1.0 Introduction

According to the World Health Organization (WHO), about 450 million people worldwide suffer from mental, behavioural and neurological and substance use disorders (WHO, 2010). The dopamine (D) and serotonin (5-hydroxytryptamine; 5-HT) pathways in the central nervous system (CNS) are putative drug targets for treating neuropsychiatric disorders, including depression, anxiety, bipolar disorder, schizophrenia and drug abuse.

Antipsychotic drugs (APDs) are the main therapeutic intervention in psychotic illness. Notably, the atypical antipsychotic drugs, such as clozapine, risperidone, olanzapine and ziprasidone are commonly used treatments for schizophrenia (Newman-Tancredi *et al.*, 2007). Meltzer *et al.*, (2003) have suggested that antagonism of both D_2 and 5-$HT_{2A/2C}$ receptors is associated with lowered extrapyramidal effects (EPS) such as neuromuscular disturbance liability and improved capacity to alleviate negative symptoms (alogia, flattening affect, social withdrawal) and cognitive impairments (sensory-gating, working, verbal memory). Although the introduction of APDs was a major advance in the treatment of schizophrenia, there is still a need for improvement, as they have a tendency to cause weight gain and a significant number of patients remain resistant to treatment.

Naturally occurring aporphine alkaloids and their synthetic derivatives have served as leads for the development of new neurotropic drugs. Of these, (*R*)-apomorphine (APO) and its derivatives have high affinity and specificity for dopaminergic receptors and may be a valuable template for the search of subtype selective ligands for diseases like Parkinson's disease, schizophrenia, attention-deficit

hyperactivity disorder and erectile dysfunctions (Sipos *et al.*, 2008). However, despite its high intrinsic agonism activity and rapid onset of anti-parkinsonian effects, APO suffers from poor bioavailability, metabolic instability and potential central emetic side-effects (Liu *et al.*, 2008).

In contrast, compounds such as bulbocapnine, corytuberine, boldine and glaucine that share the (*S*)-configuration exhibit neuroleptic-like actions in mice upon subcutaneous injection, suggesting that they may act as dopamine antagonists (Asencio *et al.*, 1999). However, orally administered boldine has very short plasma half life and is rapidly glucuronidated in the liver, a behavior that may explain its relatively weak and short-lived systemic actions and certainly limits its potential clinical usefulness (Sobarzo-Sanchez *et al.*, 2000).

In the present study, a combination of both computational and biomolecular screening approaches have been used to evaluate the affinity of aporphines at dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptors to identify selective monopotent as well as multipotent ligands for dopaminergic and serotonergic receptors. To accomplish this, a homology model of the rat 5-HT_{2A} receptor was built based on the crystal structure of the human β_2-adrenergic receptor followed by *in silico* screening of potential compounds using aporphines as the reference point. Subsequently, filtration based dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) radioligand receptor binding assays were optimised to test the compounds selected from the docking studies across the dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptors. Thus, findings in this study could have important implications for the treatment of dopamine and serotonin circuitry dysfunction.

1.1 Aim and Objectives of the Research

The aim of the research is to identify active aporphines with significant activity at the receptors that are involved in central nervous system (CNS) disorders such as schizophrenia and depression. The specific objectives are:

1. To construct a 3D molecular model of the rat 5-HT_{2A} receptor against the known crystal structure of the human β_2-adrenergic receptor (PDB ID: 2RH1) and to investigate the ligand-receptor interactions of standard 5-HT_{2A} ligands.
2. To search for potential 5-HT_{2A} receptor active ligands based on aporphines.
3. To optimise assays for the characterization of dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptor binding activity.
4. To evaluate the affinity of aporphines at dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptors to identify selective monopotent as well as multipotent ligands.

2.0 Literature Review

2.1 G-Protein Coupled Receptors (GPCRs)

2.1.1 G-Protein Coupled Receptor (GPCR) Overview

Currently, almost 30% of the marketed prescription drugs act on GPCRs as agonists or antagonists by activating or inhibiting the receptors, respectively (Overington *et al.*, 2006) **(Table 2.1)**. They still remain among the most profitable therapeutic targets (Hopkins and Groom, 2002). GPCRs have been categorized into six classes based on the nature of their ligands and sequence similarity. They are Class A (Rhodopsin-like), Class B (Secretin receptor family), Class C (Metabotropic glutamate/pherormone), Class D (Fungal mating pheromone receptor), Class E (Cyclic AMP receptor) and Class F (Frizzled/Smoothened) (George *et al.*, 2002; Kolakowski, 1994; Lagerstrom and Schioth, 2008; Pierce *et al.*, 2002). Of these, class A (Rhodopsin-like) receptors containing biogenic amines, nucleotides, odorants, lipids, proteases and neuropeptides hormones have been studied extensively.

GPCRs are integral membrane proteins composed of seven membrane-spanning segments helices connected by intracellular and extracellular loop segments, as well as an extracellular N-terminus and an intracellular C-terminus **(Figure 2.1)**. There are several highly conserved residues or sequence motifs across the GPCRs including the LXXXD motif in the transmembrane 2, the DRY motif at the end of the transmembrane 3 and the NPXXY motif on the transmembrane 7 (De La Nuez Veulens and Rodríguez, 2009). Upon binding to a GPCR, a ligand induced conformational change promotes its coupling to a heterotrimeric G-protein (alpha (G_α), beta (G_β), and gamma (G_γ) subunits), leading to the activation of various effector proteins that allow the release of second messenger molecule.

Table 2.1: Examples of some prescription drugs which target GPCRs for the indicated condition or disease state (adapted from Leifert *et al.*, 2010).

Brand name	**Generic name**	**G-protein coupled receptors**	**Disease/Indication**
Zyprexa	Olanzapine	Serotonin 5-HT_2 and Dopamine	Schizophrenia, Antipsychotic
Risperdal	Risperidone	Serotonin 5-HT_2	Schizophrenia
Claritin	Loratidine	Histamine H_1	Rhinitis, Allergies
Imigran	Sumatriptan	Serotonin 5-$HT_{1B/1D}$	Migraine
Cardura	Doxazosin	α-adrenoceptor	Prostate hypertrophy
Tenormin	Atenolol	β_1-adrenoceptor	Coronary heart disease
Serevent	Salmeterol	β_2-adrenoceptor	Asthma
Duragesic	Fentanyl	Opioid	Pain
Imodium	Loperamide	Opioid	Diarrhea
Cozaar	Losartan	Angiotensin II	Hypertension
Zantac	Ranitidine	Histamine H_2	Peptic ulcer
Cytotec	Misoprostol	Prostaglandin PGE_1	Ulcer
Zoladex	Goserelin	Gonadotrophin releasing factor	Prostate cancer
Requip	Ropinirole	Dopamine	Parkinson's disease
Atrovent	Ipratropium	Muscarinic	Chronic obstructive pulmonary disease

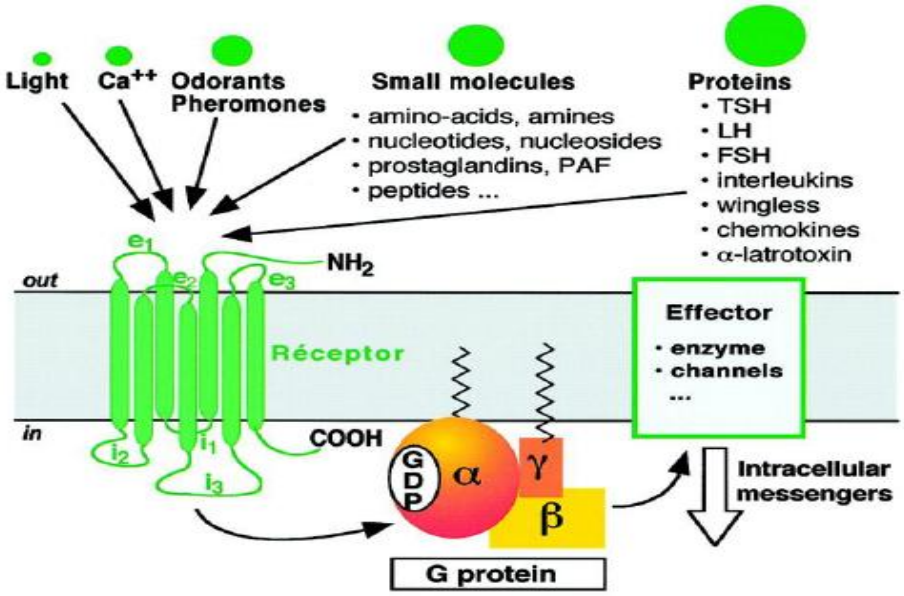


Figure 2.1: Schematic diagram of GPCRs with seven transmembrane regions (TM 1–TM 7) connected by intracellular and extracellular loop segments, as well as an extracellular N-terminus and an intracellular C-terminus (adapted from Bockaert and Pin *et al.*, 2010).

2.1.2 G-Protein Coupled Receptor (GPCR) Crystal Structures

Structural designation of GPCRs, particularly the heptahelical transmembrane region, is very challenging as their greasy hydrophobic surfaces do not readily make the regular intermolecular contacts required for high quality crystallization (Kanagarajadurai *et al.*, 2009). To date, relatively a few three dimensional (3D) crystal structures of GPCRs have been obtained **(Table 2.2)**. The structural data from these studies provide basis information on the drug interactions at molecular level and crystallization of other GPCRs which allow rational approach for drug design.

Table 2.2: List of the three dimensional crystal structures of GPCRs.

