then a conformational change occurs in the GPCR, and the GPCR has heightened affinity for the αβγ trimer of the G protein. At the point of association with the GPCR, displacement of GDP occurs and it is replaced by GTP. At this point the generally held view is that the trimer also dissociates and the αβγ trimer subunits separate and diffuse into the membrane. It is at this stage that the G protein is active.

The switching on/ switching off process is self limiting , and the α subunit restores GDP to the binding site via GTPase activity. The trimer subunits again re-unite on the membrane, dissociated once again from the GPCR

1.5 Ligand Binding

Ligands can be classified according to whether they have either agonistic or antagonistic properties. The words agonist and antagonist come from the Greek *Agonistes* (combatant) and *Antagonistes* (rival) (Park et al.,2008). Agonists binding to promotes cellular responses with either full efficacy (full agonist) or with less than maximal efficacy (partial agonist).Antagonists oppose the action of agonists and in the case of competitive antagonists this occurs by blocking the binding of an agonist to the receptor, thereby preventing the cellular response (Park et al.,2008).

In addition there are Inverse agonists and they function to reduce basal activity. The concept of constitutive activity was first put forward by Andre Delean and co workers in 1980 (DeLean et al., 1980), when they suggested an extended ternary complex model. By studying the beta-adrenergic receptor the looked to describe affinity states and intrinsic activity. In the frog erythrocyte system they found there to be high levels of basal activity for adenylate cyclase, and spontaneous complex formation between R and G = RG , a collision which is normally agonist initiated. An equilibrium exists between the active and inactive confirmations, and the active confirmation can modulate cellular events in the absence of a ligand (Aloyo et al.,2009). This phenomenon could not be accounted for in the basic ternary complex model. In 1989 Costa & Herz (Costa & Herz 1989) carried out a series of experiments to test this hypothesis, and in doing so established a new concept of inverse agonism.

The ability to study detailed pharmacological properties of GPCRs by definition relies on the availability of compounds that can selectively agonise and antagonise responses mediated by a particular GPCR (Hill,2006).

1.51 Radioligand binding studies

Throughout the decades of the 1960's and 1970's, radioligand binding studies were introduced and developed. These studies provided a means by which new insights into the molecular identity and properties of GPCRs were achieved (Hill, 2006).

The 1st study was carried out in 1965 by William Paton and Humphrey Rang (Paton and Rang, 1965), looking at binding of tritiated atropine to muscarinic receptors of the guinea pig ileum.

Binding studies using radiolabelled agonist and antagonists to GPCRs then began to follow on from this. An emerging feature of many studies of this nature was that displacement of radiolabelled antagonist binding to cell membranes by an agonist produced data consistent with the agonist binding to sites with high and low affinity components, characteristically consistent with only a single receptor state. By contrast antagonists displaced the binding of radiolabelled antagonists.

1.6 GPCR regulation

There are several regulatory mechanisms in place with regards to GPCRs.

Desensitisation is a short term mechanism of regulation adopted by the cell, in response to repeated exposure to a specific stimulus. Two types of desensitisation can occur 1) Homologous : decrease of response to an agonist after repeated/prolonged exposure of the same agonist 2) Heterologous : phosphorylation is mediated by second messenger kinases. Repeated exposure of one agonist leads the cell to have diminished responsiveness to a host of other agonists (Gray and Roth,2001). GRKs and arrestins are thought to be responsible for homologous desensitisation (Allen et al.,2001, Gray and Roth,2008). Agonists promote the interaction of a receptor with GRKs , phosphorylating specific Ser/The residues, and recruit arrestins with high affinity. G protein-coupled receptor kinases GRK2 + GRK3 regulate the desensitisation process, by uncoupling the G protein

from the receptor, an essential process to ensure G protein signalling does not occur continuously. Three steps occur in the desensitisation regulatory process:

Desensitisation occurs first. This involves the uncoupling of the receptor/effector complex once agonist binds and receptor phosphorylation occurs, followed by *sequestration*, where the agonist activated receptor is removed from the cell surface and into an intracellular compartment and then finally *down regulation* occurs, resulting in a reduction in protein synthesis and lysosomal degradation. These stages occur consecutively. This allows for uncoupling of receptor from its G protein bound complex , and removal from membrane for either degradation or recycling. Phosphorylated and desensitised receptors are gathered in clathrin coated pits where dynamindependant endocytosis occurs, leading to internalisation of the receptor in a clathrin coated vesicle. These vesicles then transport the receptor to endosomes. Various types of endosomes exist, early endosomes being the receptors first port of call, and sorting of receptors occurs here, determining whether the receptor goes onto the recycling endosomes to allow resensitisation and return to the plasma membrane to take place, or alternatively the GPCR may relocate into late endosomes for degradation or a slow recycling process. These steps are regulated by the Rab/Ras family of GTPases.

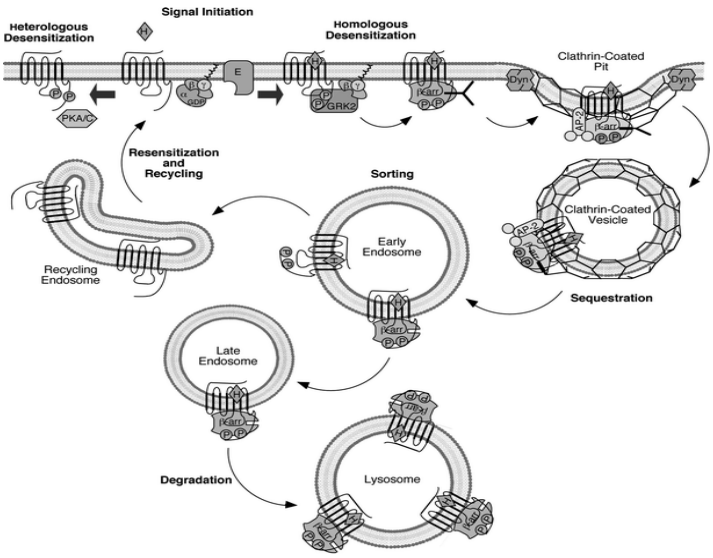


Fig 1.3 Diagram taken from Louis M. Luttrell Mol Biotechnol (2008). Desensitization, sequestration, and recycling of GPCRs.

1.61 GPCRs and the dimeric state

The concept of GPCRs existing in a dimeric state was first broached in the late 1990's (Milligan, 1998). however at the time analysis was challenging as a little range of techniques were vailable to address the questions.. In the last decade dimerisation has become a topic of considerable debate within the subject of GPCRs (Milligan, 2003 , Milligan, 2004 , Milligan, 2006 , Milligan, 2007, Park and Palczewski ,2004, Kunishima et al.,2000, Lee et al 2004).

For many years GPCRs were thought to exist only as monomers. The latest technologies have paved the way for a greater understanding of this subject and the existence of both homodimers and heterodimers. Based on the application of techniques involving fluorescence energy transfer (FRET) and bioluminescence transfer (BRET), in particular time resolved FRET which allows detection of dimers trafficked successfully to the cell surface (Milligan, 2007), formation of dimers is known to occur during synthesis in the endoplasmic reticulum, and for some receptors is a requirement for passing control points during synthesis (Bulenger,2005).

Subsequent to demonstration of homodimerisation , it became apparent that certain GPCRs displayed an ability to heterodimerise when co-expressed together (Milligan,2006 a,b). Heterodimerisation has been reported to have effects on cellular trafficking, cell surface delivery, pharmacology and signalling (Milligan,2006, Bulenger,2005). An example of this the enhancement of cell surface expression of the mouse 71 olfactory receptor when in the presence of the β_2-adrenergic receptor, an event inefficient at occurring when expressed independently (Hague et al.,2004). It is thought that prospects of GPCR heterodimers may be novel drug targets if heterodimer specific ligands, expanding on already established therapeutic targets. Dopamine is a neurotransmitter whose dysfunction is involved in disorders such as schizophrenia and Parkinsons disease. Recently it has been discovered in the lab of Susan George that dopaminergic signalling can occur via Gαq11. This is generally not associated with any single dopamine receptor subtype. However in

cells co-expressing D1 and D2 receptors ,the two subtypes showed to be able to heterodimerise, coactivating the receptors leads to signalling via this pathway (Lee et al.,2004,Rashid et al., 2007 a.b). These complexes have been linked to an elevation of BDNF (Brain-derived neurotrophic factor) expression and neuronal growth (Hasbi et al.,2009) and exist in a unique population of neurones expressing both neuropeptides DYN (dynamin) and ENK(enkephalin)(Perreault et al.,2010) D1 and D2 receptors are coexpressed and colocalising in both human and rodent brain within the nucleus accumbens and striatum (Rashid et al.,2007b), and the D1/D2 complex has now been confirmed as a physical entity by 3 distinct approaches: immunocytochemistry, confocal FRET and co-immunoprecipitation (Hasbi et al.,2009). PLC activity and calcium signalling associated with Gαq/11 coupling appears to be present in tissue systems coexpressing this D1/D1 complex as opposed to heterologous expression systems expressing either one or the other (Lee et al.,2004). In vivo work by the George group in 2010 implicated this heteromer in reward pathways, and an upregulation of sensitivity of the D1/D2 complex in rat striatum following chronic administration of amphetamine has linked the heteromer to other disorders associated with elevated dopamine transmission (Perreault et al.,2010). Since discovering the existence of this complex this group has managed to demonstrate the separation and reformation of this cell surface heteromer, finding also that D1 homo-oligomers were present within the same cell systems.

Another recent advancement has been the discovery of a serotonin/glutamate receptor complex, which is thought to be implicated in psychosis (Maeso et al., 2008). Uniquely this complex formation is between 2 receptors from different subclasses. Targeting the complex with hallucinogenic drugs which action at the 5-HT_{2A} receptor (a class A rhodopsin family GPCR) then activates mGluR2 (a family C GPCR) consequently abolishes hallucinogenic signalling and behaviour (Gonzalez-Maeso et al.,2008). It is

hoped that multi target therapy such as this hetromer can be aimed at treating a larger scale of symptoms than traditional single target models.

1.7 Serotonin (5-HT) and the serotonin receptors

Serotonin is a neurotransmitter of the brain and periphery which can be found in the blood, the CNS and wall of the intestine (aiding gastrointestinal motility). In the CNS serotonin is an important transmitter, and is known to exert control over a variety of functions including appetite, sleep, mood, hallucinations, behaviour, pain perception and even vomiting. Modification in serotonin function can lead to development of health problems including migraines and mental health and mood disorders.

The precursor of serotonin is the amino acid tryptophan (an essential amino acid), not produced by the body, but instead obtained as a dietary source. In mammals less than 1% of the dietary tryptophan is converted to 5-Hydroxytryptamine (5-HT), and only 10% of that conversion occurs in the brain (Russo et al., 2009). Synthesis of the neurotransmitter occurs via the enzyme tryptophan hydroxylase , a rate limiting stage in the process being:

L-Tryptophan + Tetrahydrobiopterin + O2 ⟷ *5- Hydroxytryptophan (5-HTP) + 4a-hydroxytetrahydrobiopterin*

5-hydroxytryptophan (5-HTP) is an intermediate in the synthesis of serotonin. This 5-HTP is subject to decarboxylation by another enzyme – decarboxylase, to create serotonin (see fig1.4.).

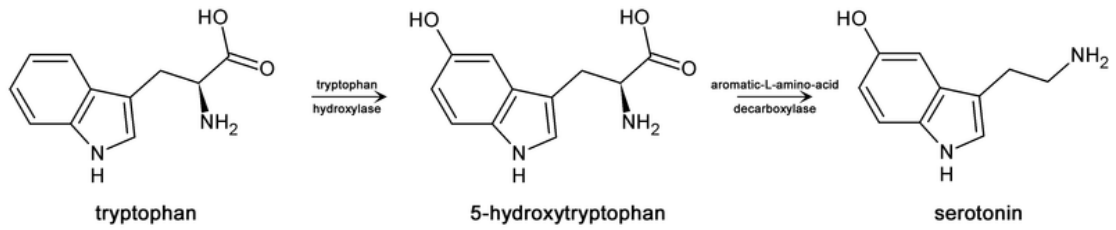


Fig 1.4.Conversion of tryptophan to serotonin.

Serotonin is incapable of crossing the blood brain barrier. Synthesis within the CNS is is heavily dependant upon the availability of L-Tryptophan within the CNS (Birdsall, T.C., 1998).

1.71 Early discoveries

Gaddum and Picarelli in 1957 (Gaddum and Picarelli, 1957), embarked upon the initial classification of serotonin receptors. They found that although 5-HT could cause contraction of guinea pig ileum, the response occurred via 2 different pathways as application of antagonists caused different results for different tissue preparations. Their experiments were carried out on the basis of previous observations of drugs antagonising the 5-HT receptor by both Gaddum and Hameed in 1954 (Gaddum and Hameed,1954), and Cambridge and Holgate in 1955 (Cambridge and Holgate,1955), where both partnerships found concentrations of known drugs (0.1μg/ml LSD in the case of Gaddum, and 0.01μg/ml Atropine in the case of Cambridge) diminished the response of 5-HT by 50%, however, further increases of atropine lead to a plateau effect, and at 100x greater concentration the observation was that it had no more effect than that of the smaller administered dose. Further experiments carried out by Gaddum and Picarelli (Gaddum and Picarelli ,1957), lead to the proposal of the D and M type receptors, thought to be muscular tissue receptors and nervous tissue receptors respectively. Drugs seen to act at and block the D type receptors antagonised the effect of 5HT at smooth muscle receptors in experiments on the rat uterus, rabbit ear and guinea pig ileum, whilst the presence of the M type receptor effected the nerve ganglia and fibres. From such studies it was then inferred two types of 5-HT receptor could co-exist in the same tissue, with distinct sensitivities for different drugs.

This important landmark in the 5-HT receptor history spurned further experimental work, giving rise to the definite conclusion that more than one type of serotonin receptor exists, opening the door for future discoveries.

There are currently 15 known G-protein-coupled serotonin receptors, split into 7 families (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5 , 5-HT_6 AND 5-HT_7) with several families containing multiple members (e.g. 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}) (Kroeze and Roth,1998, Roth et al.,2004). The 5-HT_7 receptor was first discovered in the 80s and initially was classified as 5-HT_1 (Fenuik et al.,1983), however after cloning and recharacterisation some 10 years later it was reclassified as 5-HT_7 (To et al.,1995).One of the most newly discovered serotonin receptors is 5-HT_6. This receptor was discovered in 1993 by 3 separate groups, Monsma, Plassat and Ruat (Monsma et al., 1993, Plassat et al., 1993 and Ruat et al.,1993). They cloned this receptor from the rat striatal cDNA. Cloning of the 1st human 5-HT_6 receptor occurred In 1996 by Kohen and colleagues (Kohen et al.,1996). The majority of serotonin receptors are implicated in psychosis and antipsychotic drug action, including 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_6 and 5-HT_7 (Meltzer and Nash.,1991).

As cloning strategies confirmed the existence of multiple, distinguishable serotonin receptors, it became obvious that a system must be put in place for the classification and nomenclature of all the various serotonin receptor types and subtypes. What also had to be taken into account was the physiological relevance of newly identified gene products. It should be realised that these two issues cannot be dealt with independently, and are, in fact intimately related (Hoyer & Martin,1996).

Hoyer and Martins efforts, alongside other members of the serotonin nomenclature committee, in constructing a system where by present discoveries and needs are taken care of, but is also open to and anticipates future requirements, led to the development of a well structured and thought out classification system(www.**iuphar**.org), which is know to

scientists today. Particular attention was paid to the newly emerging gene products so that the nomenclature could be easily expanded. Criteria for naming a receptor focussed on 3 characteristics-its operational, structural and transductional characteristics. Nomenclature for all newly described recombinant receptors followed the guidelines of NC IUPHAR (www.**iuphar**.org).

All serotonin receptors with the exception of 5-HT_3 (Kroeze and Roth,1998) which is a ligand gated ion channel containing 4 transmembrane regions conform to the classic 7 Transmembrane spanning α-helical region architecture of GPCRs, with an extracellular N terminus and intracellular C terminus with the exception of 5-HT_3 (Kroeze and Roth,1998) which is a ligand gated ion channel containing 4 transmembrane regions. Serotonin receptors contain several conserved residues, many within the transmembrane helices. TMII has, TMIII has 4, TMVI has five and TMVII has seven (Kroeze and Roth,1998).

1.72 The 5-HT_{2A} receptor

The 5-HT_{2A} receptor belongs to a group of receptors which includes 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. It was not until the late 1980s that that the Rat 5-HT_{2A} was successfully cloned by Pritchett and colleagues in 1988 (Pritchett et al.,1988) and 2 years later in 1990, Julius and colleagues (Julius et al.,1990), marked the identification of the human receptor. Homology between the rat and human receptor is some 87%, and chromosomal mapping of human 5-HT_{2A} has located the receptor to chromosome 13,13q14-q21 (Hsieh et al., 1990).

Distribution of 5-HT_{2A} receptor in the human brain was studied using light microscopic audioradiography techniques by Pazos and colleagues in 1987 (Pazos et al.,1987). They observed high levels were

localized over layers III and V of several cortical areas, including the frontal, parietal, temporal and occipital lobes and the hypothalamus. Intermediate concentrations were found over the hippocampus, the caudatus, putamen and accumbens nuclei among other

structures and an observation of low levels in the brain stem, thalamus, cerebellum and spinal cord.

The distribution of 5-HT_{2A}-immunolabeled soma/dendrites was characterized in cerebral cortex, olfactory system, septum, hippocampal formation, basal ganglia, amygdala, diencephalon, cerebellum, brainstem, and spinal cord of the adult rat (Cornea-Herbert et al.,1999).

The 5-HT_{2A} receptor is one of the main excitatory serotonin receptors and it mediates processes such as neuronal excitation, behavioural effects, learning and anxiety. It is coupled to the Gq/11 pathway, and in the majority of tissues stimulates the activation of PLC and causes an increase of inositol phosphates and elevation of calcium levels (Roth et al., 1994).. It is not only PLC which is activated, but also other phospholipases, PLA2 for example. Another pathway linked to the 5-HT_{2A} receptor is that of the ERK MAP Kinase pathways in contractile cells, such as vascular smooth muscle (Watts, 1998).

1.73 Phosphoinositides and calcium signalling

Many studies emerged in the 1980's, drawing focus to calcium signalling in the endoplasmic reticulum and, in particular pinpointing inositol 1,4,5-trisphosphate (IP_3)as being the key messenger in the induction of calcium signalling in a host of cell lines and native tissues (Burgess et al.,1984, Irvine et al.,1984, Ueda et al.,1986). Calcium signalling appeared to occur specifically via inositol trisphosphate as opposed to other phosphoinositides (Berridge et al 1984a,b).

Michael J.Berridge spent a lot of time evaluating already established but unfinished theories involving the incorporation of lithium ion into experiments to aid understanding of the IP response, as lithium acts in an inhibitory manner, preventing the hydrolysis of inositol monophosphate into inositol (Fig 1.5). A model was formed detailing the exact

sequence of events along with the proposal that IP3 might be the diffusible messenger that coupled receptor activation to mobilisation of internal calcium.

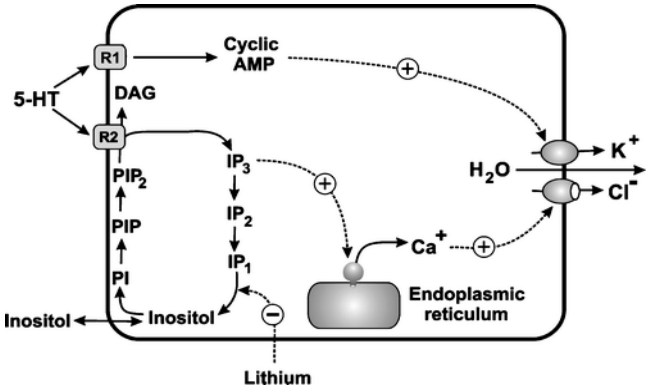


Figure 1.5 Diagram (taken from Berridge., 2008) , detailing the inositol pathway based on findings from previous research. Introduction of lithium causes response to halt at IP1 and accumulation of IP1 to occur.

A second pathway linked to the 5-HT_{2A} receptor via activation of phospholipase C (PLC) is the production of of diacylglycerol (DAG) which results in the stimulation of protein kinase C (PKC), and also the generation of arachadonic acid (AA).

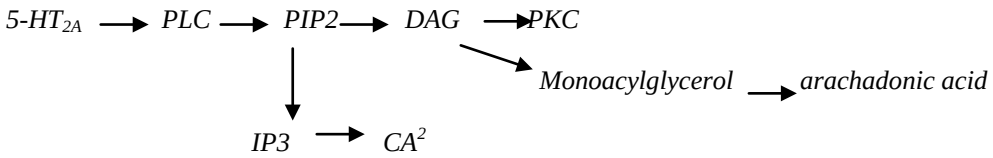


Fig 1.6 Diagram detailing a second pathway in which the inositol's signals through. Hydrolysis of Phosphatidylinositol 4,5-bisphosphate **(**PIP2) results in signalling via calcium, protein kinase C and arachadonic acid accumulation.

PLC signalling: PLA_2 activation and 2-arachidonylglycerol release

5-HT_{2A} receptor mediated release of PLA_2-AA (phospholipase A2 mediated arachidonic acid) and PLC-IP (phospholipase C mediated inositol phosphate) accumulation occurs independently of one another (Kurrasch-Orbaugh et al 2003). These studies supported previous work by Berg and colleagues (Berg et al.,1998) in which they established that efficacies of agonists differed depending on which signal transduction pathway was being measured. A phenomenon not seen in linked pathways (calcium and inositol phosphate). What the Kurrasch-Orbaugh group wanted to know was if structurally distinct ligands

could preferentially activate PLA_2-AA over PLC-IP accumulation, or vice versa. Initial studies established that PLA_2-AA and PLC-IP release could occur both through stimulation of NIH3T3 cells heterologously expressing the 5-HT_{2A} receptor with endogenous ligand serotonin. The 5-HT_{2A} specific antagonist ketanserin was used to ensure mediation of these responses occurred solely through the 5-HT_{2A} receptor and indeed abolished both responses. Inhibitors were used at various steps in both signalling pathways in order to establish the roles of PLC and PLA_2 in AA release and IP accumulation, and the cross talk that may occur between these pathways.

The group also established that coupling to each of the pathways did not occur in an equal manner, and by use of an alkylating agent phenoxybenzamine (PBZ) to inactivate the 5-HT_{2A} receptor and various functional studies they found PLA_2 to have a larger receptor reserve. A much higher concentration was required to prevent PLA2-AA release upon stimulation by the endogenous ligand 5-HT, only partially inhibiting its release. Another phenomenon which could be seen was that some agonists preferentially activated PLA_2 over PLC with as great as a 10fold difference in potency, mimicking the noted observation of receptor reserve differences.

1.74 5-HT_{2A} and psychosis

Over recent years the 5-HT_{2A} receptor has been the main focus of investigations into the nature of antipsychotic drugs, and has been classified as the principal binding site of hallucinogens acting on the central nervous system. Ismaiel and colleagues (Ismaiel et al., 1993) undertook investigations using selective 5-HT_{2A} antagonists in order to block the stimulant effects of hallucinogenic compounds which are structurally related to LSD, and successfully blocked this phenomenon with these antagonistic compounds. A few years later, sites on the 5-HT_{2A} receptor were pin pointed which were important to hallucinogenic binding (Roth et al., 1997) Other findings by Roth and colleagues highlighted several conserved aromatic residues (W76, W200, F340, W367 and Y370)

which were important in the binding of hallucinogens (Roth et al., 1993, Roth et al., 1995 , Roth et al., 1997), with 1 residue in particular (F340) being responsible for the binding of the compound 4-methoxy-2,5-di-methoxyphenylisopropylamine (DOI) which is structurally related to LSD. Atypical antipsychotics act as potent 5-HT_{2A} receptor antagonists. These drugs were developed as a new generation of drug with enhanced therapeutic and limited side effect potential, unlike the first generation typical antispychotics. Drugs such as mianserin and ritanserin acting at this receptor have been found to beneficially effect both positive and negative symptoms of schizophrenia such as disordered thoughts, delusions and inability to experience pleasure, making the 5-HT_{2A} receptor a therapeutic target for the treatment of psychotic disorder.