Receptor structure	PDB ID	Resolution (Å)	References
Bovine rhodopsin	IF88	2.8	Palczewski *et al.*, 2000
Bovine rhodopsin	1HZX	2.8	Teller *et al.*, 2001
Bovine rhodopsin	1L9H	2.6	Okada *et al.*, 2002
Bovine rhodopsin	1U19	2.2	Okada *et al.*, 2004
Bovine rhodopsin	1GZM	2.65	Li *et al.*, 2004
Bovine rhodopsin photoproduct	2l35-37	4.15	Salom *et al.*, 2006
Bovine bathorhodopsin	2G87	2.6	Nakamichi and Okada, 2006a
Bovine lumirhodopsin	2HPY	2.8	Nakamichi and Okada, 2006b
Bovine isorhodopsin	2PED	2.95	Nakamichi *et al.*, 2007
Bovine rhodopsin	2J4Y	3.4	Standfuss *et al.*, 2007
Human β_{2}-adrenoceptor	2RH1	2.4	Cherezov *et al.*, 2007
Human β_{2}-adrenoceptor	2R4R/S	3.4/3.7	Rasmussen *et al.*, 2007
Squid rhodopsin	2Z73	2.5	Murakami and Kouyama, 2008
Squid rhodopsin	2ZIY	3.7	Shimamura *et al.*, 2008
Human β_{2}-adrenoceptor	3D4S	2.8	Hanson *et al.*, 2008
Turkey β_{1}-adrenoceptor	2VT4	2.7	Warne *et al.*, 2008
Bovine rhodopsin	3CAP	2.9	Park *et al.*, 2008
Bovine rhodopsin	3DQB	3.2	Scheerer *et al.*, 2008
Human adenosine$_{2A}$	3EML	2.6	Jaakola *et al.*, 200
Human dopamine D_3	3PBL	2.89	Chien *et al.*, 2010
Human CXCR4	3ODU/EO/E6/ E8/E9	2.5/2.9/3.2/ 3.1/3.1	Wu *et al.*, 2010
Human histamine H_1	3RZE	3.1	Shimamura *et al.*, 2011
M_3 muscarinic acetylcholine	4DAJ	3.4	Kruse *et al.*, 2012
Human M_2 muscarinic acetylcholine	3UON	3.0	Haga *et al.*, 2012

2.2 Dopamine (D) and Serotonin (5-hydroxytryptamine; 5-HT) Receptors

2.2.1 Dopamine (D) Receptors

Dopamine functions as a catecholamine neurotransmitter and neurohormone, are found extensively in central nervous system, and constitute about 80% of the total brain content (Vallone *et al.*, 2000). Dopamine receptors are G-protein-coupled receptors that can be further classified into D_1-like (D_1, D_5) and the D_2-like (D_2, D_3, D_4) subfamilies, based on their pharmacological, structural and biochemical properties (Andersen *et al.*, 1990; Niznik and Van Tol, 1992; Vallone *et al.*, 2000). The D_1-like receptor subtypes exert its signal by activating G_s proteins, which stimulate adenylyl cyclases and increases cyclic adenosine monophosphate (cAMP) accumulation (Monsma *et al.*, 1990; Sunahara *et al.*, 1991), whereas the D_2-like receptor subtypes mediate its actions by activating $G_{i/0}$-proteins, which inhibits adenylyl cyclases and decreases cyclic adenosine monophosphate (cAMP) accumulation (Bunzow *et al.*, 1988 ; Sokoloff *et al.*, 1990 ; Van Tol *et al.*, 1991). Among the many types of dopamine receptors, D_1 and D_2 are widely expressed in the brain (Dziedzicka-Wasylewska, 2004).

2.2.2 Serotonin (5-hydroxytryptamine; 5-HT) Receptors

Serotonin (5-hydroxytryptamine; 5-HT) is predominantly located in the periphery as a neuromodulator of blood vessel tone and gastrointestinal tract, and only about 1–2% of the body's serotonin is found in the brain as the tryptaminergic neurotransmitter (Skop *et al.*, 1994). On the basis of their genetic, pharmacological and functional properties, serotonin receptors are clustered into seven main families, comprising a total of 14 distinct subtypes (Barnes and Sharp, 1999; Hoyer *et al.*, 2002; Peroutka, 1993). With the exception of the ionotropic 5-HT_3 receptor (Derkach *et al.*,

1989; Maricq *et al.*, 1991), all of the other serotonin receptors are G protein-coupled metabotropic receptors and act through intracellular signaling pathways to hyperpolarize (5-HT_1) or depolarize (5-$HT_{2/4/5/6/7}$) their host cells (Barnes and Sharp, 1999). Of the different subclasses of serotonin receptors, 5-HT_{1A} and 5-HT_{2A} are distributed extensively in many regions of the brain (Hoyer *et al.*, 1994).

2.2.3 Clinical Significance of Dopamine and Serotonin Receptors

The biogenic amine neurotransmitters, dopamine and serotonin are responsible for regulating diverse array of psychological and physiological functions including control of appetite, behaviour, cardiovascular system, cognition, emotion, endocrine secretion, motor, memory, mood, perception and reward (Emilien *et al.*, 1999; Jones and Blackburn, 2002; Missale *et al.*, 1998; Nichols and Nichols, 2008). Alteration in dopaminergic and serotonergic neurotransmission in central nervous system have been proposed to play a fundamental role in treating neuropsychiatric disorders including schizophrenia, depression, anxiety, Parkinson's Disease and related disorders (De Almeida *et al.*, 2008; Giovanni *et al.*, 2010). Numerous psychotropic drugs including antidepressants, antipsychotics, anxiolytics and hallucinogens have been implicated as the main therapeutic intervention for psychotic illness which exert their actions via interactions with dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptors (Alex and Pehek, 2007).

Several D_2 agonists including apomorphine, pramipexole, ropinirole, piribedil, rotigotine and the ergot alkaloids pergolide, bromocriptine and cabergoline have been used in the treatment of Parkinson's diseases (Emilien *et al.*, 1999; Güldenpfennig *et al.*, 2005 ; Morgan and Sethi, 2006; Rascol *et al.*, 2006). D_1 receptors agonist also has

been targeted in the treatment of Parkinson's diseases although its use is limited by the occurrence of dyskinesias (Mailman *et al.*, 2001). In both preclinical models and clinical trials, the role of 5-HT_{1A} agonists and 5-HT_{2A} antagonists has been implicated as potential antiparkinsonian and antidyskinetic agents (Huot *et al.*, 2011).

First generation antipsychotics like chlorpromazine and haloperidol act primarily as D_2 antagonists are ineffective against negative and cognitive symptoms and inducing extrapyramidal motor side effects (EPS). Second generation antipsychotics including clozapine, risperidone, olanzapine, sertindole, quetiapine and ziprasidone are antagonists at both 5-HT_{2A} and D_2 receptors with minimal EPS burden and effective in alleviation of negative symptoms and cognitive functions. Third generation antipsychotics such as perospirone, aripiprazole, lurasidone, bifeprunox and cariprazine possessing combined D_2 partial agonism or antagonism and 5-HT_{1A} agonism is also associated with a lower EPS liability and better efficacy in treating negative and cognitive symptoms (Abi-Dargham and Laruelle, 2005; Akhondzadeh, 2006; Gardner *et al.*, 2005; Mailman and Murthy, 2010; Meltzer *et al.*, 2003; Miyamoto *et al.*, 2005; Newman-Tancredi and Kleven, 2010).

Blockade of 5-HT_{2A} receptors by antidepressant drugs such as trazodone, nefazodone and mirtazapine are widely used treatment for depressive illness (Celada *et al.*, 2004; Richelson, 2001). Activation of 5-HT_{1A} receptors by 8-OH-DPAT, buspirone, gepirone and tandospirone revealed a significant antidepressant effects in various animal models (Koek *et al.*, 2001; Matsuda *et al.*, 1995; Schreiber *et al.*, 1994; Wieland and Lucki, 1990). Additionally, azapirone class of drugs including buspirone and its analogs ipsapirone, gepirone and tandaspirone also exert anxiolytic actions via activation of 5-HT_{1A} receptors (Barradell and Fitton, 1996; Boyer and Feighner, 1993;

Csanalosi *et al.*, 1987; Goa and Ward, 1986). Also, amoxapine is an antidepressant drug which act as weak competitive antagonist of D_2 receptors (Richelson, 2001).

2.3 Computer Aided Drug Discovery

2.3.1 Structure-based Drug Design (SBDD)

Structure-based drug design (SBDD) has emerged as a valuable pharmaceutical tool in medicinal chemistry. Remarkable contributions have been made by SBDD in the field of cancer chemotherapy, drug resistant infections and neurological diseases. The general process of SBDD involves both the synthesis of new derivatives and the evaluation of their binding to the target structure either through computational docking or elucidation of the target structure as a complex with the lead compound (Grey and Thompson, 2010). A prerequisite for this approach is a high-resolution (< 2.5Å) three-dimensional structure of the target, obtained through X-ray crystallographic analysis, in the presence or absence of the drug candidate (Grey and Thompson, 2010).

2.3.2 Homology Modelling

The process of homology or comparative modelling involves identification of the known 3D structure of a protein template, sequence alignment of the protein to be modelled (target) and the template protein, model building for the target based on the 3D structure of the template and the alignment and refining, validation or evaluation of the models (Marti-Renom *et al.*, 2000). The rate of success, quality and the reliability of the homology models depend on the level of sequence identity between the template and the target (Chothia and Lesk, 1986; Moult *et al.*, 1999; Venclovas *et al.*, 2001). As

a rule of thumb, the target protein should share at least 30% of sequence identity with the template structure **(Figure 2.2)** (Baker and Sali, 2001).

For the majority of the last decade, rhodopsin served as the template for homology modelling of other GPCRs. Rhodopsin is structurally stabilized by being covalently bound to a prosthetic group, 11-*cis*-retinal that sustains the receptor in the inactive conformation (Okada and Palczewski, 2001). In contrast, all other GPCRs are activated by diffusible ligands, expressed at relatively low levels in native tissues and having greater functional and structural plasticity (Cherezov *et al.,* 2007). Thus, functionally and structurally more reliable templates are desired to obtain GPCR models which can give further insight into ligand binding and activation mechanisms. Hence, with the acquisition of the structure of the first human β_2-adrenoceptor, the world of GPCR modelling has undergone tremendous changes especially with respect to more authentic approximations of the ligand binding sites of biogenic amine receptors.

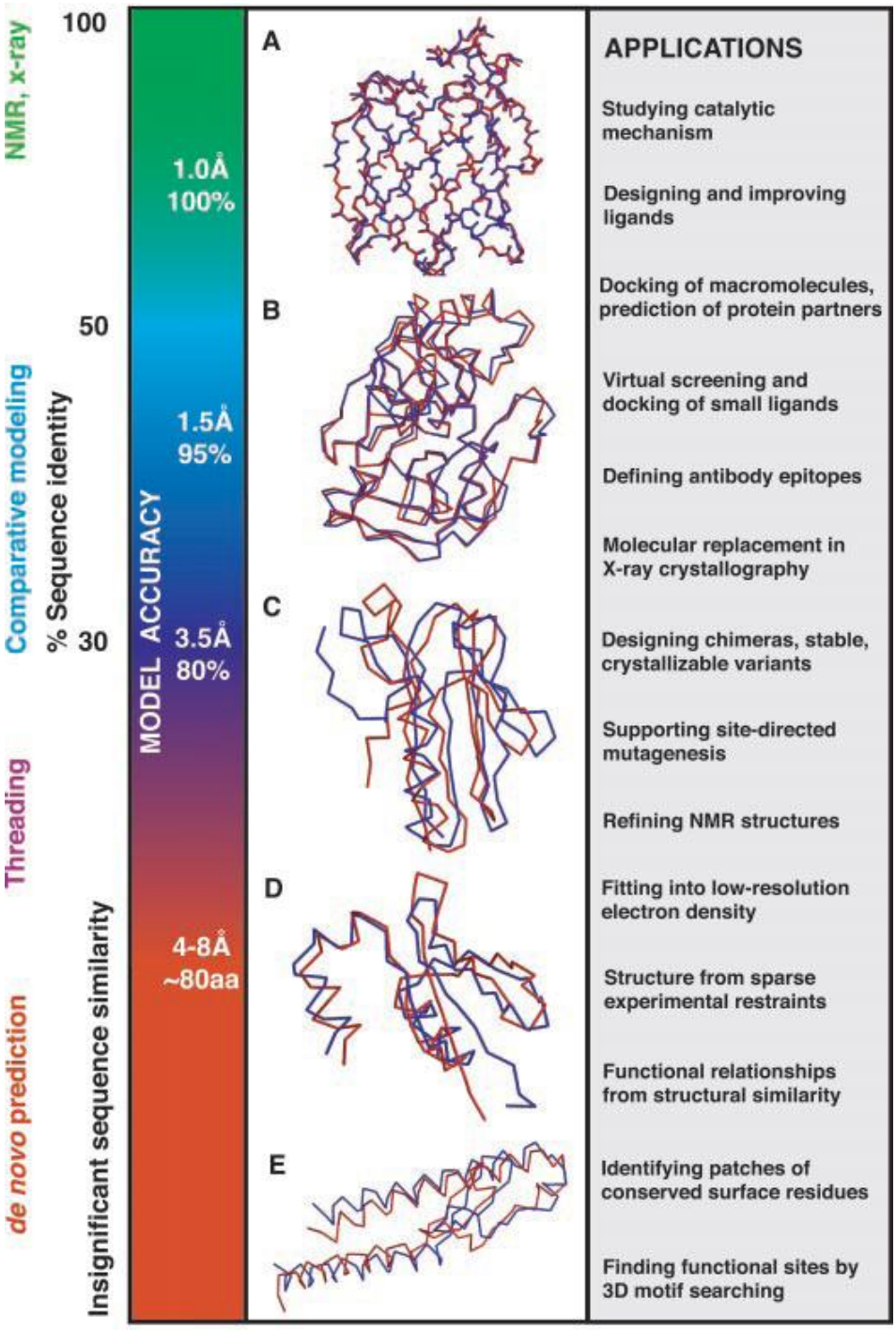


Figure 2.2: Accuracy and application of protein structure models. Shown are the different ranges of applicability of comparative protein structure modelling, threading and *de novo* prediction; the corresponding accuracy of protein models; and their sample applications (adapted from Baker and Sali, 2001).

2.3.3 Molecular Docking

Molecular docking is a computational process of searching for a ligand that is able to fit both geometrically and energetically the binding site of a protein (Teodoro *et al.,* 2001). In general, the best fit for the ligand-protein complex relies on several parameters obtained from the protein and ligand atomic coordinates, such as geometrical complementarities, atomic van der Waals radius, charge, torsion angles, intermolecular hydrogen bonds and hydrophobic contacts, which results in numerous possible conformations or poses of a ligand in the target's binding site. As such, the best docking position is chosen considering the scoring functions, which are able to evaluate intermolecular binding affinities or binding free energies and the population of the cluster (number of conformations belonging to the same cluster, as such repetition is considered a signal of a local minimum) of the ligand-protein complex (De Azevedo Jr and Dias, 2008).

2.4 Receptor Binding Assays

2.4.1 Fundamental of Receptor–ligand Interactions

According to the law of mass action, the binding of a radioligand to a receptor can be described as a biomolecular association reaction, in which a radoligand [L] binds reversibly to a single binding site on the receptor [R]. In the absence of analyte [A], the radioligand [L] binds to the receptor [R] to form a radioligand receptor complex [RL] **(Equation 1)**. At equilibrium, the ratio of k_{-1}/k_{+1} is denoted as the dissociation constant (K_d), which is inversely proportional to the ligand affinity towards the receptor. Binding occurs when the radioligand and receptor collide due to diffusion with the proper collision energy and orientation. The radioligand receptor complex remains bound

together over a certain time period depending on the affinity of the receptor and radioligand for one another.

Equation 1:

$$[R] + [L] \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} [RL]$$

The origin of radio-receptor binding assays depends on the competition between an analyte [A] and the radioligand for binding to a receptor (De Jong *et al.*, 2005). As shown in **Equation 2**, the presence of the analyte [A] leads to the formation of two receptor complexes, the radioligand receptor complex [RL] and the analyte receptor complex [RA]. The analyte competes with the radioligand for the same binding site on the receptor. The concentration at 50% inhibition (IC_{50}) value, which indicates the analyte [A] concentration that displaces 50% of the bound radioligand can be determined, if the analyte concentration is varied and both the receptor and radioligand concentrations are kept constant. The inhibition constant (K_i) of the analyte can be calculated using Cheng- Prusoff equation **(Equation 3)** (Cheng and Prusoff, 1973).

Equation 2:

$$[R] + [L] + [A] \leftrightarrow [RL] + [RA]$$

Equation 3:

$$K_i = IC_{50} / (1 + [L] / K_d)$$

2.4.2 Radioligand Receptor Binding Assays

Radioligand receptor binding assays have been utilized in drug discovery and development to identify potential lead compounds. The first example of a quantitative radio-receptor binding assay was published by Lefkowitz *et al.*, (1970) based on the

same principle as the radioimmunoassay described by Yalow and Berson (1959). Traditionally used *in vivo* or whole animal assays as high volume screening tools have been replaced by receptor binding assays due to their reliability, cost-effectiveness and rapid nature (Sweetnam *et al.*, 1993). The basic principle of receptor binding studies is to assess the ability of potential drugs to interfere with the specific binding of a radioligand to its receptor. After attaining equilibrium with receptors, excess free radioligand must be separated rapidly from the bound radio-receptor complex. In contrast to centrifugation and other techniques, filtration is the most efficient and convenient method of separating free from bound radioligand, because it requires less handling and manipulation of samples (Mckinney, 1998). The bound complex is retained on glass fiber filters and the free radioligand passes through. The filter-bound radioactivity was counted using microplate scintillation.

2.5 Aporphine Alkaloids

2.5.1 An Overview of Aporphines

The naturally occurring aporphine alkaloids constitute a broad subgroup of benzylisoquinoline compounds, with more than 500 structures reported to date (Zhang *et al.*, 2007). Examples of different classes are aporphines, proaporphines, secoaporphines, oxoaporphines, dehydroaporphines, 7-hydroxyaporphines, aporphine dimers and aristolactams. They are widely distributed in many plant species including *Annonaceae, Lauraceae, Monimiaceae, Menispermaceae, Hernandiaceae, Ranunculaceae* and others.