1.75 5-HT_{2A} antagonist functional states and trafficking

In 2009 Brea et al looked at the ability of the 5-HT_{2A} receptor to form homodimers based on previous research findings where recombinant human 5-HT_{2A} receptor lacking constitutive activity was subject to competition binding by an antagonist producing biphasic curves (Lopez Gimez et al., 2001). There had been little information on 5-HT_{2A} dimerisation, and so the group set out to gain more insight into the possibility of this. Initial studies by Brea and colleagues (Brea et al.,2009) observed the ability of the receptor to form homo-complexes by co-expressing N terminally c-myc and Flag tagged 5-HT_{2A} receptors, immunoprecipitating with anti Flag antibodies and immunoblotting with c-myc antibodies. A polypeptide of 55kDa, the anticipated receptor size was observed. Controls of cells expressing each construct individually and then mixed together could not replicate any results (bands), FRET studies were employed and a specific FRET signal could be detected for $5HT_{2A}$CFP + 5-HT_{2A}YFP , indicating two forms of the receptor have to be co-expressed to result in co-immunoprecipitation.

The group explored the possibility of a homodimers ability to adopt multiple active states. Antipsychotic drugs are known to work in a variety of means, some targeting the receptor

with high and low affinities (biphasic) while competing with radio labelled antagonists, whilst others displayed monophasic binding patterns. The Brea group (Brea et al.,2009) proposed biphasic curves bore no relation to the possible presence of another receptor species, due to application of selective liagnds for the 5-HT_{2A} receptor (Lopez-Gimenez,2001). They proposed that dimerisation occurred and ligand binding to the dimer resulted in negative co-opperativity. By assessing functional behaviour via second messenger systems (IP and AA release) which has previously been seen to occur independently of one another, they explored effects of different ligands on these pathways. Clozapine mediates receptor function in a pathway specific manner. For the IP pathway every antagonist bound in a monophasic manner however all antagonists that displayed signs of negative co-opperativity (possible homodimerisation) in binding assays also antagonised 5-HT induced AA release in a biphasic manner with the exception of haloperidol and mesguline. This taken together with the previous FRET studies was thought to indicate homo dimer existence.

Many drug profiling studies have led to the common indication that the 5-HT_{2A} receptor is a target of antagonistic agents acting as anti-psychotics. The 5-HT_{2A} has wide CNS distribution, and is targeted by many anti-psychotic drugs. The hallucinogen LSD is an agonist at the 5-HT_{2A} receptor and early discoveries showed LSD to exert pyschomimetic effects. This ligand is structurally related to serotonin. Studies involving hallucinogens at the 5-HT_{2A} receptor revealed they provoke symptoms that mirror those of schizophrenia. These effects include perceptual disturbances, sensory processing, cognition, changes in brain metabolism and self representation (Gonzalez-Maeso and S. Sealfon, 2009). In animal models schizophrenia-like symptoms caused by hallucinogens can be mirrored and observed by monitoring head twitch responses. It is now known that the reason for some compounds being hallucinogenic, and other structurally related and very similar compounds being non hallucinogenic is due to receptor trafficking different pathways. Gonzalez-Maeso and colleagues (Gonzalez-Maeso et al., 2003) proposed that structurally

similar compounds targeting this receptor can signal through different pathways due to alteration of receptor conformation, which occurs specifically in relation to each pathway. Hallucinogenic drugs act through the Gq/11 and Gi/o G-protein subtypes, increasing expression of 3early genes: erg-1, erg-2 and period 1, as a consequence. These 3 genes contribute to the observed head twitch response in animal models (Gonzalez-Maeso et al., 2003).The structurally similar non hallucinogenic drugs target Gq/11 only.

1.76 5-HT_{2A} receptor agonist binding site and ligand selectivity

The 5-HT_2 receptor family all stimulate PLC, each thought to mediate specific physiological functions, however lack of truly specific agonism means that this is not definite (Knight et al.,2004). They are very similar in structural homology for both primary and secondary structures (Hoyer et al.,1994) and also in pharmacology (Jerman et al.,2001). Knight and colleagues (Knight et al.,2004) studied the agonist preferring, high affinity binding site of the 5-HT_2 family of receptors (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}). They set up binding experiments using radiolablled antagonists [^{3}H[ketanserin for 5-HT_{2A} and [^{3}H]mesulergine for 5-HT_{2B} + 5-HT_{2C}, which have high affinity for the receptors, and bind to a larger population of receptors than the radiolabelled agonists [^{125}I]DOI for 5-HT_{2A} and [^{3}H]5-HT for 5-HT_{2B} + 5-$HT_{2C.}$ Competition experiments are used to select for highly competitive antagonists to allow study into each receptor subtype. They highlight a lack of highly selective agonists for each subset, with DOI displaying at most, a moderate selectivity for the 5-HT_{2A} receptor. The locus responsible for selectivity of ligands has been determined for 5-HT_{2A} and 5-HT_{2C} (Barbara et al,1996). Looking at both receptor subtypes, there was a difference of 1 amino acid residue , an alanine at residue 5.46 on the 5-HT_{2A} receptor and a serine at residue 5.46 on the 5-HT_{2C} receptor. The group decided to carry out site directed mutations at these points, swapping the alanine for a serine in the 5-HT_{2A} receptor and the serine for an alanine in the 5-HT_{2C} receptor, and then used agonist ligands to determine if these residue differences were important to how ligands bind. Some

ligands were effected more than others. The indoleamine ligands bind differently to the two receptor subtypes, and a change at the 5-HT_{2A} receptor decreased affinity for all the indoleamic ligands. This site difference between the two receptor subtypes only occurs in humans, with no difference in rat models (Barbara et al.,1996). The 5-HT_{2A} receptor has a side chain in which direct interaction with ligands occurs, and is involved in ligand selectivity for ergolines.

1.77 5-HT_{2A} receptor regulation

As previously discussed for GPCRs in general receptors can undergo desensitisation. This can occur homologously in an agonist-specific manner and heterologously , which is agonist-non specific.

Kinases : kinases play a role in regulation, specifically PKA and PKC, and are involved in heterologous desensitization. A more novel idea surrounding kinase regulation is the involvement of p90 ribosomal S6 kinase 2 (RSK2) (Allen et al., 2008). This serine/theonine kinase is activated much further downstream than other signalling pathways, and plays a role in transcriptional regulation. It is thought that RSK2 directly phosphorylates the 5-HT_{2A} receptor.

Scaffolding proteins: PDZ domains (a protein-protein interaction motif) acts as scaffolding at cell membranes to regulate larger molecule complexes. PSD95, classically known to interact with proteins including glutamate receptors has been shown to interact also at 5-HT_{2A} receptors. An enhancement of serotonin induced inositol phosphate accumulation could be seen in co-transfection studies with PSD95 (Allen et al., 2008)

Down regulation by antagonists: 5-HT_{2A} receptors are unusual among GPCRs in that prolonged exposure to antagonists can result in down regulation. Many studies have studied this phenomenon, including antagonists used as anti depressives (Gray and Roth 2001). It is thought that a change in gene transcription is responsible, however studies have been contradictory. Another mechanism proposed by Gray and Roth (Gray and Roth 2001)

is one in which antagonists actually induce receptor internalisation and therefore receptor degradation, which would result in the down regulation of the receptor. A suggestion put forth by Willins and colleagues (Willins et al.,1999) was that the therapeutic effect of antipsychotics was due to antipsychotic-induced sequestration of the 5-HT_{2A} receptor as antipsychotics with no antagonistic relation to 5-HT_{2A} had no effect on either regulation or subcellular distribution of this receptor.

1.78 Co-expression of serotonin receptors

The 5-HT_{2A} receptor in particular has been shown in several studies to be co-expressed and co-localise alongside other receptors, such as mu opioid, dopamine and other serotonin receptors (Vysokonov et al.,1998, Frederick and Woodruff 1999, Zhukovska and Neumaier,2000, Roga et al.,2009, Lopez-Gimenez et al.,2008 and Lorke et al.,2006). These studies involved the use of clozapine. Clozapine appears to lack receptor selectivity, as highlighted by expression studies looking at 5 clozapine sensitive receptors expressed at interneurons and pyramidal neurones , the receptors involved being (D4, M1, 5-HT_{2A}, 5-HT_{2C} and 5-HT_7) (Vysokonov et al.,1998). Through single cell RT-PCR profiling of rat prefrontal cortex neurones this group were able to detect co-expression of 5-HT_{2A} alongside M1 and 5-HT_{2C} receptors, each also Gq coupled and regulating intracellular Ca^{2+} metabolism in response to either serotonin or acetylcholine. Clozapine is also implicated in the downregulation of 5-HT_6 (Frederick and Woodruff,1999, Zhukovska and Neumaier, 2000). Cat spinal locomotor neurone studies looking at c-fos immunoreactive neurones and their co-localisation with various serotonin receptors (5-HT_7, 5-HT_{2A} and 5-HT_{1A}) indicate that c-fos immunoreactive neurones co-localise with 5-HT_7 receptors, a majority of 60-80% co-localising with 5-HT_{2A} and a more poorly defined 30% or more with 5-HT_{1A}, leading to the conclusion that to some degree these receptor subtype can be found together (Roga et al.,2009). Co-expression has also been seen between 5-HT_{2A} and 5-HT_6, with immunohistochemical methods on brain tissue samples of the prefrontal cortex revealing

expression of both in pyramidal and stellate shaped cells in cortical layers (Lorke et al.,2006).

The 5-HT_{2A} receptor has the ability to form a heteromeric unit with other receptors such as the dopamine D2 receptor (Borroto-Escuela et al.,2010). Findings suggest that 5-HT_{2A} signalling via the Gq/11 pathway is enhanced in the heteromer when co-activation of the D2 receptor occurs, however there is a decrease in signalling of the D2 through Gi/o when the heteromer counterpart 5-HT_{2A} is activated by 5-HT (Borroto-Escuela et al.,2010). As previously outlined there has been the discovery of the potential schizophrenia target 5-HT_{2A}-mGluR2 heteromeric complex (Maeso et al., 2008) and at the same time emergence of 5-HT_{2A} co-expression with the mu opioid receptor (Lopez-Gimenz et al.,2008) with 5-HT_{2A} facilitating the desensitization, internalisation and subsequent down regulation of the mu opioid receptor when co-activation occurs.

1.8 Schizophrenia

Schizophrenia was described by the American Psychiatric association in 2000 as a "disorder", however many feel this is looser terminology and prefer the more descriptive term "disease". Those in preference of this terminology include Tandon and colleagues, authors of the Science Direct series of Schizophrenia research (2008). They feel it should be termed as a disease related to brain abnormalities that are the final common pathway caused by an assortment of specific and/or environmental factors.

The annual incidence of schizophrenia averages 15 per 100,000 (Tandon et al., research 102, 2008). The development of schizophrenia has been linked to a variety of risk factors of environmental and genetic risk factors , however no single factor has been singled out as the key cause of developing schizophrenia.

1.81 Symptoms

Schizophrenia is characterised by a mixture of positive , negative, cognitive, mood and motor symptoms whose severity varies across patients (Tandon et al., research 110, 2009).

Positive symptoms: Hallucinations including sight, sound, touch, taste and smell. They are perceptions that do not exist. Delusional states are another common key positive symptom and are of grandeur or paranoid nature.

Measuring positive symptoms when evaluating the effectiveness of antipsychotics poses difficulties. In animal models agitation, hyperactivity and stereotypical behaviour which relate to psychosis can be observed eg. LSD induces visual hallucinations in humans, and can cause characteristic head twitching behaviours in mouse models. When DOI (2,5-dimethoxy-4-iodoamphetamine), another hallucinogenic ligand is administered to animals this results in “wet dog shakes”.

Negative symptoms: These are debilitating symptoms, where the patient loses interest in both themselves and the things around them. They seem to lack thought. Speech and thought are human characteristics and so therefore indeterminable in an animal model. Phenomena such as anhedonia (a decrease in ability to feel pleasure) is another human characteristic. Social interaction is the mechanism relied upon to measure exhibition of negative symptoms.

Cognitive deficits: These include an inability to hold attention and working memory impairment, simple tasks such as planning and decision making become impossible. Depressive symptoms are also commonly displayed, and other anxiety related disorders such as OCD can creep through as co-morbid disorders. Suicide attempts and deaths in relation to suicide rise in schizophrenic patients.

‘Approximately one third of individuals with schizophrenia attempt suicide one or more times and 5% of individuals die of suicide’ (Tandon et al., research 110 2009).

Limitations of animal models: Creating animal models is difficult, partially due to the likelihood of symptoms being heterogeneous and shared by other disease states. The most obvious problem is a lack of unique behavioural abnormalities found in humans can be seen in a mouse model, such as guilt or suicide. Emotional reactivity and social behaviour tests lack specificity. Discovery of genes linked to schizophrenia may not be specific to the characteristics of this disorder, with variations of genes occurring in a host of disorders including schizophrenia, bipolar and autism where there is reported to be a genetic overlap involving rare copy number variants as well as common single nucleotide polymorphisms which are associated with developmental risk . A number of genes are most likely to be implicated (Carrol and Owen 2009).

The social defeat model: It was proposed that risk of schizophrenia could be increased by chronic and long term experience of social defeat, subjecting a rat/animal model to a social defeat situation where by introduction of an intruder leads to submissive behaviour and stress causing an elevation in dopaminergic hyperactivity, thought to heighten the risk of schizophrenia development (Selten and Cantor 2005). Such models have been implicated in depression (Nestler and Carlezon 2006) and it has been shown that the model can segregate between vulnerable and nonsusceptible populations of mice due to signalling adaptations within the mesolimbic dopamine circuit, long lasting social avoidance occurs depending upon BDNF signalling in the ventral tegmental regions which are associated with reward and emotion (Krishnan et al.,2007). These models have proven difficult to ascribe molecular changes to a particular social defeat, as the behaviours mimic more than 1 disorder symptom.

Learning and memory, when evaluating the effectiveness of antipsychotics can be studied in animal models by subjecting them to a variety of tasks such as 1) object recognition- by administering rats with antipsychotics and subjecting it to the same object twice, the animals show a loss of interest in the familiar object and 2) Morris water maze- rat is

placed in a tank with water and an escape platform which is not visible to them. Animals must find this platform, and repeated exposure should result in a more rapid performance. When considering newly produced antipsychotics for therapy, their ability to induce EPS (extra pyramidal symptoms) is important. Classical antipsychotics ie; the typical antipsychotic haloperidol provokes EPS with human patients displaying signs of Parkinsonism (tremor, rigidity), dystonia (involuntary muscle spasms) and rats display related behaviours of purposeless chewing and akathisia (restlessness) causing the rats to pace and march on the spot.

1.82 Neurotransmitter involvement

There are several pathways involved in schizophrenia

1) The dopamine pathway-over activity has been observed, as seen with neuroimaging studies using amphetamines to induce psychosis, whilst antipsychotic drugs generally antagonise the dopamine D2.
2) The glutamate pathway (NMDA receptors: *N*-methyl-D-aspartate receptor)-hypofunction at these receptors results in negative and positive symptoms. Trials have -looked at how antagonistic NMDA drugs such as ketamine effect this condition.
3) GABA (Gamma Amino Butyric Acid) receptors appears to have reduced numbers along with abnormalities in the distribution of GABAergic neuronsin the pre frontal cortex of Schizophrenic patients.
4) Serotonergic pathways- upregulation of these pathways is known to have a knock on effect on the dopaminergic system, resulting in negative symptoms. Many antipsychotic drugs target the 5-HT $_{2A}$ receptor.

1.83 The role of serotonin in schizophrenia

Two key serotonin pathways are involved in schizophrenia, these being projections from the dorsal raphe nuclei to the substantia nigra , and projections from the rostal raphe nuclei to the cerebral cortex, limbic regions and basal ganglia. Hypofunction of thedopaminergic system occurs as a result of up regulation of these pathways.

Initial findings by Wooly & Shaw and Gaddum looked at LSD and found it to have psycho mimetic effects. LSD is belongs to the ergoline family. It is an agonist by nature at serotonin receptors and their activation results in visual hallucinations, in contrast to antipsychotic agents.. Serotonin plays a role in a host of behaviours related to the condition of schizophrenia, including cognition, perception and attention, mood, pain sensitivity and appetite. Particularly the positive and negative symptoms associated with the condition.

According to Meltzer & Roth 'functional alterations in the serotonergic system (including both pre and post synaptic function) affect multiple neurotransmitter systems' , and in doing so lead to exhibition of various behaviours. They believe that by pharmacologically manipulating the serotonergic system , causing exacerbation or reduction in positive and negative symptoms and cognitive functions, with ability to modulate extrapyramidal function (Roth & Meltzer 1995).

Many available antipsychotic drugs are aimed at targeting various serotonin receptors, in particular 5-HT_{1A} and 5-HT_{2A}, the most studied and known to be the most effective targets, but also now it is becoming accepted that 5-HT_4 and 5-HT_6 are emerging as highly therapeutic targets, with interest also surrounding 5-HT_{2C} and 5HT_7 (Meltzer et al.,2003, Roth.B.L.,2004 and Meltzer et al.,2006).

In my thesis I hope to explore the pharmacology of the 5-HT2A receptor and demonstrate the difficulty surrounding functional studies with single target models.

2. Materials and methods

2.1 Reagents and kits

BDH, Lutterworth, Leicestershire, UK

22mm cover slips, KOH

CisBio assays (HTRF) , France

IP-oneTb assay kits

Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK

$CaCl_2$, D-glucose, ethanol, ethylene glycol, isopropranolol, glycerol, glycine, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), KCl, methanol, 3-(N-Morpholino)propanesulfonic acid (MOPS), NaCl, sodium dodecyl sulphate (SDS), sucrose, $MnCl_2$.

Flowgen Biosciences Ltd., Nottingham, UK

Agarose

Invitrogen Ltd., Paisley, UK

NuPage Novex pre-cast 4-12 % Bis-Tris gels, NuPage MOPS SDS running buffer

Konica Europe, Hohenbrunn, Germany

X-ray film

Merck Chemicals Ltd., Beeston, Nottingham, UK

Luria-Bertani (LB)-agar, LB-bouillon

New England Biolabs, Herts, Uk

Prestained Protein Marker, Broad Range (7-175 kDa)

Perbio Science UK Ltd., Cramlington, Northumberland, UK

Supersignal West Pico chemiluminescent substrate (ECL)

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Complete EDTA-free protease inhibitor tablets

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Ampicillin, BCA solution A, bromophenol blue, bovine
serum albumin (BSA), deoxycholic acid (sodium salt), dithiothreitol (DTT),
dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA),
ethidium bromide, ethylene glycol, FURA-2AM, glycerol, HCl, 3-isobutyl-1-methylxanthine (IBMX),
lithium chloride, $MgCl_2$, $RbCl_2$, Na_2HPO_4, NaCl, NaF, NaOH, Tween-20, Triton x 100,
Tris-base

Whatman International Ltd., Maidstone, UK

Protran® nitrocellulose transfer membrane

2.2 Antibodies and antisera

Cell signalling technology, Danvers, Ma

C-myc anti-mouse, C-myc anti rabbit antibodies

GE health care Uk and Ireland, Buckinghamshire

Anti-mouse IgG-horse radish peroxidase (HRP) conjugate from sheep, Anti-rabbit IgG (HRP) conjugate from donkey

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

ANTI-FLAG antibody produced in rabbit, Rabbit anti-goat IgG-HRP conjugate

GFP antisera and all G protein antisera were produced in-house

2.3 Pharmacological compounds

Schering-Plough research institute

Company donated ligands: MDL100.907, Melperone, SB742.457, SB399.885, Spiperone, Ziprasidone, WAY257.561

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Amoxapine, Chlorpromazine hydrochloride, Clozapine, Dihydroergotamine methanesulfanate salt, Mianserin hydrochloride, 5-Benzyloxytryptamine

2.4 Radiochemicals

Perkinelmer, Cambridgeshire, UK

Ketanserin Hydrochloride (R41 468), [Ethylene-^{3}H]-

2.5 Tissue culture disposables, reagents and plates

Costar, Cambridge, MA., USA

5 mL, 10 mL and 25 mL pipettes, 75 cm^2 vented tissue culture flasks, 6 well plates, 12 well plates, 96 well plates

Invitrogen BV, Groningen, The Netherlands

Blasticidin, Flp-In TREx HEK293 cell line, dialyzed foetal bovine serum (dialyzed FBS), L-glutamine (200 mM), Hank's buffered saline solution (HBSS), new born calf serum (NBCS), 100 x, penicillin-streptomycin mix, Versene,

Roche Applied Science, Lewes, East Sussex, UK

Hygromycin B

Sigma-Aldrich Company Ltd., Dorset, UK

DMEM (- sodium pyruvate, + L-glutamine, + 4.5 g/L glucose) 0.25 % trypsin-EDTA, pertussis toxin, poly-D-lysine

2.6 Buffers and solutions

Binding buffer

75 mM Tris 45.4g

5 mM EDTA 9.30g

12.5 mM $MgCl_2$

Make up to 5L with H_20 pH 7.4 with conc HCL

Blocking buffer

500ml PBS + 0.05% Tween 20, 3-5% Marvel

DNA loading buffer (6 x) 10MLS

1.25ml saturated bromophenol blue,

4g sucrose

Dissolved in sterile H_20

Laemmli buffer (5 x) pH 6.8 50mls

1.9g Tris, 25mls Glycerol pH6.8 then on heat stirrer add:

1.95g DDT , H_20 >50mls, pinch of bromophenol blue.

Buffer was stored at –20 ºC

Microscope buffer

NaCl 7.6g, KCl 0.373g, Hepes 4.766g, Glucose 1.8g, 1M $MgCl_2$ 1ml, make up to 1L H_20

pH 7.2. Buffer was stored at 4 °C

Phosphate buffered saline (PBS) (10 x)

137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10.2 mM Na_2HPO_4; pH 7.4. 1 x PBS was prepared by diluting the stock 1:10 in H2O

2 x RIPA

11.9g 100 mM HEPES

8.8g 300 mM NaCl

10mls 2 %(v/v) Triton X-100,

5g1 %(w/v) sodium deoxycholate

1g 0.2 % (w/v) SDS

pH 7.4 >500ml H_20

1 x RIPA

25mls 2x buffer + 25mls H_2O – this was supplemented with

500ml 1M sodium fluoride

500ml 0.5 M EDTA

500ml 0.2 M $NaPO_4$

2.5mls 5 %(v/v) ethylene glycol and 1 x EDTA-free protease inhibitor tablet

Tris-EDTA (TE) buffer

10 mM Tris-base, 0.1 mM EDTA; pH 7.4. Buffer was stored at 4 °C

Transfer BUFFER 5X 1L

72g Glycine

15.5g Tris

Wash buffer

500ml PBS + 0.05% Tween 20

2.7 Assays and methods

Tissue culture

Maintenance of Flp-In™ T-REx™ HEK293 inducible cells stably expressing Serotonin2A receptor

Cells were maintained in DMEM (with 4.5 g/L glucose, L-glutamine, - sodium pyruvate). The media was supplemented with heat inactivated 10 %(v/v) dialyzed FBS, 1 %(v/v) penicillin/streptomycin mix, 1mg/mL blasticidin and 2.2mls hygromycin.Cells were grown in a humidified incubator (95 % air/5 % CO2; 37 °C).

Passage of cells

Confluent Flp-In™ T-REx™ HEK293 cells stably expressing Serotinin 2A receptor were treated with Sterile 0.25 % trypsin-EDTA solution and flasks were gently rotated to cover the monolayer and placed in the incubator (for up to 5 min) until cells became detached. Once detached, 7 mL of fresh media was added to inactivate the trypsin and the cells were centrifuged at 288 x *g* (5 min;25 °C). The pellet was resuspended in fresh media and the suspension split into Flasks or plates.

Preparations, Assays and Analysis

Bicinchoninic acid protein quantification

The protein concentration of samples was quantified using the BCA assay. This assay Incorporates bicinchoninic acid (BCA) and copper sulphate solutions. Protein reduces Cu(II) ions to Cu(I) ions in a concentration-dependent manner. Reduced Cu(I) can be bound by BCA which causes a colour change that has an absorption maximum of 562 nm. BSA of known concentrations (0.2-2.2 mg/mL) was used to construct a standard curve, which allows the concentrations of unknown samples to be extrapolated. A 1:50 ratio of reagent A(1 %(w/v) BCA, 2 %(w/v) Na_2C0_3, 0.16 %(w/v) sodium tartrate, 0.4 % (w/v) NaOH, 0.95 %(w/v) $NaHCO_3$; pH 11.25) : reagent B (4 %(w/v) CuSO4) was mixed and 200 µL of this solution added to 10 µL of protein standard or unknown sample in a 96-well ELISA plate. The assay was incubated (30 min; 37 oC) before the absorbance was read at 600 nm.

Cell harvesting

Stably transfected Flp-In TREx HEK293 cells were harvested after 16 h doxycycline treatment. The media was removed and cells were washed three times in 5 mL ice cold PBS. Cells were scraped from the dish using a disposable cell scraper and transferred to a

15 mL centrifuge tube and centrifuged (288 x *g*; 5 min; 4 oC). After discarding the supernatant, the cell pellet was frozen at –80 oC until required.

Cell lysates prep

Cells were grown on 6-well sterile tissue culture plates. 1x radioimmunoprecipitation (RIPA) buffer was made fresh on the day of the assay by diluting 2 x RIPA (100 mM HEPES, 300 mM NaCl, 2 %(v/v) Triton X-100, 1 %(w/v) sodium deoxycholate, 0.2 % (w/v) SDS; pH 7.4) buffer in H2O – this was supplemented with 0.5 M sodium fluoride, 0.5 M EDTA, 0.2 M $NaPO_4$, 5 %(v/v) ethylene glycol and 1 x EDTA-free protease cocktail inhibitor tablet. Cells were washed 3 x 5 min with PBS before the addition of 200µL 1 x RIPA buffer. The mixture was rocked for 30mins at 4 ^{o}C on a plate rocker before aliquoting out and centrifugation (max 4 ºC; 15 min) to pellet cellular debris. Protein concentration was determined (as above for BCA method). Lysates were stored at –20 ºC. When ready for loading into a gel for SDS page(see below) lysates were added 50/50 with 2xLaemmli buffer and appropriate concentration obtained , loading into wells no less than 20µg/ well.

Cell membranes prep

Harvested pellets were thawed and re-suspended in Tris-EDTA buffer. The cells were homogenised (50 passes of a Teflon-in-glass homogeniser) and the resulting suspension centrifuged (288 x *g*; 10 min; 4 °C) to remove unbroken cells and nuclei. The supernatant was ultracentrifuged (50,000 x *g*; 30 min; 4 °C) in an Optima TLX Ultracentrifuge (Beckam Coulter, Palo Alto, CA). The resulting pellet was re-suspended in Tris-EDTA buffer and passed 10x through a 25-gauge needle. The protein concentration was determined as detailed in the BCA method and the membranes stored at –80 oC until required. When ready for loading into a gel for SDS page (see below) lysates were added

50/50 with 2xLaemmli buffer and appropriate concentration obtained , loading into wells no less than 20μg/ well.

Calcium mobilization (dose response and schild)

Cells were split, resuspending the pellet in 25-30mls of growth medium, and plated out 100μls cells/well. Dox induce relevant wells and incubate at 37°c, 5% CO2 over night. On the day treat with FURA 2AM (33μl in 11mls of media, and plate out 100μls/well, incubating for 45minutes 37°c, 5% CO_2. Wash 2x with 100μl/well of microscope buffer. Prepare agonist drug plates, and a range of antagonist concentrations in microscope buffer during incubation time. Each concentration should be plated out in triplicate. Ec50 values could be established once data input into graphpad prism, indicating the ligands potency.

****Dose response curves:*** stimulate with antagonist for 15minutes then run on the flex station.

****Schild experiments:*** 4 curves set up in triplicate, first curve being the agonist dose response control curve and the other 3 curves for an agonist dose response in the presence of set antagonist concentrations. The plate is set up, as above, and a third wash of microscope buffer is added to the plate and left to incubate for 15mins. The drug plates were set up to contain the relevant combinations of ligand (co-administering the drugs). After 15minutes the assay was ran on the flex station. Values from the shift in potency (Ec50) were then used in graphpad prism to extrapolate the dose ratio's and PA2 values.

Cell surface ELISA

Cells were split, resuspending the pellet in 25-30mls of growth medium, and plated out 100μls cells/well into a 96well clear plate and incubate at 37°c, 5% CO_2 over night. After 24hrs dox induce relevant wells and incubate at 37°c, 5% CO_2 over night. On the day remove media and add agonist to wells in normal media, fresh media only for agonist

exempt cells. Remove all media, and treat with antibody in final volume of 50µl in growth medium, Anti-Myc 1:500 dilution. Incubate for 30mins 37^{o}c, 5% $_{CO2}$. Remove media completely, wash with DMEM-HEPES (100µl) 1x then treat with secondary antibody + hoescht mixture (Anti-rabbit 1:1000 + hoescht 1:1000, total volume 100µl/cell growth medium). Incubate for 30mins 37^{o}c, 5% $_{CO2}$ in the dark. Warm PBS and TMB substrate (100µl/well). Wash 2x with PBS, on second wash perform hoetsch readout then remove PBS and dry wells gently, add TMB and cover for 5-10mins, read at 620nm.

IP-ONE tb (cis bio kit)

This kit is intended for the direct quantitative determination of myo-Inositol 1 phosphate (IP1), and has been optimized in order to measure IP1 directly on cultured cells.
This assay is based on a monoclonal antibody specific for IP1 labeled with Eu Cryptate, competing with both native IP1 produced by cells and IP1 coupled to the dye d2. The specific signal is inversely proportional to the concentration of IP1 in the calibrator or in the cell lysate. As for all other HTRF® assays, data reduction using the fluorescence ratio (665nm/620 nm) eliminates possible photophysical interference and means the assay is unaffected by the usual medium conditions and colored compounds.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were resolved using SDS-PAGE. Precast NuPage Novex Bis-Tris gels with a 4-12 % acrylamide concentration were locked into an XCell Surelock mini-cell gel tank and covered in NuPage MOPS SDS buffer. To allow estimation of protein weight, samples were compared with the full range molecular weight marker. The gel was run at 200 V until the dye front reached the base of the gel.

Western blotting

Following separation of samples by SDS-PAGE proteins were electrophoretically transferred onto nitrocellulose using the XCell II blot module. Proteins were transferred at 30 V (1 h) in transfer buffer (0.2 M glycine, 25 mM Tris and 20 % (v/v) methanol). To block non-specific binding sites, the membrane was incubated on a rotating incubator for 1h at room temperature in 5 %(w/v) low-fat milk, PBS+ 0.1 %(v/v) Tween 20 (PBS-Tween). The membrane was incubated (over night; 4 °C) with primary antibody in 5 %(w/v) low fat milk dissolved in PBS-Tween containing the required antibody dilution (Table 2-1). The membrane was washed three times for 5 min in PBS-Tween. Secondary antibody linked to horseradish peroxidase was diluted in 5 %(w/v) low fat milk dissolved in PBSTween and incubated with the membrane at room temperature for 1 h. The membrane was washed three times for 5 min in PBS-Tween followed by incubation with ECL solution for 5 min and exposure to blue Kodak film.

Antibody treatments for western blotts

Primary antibody	**Secondary antibody**
α cMYC 1:1000	α Rabbit 1:2000
α GFP (sheep) 1:5000	α Goat 1:10000
Pp44/42 phospho 1:1000	α Mouse 1:5000
P44/42 kinase total 1:1000	α Rabbit 1:5000

Table 2.1 Western blot antibody treatment dilutions.

Radioligand binding studies

****Specific:*** specific radioligand binding over a range of concentrations. Triplicate mixtures containing 24μg protein and a range of radioligand concentrations from 0.2nM to 10nM , including a zero radioligand tube. Non specific binding was determined by addition of 10^{-}

^{4}M mianserin (final concentration 10^{-5}M). Samples were added to tubes which had been chilling over ice.

Each tube contains a total of 500 [illegible]:

300ml binding buffer(ice cold)

50ml non specific ligand or buffer

50ml radio ligand or buffer

100ml membranes

Incubate for 1hour at 25°C.

Filter paper was pre soaked in the buffer prior to filtration on the brandel. After harvesting the filters were allowed to dry briefly, and then the filter discs were placed inside a tube with 3mls scintillant and vortexed before radioactivity was measured on the top counter (Beckman Ls6500 multi purpose scintillation counter).
Specific binding was determined by subtracting non specific counts from total counts, plotting against radioligand concentration. Graphpad prism was used to determine receptor expression (Bmax) and Kd (dissociation constant) was also calculated.

Competition: Triplicate reaction mixtures set up to contain 24µg protein, radioligand at Kd equivalent concentration 10x, so 11.2 nM [3^{H}] Ketanserin and a range of concentrations of cold ligand ranging from 10^{-11}M to 10^{-4}M, including a zero. Non specific triplicates were also included to contain 10^{-5}M Mianserin.

Each tube contains a total of 500 µl:

300µl binding buffer(ice cold)

50µl cold ligand/ non specific ligand/ buffer

50µl radio ligand

100µl membranes

Incubate for 1hour at 25°C.

Filtration occurred as with the specific binding above. Graphs were then plotted to determine an EC50(equivalent to IC50) value using non linear regression constraints, and then the Ki of the cold ligand was calculated using the Cheng-Prusoff equation.

3. Results

3.1 Receptor expression and internalisation studies

The human 5-HT_{2A} receptor was expressed stably in cell lines by Juan F Lopez-Gimenez, a post doctoral fellow in the lab. He modified the receptor so that it contained a cMyc epitope in the N-terminal domain and a CFP fluorescent tag linked to the C-termial tail. The receptor construct was then cloned into an inducible Flp-IN-TRex 293 cell line, allowing generation of a stable cell population in which receptor expression can be controlled by application of the antibiotic tetracycline or its analogue doxycycline (Lopez-Gimenez, et al 2008, González-Maeso,et al 2008). As the receptor is linked to the cMyc epitope at the N terminal, it is possible to detect expression with anti-Myc antibodies and performing Western blots. Expression of the receptor would be identified as a band on the gel at a size anticipated to be 59.6kDa due to the anti-body binding to the cMyc epitope, if present. If no receptor expression occurs then no anti-Myc reactivity should be detected.

3.1.1 Receptor expression using Western blotting

Firstly it had to be ensured that receptor expression only occurred when induced by addition of doxycycline. In order to do so cell lysates were generated and SDS-PAGE performed. It can clearly be seen in Figure 3.1a that expression of the 5-HT_{2A} receptor only occurs when induced by doxycycline.

Subsequently various concentrations of doxycycline were used to assess how this would impact receptor expression (Fig 3.1b). With zero as a control the concentrations of doxycycline ranged from 1ng/ml to 1μg/ml. Figure 3.1b shows that over this range of concentrations of doxycycline there was not a substantial impact on the amount of receptor expressed.

I also assessed if receptor expression was influenced by cell passage number. Various passages of the cells were harvested including/excluding doxycycline, and SDS-PAGE and immunoblotting used to observe potential variation in expression. It can clearly be seen that over the range used for subsequent studies the passage of cells did not greatly impact on receptor expression (Fig 3.2).

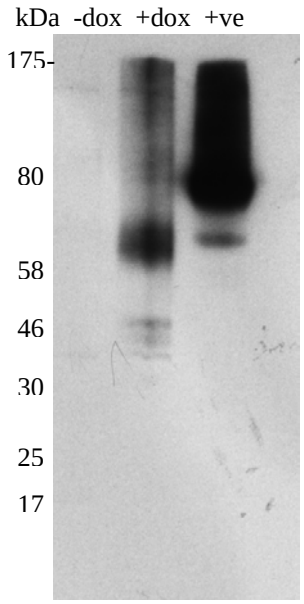


Fig.3.1a

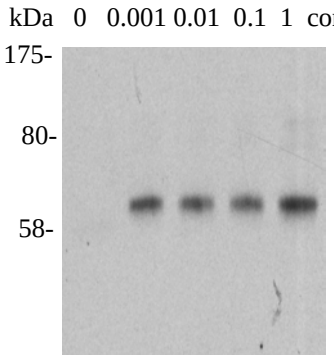


Fig.3.1b

Fig 3.1a Flp-IN-TRex cells harbouring c-Myc-5-HT_{2A}-CFP were maintained without doxycycline and induced with doxycycline(1µg/ml) and a control (+ve) expressing c-Myc. Lysates were prepared and Western blotting carried out. Detection by primary antibody anti-myc(rabbit) and secondary antibody (rabbit).
Fig 3.1b Cells induced with varying concentrations of doxycycline , lysates prepared and Western blot carried out. Detection by primary antibody anti-myc (rabbit) and secondary antibody (anti-rabbit).

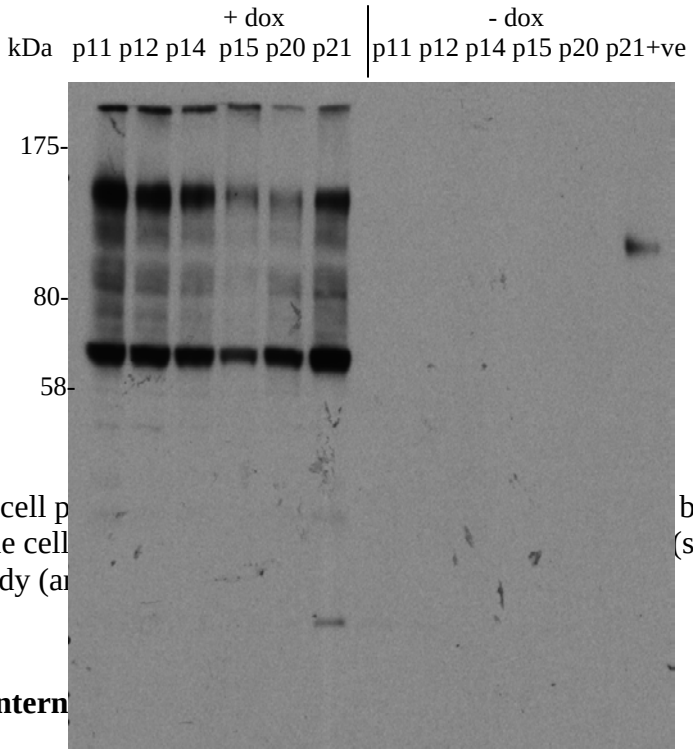


Fig.3.2 Effect of cell p both +doxycycline and – doxycycline cell (sheep) and secondary antibody (a

3.1.2 Receptor intern

Cell surface ELISA was performed to observe what effect applying agonist ligands had on cell surface levels of the 5-HT_{2A} receptor construct (Fig 3.3).

In the absence of receptor induction some level of anti-Myc binding was observed. Basal levels appear to not be at zero, and instead are slightly elevated, indicating non specific interaction. Treatment with doxycycline resulted in approximately a 3 fold increase in signal. This difference represents cell surface c-Myc-5-HT_{2A}-CFP. On application of EC_{80} concentrations (derived from calcium experiments , representing the concentration of ligand which gives maximal response) of 4 agonist ligands (5-HT, DOI, quipazine and 5-benzyloxytryptamine) , the cell surface expression of 5-HT_{2A} was reduced to similar levels by each agonist, although basal level was never achieved.

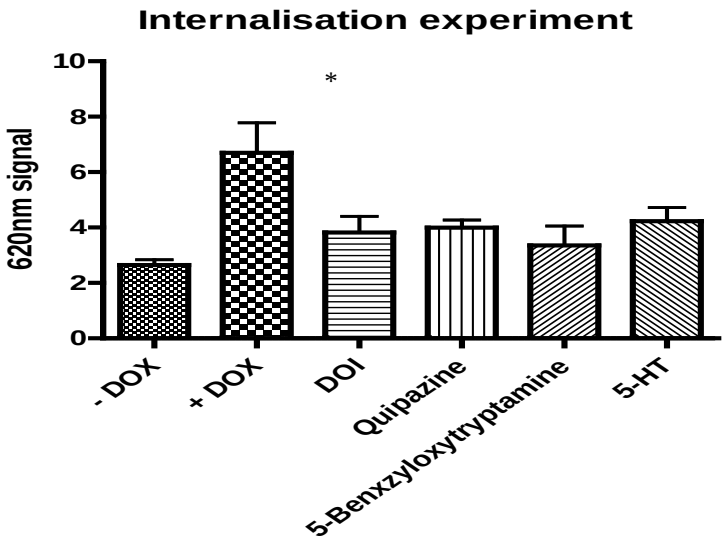


Fig.3.3 The effect of 5-HT_{2A} ligands on internalisation of c-Myc-5-HT_{2A}-CFP expressed in Flp-IN TREx 293 cells. P= 0.033 between – doxycycline and + doxycyline. No significant difference could be established between +doxycline cells and application of agonist ligands.

3.2 Radioligand binding studies

Radiolabelled ligands can provide both a direct measurement of the presence of a receptor and the affinity of interaction between the two. A series of such experiments was carried out using the tritiated radioligand, [^{3}H]ketanserin which is reported to bind selectively and with high affinity to the 5-HT_{2A} receptor.

3.2.1[^{3}H]ketanserin saturation binding assays

Radioligand saturation binding allows the establishment of 2 parameters. Firstly the B_{max} can be determined. This is the density of receptors present. Secondly the K_D which reflects the affinity of the radioligand for the receptor can be established (Davenport and Russell, 1996). From the cells described in 3.1, cell membranes were prepared after treatment with or without 1μg/ml doxycycline for 24hours to express the 5-HT_{2A} receptor before harvesting. Experiments were established using increasing concentrations of [^{3}H]ketanserin (Fig3.4a). Ketanserin is a selective ligand for the receptor, however, even such a selective ligand may bind to other sites apart from the receptor of interest. Application of a high concentration of a non-radioactive, competitive inhibitor (mianserin) occupies the specific receptor sites otherwise available for [^{3}H]ketanserin to bind to, and therefore any binding of [^{3}H]ketanserin in the presence of mianserin reflects such non-specific binding. Non specific binding is linear [^{3}H]ketanserin and subtracted from total binding to give the specific binding. From such studies a B_{max} (489.5fmol/mg) and K_D (1.4nM) could be defined (Fig 3.4b).

Without doxycycline there was no specific binding, which indicates a lack of receptor expression. This reconfirms previous expression studies, Figure 3.1.

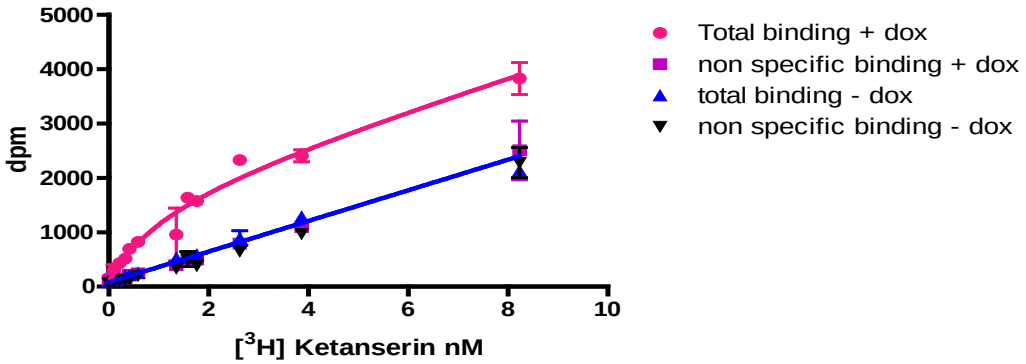
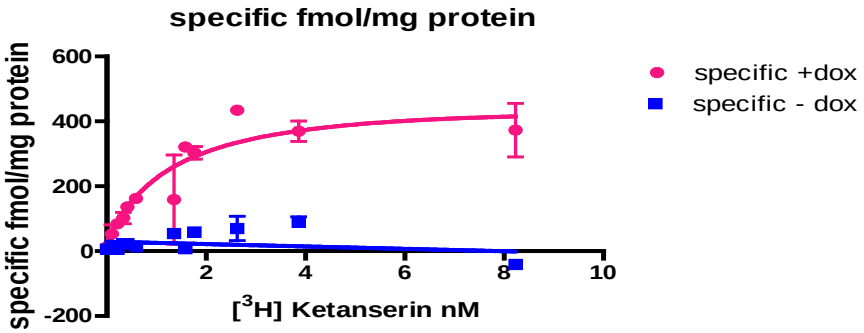


Fig.3.4a



	A - B	C - D
One site -- Total and nonspecific binding		
Best-fit values		
Bmax	489.5	(not used)
Kd	1.451	(not used)

Fig.3.4b

Fig.3.4a The binding of varying concentrations of [^{3}H]ketanserin was assessed in membranes of Flp-In TREx 293 cells either untreated, or induced to express the 5-HT$_{2A}$ receptor in the presence (non specific binding) and absence (total binding) of a fixed concentration of mianserin (10^{-5}M).(3.4b) specific binding of [^{3}H]ketanserin is shown in each case. B_{max} value: fmol/mg protein, K_D value: nM.

3.2.2 [^{3}H]Ketanserin competition binding studies

Also known as displacement binding studies, experiments of this form allow the affinity of non-traceable (non-radiolabelled) ligands to be assessed by analyzing their competition with the traceable radioligand.

Flp-In TREx 293 cell membranes were prepared after inducing the cells with 1μg/ml doxycycline to express the 5-HT_{2A} receptor before harvesting. Each antagonist ligand was used in a concentration dependent manner to compete with [^{3}H]ketanserin for the 5-HT_{2A} binding sites. From this inverse sigmoidal curves were generated (Fig 3.5(a-k)) and inhibition values were obtained. The inhibition values (IC_{50}) are the half maximal (50%) inhibitory concentration of competitor which is used to define the concentration of ligand required to inhibit the specific binding of [^{3}H]ketanserin . The Cheng-Prusoff equation was then used (see equation 1) in order to define absolute affinity values, known as Ki (Table 3.1).

In the majority of experiments the competition binding worked very well, and produced some curves of the expected characteristics. The results obtained are fairly consistent with the published values on the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (IUPHAR) website (www.**iuphar**.org). Each of the antagonist ligands were seen to compete with the radioligand to occupy the receptor site, therefore reducing the binding of [^{3}H]ketanserin in a concentration dependent manner, represented by an inverted sigmoidal curve. With regards to the amoxopine curve, the dose range is not adequate to cover a concentration dependant curve, and instead shows inhibition to occur immediately after only a low concentration of antagonist is added, and perhaps experimental error has occurred here during sample preparation.