2.5.2 Structural Diversity of Aporphines

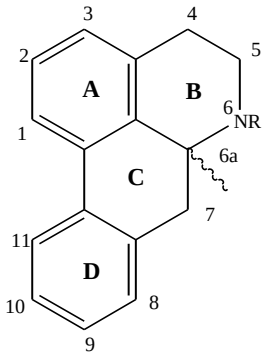


Figure 2.3: General structure of aporphines

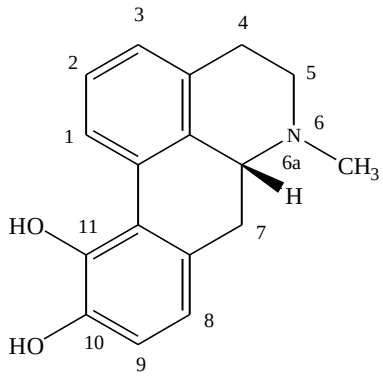
. Aporphines are a diverse group of tetracyclic bases derived by direct bonding between the A and D aromatic rings of the benzylisoquinoline nucleus (**Figure 2.3**). Many natural aporphines are exist as *R*-(-) or *S*-(-) configuration, depending on the stereochemistry of C-6a and usually substituted at position 1 and 2 by hydroxyl, methoxy or methlenedioxy groups. The tetracyclic skeleton can also be substituted at position 9, 10 and 11, and less frequently at positions 3 and 8 and in a few cases, the position 7 or 4 is oxygenated. The nitrogen atom at position 6 is usually tertiary in the base form but may also be quaternary, less frequently acetylated or formylated (Stevigny *et al.*, 2005).

2.5.3 Pharmacological Properties of Aporphines

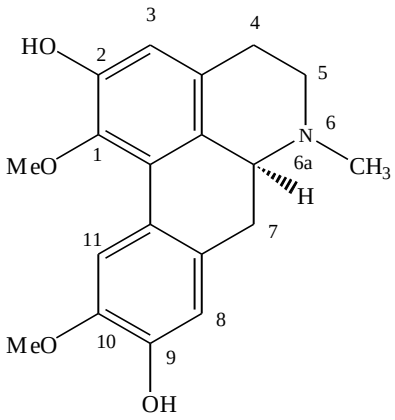
Aporphines exhibit a wide range of interesting pharmacological properties including antioxidant, antiplatelet, antitumor, anticonvulsant, antiplasmodial, antineoplastic, antimalarial, antiprotozoal, antipoliovirus, cytotoxic and antiparkinsonian effects (Guinaudeau *et al.*, 1994; Rios *et al.*, 2000). Of these, *R*-(–)-Apomorphine **(APO; Figure 2.4)**, the semisynthetic or total synthetic prototypical

aporphine (Neumeyer *et al.*, 1970), is a well established dopamine agonist and has been marketed for the treatment of Parkinson's disease and erectile dysfunction (Picada *et al.*, 2002). Despite its high intrinsic agonist activity and fast onset of anti-parkinsonian effects, APO suffers from poor bioavailability, short duration of action and potential central emetic side-effects due to its oxidation potential (Subramony, 2006). In addition, although the catechol moiety contributes to the high dopaminergic activity by H-bonding with dopamine receptors it is not physico-chemically stable (Liu *et al.*, 2008). Thus, to improve APO's pharmacological profile, there is a need for further development of novel compounds by bioisosterically replacing the catecholic component with a more stable functional group.

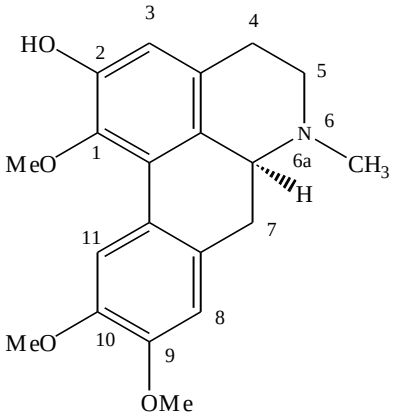
Likewise, *S*-(+)-boldine **(Figure 2.4)**, the major active alkaloidal constituent in the leaves and bark of the Chilean boldo tree (*Peumus boldus* Molina, Monimiaceae) (O'brien *et al.*, 2006) has been commercially used for hepatic dysfunction and as an ingredient in choleretics and laxatives. In spite of the greater *in vitro* binding affinity of boldine, its *in vivo* activities are weaker than those of glaucine, which is interpreted as a result of its poor access to the central nervous system. Therefore, it has been suggested that halogenation of boldine derivative would be expected to give a better absorption and distribution properties, being more lipophilic, as well as being more stable metabolically (Asencio *et al.*, 2005). *O*-methylation of one or both of the hydroxyl groups of boldine as exhibited by predicentrine and glaucine also enhances the pharmacokinetic profile **(Figure 2.4)**. Chung *et al.*, (2009b) also reported that boldine shows competitive binding to 5-HT_{2A} receptors.



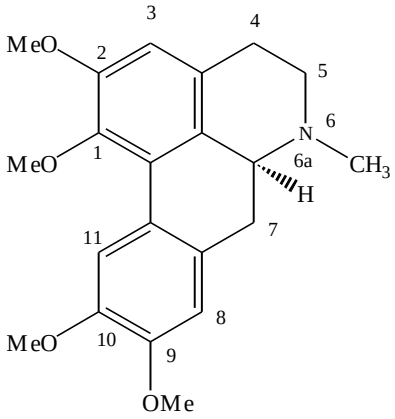
R-(–)-apomorphine



S-(+)-boldine



S-(+)-predicentrine



S-(+)-glaucine

Figure 2.4: Structures of aporphines

3.0 Computational Investigation of the 5-HT_{2A} Receptor Binding Interactions of Standard Ligands and Aporphines

3.1 Introduction

3.1.1 5-HT_{2A} Receptor (5-HT_{2A}R) Structure

The 5-HT_2 receptor family consists of three subtypes, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}, which are similar in terms of molecular structure, pharmacology and signal transduction pathways (Barnes and Sharp, 1999). All three subtypes are single protein molecules with a high degree of homology within the seven transmembrane domains and coupled via heterotrimeric GTP binding protein of the $Gq_{/11}$ type to increase the hydrolysis of inositol phosphates and elevate intracellular calcium (Hoyer *et al.*, 2002). However, due to high sequence conservation within the transmembrane region, it has been historically difficult to develop subtype selective ligands. This task has been made even more challenging in the absence of X-ray crystal structures for any of serotonin receptors. 5-HT receptors share a common molecular architecture with other members of the GPCR. The 5-HT_{2A} receptor structure consists of an extracellular domain, a membrane domain and an intracellular domain. The extracellular domain comprises the extracellular loops (ECL) ECL1, ECL2 and ECL3 between transmembrane (TM) helices TM 2 and 3, TM 4 and 5, and TM 6 and 7, respectively. The membrane domains possess seven transmembrane helices, TM1–TM7. The intracellular domain comprises the C-terminus and the intracellular loops (ICL) ICL1, ICL2 and ICL3 between TM 1 and 2, TM 3 and 4, and TM 5 and 6, respectively, and a highly conserved disulfide bond linking TM3 and ECL2 in nearly all GPCRs.

Site-directed mutagenesis studies associated with molecular modelling studies have led to the identification of a number of residues that are probably involved in

ligand binding and effector coupling features of the 5-HT_{2A} receptor (Baxter *et al.*, 1995; Boess and Martin, 1994; Saudou and Hen, 1994). A number of 5-HT_{2A} receptor models have been constructed based on analogy with rhodopsin and bacteriorhodopsin and subjected to site directed mutagenesis (Almaula *et al.*, 1996a; Almaula *et al.*, 1996b; Choudhary *et al.*, 1993; Holtje and Jendretzki, 1995; Kristiansen and Dahl, 1996; Roth *et al.*, 1997a; Westkaemper and Glennon, 1993). Another X-ray crystal structure of GPCR, human β_2-adrenergic receptor that belongs to amine subfamily has also been used as a template to build 5-HT_{2A} homology model (Kanagarajadurai *et al.*, 2009; Pecic *et al.*, 2010).

Most distinct models of the 5-HT_{2A} receptor structure share the following properties: an electrostatic interaction between the positively charged amine moiety of the agonists and antagonists with a negatively charged aspartic acid residue Asp155(3.32) (Wang *et al.*, 1993), interactions with polar/charged side chain groups including Ser159(3.36) (Almaula *et al.*, 1996b) and Ser239(5.43) (Braden and Nichols, 2007) and van der Waals interactions with aromatic residues such as Trp151(3.28), Phe243(5.47), Phe244(5.48), Trp336(6.48), Phe339(6.51), Phe340(6.52), Trp367(7.40) and Tyr370(7.43) which form a hydrophobic pocket surrounding the agonist or antagonist molecule (Choudhary *et al.*, 1995; Kroeze *et al.*, 2002; Roth *et al.*, 1997b; Shapiro *et al.*, 2000). Structural information from these studies has shown the importance of the conserved residues in the binding mode of both agonists and antagonists and also provides detailed knowledge about the ligand-receptor interactions.

3.2 Materials

Silicon graphics workstations, Sybyl modelling software, Autodock4.0 docking, Discovery Studio 3.1 and LigPlot 4.4.2 softwares.

3.3 Methodology

3.3.1 Sequence Alignments

FASTA sequences used in the analysis were retrieved from the European Bioinformatics Institute website (http: //www.ebi.ac.uk) database. Sequence alignments were generated with CLUSTALW2 software (http://www.ebi.ac.uk/Tools/clustalw2/) (Thompson *et al.*, 1994). Conserved residues among the 5-HT family with respect to 5-HT_{2A} receptor were resolved through sequence comparison.

3.3.2 Model Construction

A homology model for the rat 5-HT_{2A} receptor was constructed using Sybyl 7.1 (Tripos, St. Louis, MO, USA) on a Silicon Graphics O_2 station. The FASTA sequence rat 5-HT_{2A} receptor (ID: P14842) was submitted for BLASTp analysis to search for a suitable template. The template and target sequences were aligned using CLUSTALW2. The alignment was then manually refined using JALVIEW 2.4 to ensure alignment of the known conserved motifs and minimise gaps in the transmembrane regions. The final alignment was used as the input to the Sybyl 7.1 software package to build the model. Molecular mechanics minimisations were performed using CHARMM (Brooks *et al.*, 1983) with a nonbonded interaction cutoff = 8 Å and a distance dependent dielectric constant $\varepsilon = 4$. The output structure was validated using PROCHECK

(http://nihserver.mbi.ucla.edu/SAVES/) (Laskowski *et al.*, 1993) to assess the stereochemical quality by Ramachandran plot analysis.