Fig.3.5a

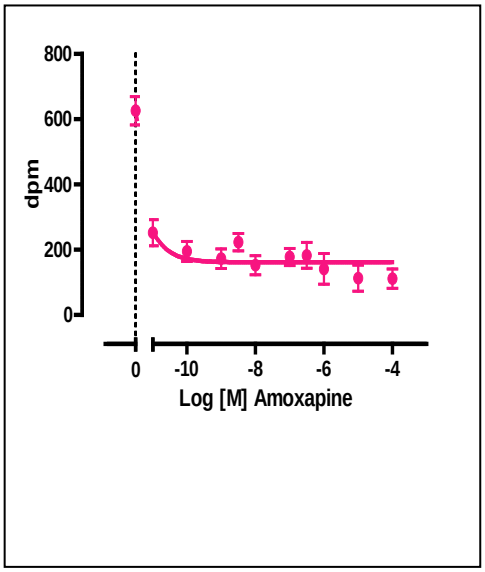


Fig.3.5b

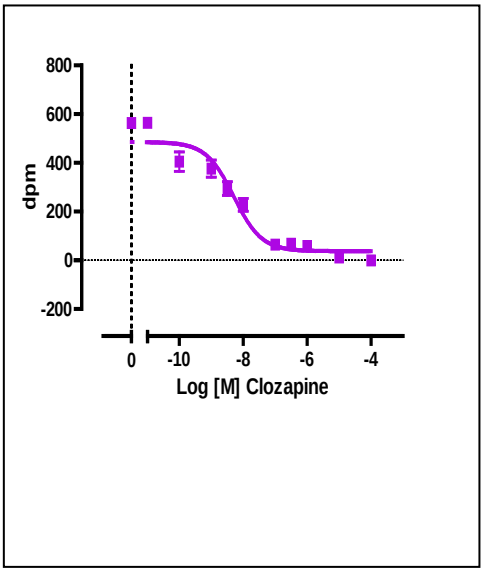


Fig.3.5c

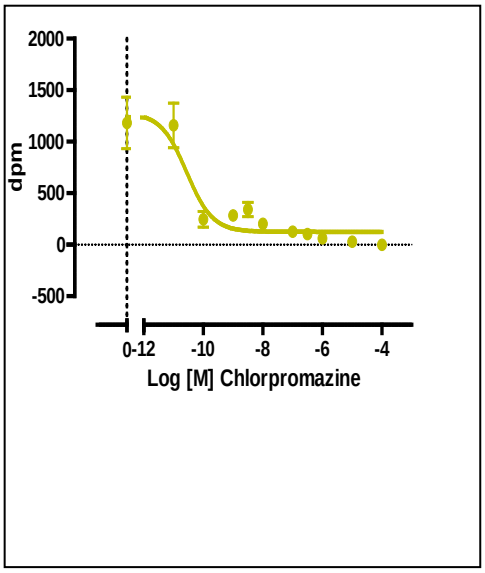


Fig.3.5d

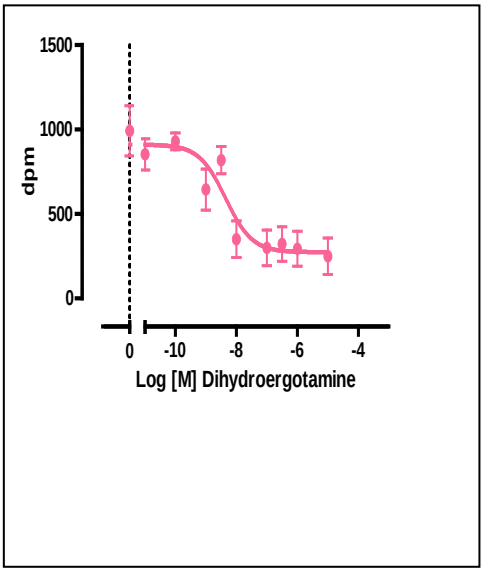


Fig.3.5e

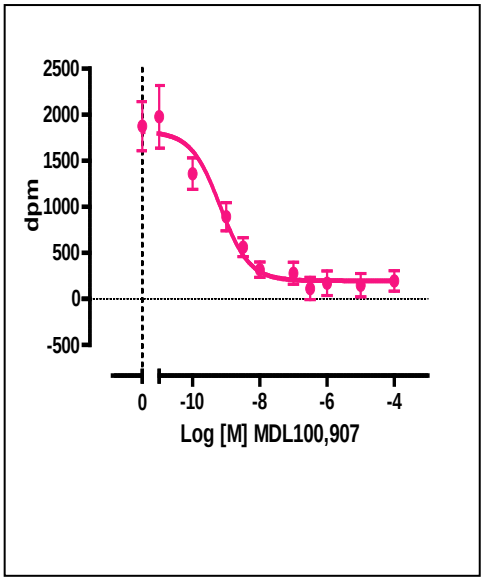


Fig.3.5f

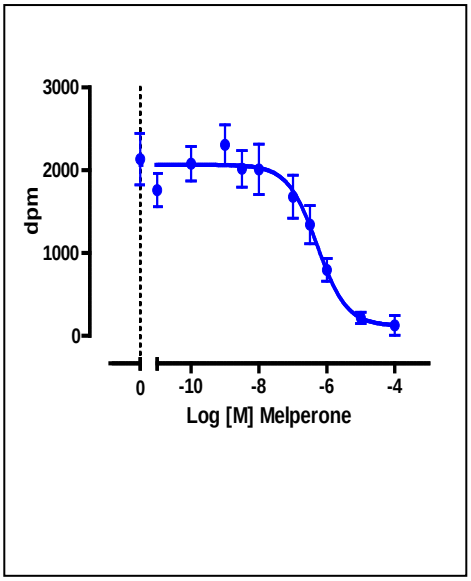


Fig.3.5g

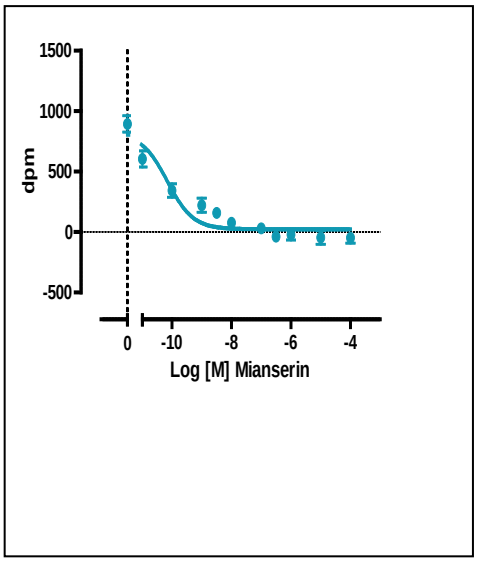


Fig.3.5h

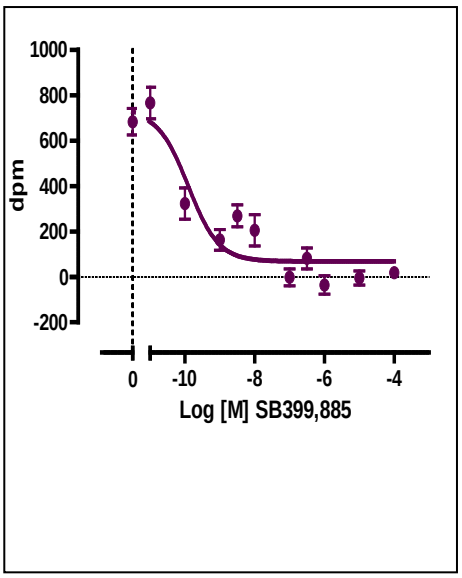


Fig.3.5i

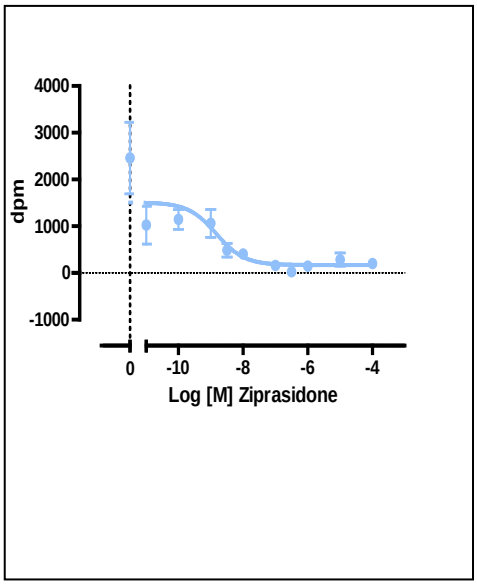


Fig.3.5j

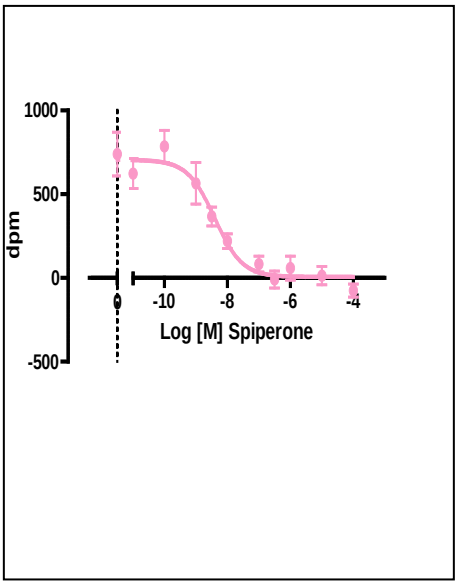


Fig.3.5k

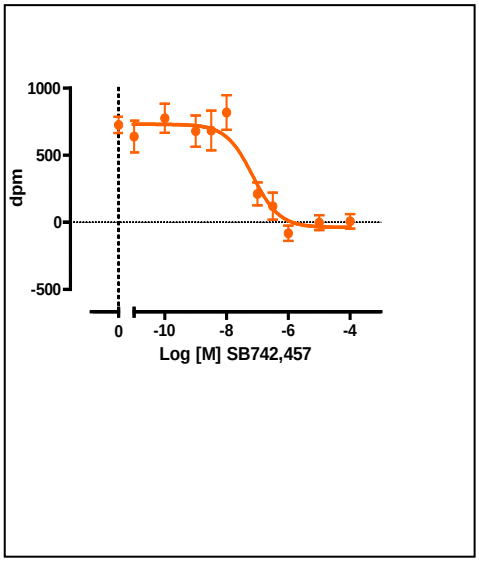


Fig.3.5(a-k) Competition binding curves in Flp-In TREx 293 cell membranes induced to express the 5-HT_{2A} receptor assessed using various ligands; amoxapine (a), clozapine (b), chlorpromazine (c), dihydroergotamine (d), MDL100,907(e), melperone (f), mianserin (g), SB399,885 (h), ziprasidone (i), spiperone (j) and SB742,457 (k) using 11.2 nM [^{3}H]ketanserin ,each taking into account non-specific binding determined by 10^{-5}M Mianserin. pK_i values were determined and are shown in table 2.1 to follow. Data are means ± SEM, n=3.

Ligand	Affinity (pKi)	Std. Error S.E.M
Amoxapine	nd	nd
Chlorpromazine	10.0	1.12
Clozapine	8.9	0.23
Dihydroergotamine	9.0	0.76
Mianserin	9.7	0.30
MDL100,907	9.2	0.17
Melperone	6.4	0.09
SB-742,457	7.8	0.05
SB-399,885	9.6	0.06
Spiperone	8.7	0.24
Ziprasidone	nd	nd

Table 3.1 Affinity values (pKi) derived from competition binding experiments using [^{3}H]Ketanserin, Data are means ± SEM, n=3. Amoxapine and ziprasidone could not be determined.

Antagonist order of affinity: Chlorpromazine > Mianserin > SB399,885 > MDL100,907 > Dihydroergotamine > Clozapine > Spiperone > SB742,457 > Melperone

3.3 Calcium mobilisation experiments

The serotonin 5-HT_{2A} receptor is an important and well known class A receptor which couples, at least in part to the Gq_{11} pathway, stimulating Phospholipase C. This results in a marked increase of inositol phosphate production and elevated calcium levels (Burgess et al.,1984,Roth et al.,1994). Therefore an appropriate means to investigate the pharmacology of this receptor is based on calcium mobilisation experiments. This was achieved by using a calcium sensitive dye (FURA 2AM) in these experiments to assess calcium mobilisation.

3.3.1 Concentration-response experiments

A series of concentration-response experiments was performed using a number of agonist and antagonist ligands. The presence in the cell medium of doxycycline turns on receptor expression. The antagonist ligands were added in varying concentrations alongside a single concentration of 5-HT (10^{-6}M), equating to the concentration of 5-HT which alone produced EC_{80} response.

Agonists cause elevation of [calcium] in cells expressing the 5-HT_{2A} receptor (Fig.3.6(a-c)). A rank order of potency could be observed for both agonist and antagonist ligands. EC_{50} and IC_{50} values from these studies are detailed in Fig.3.6 (a-c) and Fig.3.7 (a-l) and the corresponding tables 3.2 and 3.3.

DOI was the most potent agonist of the group followed by the endogenous ligand 5-HT, slightly less potent but still a full agonist. Quipazine appeared to be a partial agonist at the 5-HT_{2A} receptor, not able to generate maximal response values, and 5-benzyloxytryptamine was a weak but full agonist, with a lower EC_{50} than the endogenous ligand.

A rank order of potency was also determined for the antagonists, based upon their ability to reduce the response of the 5-HT stimulation from maximal to basal from (Fig.3.7(a-l) and table 3.3).

Fig.3.6a

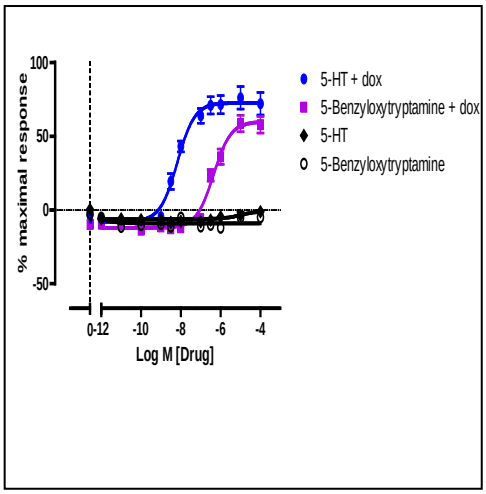


Fig.3.6b

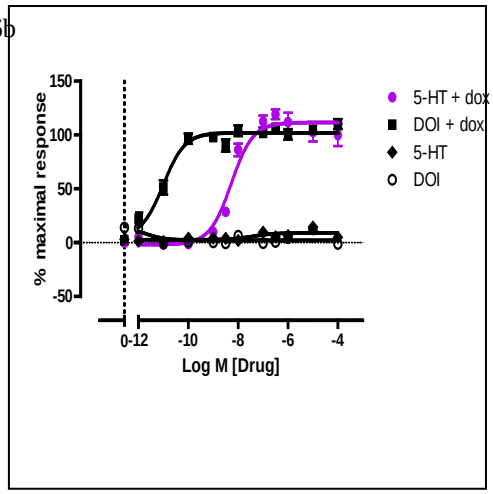


Fig.3.6c

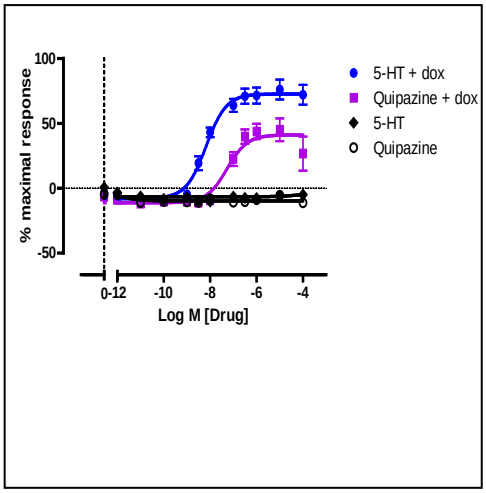


Fig.3.6(a-c). Calcium mobilisation experiments were performed using the ligands 5-benzyloxytryptamine (a), DOI (b) or quipazine (c). The endogenous ligand 5-HT in each case was employed as an internal reference. Data are means ± SEM, n=3.

Ligand	**pEC_{50}**	**Std. Error S.E.M**
5-HT	8.2	0.21
Quipazine	7.1	0.25
DOI	11.4	0.24
5 Benzyloxytryptamine	6.4	0.17

Table 3.2 Potency values for the agonist ligands in calcium mobilisation experiments. Data are means ± SEM, n=3.

Agonist order of potency: DOI > 5HT > quipazine > 5-benzyloxytryptamine

Fig.3.7a

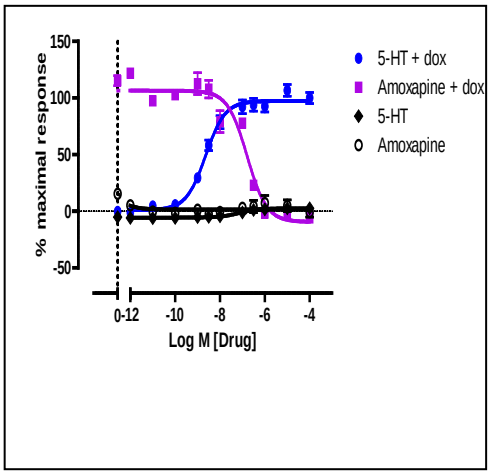


Fig.3.7b

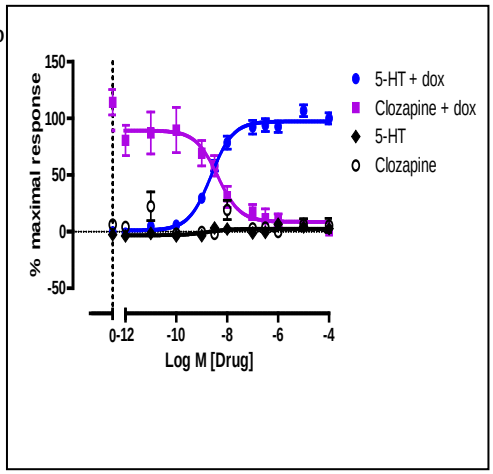


Fig.3.7c

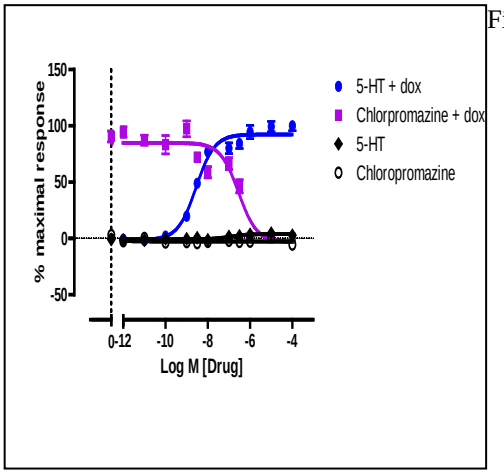


Fig.3.7d

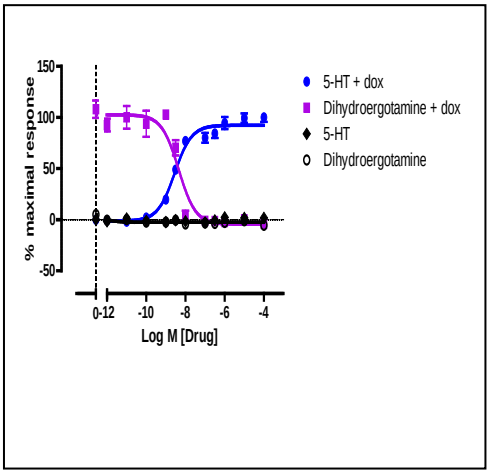


Fig.3.7e

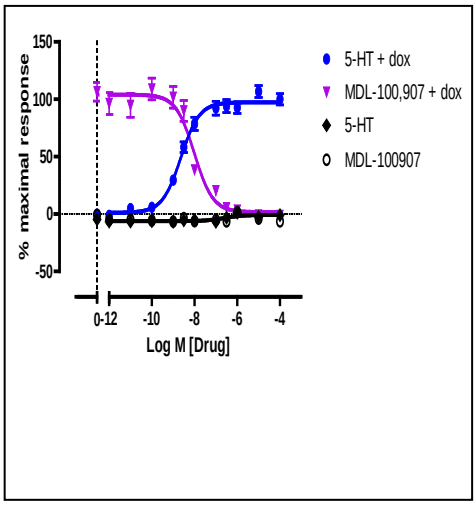


Fig.3.7f

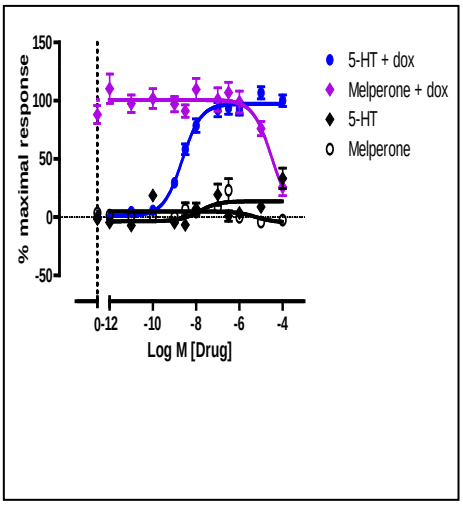


Fig.3.7g

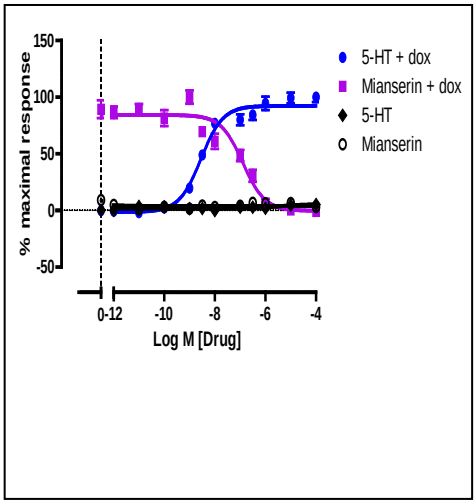


Fig.3.7h

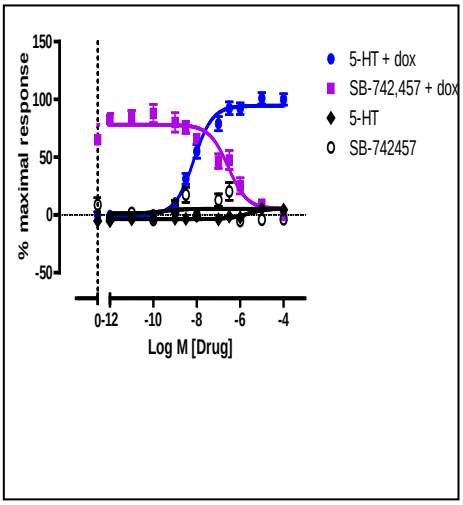


Fig.3.7i

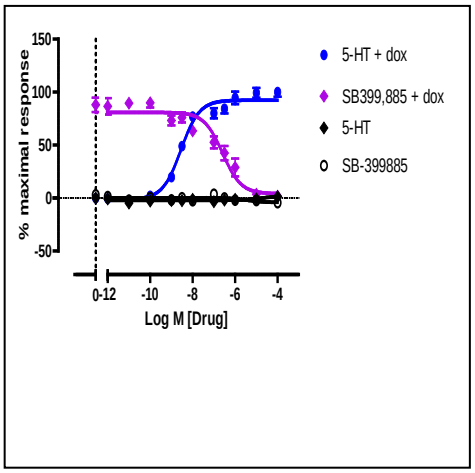


Fig.3.7j

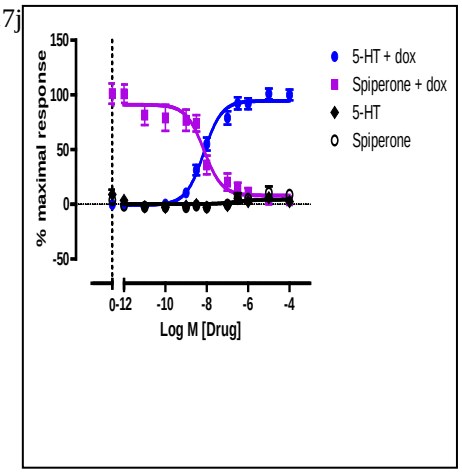


Fig.3.7k

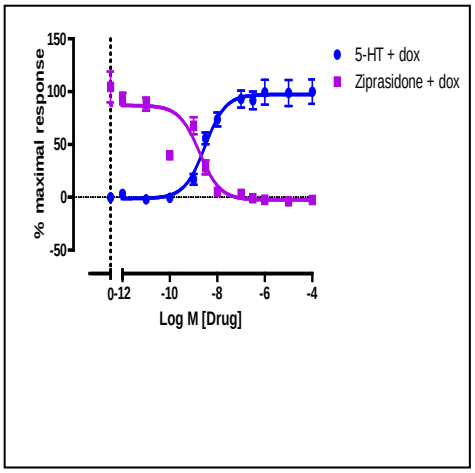


Fig.3.7l

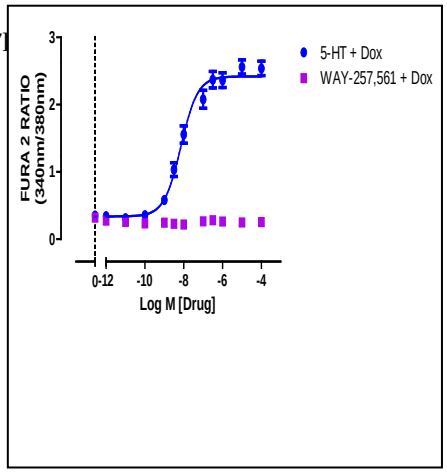


Fig.3.7 (a-l) Effect of a series of antagonist ligands on 5-HT(10^{-6}M) stimulated calcium mobilisation ; amoxapine (a), clozapine (b), chlorpromazine (c), dihydroergotamine (d), MDL100,907(e), melperone (f), mianserin (g), SB742,457 (h), SB399,885 (i), spiperone (j), ziprasidone (k) and WAY-257(l). The endogenous ligand 5-HT in each case was employed as an internal reference. Data means ± SEM, n=3.

Ligand	pIC_{50}	Std. Error S.E.M
Amoxapine	6.7	0.04
Chlorpromazine	6.5	0.14
Clozapine	8.6	0.14
Dihydroergotamine	7.7	0.72
Mianserin	7.0	0.14
MDL100,907	8.2	0.11
Melperone	4.5	0.06
SB-742,457	6.6	0.51
SB-399,885	6.7	0.47
Spiperone	8.9	0.63
Ziprasidone	9.5	0.25

Table.3.3 Corresponding pIC_{50} potency values of the antagonists in the Ca^{2+} mobilisation experiments from Fig.3.7(a-k). Data are means ± SEM, n=3.

Antagonist order of potency: Ziprasidone > Spiperone > Clozapine > MDL100,907 > Dihydroergotamine > Mianserin > Amoxapine > SB399,885 > SB742,457 > Chlorpromazine > Melperone

3.3.2 Calcium mobilisation: Schild regression experiments

The calcium mobilisation studies were used to generate Schild regression data for the antagonists. Surprisingly this generated data indicating that they were acting in an apparently non-competitive and insurmountable fashion (Fig.3.8). This however was considered unlikely based on various literature searches. In these studies cells were pre-incubated with an antagonist then signal measured immediately upon agonist addition. This may not allow for maximal response generation, possibly due to non-equilibria reflecting slow antagonist off rates. The prospect of hemi-equilibrium had to be explored.

'A traditional hallmark of competitive antagonism in functional assays is the ability of the antagonist to produce parallel dextral shifts of the agonist concentration response curve, with no change in the maximum agonist response' (Christopoulos et al.,1999). The Christopoulos group were exploring muscinarinic M1 acetylcholine receptor antagonists (atropine and pirenzepine), and found them to exhibit insurmountable antagonism of receptor-mediated Ca^{2+} mobilisation in CHO cells, in the presence of the agonists carbachol and xanomeline. Maximal response of the agonist was unobtainable, and rightward shifted in a concentration- dependent manner in relation to the antagonist. The underlying problem stemmed from the desired state of equilibrium not being achieved, due to the relative slower association-dissociation kinetics of the ligands.

Fig.3.8 is an example of initial experiments in which an agonist pre-incubation step was included, and clearly maximal response of agonist was not obtained in this situation. Hemi-equilibrium appears to occur when there is pre incubation with an antagonist, and once the agonist is applied the equilibrium shift is to slow to be detected before or during response measurement.

To potentially overcome this phenomenon the experiments were then adapted such that the antagonist and agonist ligands were co-administered (Fig 3.9 a-k).

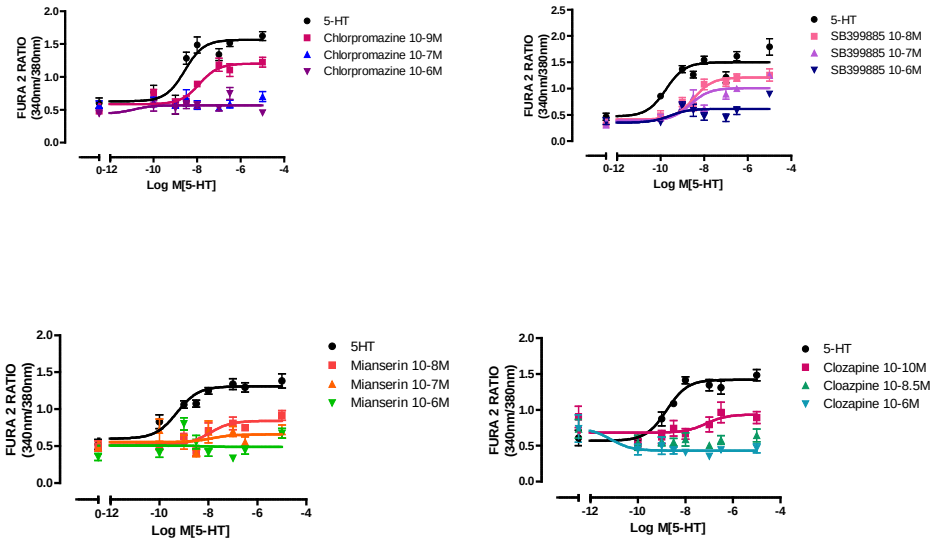
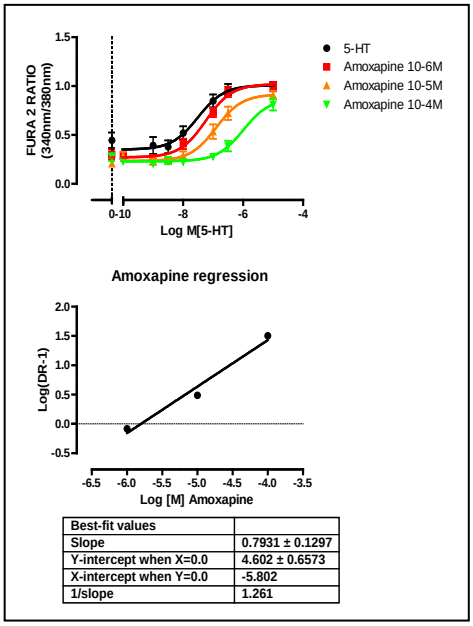


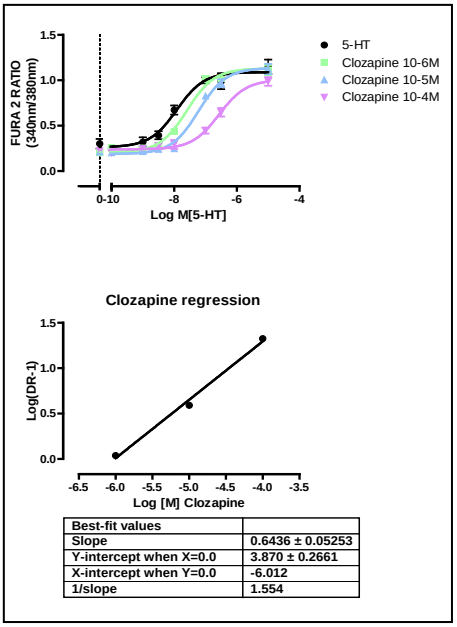
Fig.3.8 The ability of varying concentrations of 5-HT to elevate [Ca^{2+}] was assessed in Flp-In TREx 293 cells induced to express the 5-HT_{2A} receptor. Such experiments were performed in the presence of varying concentrations of different antagonists (a) chlorpromazine, (b) SB-399,885, (c) mianserin, (d) clozapine. Each curve should display a rightward shift, and maximal response of agonist should always be achieved even if at the highest concentration of antagonist if the antagonist is acting in a competitive manner.

Fig.3.9a



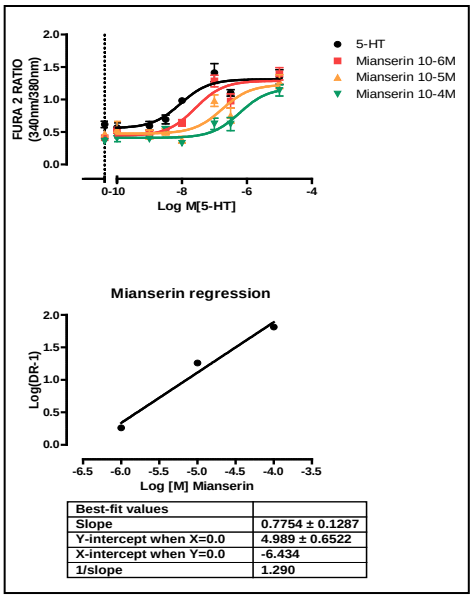
Best-fit values	
Slope	0.7931 ± 0.1297
Y-intercept when X=0.0	4.602 ± 0.6573
X-intercept when Y=0.0	-5.802
1/slope	1.261

Fig.3.9b



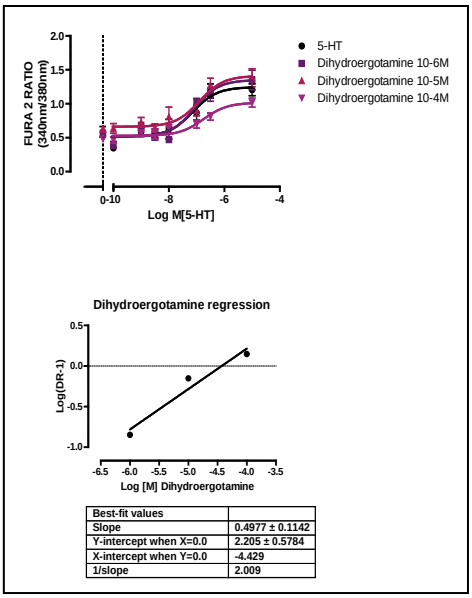
Best-fit values	
Slope	0.6436 ± 0.05253
Y-intercept when X=0.0	3.870 ± 0.2661
X-intercept when Y=0.0	-6.012
1/slope	1.554

Fig.3.9c



Best-fit values	
Slope	0.7754 ± 0.1287
Y-intercept when X=0.0	4.989 ± 0.6522
X-intercept when Y=0.0	-6.434
1/slope	1.290

Fig.3.9d

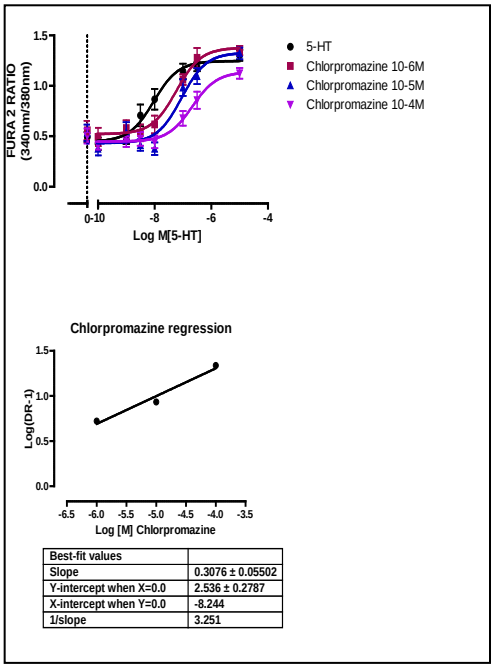


Best-fit values	
Slope	0.4977 ± 0.1142
Y-intercept when X=0.0	2.205 ± 0.5784
X-intercept when Y=0.0	-4.429
1/slope	2.009

Fig.3.9(a-d) The ability of varying concentrations of 5-HT to elevate [Ca^{2+}] was assessed in Flp-In TREx 293 cells induced to express the 5-HT_{2A} receptor. The effect of of increasing concentrations of (a) amoxapine, (b) clozapine, (c) mianserin and (d) dihydroergotamine on this is shown. Data are means ± SEM, n=3.

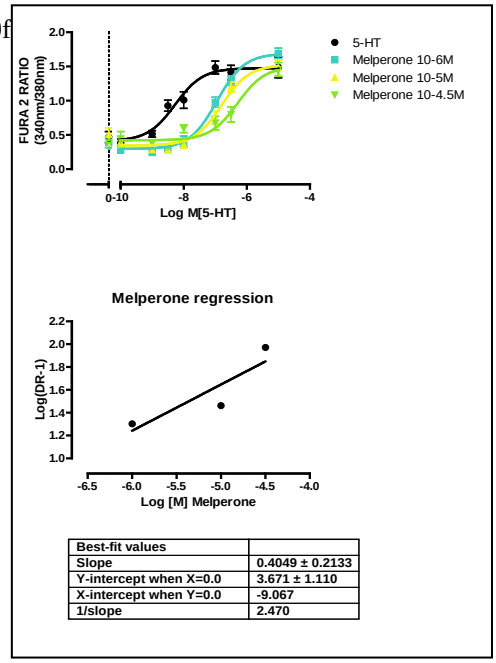
These experiments (Fig 3.9a-d) worked well, however the results to follow are all results i would class as being poor quality data.

Fig.3.9e



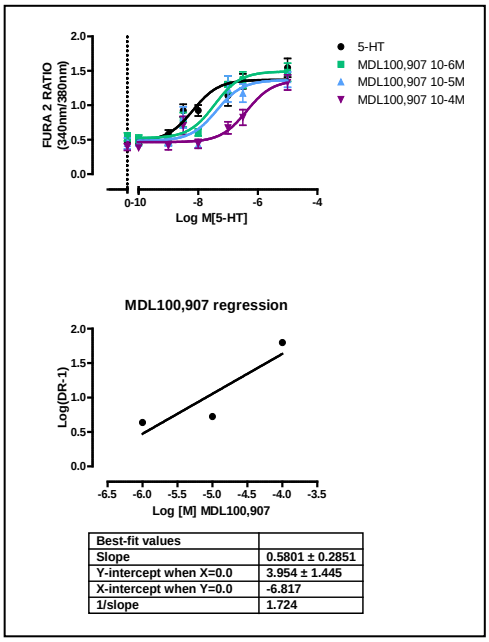
Best-fit values	
Slope	0.3076 ± 0.05502
Y-intercept when X=0.0	2.536 ± 0.2787
X-intercept when Y=0.0	-8.244
1/slope	3.251

Fig.3.9f



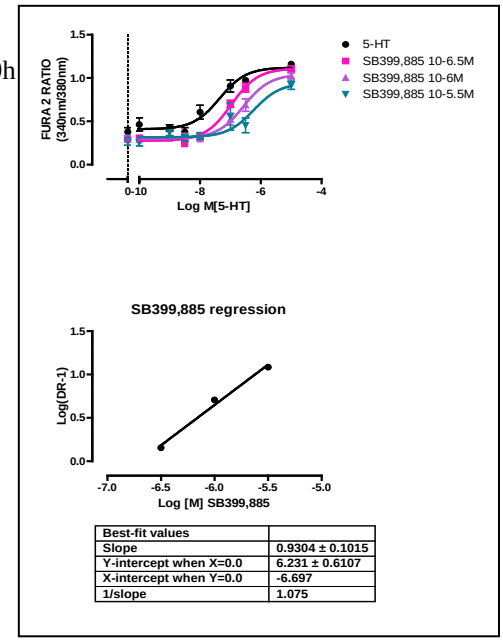
Best-fit values	
Slope	0.4049 ± 0.2133
Y-intercept when X=0.0	3.671 ± 1.110
X-intercept when Y=0.0	-9.067
1/slope	2.470

Fig.3.9g



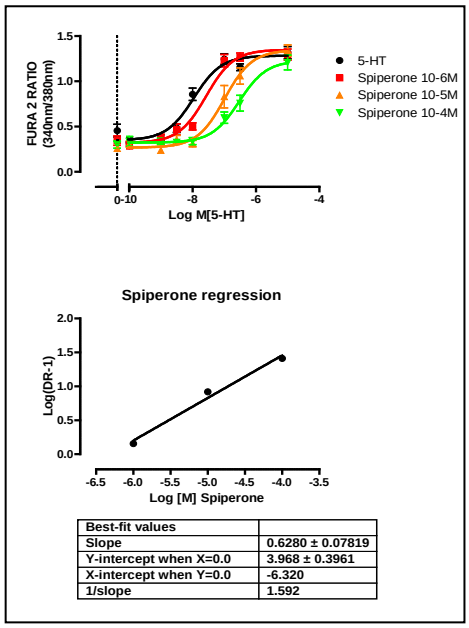
Best-fit values	
Slope	0.5801 ± 0.2851
Y-intercept when X=0.0	3.954 ± 1.445
X-intercept when Y=0.0	-6.817
1/slope	1.724

Fig.3.9h



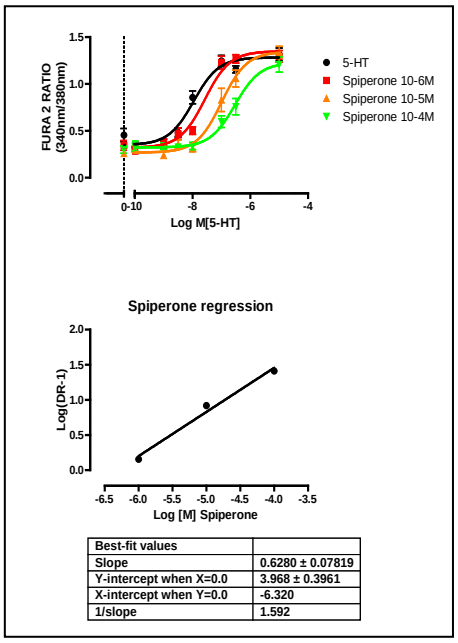
Best-fit values	
Slope	0.9304 ± 0.1015
Y-intercept when X=0.0	6.231 ± 0.6107
X-intercept when Y=0.0	-6.697
1/slope	1.075

Fig.3.9i



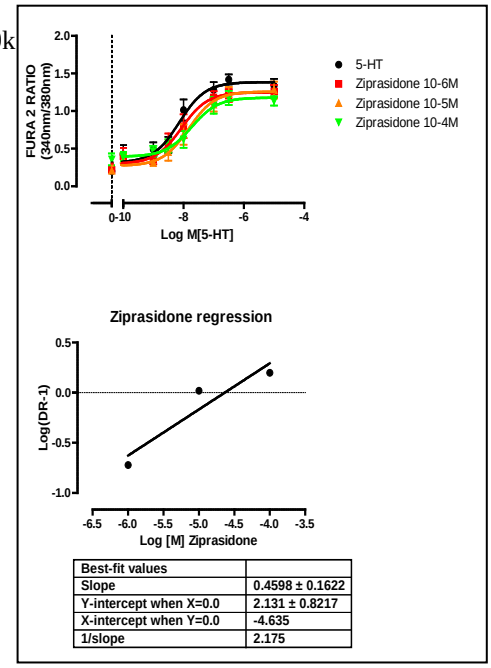
Best-fit values	
Slope	0.6280 ± 0.07819
Y-intercept when X=0.0	3.968 ± 0.3961
X-intercept when Y=0.0	-6.320
1/slope	1.592

Fig.3.9j



Best-fit values	
Slope	0.6280 ± 0.07819
Y-intercept when X=0.0	3.968 ± 0.3961
X-intercept when Y=0.0	-6.320
1/slope	1.592

Fig.3.9k



Best-fit values	
Slope	0.4598 ± 0.1622
Y-intercept when X=0.0	2.131 ± 0.8217
X-intercept when Y=0.0	-4.635
1/slope	2.175

Fig 3.9(a-k) Agonist and antagonist ligands co-administered. The ability of varying concentrations of 5-HT to elevate [Ca^{2+}] was assessed in Flp-In TREx 293 cells induced to express the 5-HT_{2A} receptor. Such experiments were performed in the presence of varying concentrations of different antagonists; chlorpromazine (e), melperone (f), MDL100,907(g), SB399,885 (h), SB742,457 (i), spiperone (j) and ziprasidone (k). Data are means ± SEM, n=3.

These experiments allowed for the generation of pA_2 values, which in a functional experiment is a measure of affinity (table3.4) and a rank order of affinity derived.

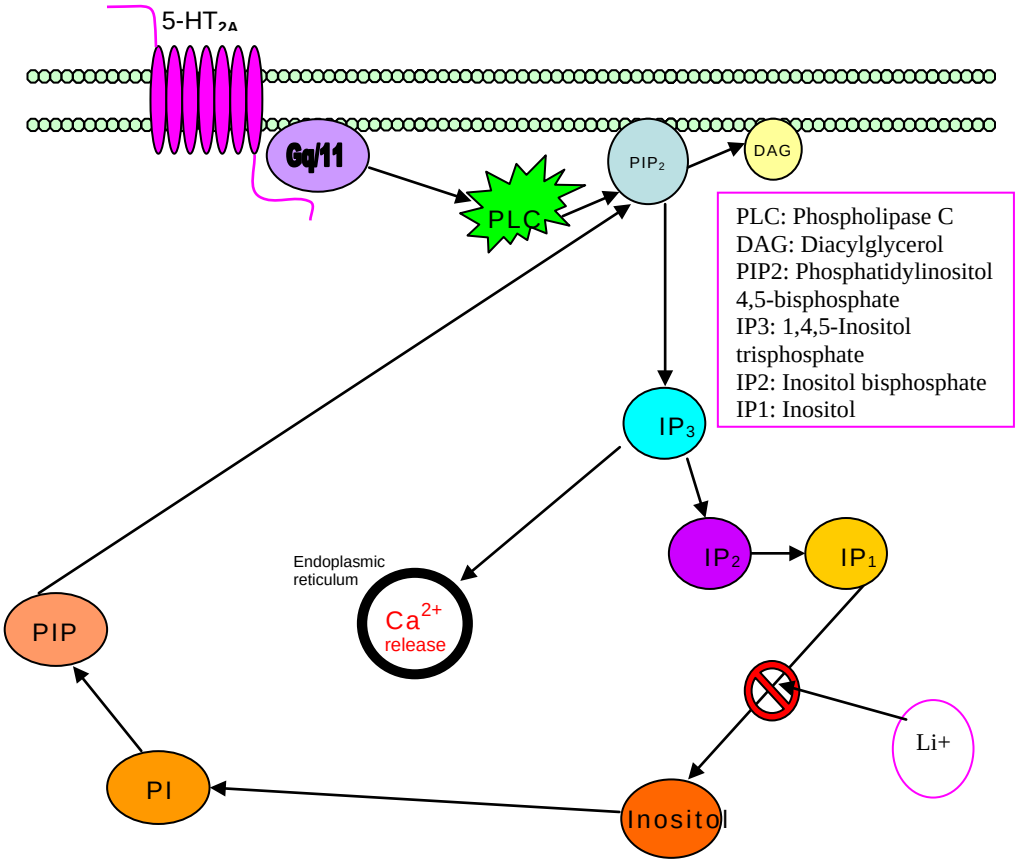
Ligand	**pA_2**	**Std. Error S.E.M**
Amoxapine	**5.7 (slope: 0.79)**	**0.23 (0.13)**
Chlorpromazine	5.8 (slope:0.31)	0.38 (0.05)
Clozapine	**5.6 (slope: 0.64)**	**0.96 (0.05)**
Dihydroergotamine	6.1 (slope: 0.50)	0.92 (0.11)
Mianserin	**6.4 (slope: 0.77)**	**0.30 (0.13)**
MDL100,907	5.8 (slope:0.51)	0.38 (0.28)
Melperone	7.1 (slope:0.41)	0.69 (0.21)
SB-742,457	8.8 (slope:0.53)	0.58 (0.10)
SB-399,885	**-6.4 (slope: 0.93)**	**0.23 (0.10)**
Spiperone	6.3 (slope: 0.63)	0.36 (0.08)
Ziprasidone	5.0 (slope: 0.46)	1.07 (0.16)

Table 3.4 Affinity values derived from Schild plots for antagonist ligands, measured via Ca^{2+} mobilisation. The highlighted ligands are the ligands which work well (Fig 3.9a-d). Slope should be equal to or as close to 1 as possible to indicate competitive antagonism. These experiments allowed for the generation of $_pA_2$ values which in a functional experiment is a measure of affinity Data are means ± SEM, n=3.