3.3.3 Docking Procedure

Molecular docking was conducted using the Lamarckian genetic algorithm (Morris *et al.*, 1998) implemented in Autodock4.0. 3D structures of the 5-HT_{2A} standard ligands were taken from the ZINC database (http://zinc.docking.org) (Irwin and Shoichet, 2005). Prior to docking, the structures of the rat 5-HT_{2A} receptor and the compounds were prepared using AutoDock Tools interface. The polar hydrogen atoms were added to the receptor structure, non-polar hydrogen atoms were merged, missing atoms were repaired, Kollman charges were added and the resulting structure was saved as a pdbqt file. On the other hand, Gasteiger charges were added to each of the compound structure and these were then saved as separate pdbqt file. Using the Autogrid utility, the binding cavity was defined to cover the active site with a size of 128 x 128 x 128 points and a grid spacing of 0.2 Å. A genetic algorithm was calculated for 100 runs with a population size of 150 and a total number of 2,500,000 evaluations. Based on the generated clusters using the MGL Autodock tools 1.5.2, the docked conformations were visualized using the 3D window of Discovery Studio 3.1 (Accelrys, Inc., San Diego, CA, USA). Hydrogen bonds, hydrophobic contacts and distances were plotted by LigPlot 4.4.2 (Wallace *et al.*, 1995).

3.3.4 Compound Selection for Docking

The ZINC database with approximately ~3.7 million compounds was used as a source of compounds (http://zinc.docking.org) (Irwin and Shoichet, 2005). A set of 69

compounds were selected based on boldine as the backbone structure with at least 90% similarity, drug-like properties (molecular weight ≤ 500, number of rotatable bonds ≤8, number of hydrogen bond donors or acceptors ≤ 10, log P value from -4 to 5, net charges from -5 to 5, polar desolvation value from -400 to 1 kcal/mol, apolar desolvation value from -100 to 40 kcal/mol, polar surface area of 150Å^2) and purchasable. The selected compounds were further filtered by discarding compounds with unusual substitutions not found in naturally-occurring aporphines including compounds with R_3-methylamine, R_3-hydroxymethyl, R_6-dimethyl and R_6-oxoethyl substituents **(Figure 3.1)**. The remaining 52 compounds were then subjected for docking using the generated rat 5-HT_{2A} receptor model according to protocol described previously **(see Section 3.3.3)**.

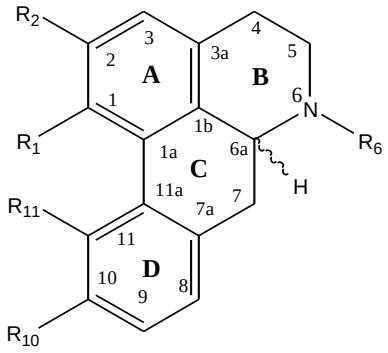


Figure 3.1: General structure of naturally-occuring aporphines with possible substitution sites indicated as R_1–R_{11}.

3.4 Results and Discussion

3.4.1 Selection of Templates

Following BLASTp query, the human β_2-adrenergic receptor structure (PDB ID: 2RH1) was selected as the template sequence. This receptor belongs to the group of GPCRs with almost 30% sequence similarity with the 5-HT_{2A} receptor, thus it is functionally connected to the family of serotonin receptors. In contrast, bovine rhodopsin shares a low sequence similarity to 5-HT_{2A} receptor with less than 20% and it is a retinal-binding protein, functionally totally different from the 5-HT_{2A} receptor (Palczewski *et al.*, 2000).

3.4.2 Sequence Analysis

Alignment of the rat 5-HT family receptor sequences revealed that they possess 19–68% identity **(Figure 8.1 in Appendix A)**. The analysis revealed the presence of motif [T/S]XSI at transmembrane 3, F[Y/F]XP at transmembrane 5, WXPFF at transmembrane 6, WXGY and SX_2NPX_2Y at transmembrane 7 and DRY at intracellular loop 2. Alignment of the multiple species of 5-HT_{2A} receptor including human, orang utan, Rhesus macaque, bovin, pig, dog, rat, mouse and Chinese hamster showed that they share 88–97% identity **(Figure 8.2 in Appendix A)**. The major discrepancies occur at the N-terminus and C-terminus where there is very little conservation of sequence. However, these regions are less likely to affect the putative ligand binding site. The rat and human 5-HT_{2A} receptors displayed 91% sequence identity **(Figure 8.3 in Appendix A)**. A detailed comparison of their sequences indicated that all the species specific residues are found in the loop regions apart from Thr82, Ile150 and Ala242 which are located in transmembrane regions 1, 3 and 5,

respectively. The rat 5-HT_{2A} receptor is closer to rat 5-HT_{2C} than rat 5-HT_{2B} with 48% and 38 % identity respectively **(Figure 8.4 in Appendix A)**.

3.4.3 Model Construction

Chain A of the human β_2-adrenergic receptor crystal structure (PDB: 2RH1) was used as a basis structure after excision of the lysozyme adduct. A rat 5-HT_{2A} receptor homology model was constructed from the substitution of amino acids in the 3D structure of the human β_2-adrenergic receptor and the insertion and/or deletion of amino acids according to the final sequence alignment using Sybyl 7.1. Internal water molecules were ignored during model building. The conserved disulfide bond between Cys148 in TM3 and Cys227 in extracellular loop 2 (ECL2) was maintained during the building of rat 5-HT_{2A} homology model. A protein loop search was used to fill the gaps in the intracellular and extracellular loops, the C-terminus and the N-terminus by the Biopolymer loop search facility in Sybyl 7.1 with suitable fragments from its protein database based on PDB structures.

3.4.4 Model Evaluation

The output structure was energy minimised using the CHARMM force field in order to remove as many as possible of any of unfavourable bond lengths, bond angles, torsion angles or non-bonded interactions that may have been produced during the modelling process. After the refinement process, the full-length structure was submitted to PROCHECK to verify the integrity of the structure. All together 89.4% of the residues were in the most favourable region, 9.9% in the additionally allowed region and 0.5% in the generously allowed region **(Figure 8.5 in Appendix B)**.

3.4.5 Docking

In order to validate the model and computational methods, docking was performed with standard 5-HT_{2A} ligands which are classified as endogenous ligands (serotonin), agonists (tryptamine, 5-methoxytryptamine (5-MeOT), (*R*/*S*)-isomers of 2, 5-dimethoxy-4-iodoamphetamine (DOI)) and antagonists (spiperone, risperidone, haloperidol, ritanserin and ketanserin).

All the standard ligands were observed to bind within the active site. Three sets of residues were involved: Asp155 forming an electrostatic interaction with the protonated amine group of the agonists and antagonists, an aromatic network consisting of the conserved residues (Trp336, Phe339, Phe340 and Tyr370) and non-aromatic residues including Val156, Ser131, Ile152, Ser159, Thr160, Leu229, Val235, Gly238, Ser239, Ala242, Asn343 and Val366. In the case of serotonin, residues from transmembrane regions 3, 5 and 6 **(Figure 8.6(a) in Appendix C)** were seen to be involved in binding interactions. The protonated amine moiety was able to form an electrostatic interaction with Asp155, whereas the hydroxyl group was hydrogen interacted with Thr160. Johnson *et al.*, (1994) reported an interaction of Ser239 with the hydroxyl group. The van der Waals interactions were also observed between the endogenous ligand and the residues Trp336, Phe339 and Phe 340.

The agonists interacted with similar residues from transmembrane regions 3, 5, 6 and 7 as for serotonin **(Figure 8.6(b)–(e) in Appendix C)**. However, the antagonists were found to bind with a wider range of residues from transmembrane regions 2,3,5,6,7 and extracellular loop 2 (ECL2) **(Figure 8.7(a)–(e) in Appendix C)** with a

number of residues in common such as Trp151, Asp155, Ser159, Thr160, Leu229, Val235, Gly238, Ala242, Trp336, Phe339, Phe340 and Tyr370.

These observations were consistent with experimental data and also indicated that the conformation of the ligand and a preference of residues in certain helices are influenced by the type of the ligand. The electrostatic interaction with the Asp155 residue has been found to be essential for agonist and antagonist binding (Wang *et al.*, 1993). Almaula *et al.*, (1996b) have suggested that Ser159 in transmembrane 3 helps in anchoring the charged amino group of serotonin. Mutation of Ser239A causes a significant reduction in affinity of serotonin, but has less effect on 5-MeOT and (*R*/*S*)-DOI affinity and no effect on ketanserin, ritanserin and spiperone affinity (Johnson *et al.*, 1997; Shapiro *et al.*, 2000; Shapiro *et al.*, 2002). Other molecular modelling studies on serotonin receptors have suggested that aromatic residues in the transmembrane region, Trp151, Phe243, Phe244, Trp336, Phe339, Phe340, Trp367 and Tyr370 involved in van der Waals interactions and formed a hydrophobic pocket surrounding the agonist or antagonist ligands (Choudhary *et al.*, 1995; Kroeze *et al.*, 2002; Roth *et al.*, 1997b; Shapiro *et al.*, 2000).