Antagonist order of affinity: Mianserin > SB399,885 > Amoxapine > Clozapine

3.4 IP1 accumulation assay

IP1 accumulation measured using HTRF technology (homogeneous time resolved fluorescence), encompassing fluorophores conjugated to antibodies. The assay kit contains an antibody specific to IP-1 which is labelled with Cryptate (linked to the rare earth element Terbium) and it is the emission of this which is measured. This antibody competes with native IP1 produced by cells and IP1 coupled to the dye d2. An essential component of this particular assay is the inclusion of Li^{+} in the stimulation buffer, which allows the IP1 being produced to accumulate rather than breaking down into myo Inositol. The effect of lithium interference in the breakdown of IP1 by inhibiting the enzyme inositol monophosphatase was explored in great detail by Berridge (Berridge et al.,1984).



Time resolved FRET (Tr-FRET) overcomes several drawbacks of Resonance Energy Transfer techniques, such as low signal to noise ratios, where the signal you observe may not be much above the interference background noise signal (possibly due to autoflourescence of cells) (Maurel et al.,2008). The combination of europium cryptate as the donor and d2 as the acceptor fluorophore increases the signal to noise ratio due to the extension of emission from the cryptate, increasing the stability of the assay, allowing for all other signals to be reduced down before readings are taken.

A brief time course experiment was set up to look at 1P-1 accumulation in relation to constitutive activity which has been previously shown to occur at the 5-HT_{2A} receptor. Should constitutive activity be present then there would be significant activity in the cells minus agonist. However from Figure 3.10 you can see that this was not to be the case.

Agonist concentration response curves set up in triplicate, included a 5-HT control in order to observe the effect of the agonists in comparison to the endogenous ligand (Fig 3.11 a-c). The presence of doxycycline turns on the receptor expression.

Antagonist curves were set up in the presence of a control curve of 5-HT to allow maximal and basal values to be determined, and then separate curves of antagonist alone and the antagonist ligand in the presence of EC_{80} concentration of 5-HT, which allows the ligands antagonistic or inverse agonism properties to be seen (Fig 3.12a-k) and inhibition values gathered.

Several details could be extrapolated from these experiments, including both the potency of ligands and affinity results (see tables 3.6 and 3.7) which are comparable with the calcium mobilisation results (tables 3.3 and 3.4).These pathways lie in close proximity to one another downstream of receptor activation and should in effect provide similar values. Interestingly the results obtained for potency differed from that of the calcium mobilisation experiments (Fig 3.7a-l).

A correction was then applied based on the IC_{50} and Hill slope values of these competition curves in order to produce an approximation of $_pK_B$ (table 3.7), see equation 2. $_pK_B$ is a functional measure of affinity, differing from $_pA_2$ values obtained from Schild and Ki values from binding studies (tables 3.4 and 3.1)

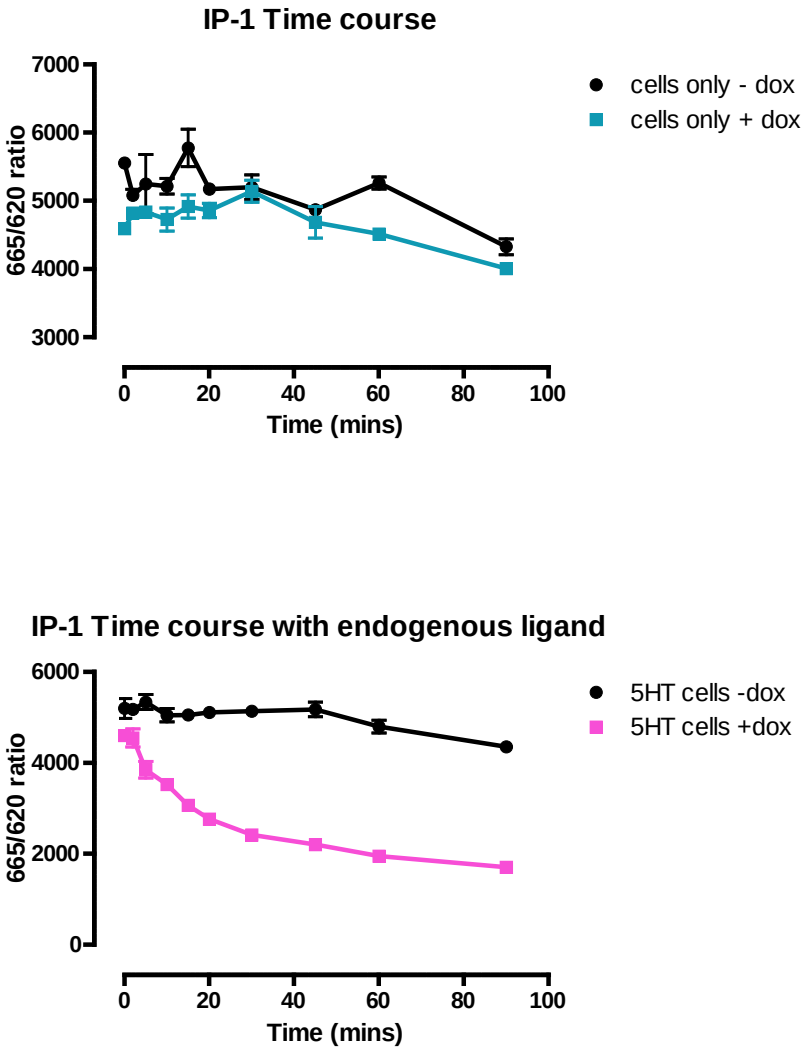


Fig.3.10 Time course experiments exploring the possibility of constitutive activity. Cells either untreated or induced with doxycycline to turn on 5-HT_{2A} receptor expression for comparison.

Fig.3.11a

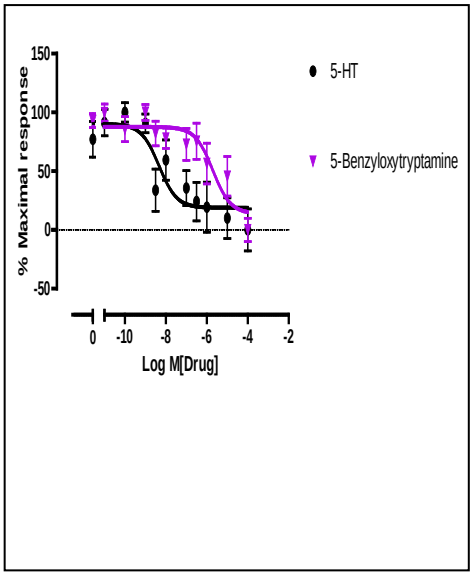


Fig.3.11b

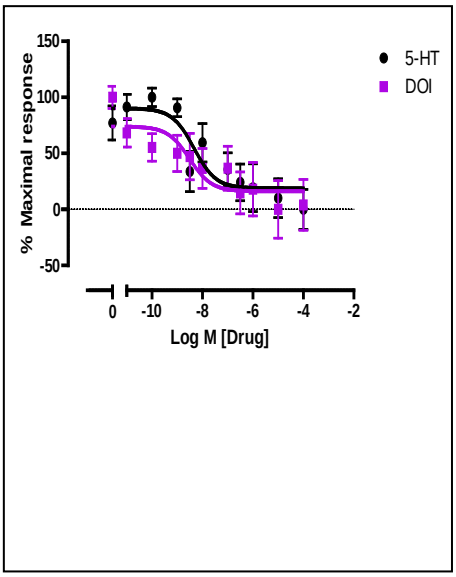


Fig.3.11c

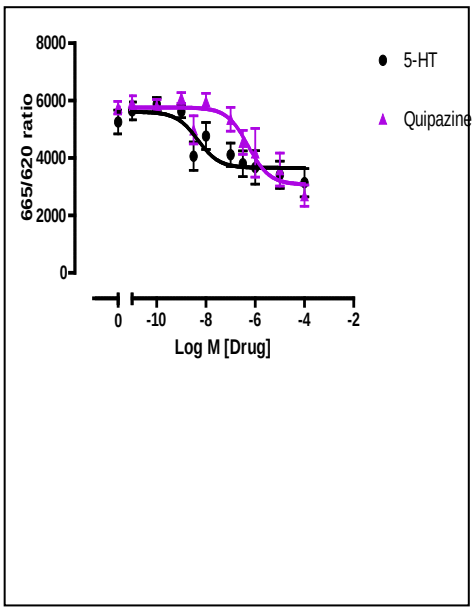


Fig 3.11 (a-c). Agonist IP-one accumulation experiments measured in Flp-In TREx 293 cells induced to express the 5-HT_{2A} receptor and using the ligands 5-benzyloxytryptamine (a), DOI (b) or quipazine (c). The endogenous ligand 5-HT in each case was employed as an internal reference. Data are means ± SEM, n=3.

Ligand	Potency (pEC50)	Std. Error S.E.M
5-Hydroxytryptamine	8.6	0.30
Quipazine	6.1	0.85
DOI	10.0	0.36
5 Benzyloxytryptamine	5.4	0.13

Table 3.5 Potency values for the agonist ligands in IP-one accumulation experiments (Fig.14a-c), Data are means ± SEM, n=3.

Agonist order of potency: DOI > 5HT > Quipazine > 5-Benzyloxytryptamine

Fig.3.12 a

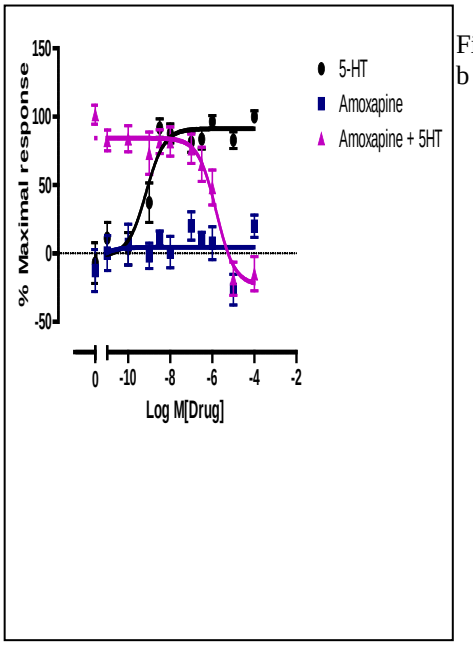


Fig.3.12 b

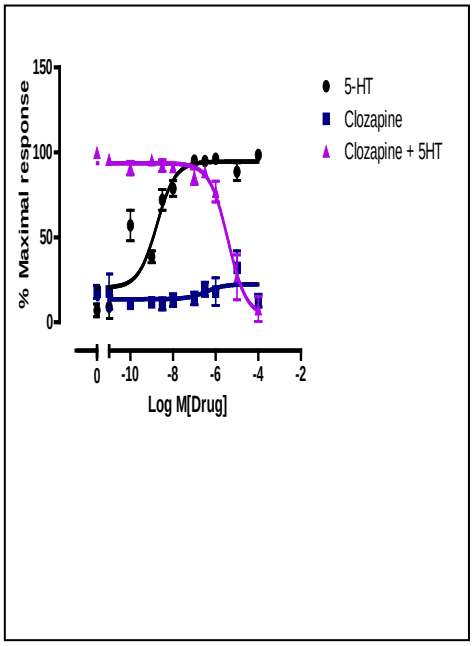


Fig.3.12 c

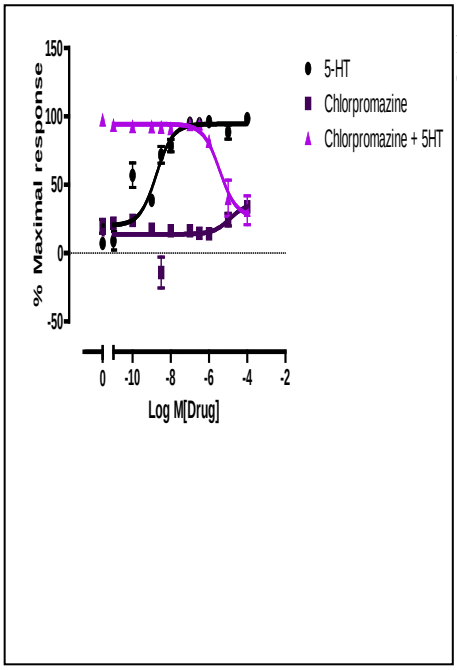


Fig.3.12 d

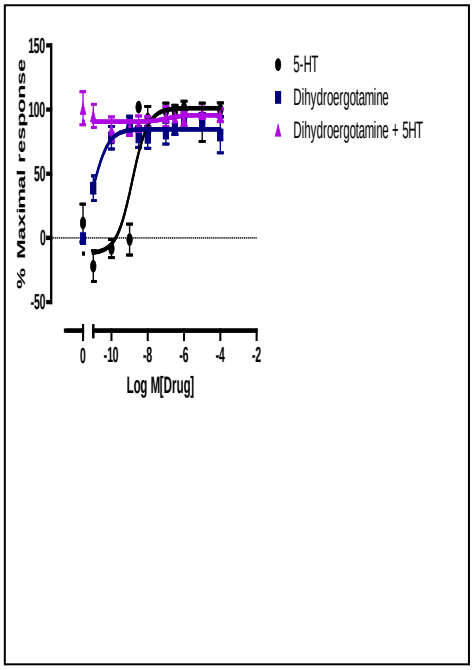


Fig.3.12e

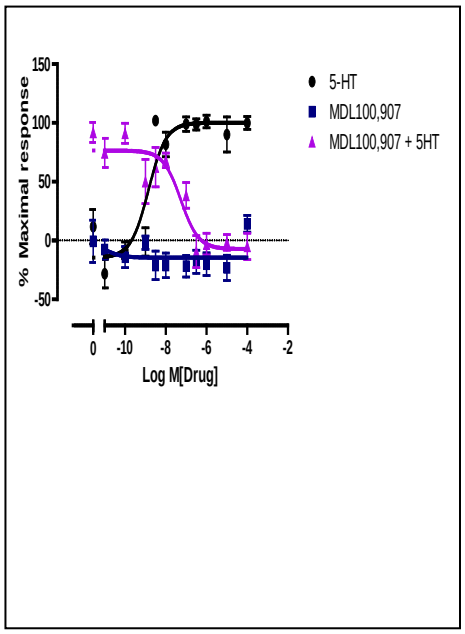


Fig.3.12f

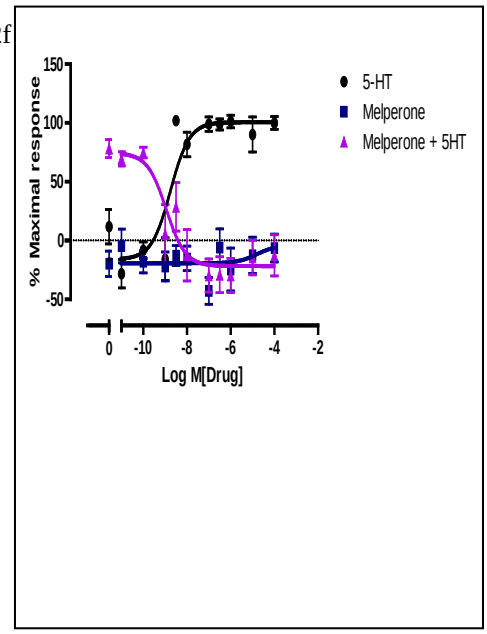


Fig.3.12g

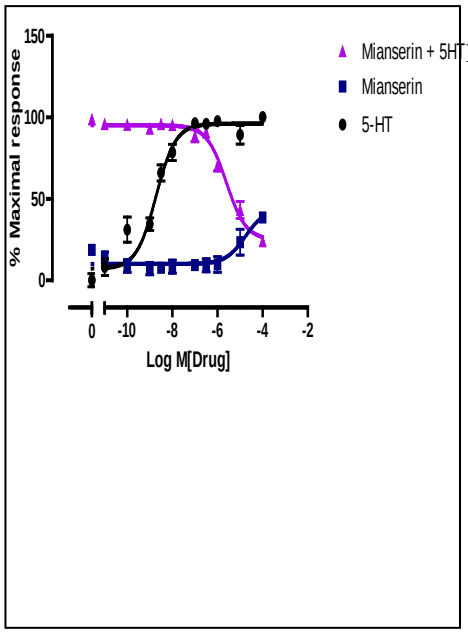


Fig.3.12h

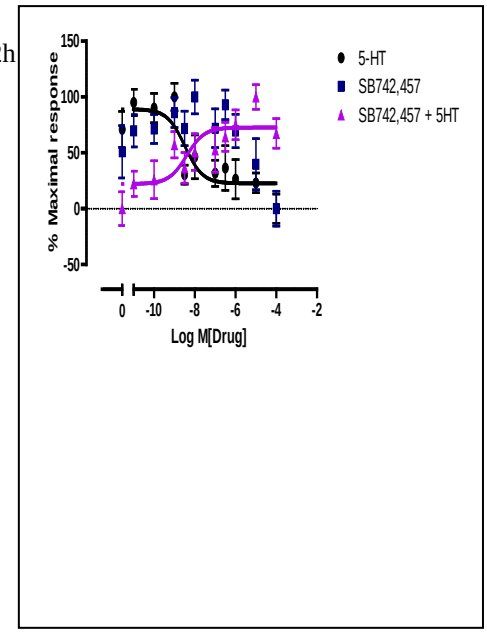


Fig.3.12i

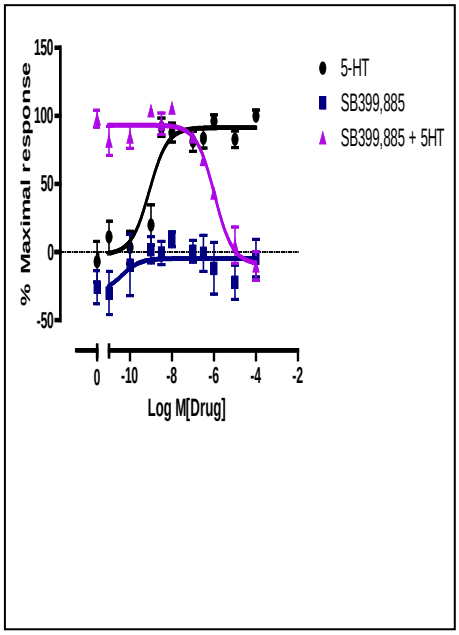


Fig.3.12j

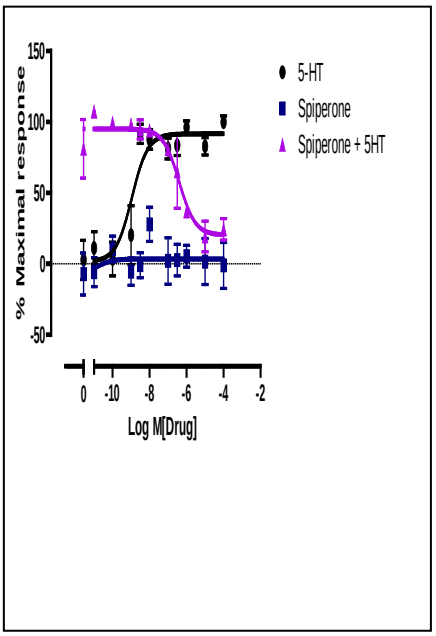


Fig.3.12k

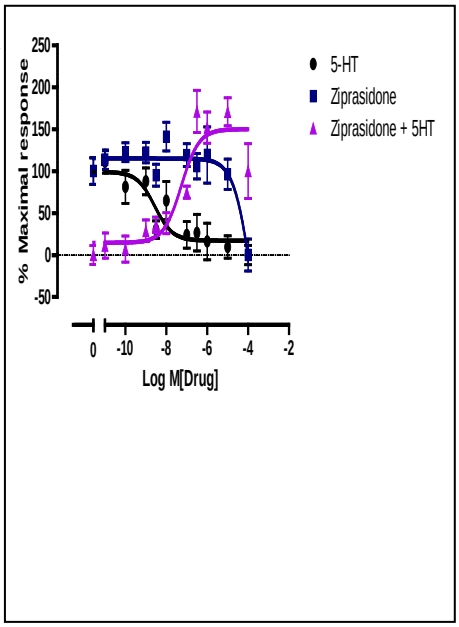


Fig 3.12 (a-k). Effect of a series of antagonist ligands on 5-HT(10^{-6}M) stimulated IP-one accumulation; amoxapine (a), clozapine (b), chlorpromazine (c), dihydroergotamine (d), MDL100,907(e), melperone (f), mianserin (g), SB742,457(h), SB399,885(i), spiperone (j) and ziprasidone (k). The endogenous ligand 5-HT in each case was employed as an internal reference . Data are means ± SEM, ,n=3.

*Several compounds are known to be “inverse agonists” (clozapine, chlorpromazine and mianserin) however based on the lack of constitutive activity in this assay shown in Fig.3.10 looking at IP-one accumulation all of these compounds appear to only act as antagonists.

Ligand	Affinity (pIC50)	Std. Error S.E.M
Amoxapine	5.9	0.06
Chlorpromazine	5.3	0.19
Clozapine	5.5	0.30
Dihydroergotamine	nd	nd
Mianserin	5.6	0.14
MDL100,907	8.1	0.69
Melperone	5.2	0.29
SB-742,457	nd	nd
SB-399,885	6.3	0.16
Spiperone	6.1	0.37
Ziprasidone	nd	nd

Table 3.6 Potency values for the antagonist ligands in IP-one accumulation experiments (Fig.15a-k), Data are means ± SEM, n=3. Dihydroergotamine, SB-742,457 and Ziprasidone could not be determined.

Antagonist order of potency: MDL100,907 > SB399,885 > Spiperone > Amoxapine > Mianserin > Clozapine > Chlorpromazine > Melperone

Ligand	Affinity (pKB)	Std. Error S.E.M
Amoxapine	8.4	0.06
Chlorpromazine	8.4	0.19
Clozapine	9.2	0.30
Dihydroergotamine	nd	nd
Mianserin	9.4	0.13
MDL100,907	11.2	0.69
Melperone	8.3	0.06
SB-742,457	nd	nd
SB-399,885	9.1	0.16
Spiperone	9.8	0.37
Ziprasidone	nd	nd

Table 3.7 Afinity values for the antagonist ligands in IP-one accumulation experiments corresponding to Fig 3.12 (a-k), after correction factor (equation 2) applied , Data are means ± SEM, n=3. Dihydroergotamine, SB-742,457 and Ziprasidone could not be determined.

Antagonist order of affinity: MDL100,907 > Spiperone > Mianserin > Clozapine > SB399,885 > Chlorpromazine > Amoxapine > Melperone

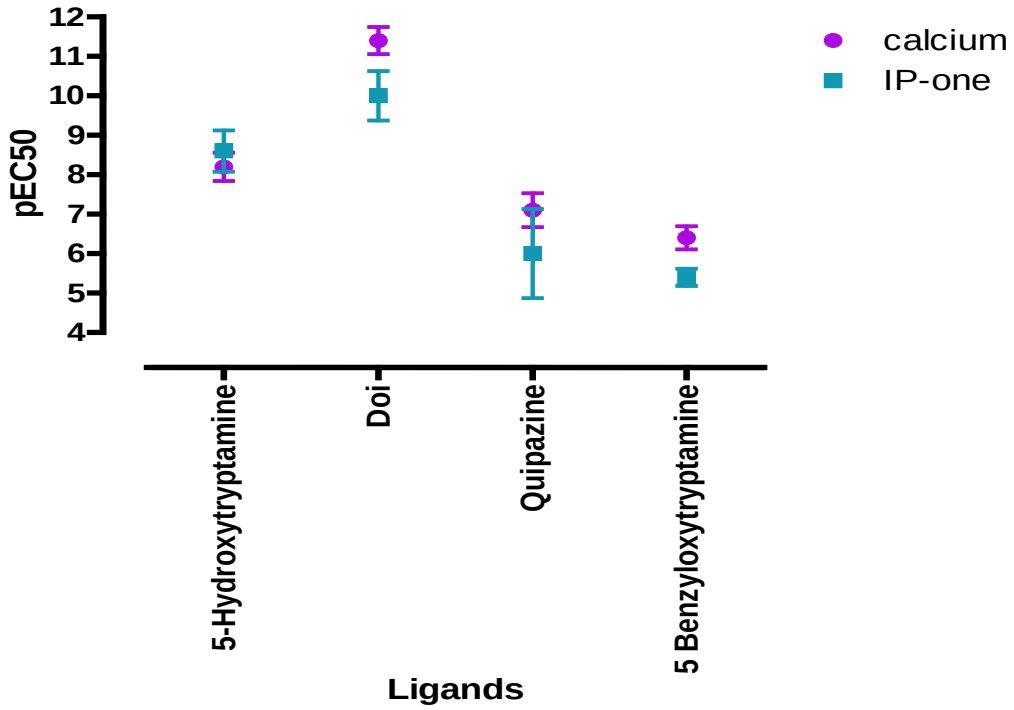


Fig 3.13 Comparison chart of potency of agonist ligands 5-HT, DOI, quipazine and 5-benzyloxtryptamine at Ca^{2+} mobilisation and IP-one accumulation assays assessed using Flp-In TREx 293 cells induced to express the $5\text{-}HT_{2A}$ receptor.

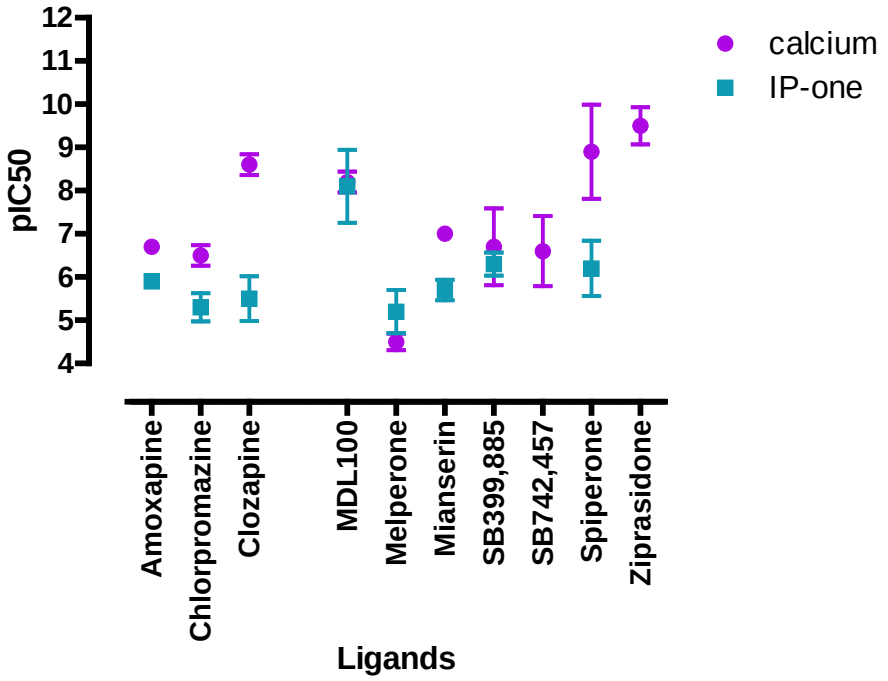


Fig 3.14 Comparison chart of potency of antagonist ligands amoxapine, clozapine, chlorpromazine , MDL100,907, melperone, mianserin, SB742,457, SB399,885, spiperone and ziprasidone at Ca^{2+} mobilisation and IP-one accumulation assays assessd using Flp-In TREx 293 cells induced to express the 5-HT_{2A} receptor

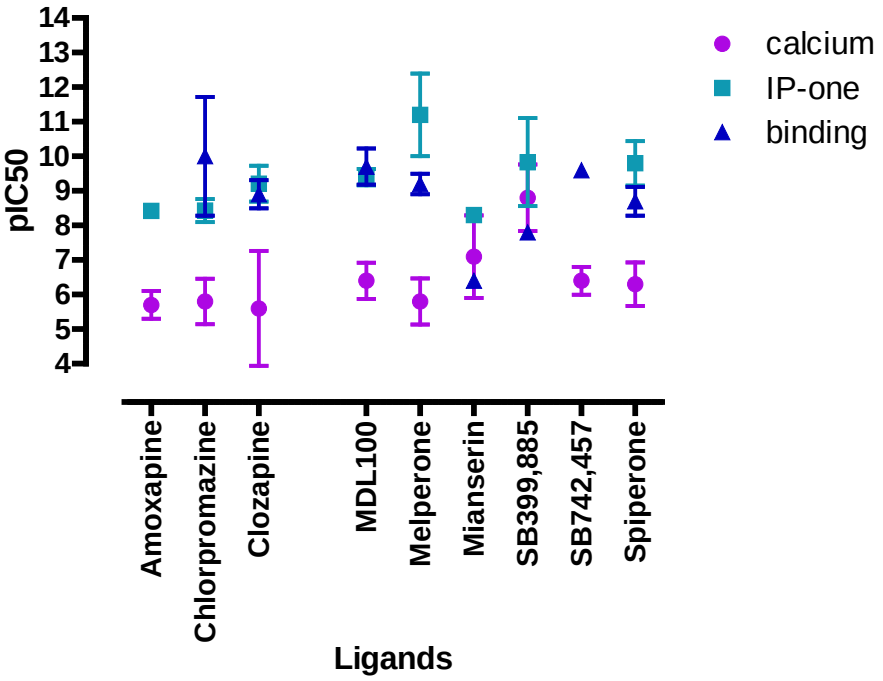


Fig 3.15 Comparison chart of affinity of antagonist ligands amoxapine, clozapine, chlorpromazine, MDL100,907, melperone, mianserin, SB742,457, SB399,885, spiperone and ziprasidone in radioligand binding, Ca^{2+} mobilisation and IP-one accumulation assays assessed in Flp-In TREx 293 cells induced to express the 5-HT_{2A} receptor.

Rank order of potency for agonists and antagonists

Rank Order of potency	Calcium experiments	IP-One experiments
1	DOI	DOI
2	5-HT	5-HT
3	Quipazine	Quipazine
4	5-Benzyloxytryptamine	5-Benzyloxytryptamine

Table 3.8 Ranks the order of potency of each agonist ligand in both Ca^{2+} mobilisation and IP-one accumulation assays

Rank Order of potency	Calcium experiments	IP-One experiments
1	Ziprasidone	MDL100,907
2	Spiperone	SB-742,457
3	Clozapine	Ziprasidone
4	MDL100,907	SB-399,885
5	Dihydroergotamine	Spiperone
6	Mianserin	Amoxapine
7	Amoxapine	Mianserin
8	SB-399,885	Clozapine
9	SB-742,457	Chlorpromazine
10	Chlorpromazine	Melperone
11	Melperone	

Table 3.9 Ranks the order of potency of each antagonist ligand in both Ca^{2+} mobilisation and IP-one accumulation assays.

Rank order of affinity for agonists and antagonists

Rank Order of Affinity	**Binding (Ki)**	**Schild $_pA_2$**	**IP-one $_pK_B$**
1	Chlorpromazine	Mianserin	MDL100,907
2	Mianserin	SB-399,885	Spiperone
3	SB-399,885	Amoxapine	Mianserin
4	MDL100,907	Clozapine	Clozapine
5	Dihydroergotamine		SB-399,885
6	Clozapine		Chlorpromazine
7	Spiperone		Amoxapine
8	SB-742,457		Melperone
9	Melperone		

Table 3.10 Ranks the order of affinity of each antagonist ligand in radioligand binding, Ca^{2+} mobilisation and IP-one accumulation assays

IUPHAR published values, taken from the IUPHAR website

Agonist Ligand	IUPHAR Potency values
5-Hydroxytryptamine	-6 .0 - -8.4
Quipazine	-6.9
DOI	-9.0 - -9.3
5 Benzyloxytryptamine	Data not available

Table 3.11 Published values taken from the IUPHAR website, detailing the potency of the agonist ligands at the 5-HT_{2A} receptor .

Antagonist Ligand	IUPHAR Affinity values
Amoxapine	-9.0
Chlorpromazine	-8.7
Clozapine	-7.6 - -9.0
Mianserin	-7.4 - -9.6
MDL100,907	-6.5 - -9.3
Melperone	-7.1
Spiperone	-7.8 - -9.4
Ziprasidone	-8.8 - -9.5

Table 3.12 Published values taken from the IUPHAR website, detailing previously reported affinity of the antagonist ligands at the 5-HT_{2A} receptor. Dihydroergotamine, SB399,885 and SB742,457 have not been listed as having any effect at the 5-HT_{2A} receptor.

Equations

1)Cheng-Prusoff correction $IC_{50} = K_B\ ([A^*]/K_D + 1)$

2) ${}_PK_B$ correction factor : Anti log ${}_PK_B = -IC_{50}/((2+([A]/EC50)^n)^{1/n} - 1)$

4. Discussion

The spectrum of human diseases treated by GPCR drug targets is extremely broad, ranging from pain and asthma to cognitive dysfunction and cardiovascular disease. Receptors have two primary functions 1) the recognition and binding of ligands 2) the generation of downstream responses. There are more than 200 known receptors transducing a physiological process and are the targets of around 50% of the drugs now commercially available, with at least another 150 orphan GPCRs expected to exist from the human genome (Wise et al 2004, Katugampola & Davenport.,2003). From the 35000-60000 human genes that have been identified from private and public databases roughly 1000 encode GPCRs (Katugampola & Davenport 2003). Drugs with action at 5-HT_{2A} receptors are used in the treatment of many disorders. The identification of an activating ligand occurs by initiation of an intracellular cascade ie: IP1 or Ca^{2+} .

Several compounds used (clozapine, mianserin and chlorpromazine) were listed as inverse agonists at the 5-HT_{2A} receptor but showed no such behaviour in functional studies such as IP-one (Figures 3.10 and 3.12). 5-HT_{2A} constitutive activity in vitro is weak without either mutating the receptor (Teitler et al.,2002) or by over expressing G proteins (Weiner et al.,2001). However constitutive activity at the 5-HT_{2A} receptor has been shown to have substantial effects in vivo (Welsh et al.,1998),Harvey et al.,1999,). To be sure constitutive activity did not occur over a period of time, a time course experiment was also carried out for the 5-HT_{2A} receptor and IP-one accumulation measured (Fig.3.10).

As discussed previously the 5-HT_{2A} receptor was incorporated into a stable, inducible cell line using HEK 293 cells and the Flp-in T-REx system, allowing receptor expression to be under the control of the antibiotic doxycycline. It could be seen clearly that expression of the receptor was nice, and clear only when dox was applied (Fig.3.1), no appropriate band could be observed in wells in which the cell preparations had no prior application of

doxycycline. Cell age did not have a major impact either on the expression of the receptor, and did not cause a decline as the cells progressed in age. The binding experiments also confirmed that expression was good, and inducible only if doxycycline was present (Fig.3.4b).

Agonist potency

There is a variation when looking at the potency of agonists in relation to calcium mobilisation and IP-one accumulation (tables 3.2 and 3.5)(Fig.3.13). The endogenous ligand would appear to be close in potency values for each, with EC_{50} values of -8.2M and -8.6M respectively, however the other agonist values are 1 log value less potent in IP-one experiments. As the pathways occur sequentially, both downstream of IP3, it is not possible to suggest agonist directed trafficking as a reason for this (Berg et al.,1998), a phenomenon by which agonist binds to receptor and promotes agonist specific receptor conformation, stimulating pathways preferentially. The pathways would be completely separate entities, existing in their own right independent of one another such as arachidonic acid and inositol phosphate (Berg et al.,1998), unlike IP-one and Ca^{2+}. The agonists follow the same order of potency for each of the experiments (table 3.8).

Antagonist potency

Several antagonistic compounds, when looking at the comparison chart (Fig.3.14) have EC50 values which lie in close proximity to one another. These compounds include amoxapine, chlorpromazine, MDL100,907, melperone, mianserin, SB399,885 and SB742,457. Clear differences can be seen for clozapine, spiperone and ziprasidone, with more than a 2 degree difference in potency (tables 3.3 and 3.6).

The order of potency for the majority of ligands is very different when looking at IP1 and Ca^{2+} experiments (table 3.9).

The SB compounds are not regarded as being an antagonist at the 5-HT_{2A} receptor in any literature, but they display a very clear antagonistic tendency at this receptor, with moderate potency and affinity values (tables 3.1, 3.3 and 3.6).

Antagonist affinity

Binding experiment values (table 3.1) and IP1 $_{P}K_{B}$ values (table 3.7) are more closely related to the published IUPHAR affinity values (table 3.12) and each other for several ligands including clozapine, mianserin, spiperone and ziprasidone (Fig.3.15) These ligands lie in close proximity to each other in the comparison chart, as do the SB compounds. There are a few obvious discrepancies including amoxapine and melperone.

The $_{P}A_{2}$ values generated for calcium Schild experiments (table 3.4) do not sit closely to the IUPHAR values (table 11), IP1 $_{P}K_{B}$ values (table 3.7) or the binding ki values (table 3.1) (fig.3.15), for all except 2 antagonists, mianserin and SB399,885. Many of the graphs display extremely badly fitting regression lines, with unacceptable or unexplainable negative values, no slopes being close to 1.0 to indicate competitive antagonism. In the case of ziprasidone there is no shift in curve, indicating that this acts very weakly at the 5-HT_{2A} receptor. The Ki values of the binding experiments follows a similar order to that of the published literature (IUPHAR website), for chlorpromazine, mainserin, MDL100,907 and spiperone. The exceptions here are clozapine and ziprasidone, and non determinable amoxpaine.

For both the Ca^{2+} and IP1 experiments there is a lot of variation between the two with regards to rank order of affinity (table 3.10). Discounting the additional ligands SSB399,885, SB742457 and dihydroergotamine , the ligands are binding with different affinities at each pathway and also differing from the Ki values. A reason for this could be the formation of complexes with other proteins, allowing downstream complexes to have their own pharmacology (Baker & Hill.,2007) Scaffolding of GPCRs, G proteins, effectors and down stream elements are all thought to play a pivotal role in downstream signalling, with intracellular domains interacting with cellular proteins involved in each signalling cascade independent of the g protein (Brady and Limbird, 2001).

A clear cause for thought at this point would be the use of calcium assays in pharmacological measurements and comparisons.

Another aspect to look at had there been more time, would have been receptor reserve. As these pathways both have the same starting point, branching off upon IP3 breaking down it would be interesting to know what occurs between this point and the formation of each biological product.

Antagonists at 5-HT_{2A} not only inhibit agonist induced and constitutive activities but could be responsible for the deactivation of GPCR responses in a pathway specific manner (Brea et al.,2009). Antagonist induced receptor internalisation can result in the activation of extracellular signal-regulated kinase mitogen activated protein kinase occurring via β-arrestin signalling. β arrestins act as scaffolding proteins and can activate a subset of signal networks (ERK1/2) independent of G protein signalling (Wisler et al.,2007).

5-HT_{2A} receptor antagonists can induce internalisation of the receptor (Willins et al.,1999, Gray and Roth 2001). It is possible that this then stimulates the ERK1/2 pathways, and time permitting could have been a way to look at receptor reserve.

The validity of using calcium assays as a measure of potency and affinity

Another consideration is the assay type itself. Particularly with regards to the calcium assay where there is reliance on a light signal which is not stable for a prolonged period of time. Fura-2AM being a ratiometric dye, has a less bright emittance than other available single length indicators. Typical assay set up involves moving from low to high concentrations, but due to the nature of the plate reader on the flex station, where upon it reads a well at a time in order from row 1 to row 12, the entire plate read out takes 45minutes, perhaps having an impact on the readout. Particularly with the Schild experiments this seemed to be problematic as the larger concentrations of antagonist was applied to the receptor for over 1 hour when the pre incubation step was included initially, upsetting the off rate of antagonist and the agonists ability to compete and knock off the antagonist.

Calcium is released from the endoplasmic reticulum upon stimulation of the inositol 1,4,5-trisphosphate (InsP3) receptor by IP3 (Foskett.J.K.,et al 2007).

Calcium signal generation is rapid and complex and therefore unlikely to reflect equilibrium binding conditions (Charlton and Vauquelin,2010). Schild analysis assumes that equilibrium for binding is reached, but this however is not the case. There is a short time lapse between the addition of the agonist and the peak response being measured, which is not favourable of equilibrium establishment. Equilibrium is achieved for the antagonist by pre incubating with the ligand, however true equilibrium is not achieved for the agonist and a reduced maximal response is generated, even in situations where the antagonist is fully surmountable in other assays. The antagonist dissociation is slow and results in only part of the receptor being available for occupancy by the agonist at point of administration. It is not possible to allow for complete equilibrium to occur due to the speed and sensitive nature of the calcium experiments (Charlton and Vauquelin,2010). In the case where antagonists appear insurmountable , co stimulation is a favoured method, although still not entirely favouring the equilibrium requirements.

Apparent insurmountable antagonism

Surmountable antagonists produce a parallel rightward shift of agonist dose response curves without an effect on the maximal response. Insurmountable antagonists however depress the maximal response. Insurmountable antagonism is not only drug related and it may also depend on the tissue, species and experimental design (Vauquelin et al.,2002). The generation of a biphasic curve in experiments in which the dose response of antagonists were being looked at would be an indicator of partial insurmountable antagonism (Vauquelin et al.,2002). As said previously , in a Schild experiment antagonist concentrations should shift the agonist response curve parallel to the right. The degree of shift is then measured for each antagonist concentration in comparison to the control agonist only curve and the logarithm plotted. The regression slope should not differ greatly from 1 for the antagonist to be competitive. A widely used technique, thought to be flawed and leading to error in the estimation of antagonist affinity (Hill and Langmead, 2010).

Calcium assays, due to their sensitive nature are favourable when initially screening ligands to determine coupling to GαQ but require careful consideration when using for absolute values for compounds.

References

Allen JA, Yadav PN and Roth BL (2008) Insights into the regulation of 5-HT2A serotonin receptors by scaffolding proteins and kinases. Neuropharmacology 55(6):961-968.

Almaula N, Ebersole BJ, Ballesteros JA, Weinstein H and Sealfon SC (1996) Contribution of a helix 5 locus to selectivity of hallucinogenic and nonhallucinogenic ligands for the human 5-hydroxytryptamine2A and 5-hydroxytryptamine2C receptors: direct and indirect effects on ligand affinity mediated by the same locus. Mol Pharmacol 50(1):34-42.

Aloyo VJ, Berg KA, Spampinato U, Clarke WP and Harvey JA (2009) Current status of inverse agonism at serotonin2A (5-HT2A) and 5-HT2C receptors. Pharmacology & Therapeutics 121(2):160-173.

Amargos-Bosch M, Bortolozzi A, Puig MV, Serrats J, Adell A, Celada P, Toth M, Mengod G and Artigas F (2004) Co-expression and in vivo interaction of serotonin1A and serotonin2A receptors in pyramidal neurons of prefrontal cortex. Cereb Cortex 14(3):281-290.

Baker JG and Hill SJ (2007) Multiple GPCR conformations and signalling pathways: implications for antagonist affinity estimates. Trends Pharmacol Sci 28(8):374-381.

Ballesteros J, Kitanovic S, Guarnieri F, Davies P, Fromme BJ, Konvicka K, Chi L, Millar RP, Davidson JS, Weinstein H and Sealfon SC (1998) Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. J Biol Chem 273(17):10445-10453.

Ballesteros JA, Shi L and Javitch JA (2001) Structural mimicry in G protein-coupled receptors: implications of the high-resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors. Mol Pharmacol 60(1):1-19.

Ballesteros JA, Weinstein H and Stuart CS (1995) [19] Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors, in Methods in Neurosciences pp 366-428, Academic Press.

Berg KA, Maayani S, Goldfarb J and Clarke WP (1998a) Pleiotropic behavior of 5-HT2A and 5-HT2C receptor agonists. Ann N Y Acad Sci 861:104-110.

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P and Clarke WP (1998b) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. Mol Pharmacol 54(1):94-104.

Berridge MJ (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu Rev Biochem 56:159-193.

Berridge MJ (2005) Unlocking the secrets of cell signaling. Annu Rev Physiol 67:1-21.

Berridge MJ, Heslop JP, Irvine RF and Brown KD (1984) Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. Biochem J 222(1):195-201.

Birdsall TC (1998) 5-Hydroxytryptophan: a clinically-effective serotonin precursor. Altern Med Rev 3(4):271-280.

Borroto-Escuela DO, Romero-Fernandez W, Tarakanov AO, Marcellino D, Ciruela F, Agnati LF and Fuxe K Dopamine D2 and 5-hydroxytryptamine 5-HT(A) receptors assemble into functionally interacting heteromers. Biochem Biophys Res Commun 401(4):605-610.

Brady AE and Limbird LE (2002) G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. Cell Signal 14(4):297-309.

Brea J, Castro M, Giraldo J, Lopez-Gimenez JF, Padin JF, Quintian F, Cadavid MI, Vilaro MT, Mengod G, Berg KA, Clarke WP, Vilardaga JP, Milligan G and Loza MI (2009) Evidence for distinct antagonist-revealed functional states of 5-hydroxytryptamine(2A) receptor homodimers. Mol Pharmacol 75(6):1380-1391.

Bulenger S, Marullo S and Bouvier M (2005) Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. Trends Pharmacol Sci 26(3):131-137.

Burgess GM, Godfrey PP, McKinney JS, Berridge MJ, Irvine RF and Putney JW, Jr. (1984) The second messenger linking receptor activation to internal Ca release in liver. Nature 309(5963):63-66.