3.4.6 Compound Selection from Docking Studies

It is well known that aporphinods can bind to at least three types of neurotransmitters including adrenergic, dopaminergic and serotonergic receptors. Therefore, identification of compounds that have favourable affinity and selectivity for 5-HT_{2A} receptor as therapeutic drugs and chemical leads is pivotal. Hence, in this study, boldine based on aporphine skeleton with *S*-configuration was selected as the reference

point because it was thought to bind with high affinity to 5-HT_{2A} receptor (Chung *et al.*, 2009b).

An investigation into the ligand-receptor interactions for the 5-HT_{2A} receptor revealed the formation of an electrostatic interaction and hydrogen bonding between the protonated amine group of (*S*)-boldine and the carboxylate group of the conserved Asp155 **(Figure 3.2)**. This observation was in agreement with the previous work reported by Indra *et al.*, (2002) who had also demonstrated that the conserved Asp155 in transmembrane region 3 forms a strong reinforced electrostatic interaction with the protonated amine group of aporphines. On the other hand, the hydrophobic part of the (*S*)-boldine that is defined by tetracyclic core (ring A, B, C, D) was surrounded by residues from transmembrane domains 3, 5, 6 and 7 and extracellular loop 2 including the non-aromatic residues (Val156, Ser159, Thr160, Leu229, Val235, Ser239, Ala242 and Val366) and aromatic residues (Phe234, Trp336, Phe339, Phe340 and Tyr370). Of these, Val156, Ser159, Thr160 and Phe234 involved in polar interactions or hydrogen bonding and Leu229, Ser239, Ala242, Trp336, Phe339, Phe340 and Tyr370 involved in van der Waals interactions. This is consistent with the work by (Braden *et al.*, 2006; Choudhary *et al.*, 1993; Roth *et al.*, 1997b) who reported the importance of the conserved aromatic residues (Trp336, Phe339, Phe340 and Tyr370) in the binding of 5-HT_{2A} receptor.

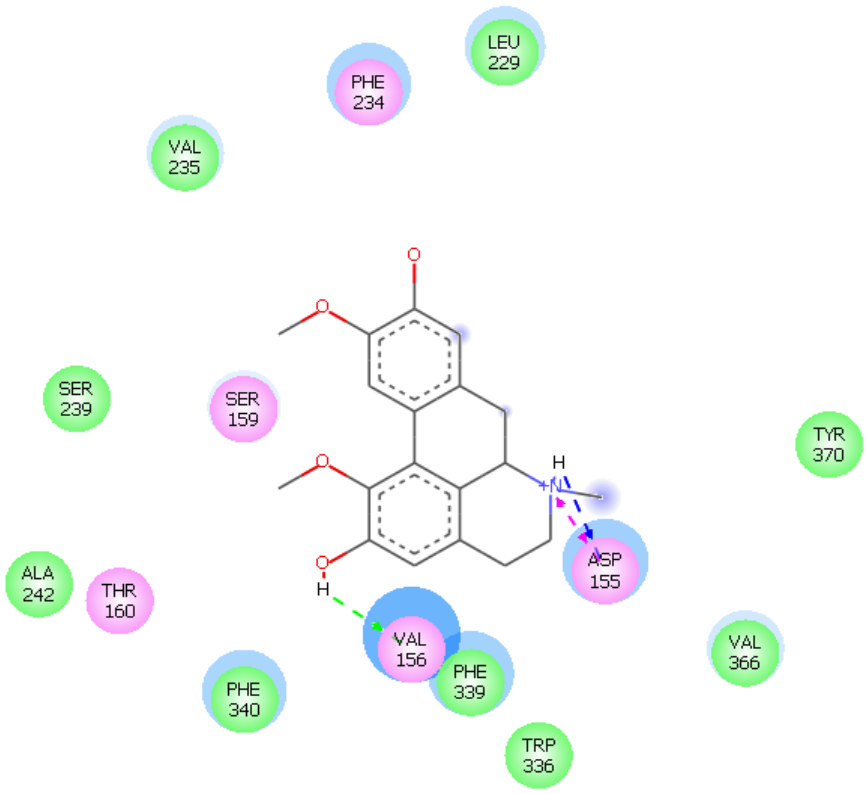


Figure 3.2: Proposed binding mode of (*S*)-boldine in the 5-HT_{2A} receptor. The residues involved in hydrogen bonding, electrostatic or polar interactions were represented in pink circles with their hydrogen bonds with their main chains (blue dashed arrow), side chains (green dashed arrow), charge interactions (pink dashed double-ended arrow) and residues involved in van der Waals interactions (green circles).

Through virtual screening against the 5-HT_{2A} receptor model, 13 purchasable compounds with higher or comparable activity to (*S*)-boldine were identified **(Table 3.1)**. Of these, four of them ((*R*) and (*S*)-laureline, (*S*)-nuciferine and (*S*)-isothebaine) were only available in racemic form. The selected compounds were assayed to determine their binding affinities towards dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptors.

Table 3.1: Computed K_i values of the selected compounds.

Compounds	6a	R_1	R_2	R_6	R_9	R_{10}	R_{11}	K_i (μM)
Laureline	*S*	O-CH_2-O		Me		OMe		2.81
2-OH-NPA	*R*		OH	Pr		OH	OH	5.45
Roemerine	*R*	O-CH_2-O		Me				5.82
Bulbocapnine	*S*	O-CH_2-O		Me		OMe	OH	6.82
NPA	*R*			Pr		OH	OH	6.73
Laureline	*R*	O-CH_2-O		Me		OMe		6.79
Isothebaine	*S*	OH	OMe	Me			OMe	10.94
Apomorphine	*R*			Me		OH	OH	11.43
Nuciferine	*S*	OMe	OMe	Me				12.51
Apomorphine	*S*			Me		OH	OH	13.70
Boldine	*S*	OMe	OH	Me	OH	OMe		15.32
Isocorydine	*S*	OMe	OMe	Me		OMe	OH	19.28
Corydine	*S*	OH	OMe	Me		OMe	OMe	25.07
Corytuberin	*S*	OH	OMe	Me		OMe	OH	28.80

NPA=*N*-propylapomorphine; 2-OH-NPA=2-hydroxy- *N*-propylapomorphine

Conclusion

A 3D molecular model of the rat 5-HT_{2A} receptor was constructed by homology modelling against the human β_2-adrenergic receptor. All standard 5-HT_{2A} receptor ligands were accommodated well at the putative ligand binding cleft, however there were slight variations in their interactions due to the different functional groups present. The constructed model is competent to be used as the starting structure to predict ligand-receptor interactions at the molecular level and future drug design. A set of 13 compounds were selected to determine their activities across the dopamine and serotonin receptors using (*S*)-boldine as the backbone structure.

4.0 Optimisation and Evaluation of Filtration Based Receptor Binding Assays for Dopamine (D_1 and D_2) and 5-Hydroxytryptamine (5-HT_{1A} and 5-HT_{2A})

4.1 Introduction

4.1.1 Properties of Receptor Binding Assays

Among the heterogeneous assays, filtration based receptor binding assays have been used widely for the biochemical identification and pharmacological characterization of the receptors. This assay is a traditional and trusted method which offer the advantages of less colour quenching, larger sample volumes, higher efficiency than the scintillation proximity assays and with a fewest development steps (De Jong *et al.*, 2005). There are two components of the radioligand binding including the specific binding to a targeted receptor sites and nonspecific binding to other receptor sites. The nonspecific binding is determined in the presence of excess unlabelled ligand to block the receptor binding sites of interest. Specific binding is defined as the difference between total binding observed in the absence of the unlabelled ligand and nonspecific binding.

It is important to meet the desired binding criteria for validating the radioligand binding assays (Mckinney, 1998; Scheinin *et al.*, 1994). These are the following criteria:

- the binding should be reversible and saturable with low ligand concentrations.
- nonspecific binding should be lower (less than 20% of the total binding).
- total binding should be equal or less than 10% of the radioligand added.
- more than 80% of the specific binding observed at K_d concentration of the radioligand.

- the binding should reach equilibrium and stable at the each concentration of the radioligand.
- the tissue and subcellular distribution of the specific binding should be consistent.
- correlation between the drug affinity *in vitro* and pharmacological potency *in vivo*.
- characteristic drug displacement with both agonists and antagonists.

The binding parameters of a ligand to its receptor also might be altered by other assay conditions including incubation time and temperature, buffer pH, filter plate type, washing times and concentration of radioligand, unlabelled ligand and membrane.

4.1.2 Selection of Receptor Source

In the brain, the D_1 and D_2 receptors are highly concentrated in the striatum and are also present at lower levels, in several other brain regions including the amyglada, cortex, globus pallidus, (Jackson and Westlind-Danielsson, 1994) hippocampus, hypothalamus, thalamus, substantia nigra and ventral tegmentum (Beaulieu and Gainetdinov, 2011; Missale *et al.*, 1998; Weiner *et al.*, 1991). The D_2 receptors are observed to be lower in cortical areas (De Keyser *et al.*, 1988; Hall *et al.*, 1991; Lidow *et al.*, 1989). Conversely, D_2 receptors are expressed at higher level in thalamus, hippocampus and brainstem (Hurd *et al.*, 2001; Jackson and Westlind-Danielsson, 1994; Weiner *et al.*, 1991). There is only a minimal amount of D_1 and D_2 receptors in the cerebellum (Beaulieu and Gainetdinov, 2011; Camps *et al.*, 1989). Radioligand-binding studies have shown that rat striatum is used widely as a D_1 and D_2 receptor source, where they are distributed abundantly (Billard *et al.*, 1984 ; Lo, 2007). Thus, this study

was carried out using striatum from male Sprague Dawley (SD) rat brain as D_1 and D_2 receptor source.

Within the brain, 5-HT_{1A} receptors are mainly found both pre- and postsynaptically, particularly with highest expression in the cerebral cortex, hippocampus, septum, amyglada and raphe nucleus and with lower levels of expression in the basal ganglia and thalamus (Barnes and Sharp, 1999; El Mestikawy *et al.*, 1991; Hoyer *et al.*, 2002; Pazos and Palacios, 1985a). On the other hand, 5-HT_{2A} receptors are primarily located postynaptically, where they are most densely distributed throughout the layers of cortex, notably in the neocortex, but they are found with much lower levels in brainstem, hippocampus and striatum (Barnes and Sharp, 1999; Cornea-Hebert *et al.*, 2002; Hoyer *et al.*, 2002; Pazos *et al.*, 1985b; Xu and Pandey, 2000). Radioligand binding studies have shown that either rat cerebral cortex or hippocampus is used as a 5-HT_{1A} receptor source (Arro *et al.*, 2001; Chung *et al.*, 2009a; Hall *et al.*, 1985; Nénonéné *et al.*, 1994; Nénonéné *et al.*, 1996) in contrast to either rat prefrontal cortex or cerebral cortex as receptor source for 5-HT_{2A} receptors (Chung *et al.*, 2009b; Harms *et al.*, 2000; Hoyer *et al.*, 1985). This study was conducted using the cerebral cortex from male Sprague Dawley (SD) rat brain as the 5-HT_{1A} and 5-HT_{2A} receptor source due to the higher yield of cerebral cortex.

4.1.3 Selection of Radioligand

A number of *in vitro* binding assays with radiolabelled drugs containing tritium, [^{3}H] or iodine, [^{125}I] have previously been developed to characterise the interaction of ligands with different types of receptors. Tritium is the radiolabel most commonly used because it has a long half life and is less hazardous, meaning that fewer handling precautions are required and larger quantities can be stored and disposed of. The choice

of radioligand used depends on if possessing high specific activity, affinity and selectivity toward the receptor being studied. The radioligand also should be pure and chemically stable in the assay medium used during incubation.

Quantitative autoradiographic as well as *in vitro* and *in vivo* binding studies have revealed the specificity and selectivity of [^{3}H] SCH 23390 in labelling D_1 receptors (Andersen and Groenvald, 1986a; Billard *et al.*, 1984 ; Dawson *et al.*, 1986). Thus, [^{3}H] SCH 23390 is designated as the most potent and selective antagonist radiotracer in ascribing the pharmacological and regional characteristics of D_1 receptors (Hyttel, 1983; Iorio *et al.*, 1983).

On the other hand, in case of D_2 receptors, [^{3}H] spiperone has been used in both *in vitro* and *in vivo* binding experiments as a potent antagonist radioligand with reasonable selectivity over D_2 receptors (Bischoff and Gunst, 1997; Chung *et al.*, 2005; Kalkman *et al.*, 2001; Lo, 2007; Madras *et al.*, 1988). In addition, [^{3}H] spiperone is also used for the visualization of D_2 receptor in rat brain using quantitative autoradiography (Boyson *et al.*, 1986).

For 5-HT_{1A} receptors, the prototypical radiolabelled agonist, [^{3}H] 8-OH-DPAT has been used extensively to characterize their physiological, biochemical and behaviours properties (Gozlan *et al.*, 1983; Hall *et al.*, 1985). It has also been widely used as a specific radioligand for both *in vitro* and *in vivo* studies of the 5-HT_{1A} receptor (Assie and Koek, 1996; Chung *et al.*, 2005; Chung *et al.*, 2009a; Hamon *et al.*, 1984). and for quantitative autoradiographic analysis to determine the regional distribution of 5-HT_{1A} receptors in rat brain (Pazos and Palacios, 1985a).

In the case of 5-HT_{2A} receptors [^{3}H] ketanserin has been found to be the radioligand of choice for *in vitro, in vivo* and autoradiographical studies to investigate the neuroanatomical localization, pharmacological characterization and functions of the receptor throughout the central nervous system (Assie *et al.*, 1988; Chung *et al.*, 2009b; Harms *et al.*, 2000; Leysen *et al.*, 1982; López-Giménez *et al.*, 1997; Pazos *et al.*, 1985b).

4.2 Materials

4.2.1 Chemicals and Instrumentation

[^{3}H] 8-OH-DPAT (150 Ci/mmol), [^{3}H] ketanserin HCl (67 Ci/mmol), [^{3}H] SCH 23390 (60 Ci/mmol) and [^{3}H] spiperone (15 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Bradford protein reagent, tris(hydroxymethyl)aminomethane, bovine serum albumin (BSA, fraction V), spiperone, serotonin hydrochloride, butaclamol hydrochloride, SCH 23390 hydrochloride, buspirone hydrochloride, (±)-propranolol hydrochloride, dicyclomine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvent and reagents were of analytical grade and purchased from Fisher Scientific (Loughborough, Leicestershire, UK).

96-well flat bottom Falcon plates were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA). TopSeal-A, Bottom Seal, MicroScint-O scintillation cocktail, UniFilter GF/C plates and UniFilter GF/B plates were supplied by Perkin Elmer (Wellesley, MA, USA).

The instruments used included a Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany) and a glass-teflon motor-driven Glas-Col homogenizer (Terre Haute, Indiana, USA), an ultra high speed centrifuge (Beckman Coulter's Optima L-100 K Ultracentrifuge, Brea, CA, USA), a UV-vis spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan), an incubator (Friocell 55, MMM Medcenter Einrichtungen GmbH, Grafelfing, Germany), a FilterMate Cell Harvester, a TopCount NXT microplate scintillation and a luminescence counter (Perkin Elmer).

4.3 Methodology

4.3.1 Preparation of Dopamine (D_1 and D_2) and 5-Hydroxytryptamine (5-HT_{1A} and 5-HT_{2A}) Membrane Receptors

All animal use techniques were in accordance with the guiding principles for the care and use of research animals promulgated by the Ethics Committee on Animal Welfare and Experimentation, Faculty of Medicine, University of Malaya, Malaysia (Reference number: FAR/05/11/2007/CLY (b) (R)). Male Sprague Dawley rats weighing approximately 200–400 g were killed by cervical dislocation and their brains were removed rapidly and stored at -80°C prior to use. Either rat striatum (for D_1 and D_2 receptors) or rat cerebral cortex (for 5-HT_{1A} and 5-HT_{2A} receptors) was dissected on ice, weighed and placed in 20 volumes (weight/volume) of ice-cold 50 mM Tris-HCl buffer at pH 7.4 **(Figure 4.1)**. The tissues were homogenized using an Ultra-Turrax homogenizer and a glass-teflon motor driven Glas-Col homogenizer at 2400 rpm on ice and centrifuged at 40,000g for 45 min at 4 °C. The supernatant was then discarded and the pellet was resuspended, homogenized and centrifuged for another 45 min. The resulting supernatant was discarded and the pellet was re-homogenized and incubated at 25 °C for 20 min to destroy endogenous dopamine or serotonin before being re-centrifuged as above. The final pellet was resuspended in 3 volumes of the same buffer.