Capra V, Veltri A, Foglia C, Crimaldi L, Habib A, Parenti M and Rovati GE (2004) Mutational analysis of the highly conserved ERY motif of the thromboxane A2 receptor: alternative role in G protein-coupled receptor signaling. Mol Pharmacol 66(4):880-889.

Carroll LS and Owen MJ (2009) Genetic overlap between autism, schizophrenia and bipolar disorder. Genome Med 1(10):102.

Charlton SJ and Vauquelin G Elusive equilibrium: the challenge of interpreting receptor pharmacology using calcium assays. Br J Pharmacol 161(6):1250-1265.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318(5854):1258-1265.

Cornea-Hebert V, Riad M, Wu C, Singh SK and Descarries L (1999) Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat. J Comp Neurol 409(2):187-209.

Davenport AP (2003) Peptide and trace amine orphan receptors: prospects for new therapeutic targets. Curr Opin Pharmacol 3(2):127-134.

Davenport AP and Macphee CH (2003) Translating the human genome: renaissance of cardiovascular receptor pharmacology. Curr Opin Pharmacol 3(2):111-113.

Dubby PS and Tandon KN (1966) Adsorption indicators in the titrations of hexacyanoferrate(II) and oxalate with thorium ions. Talanta 13(5):765-766.

Eglen RM (2005) Emerging concepts in GPCR function--the influence of cell phenotype on GPCR pharmacology. Proc West Pharmacol Soc 48:31-34.

Feniuk W, Humphrey PP and Watts AD (1983) 5-Hydroxytryptamine-induced relaxation of isolated mammalian smooth muscle. Eur J Pharmacol 96(1-2):71-78.

Filipek S, Stenkamp RE, Teller DC and Palczewski K (2003) G protein-coupled receptor rhodopsin: a prospectus. Annu Rev Physiol 65:851-879.

Flanagan CA (2005) A GPCR that is not "DRY". Mol Pharmacol 68(1):1-3.

Foskett JK, White C, Cheung KH and Mak DO (2007) Inositol trisphosphate receptor Ca2+ release channels. Physiol Rev 87(2):593-658.

Fredriksson R, Lagerstrom MC, Lundin LG and Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63(6):1256-1272.

Gaddum JH and Hameed KA (1954) Drugs which antagonize 5-hydroxytryptamine. Br J Pharmacol Chemother 9(2):240-248.

Gaddum JH and Picarelli ZP (1957) Two kinds of tryptamine receptor. Br J Pharmacol Chemother 12(3):323-328.

Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, Zhou M, Okawa Y, Callado LF, Milligan G, Gingrich JA, Filizola M, Meana JJ and Sealfon SC (2008) Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature 452(7183):93-97.

Gonzalez-Maeso J and Sealfon SC (2009) Psychedelics and schizophrenia. Trends Neurosci 32(4):225-232.

Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC and Gingrich JA (2007) Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. Neuron 53(3):439-452.

Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, Weisstaub N, Hen R, Gingrich JA and Sealfon SC (2003) Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. J Neurosci 23(26):8836-8843.

Gray JA and Roth BL (2001) Paradoxical trafficking and regulation of 5-HT(2A) receptors by agonists and antagonists. Brain Res Bull 56(5):441-451.

Hague C, Uberti MA, Chen Z, Bush CF, Jones SV, Ressler KJ, Hall RA and Minneman KP (2004) Olfactory receptor surface expression is driven by association with the beta2-adrenergic receptor. Proc Natl Acad Sci U S A 101(37):13672-13676.

Hall DA and Langmead CJ Matching models to data: a receptor pharmacologist's guide. Br J Pharmacol 161(6):1276-1290.

Harvey JA, Welsh SE, Hood H and Romano AG (1999) Effect of 5-HT2 receptor antagonists on a cranial nerve reflex in the rabbit: evidence for inverse agonism. Psychopharmacology (Berl) 141(2):162-168.

Hasbi A, Fan T, Alijaniaram M, Nguyen T, Perreault ML, O'Dowd BF and George SR (2009) Calcium signaling cascade links dopamine D1-D2 receptor heteromer to striatal BDNF production and neuronal growth. Proc Natl Acad Sci U S A 106(50):21377-21382.

Hebert M, Thibeault R, Sacolax N, Tandon K, Germain M, Bruneau P and Gravel J (2007) [Clinical utility of three evaluation instruments for persons with dementia]. Can J Occup Ther 74(2):102-114.

Hill SJ (2006) G-protein-coupled receptors: past, present and future. Br J Pharmacol 147 Suppl 1:S27-37.

Horacek J, Bubenikova-Valesova V, Kopecek M, Palenicek T, Dockery C, Mohr P and Hoschl C (2006) Mechanism of action of atypical antipsychotic drugs and the neurobiology of schizophrenia. CNS Drugs 20(5):389-409.

Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR and Humphrey PP (1994) International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol Rev 46(2):157-203.

Hoyer D, Hannon JP and Martin GR (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav 71(4):533-554.

Hoyer D and Martin GR (1996) Classification and nomenclature of 5-HT receptors: a comment on current issues. Behav Brain Res 73(1-2):263-268.

Hsieh CL, Bowcock AM, Farrer LA, Hebert JM, Huang KN, Cavalli-Sforza LL, Julius D and Francke U (1990) The serotonin receptor subtype 2 locus HTR2 is on human chromosome 13 near genes for esterase D and retinoblastoma-1 and on mouse chromosome 14. Somat Cell Mol Genet 16(6):567-574.

Huezo-Diaz P, Tandon K and Aitchison KJ (2005) The genetics of depression and related traits. Curr Psychiatry Rep 7(2):117-124.

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP and Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 322(5905):1211-1217.

Jerman JC, Brough SJ, Gager T, Wood M, Coldwell MC, Smart D and Middlemiss DN (2001) Pharmacological characterisation of human 5-HT2 receptor subtypes. Eur J Pharmacol 414(1):23-30.

Ji TH, Grossmann M and Ji I (1998) G protein-coupled receptors. I. Diversity of receptor-ligand interactions. J Biol Chem 273(28):17299-17302.

Jordan MJ, Tandon K, Shaw PE and Goodner KL (2001) Aromatic profile of aqueous banana essence and banana fruit by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactometry (GC-O). J Agric Food Chem 49(10):4813-4817.

Julius D, Huang KN, Livelli TJ, Axel R and Jessell TM (1990) The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. Proc Natl Acad Sci U S A 87(3):928-932.

Katugampola S and Davenport A (2003) Emerging roles for orphan G-protein-coupled receptors in the cardiovascular system. Trends Pharmacol Sci 24(1):30-35.

Kelly P, Moeller BJ, Juneja J, Booden MA, Der CJ, Daaka Y, Dewhirst MW, Fields TA and Casey PJ (2006) The G12 family of heterotrimeric G proteins promotes breast cancer invasion and metastasis. Proc Natl Acad Sci U S A 103(21):8173-8178.

Kenakin T, Jenkinson S and Watson C (2006) Determining the potency and molecular mechanism of action of insurmountable antagonists. J Pharmacol Exp Ther 319(2):710-723.

Knight AR, Misra A, Quirk K, Benwell K, Revell D, Kennett G and Bickerdike M (2004) Pharmacological characterisation of the agonist radioligand binding site of 5-HT(2A), 5-HT(2B) and 5-HT(2C) receptors. Naunyn Schmiedebergs Arch Pharmacol 370(2):114-123.

Koshy A and Tandon K (1975) Malabsorption in children: a review. Indian Pediatr 12(10):1021-1027.

Kostenis E, Waelbroeck M and Milligan G (2005) Techniques: promiscuous Galpha proteins in basic research and drug discovery. Trends Pharmacol Sci 26(11):595-602.

Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A, Lutter M, Lagace DC, Ghose S, Reister R, Tannous P, Green TA, Neve RL, Chakravarty S, Kumar A, Eisch AJ, Self DW, Lee FS, Tamminga CA, Cooper DC, Gershenfeld HK and Nestler EJ (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 131(2):391-404.

Kroeze WK and Roth BL (1998) The molecular biology of serotonin receptors: therapeutic implications for the interface of mood and psychosis. Biol Psychiatry 44(11):1128-1142.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature 407(6807):971-977.

Kurrasch-Orbaugh DM, Watts VJ, Barker EL and Nichols DE (2003) Serotonin 5-hydroxytryptamine 2A receptor-coupled phospholipase C and phospholipase A2 signaling pathways have different receptor reserves. J Pharmacol Exp Ther 304(1):229-237.

Kutzler MA, Molnar J, Schlafer DH, Kuc RE, Davenport AP and Nathanielsz PW (2003) Maternal dexamethasone increases endothelin-1 sensitivity and endothelin a receptor expression in ovine foetal placental arteries. Placenta 24(4):392-402.

Lagerstrom MC and Schioth HB (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov 7(4):339-357.

Lee SP, So CH, Rashid AJ, Varghese G, Cheng R, Lanca AJ, O'Dowd BF and George SR (2004) Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. J Biol Chem 279(34):35671-35678.

Lopez-Gimenez JF, Vilaro MT and Milligan G (2008) Morphine desensitization, internalization, and down-regulation of the mu opioid receptor is facilitated by serotonin 5-hydroxytryptamine2A receptor coactivation. Mol Pharmacol 74(5):1278-1291.

Lopez-Gimenez JF, Villazon M, Brea J, Loza MI, Palacios JM, Mengod G and Vilaro MT (2001) Multiple conformations of native and recombinant human 5-hydroxytryptamine(2a) receptors are labeled by agonists and discriminated by antagonists. Mol Pharmacol 60(4):690-699.

Lorke DE, Lu G, Cho E and Yew DT (2006) Serotonin 5-HT2A and 5-HT6 receptors in the prefrontal cortex of Alzheimer and normal aging patients. BMC Neurosci 7:36.

Luttrell LM (2008) Reviews in molecular biology and biotechnology: transmembrane signaling by G protein-coupled receptors. Mol Biotechnol 39(3):239-264.

Mak DO, Pearson JE, Loong KP, Datta S, Fernandez-Mongil M and Foskett JK (2007) Rapid ligand-regulated gating kinetics of single inositol 1,4,5-trisphosphate receptor Ca2+ release channels. EMBO Rep 8(11):1044-1051.

Marcotte ER, Pearson DM and Srivastava LK (2001) Animal models of schizophrenia: a critical review. J Psychiatry Neurosci 26(5):395-410.

Marinissen MJ and Gutkind JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 22(7):368-376.

Maurel D, Comps-Agrar L, Brock C, Rives ML, Bourrier E, Ayoub MA, Bazin H, Tinel N, Durroux T, Prezeau L, Trinquet E and Pin JP (2008) Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. Nat Methods 5(6):561-567.

McGuffin P, Tandon K and Corsico A (2003) Linkage and association studies of schizophrenia. Curr Psychiatry Rep 5(2):121-127.

Meltzer HY, Li Z, Kaneda Y and Ichikawa J (2003) Serotonin receptors: their key role in drugs to treat schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 27(7):1159-1172.

Meltzer HY and Nash JF (1991) Effects of antipsychotic drugs on serotonin receptors. Pharmacol Rev 43(4):587-604.

Milligan G (1998) New aspects of G-protein-coupled receptor signalling and regulation. Trends Endocrinol Metab 9(1):13-19.

Milligan G (2004) Applications of bioluminescence- and fluorescence resonance energy transfer to drug discovery at G protein-coupled receptors. Eur J Pharm Sci 21(4):397-405.

Milligan G (2006) G-protein-coupled receptor heterodimers: pharmacology, function and relevance to drug discovery. Drug Discov Today 11(11-12):541-549.

Milligan G (2007) G protein-coupled receptor dimerisation: molecular basis and relevance to function. Biochim Biophys Acta 1768(4):825-835.

Milligan G (2008) A day in the life of a G protein-coupled receptor: the contribution to function of G protein-coupled receptor dimerization. Br J Pharmacol 153 Suppl 1:S216-229.

Milligan G and Kostenis E (2006) Heterotrimeric G-proteins: a short history. Br J Pharmacol 147 Suppl 1:S46-55.

Milligan G, Ramsay D, Pascal G and Carrillo JJ (2003) GPCR dimerisation. Life Sci 74(2-3):181-188.

Mirzadegan T, Benko G, Filipek S and Palczewski K (2003) Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. Biochemistry 42(10):2759-2767.

Murakami M and Kouyama T (2008) Crystal structure of squid rhodopsin. Nature 453(7193):363-367.

Naik SN, Tandon KN and Naik PV (1969) Transferrin types in Indian water buffalo (Bos bubalis). Experientia 25(10):1105-1106.

Noga BR, Johnson DM, Riesgo MI and Pinzon A (2009) Locomotor-activated neurons of the cat. I. Serotonergic innervation and co-localization of 5-HT7, 5-HT2A, and 5-HT1A receptors in the thoraco-lumbar spinal cord. J Neurophysiol 102(3):1560-1576.

O'Dowd BF, Ji X, Alijaniaram M, Nguyen T and George SR Separation and reformation of cell surface dopamine receptor oligomers visualized in cells. Eur J Pharmacol 658(2-3):74-83.

Ohmiya Y (2004) [Basic and application principle on the bioluminescence system of insect luciferases]. Seikagaku 76(1):5-15.

Palczewski K (2006) G protein-coupled receptor rhodopsin. Annu Rev Biochem 75:743-767.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science 289(5480):739-745.

Park PS, Filipek S, Wells JW and Palczewski K (2004) Oligomerization of G protein-coupled receptors: past, present, and future. Biochemistry 43(50):15643-15656.

Park PS, Lodowski DT and Palczewski K (2008) Activation of G protein-coupled receptors: beyond two-state models and tertiary conformational changes. Annu Rev Pharmacol Toxicol 48:107-141.

Pazos A, Probst A and Palacios JM (1987) Serotonin receptors in the human brainâ€”IV. Autoradiographic mapping of serotonin-2 receptors. Neuroscience 21(1):123-139.

Perreault ML, Hasbi A, Alijaniaram M, Fan T, Varghese G, Fletcher PJ, Seeman P, O'Dowd BF and George SR The dopamine D1-D2 receptor heteromer localizes in dynorphin/enkephalin neurons: increased high affinity state following amphetamine and in schizophrenia. J Biol Chem 285(47):36625-36634.

Perreault ML, O'Dowd BF and George SR Dopamine receptor homooligomers and heterooligomers in schizophrenia. CNS Neurosci Ther 17(1):52-57.

Porsolt RD, Moser PC and Castagne V Behavioral indices in antipsychotic drug discovery. J Pharmacol Exp Ther 333(3):632-638.

Prioleau C, Visiers I, Ebersole BJ, Weinstein H and Sealfon SC (2002) Conserved helix 7 tyrosine acts as a multistate conformational switch in the 5HT2C receptor. Identification of a novel "locked-on" phenotype and double revertant mutations. J Biol Chem 277(39):36577-36584.

Pritchett DB, Bach AW, Wozny M, Taleb O, Dal Toso R, Shih JC and Seeburg PH (1988) Structure and functional expression of cloned rat serotonin 5HT-2 receptor. EMBO J 7(13):4135-4140.

Pupillo M, Insall R, Pitt GS and Devreotes PN (1992) Multiple cyclic AMP receptors are linked to adenylyl cyclase in Dictyostelium. Mol Biol Cell 3(11):1229-1234.

Rang HP (2006) The receptor concept: pharmacology's big idea. Br J Pharmacol 147 Suppl 1:S9-16.

Rashid AJ, O'Dowd BF, Verma V and George SR (2007a) Neuronal Gq/11-coupled dopamine receptors: an uncharted role for dopamine. Trends Pharmacol Sci 28(11):551-555.

Rashid AJ, So CH, Kong MM, Furtak T, El-Ghundi M, Cheng R, O'Dowd BF and George SR (2007b) D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. Proc Natl Acad Sci U S A 104(2):654-659.

Raymond JR, Mukhin YV, Gelasco A, Turner J, Collinsworth G, Gettys TW, Grewal JS and Garnovskaya MN (2001) Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol Ther 92(2-3):179-212.

Robas N, O'Reilly M, Katugampola S and Fidock M (2003) Maximizing serendipity: strategies for identifying ligands for orphan G-protein-coupled receptors. Curr Opin Pharmacol 3(2):121-126.

Roth BL, Berry SA, Kroeze WK, Willins DL and Kristiansen K (1998) Serotonin 5-HT2A receptors: molecular biology and mechanisms of regulation. Crit Rev Neurobiol 12(4):319-338.

Roth BL, Craigo SC, Choudhary MS, Uluer A, Monsma FJ, Jr., Shen Y, Meltzer HY and Sibley DR (1994) Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. J Pharmacol Exp Ther 268(3):1403-1410.

Roth BL, Palvimaki EP, Berry S, Khan N, Sachs N, Uluer A and Choudhary MS (1995) 5-Hydroxytryptamine2A (5-HT2A) receptor desensitization can occur without down-regulation. J Pharmacol Exp Ther 275(3):1638-1646.

Roth BL, Shoham M, Choudhary MS and Khan N (1997) Identification of conserved aromatic residues essential for agonist binding and second messenger production at 5-hydroxytryptamine2A receptors. Mol Pharmacol 52(2):259-266.

Rovati GE, Capra V and Neubig RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. Mol Pharmacol 71(4):959-964.

Salo D, Eget D, Lavery RF, Garner L, Bernstein S and Tandon K (2003) Can patients accurately read a visual analog pain scale? Am J Emerg Med 21(7):515-519.

Selten JP and Cantor-Graae E (2005) Social defeat: risk factor for schizophrenia? Br J Psychiatry 187:101-102.

Shi L, Liapakis G, Xu R, Guarnieri F, Ballesteros JA and Javitch JA (2002) Beta2 adrenergic receptor activation. Modulation of the proline kink in transmembrane 6 by a rotamer toggle switch. J Biol Chem 277(43):40989-40996.

Sihota R, Tandon K, Betharia SM and Arora R (1996) Malignant eyelid tumors in an Indian population. Arch Ophthalmol 114(1):108-109.

Singhal GK and Tandon KN (1967a) Studies on metallochromic indicators--I. Pyrocatechol hthalein as metallochromic indicator. Talanta 14(9):1127-1129.

Singhal GK and Tandon KN (1967b) Studies on metallochromic indicators--II. Zincon and its mercury and zinc complexes as indicators in EDTA titrations. Talanta 14(11):1351-1353.

Singhal GK and Tandon KN (1968) Studies on metallochromic indicators-III Hematoxylin and hematein. Talanta 15(7):707-710.

Stephenson RP (1956) A modification of receptor theory. Br J Pharmacol Chemother 11(4):379-393.

Tandon K and McGuffin P (2002) The genetic basis for psychiatric illness in man. Eur J Neurosci 16(3):403-407.

Tandon KK and Sukheja IK (1974) Histochemical reactions on the localization of lipids and carbohydrates in the diencephalon and the mesencephalon of Barilius bendelisis (Ham.) and Barilius vagra (Ham.). Acta Anat (Basel) 89(4):560-571.

Tandon KN (1962) Evolution of cataract surgery. J Indian Med Assoc 39:587-589.

Tandon KN (1966) Complexometric determination of mercury(II) using Congo Red as indicator. Talanta 13(1):161-163.

Tandon KN and Naik SN (1976) A, B and H substances in the saliva of dogs. Indian J Exp Biol 14(2):97-99.

Tandon R, Keshavan MS and Nasrallah HA (2008a) Schizophrenia, "just the facts" what we know in 2008. 2. Epidemiology and etiology. Schizophr Res 102(1-3):1-18.

Tandon R, Keshavan MS and Nasrallah HA (2008b) Schizophrenia, "Just the Facts": what we know in 2008 part 1: overview. Schizophr Res 100(1-3):4-19.

Tandon R, Nasrallah HA and Keshavan MS (2009) Schizophrenia, "just the facts" 4. Clinical features and conceptualization. Schizophr Res 110(1-3):1-23.

Tandon RK, Patney NL, Goyal SP and Tandon K (1974) Pulmonary resection in child-bearing age and its effect on pregnancy. Indian J Chest Dis 16(4):381-385.

Teitler M, Herrick-Davis K and Purohit A (2002) Constitutive activity of G-protein coupled receptors: emphasis on serotonin receptors. Curr Top Med Chem 2(6):529-538.

To ZP, Bonhaus DW, Eglen RM and Jakeman LB (1995) Characterization and distribution of putative 5-ht7 receptors in guinea-pig brain. Br J Pharmacol 115(1):107-116.

Ueda T, Chueh SH, Noel MW and Gill DL (1986) Influence of inositol 1,4,5-trisphosphate and guanine nucleotides on intracellular calcium release within the N1E-115 neuronal cell line. J Biol Chem 261(7):3184-3192.

Vauquelin G and Van Liefde I (2006) Slow antagonist dissociation and long-lasting in vivo receptor protection. Trends Pharmacol Sci 27(7):356-359.

Vauquelin G, Van Liefde I, Birzbier BB and Vanderheyden PM (2002a) New insights in insurmountable antagonism. Fundam Clin Pharmacol 16(4):263-272.

Vauquelin G, Van Liefde I and Vanderheyden P (2002b) Models and methods for studying insurmountable antagonism. Trends Pharmacol Sci 23(11):514-518.

Virostko J, Chen Z, Fowler M, Poffenberger G, Powers AC and Jansen ED (2004) Factors influencing quantification of in vivo bioluminescence imaging: application to assessment of pancreatic islet transplants. Mol Imaging 3(4):333-342.

Vysokanov A, Flores-Hernandez J and Surmeier DJ (1998) mRNAs for clozapine-sensitive receptors co-localize in rat prefrontal cortex neurons. Neurosci Lett 258(3):179-182.

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG and Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. Nature 454(7203):486-491.

Watts SW (1998) Activation of the mitogen-activated protein kinase pathway via the 5-HT2A receptor. Ann N Y Acad Sci 861:162-168.

Weiner DM, Burstein ES, Nash N, Croston GE, Currier EA, Vanover KE, Harvey SC, Donohue E, Hansen HC, Andersson CM, Spalding TA, Gibson DF, Krebs-Thomson K, Powell SB, Geyer MA, Hacksell U and Brann MR (2001) 5-hydroxytryptamine2A receptor inverse agonists as antipsychotics. J Pharmacol Exp Ther 299(1):268-276.

Welsh SE, Kachelries WJ, Romano AG, Simansky KJ and Harvey JA (1998) Effects of LSD, ritanserin, 8-OH-DPAT, and lisuride on classical conditioning in the rabbit. Pharmacol Biochem Behav 59(2):469-475.

Willins DL, Alsayegh L, Berry SA, Backstrom JR, Sanders-Bush E, Friedman L, Khan N and Roth BL (1998) Serotonergic antagonist effects on trafficking of serotonin 5-HT2A receptors in vitro and in vivo. Ann N Y Acad Sci 861:121-127.

Willins DL, Berry SA, Alsayegh L, Backstrom JR, Sanders-Bush E, Friedman L and Roth BL (1999) Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors in vitro and in vivo. Neuroscience 91(2):599-606.

Wise A, Jupe SC and Rees S (2004) The identification of ligands at orphan G-protein coupled receptors. Annu Rev Pharmacol Toxicol 44:43-66.

Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK and Lefkowitz RJ (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci U S A 104(42):16657-16662.

Wohlgenannt M, Tandon K, Mazumdar S, Ramasesha S and Vardeny ZV (2001) Formation cross-sections of singlet and triplet excitons in pi-conjugated polymers. Nature 409(6819):494-497.

Yadav PN, Kroeze WK, Farrell MS and Roth BL Antagonist functional selectivity: 5-HT2A serotonin receptor antagonists differentially regulate 5-HT2A receptor protein level in vivo. J Pharmacol Exp Ther 339(1):99-105.