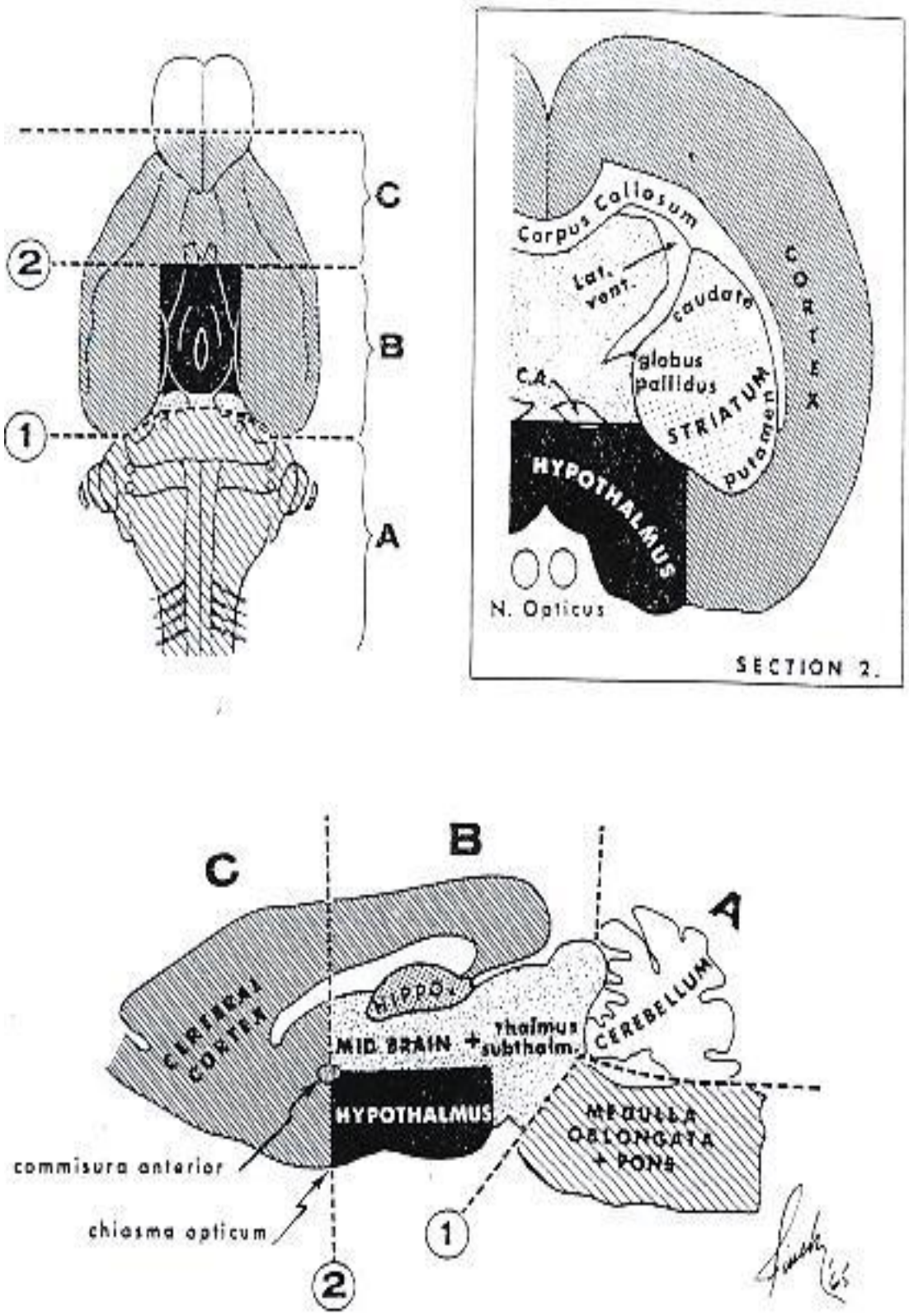


Figure 4.1: Diagrammatic representations of dissection procedure for rat brain. Dotted lines indicate positions of initial sections (adapted from Glowinski and Iversen, 1966).

4.3.2 Protein Determination

A standard curve for Bovine Serum Albumin (BSA) was constructed for the range of 0–2.0 mg/ml. The assay contained of 0.1 ml of protein standard and 0.1 ml of tissue sample, respectively. Thereafter, 3.0 ml of Bradford reagent was added and gently vortexed to ensure even mixing. The reaction was allowed to continue for 45 min at room temperature before the absorbance was measured at the wavelength 595 nm. Protein concentration estimations were calculated from the BSA standard curve plotted using PRISM® software, version 3.0 (Graphpad software, Inc, San Diego, CA, USA).

4.3.3 Receptor Binding Assays

Filtration based radioligand binding assays of dopamine (D_1 and D_2) and 5-hydroxytryptamine (5-HT_{1A} and 5-HT_{2A}) receptors were carried out according to the methods published previously (Billard *et al.*, 1984 ; Chung *et al.*, 2009b; Chung *et al.*, 2009a; Lo, 2007; Soo, 2006) with minor modifications.

4.3.3.1 Optimisation of Dopamine (D_1 and D_2) and 5-Hydroxytryptamine (5-HT_{1A} and 5-HT_{2A}) Receptor Binding Assays

All assays were performed in triplicate in a total volume of 250 µl according to the protocol summarized in **Table 4.1**. To optimise a variety of assay conditions, the reaction mixtures containing 200 µl of membrane suspension in 50 mM tris-HCl buffer at different pH values was incubated with 25 µl of radioligand for different periods of time at a variety of controlled temperatures. Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand. Incubations of the assay mixtures

were terminated by rapid filtration through GF/C or GF/B filters using a FilterMate Cell Harvester and washed with 200 µl of ice-cold 50 mM Tris-HCl buffer at pH 7.4. The filters were then dried at room temperature overnight and sealed at the bottom. Subsequently, 25 µl of MicroScint-O scintillation cocktail was added into each well and the plates were sealed on top. The radioactivity retained on the filters was quantified using a micro plate scintillation counter for 1 min per well.

4.3.3.2 Saturation Experiments

For the determination of K_d and B_{max} values by saturation binding assays, the reaction mixtures containing 200 µl of either striatum (30 µg/well (D_1) and 50 µg/well (D_2)) or cerebral cortex (100 µg/well (5-HT_{1A} and 5-HT_{2A})) were incubated with 25 µl of radioligand (0.003–1.0 nM [^{3}H] SCH 23390 for D_1 receptor, 0.003–2.0 nM [^{3}H] spiperone for D_2 receptor, 0.020–1.0 nM [^{3}H] 8-OH-DPAT for 5-HT_{1A} receptor or 0.003–0.8 nM [^{3}H] ketanserin for 5-HT_{2A} receptor). Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand (1 µM SCH 23390 for D_1, 100 µM butaclamol hydrochloride for D_2, 100 µM serotonin hydrochloride for 5-HT_{1A} or 100 µM spiperone for 5-HT_{2A}). Incubations of assay mixtures (90 min at 22 °C (D_1 and 5-HT_{2A}), 80 min at 22 °C (D_2), 90 min at 25 °C (5-HT_{1A})) were terminated by rapid filtration through a GF/C filter using a FilterMate Cell Harvester and washed with 200 µl of ice-cold 50 mM Tris-HCl buffer at pH 7.4 (4 times (D_1 and 5-HT_{2A}), 5 times (D_2), 3 times (5-HT_{1A})). The filters were then dried at room temperature overnight and sealed at the bottom. Subsequently, 25 µl of MicroScint-O scintillation cocktail was added into each well and the plates were sealed on top. The radioactivity retained on filters was quantified using a micro plate scintillation counter for 1 min per well.

4.3.3.3 Competition Experiments

For the determination of K_i values by competition binding assays, the reaction mixtures containing of 200 µl of either striatum (30 µg/well (D_1) and 50 µg/well (D_2)) or cerebral cortex (100 µg/well (5-HT_{1A} and 5-HT_{2A})) were incubated with 25 µl of 0.5 nM [^{3}H] SCH 23390 (D_1), 0.8 nM [^{3}H] spiperone (D_2), 0.4 nM [^{3}H] 8-OH-DPAT (5-HT_{1A}) or 0.5 nM [^{3}H] ketanserin HCl (5-HT_{2A}) and 25 µl of the standard unlabelled ligands or DMSO. Each of the standard unlabelled ligand or DMSO interference was tested at 9–12 varied concentrations. Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand (1 µM SCH 23390 for D_1, 100 µM butaclamol hydrochloride for D_2, 100 µM serotonin hydrochloride for 5-HT_{1A} or 100 µM spiperone for 5-HT_{2A}). The incubations were terminated, washed and dried and the radioactivity quantified as described earlier for the saturation experiments.

4.3.3.4 Z′ factor

For the determination of Z' factor, the reaction mixtures containing 200 µl of either striatum (30 µg/well (D_1) and 50 µg/well (D_2)) or cerebral cortex (100 µg/well (5-HT_{1A} and 5-HT_{2A})) were incubated with 25 µl of 0.5 nM [^{3}H] SCH 23390 (D_1), 0.8 nM [^{3}H] spiperone (D_2), 0.4 nM [^{3}H] 8-OH-DPAT (5-HT_{1A}) or 0.5 nM [^{3}H] ketanserin HCl (5-HT_{2A}). Nonspecific binding was determined by parallel incubations with 25 µl of reference ligand (1 µM SCH 23390 for D_1, 100 µM butaclamol hydrochloride for D_2, 100 µM serotonin hydrochloride for 5-HT_{1A} or 100 µM spiperone for 5-HT_{2A}). The incubations were terminated, washed and dried and the radioactivity quantified as described earlier for the saturation experiments.

Table 4.1: Summary of the values used for the different parameters involved in optimising the protocols for the radioligand binding assays.

Parameters	**Receptors**			
	D_1	**D_2**	**$5HT_{1A}$**	**$5HT_{2A}$**
Receptor source (SD rat)	striatum	striatum	cerebral cortex	cerebral cortex
Membrane (µg protein/well)	0–100	0–100	0–110	0–110
Radioligand	[^{3}H] SCH 23390	[^{3}H] spiperone	[^{3}H] 8-OH-DPAT	[^{3}H] ketanserin
Radioligand (nM)	0.003–1.0	0.003–2.0	0.020–1.0	0.003–0.8
Reference ligand	SCH 23390 HCl	butaclamol HCl	serotonin HCl	spiperone
Reference ligand (µM)	10^{-4}–10^{-13}	10^{-2}–10^{-13}	10^{-2}–10^{-13}	10^{-4}–10^{-13}
buffer pH (50 mM Tris-HCl)	7.4, 8.0, 9.0	7.4, 8.0, 9.0	7.4, 8.0, 9.0	7.4, 8.0, 9.0
Filter plate type	GF/C,GF/B	GF/C,GF/B	GF/C,GF/B	GF/C,GF/B
Incubation time (min)	0–300	0–300	0–180	0–120
Incubation temperature (°C)	4, 22, 37	4, 22, 37	4, 25, 37	4, 22, 37
Washing times	1–12	1–10	1–9	1–10
References	Billiard *et al*., 1984	Lo, 2007	Chung *et al*., 2009a Soo, 2006	Chung *et al*., 2009b Lo, 2007

4.4 Data and Statistical Analysis

The percentage of specific binding of radioligand in the presence of standard competitors or solvents was calculated using a standard data reduction algorithm as follows: ([B - NSB] / [TB - NSB]) × 100 where B is the binding in the presence of standard competitor or solvents, NSB is the non-specific binding in the presence of excess reference ligand and TB is the total binding.

The data from the radioligand binding assays was analyzed by non-linear regression and curve fitting programmes using prism Version 3.0 (GraphPad Inc., San Diego CA, USA). Equilibrium dissociation constant for the radioligand (K_d) and maximal binding capacity (B_{max}) values were determined from saturation binding assays with one-site binding hyperbola analysis using the equation, Specific binding = B_{max} × [L] / (K_d + [L]), where [L] was the concentration of radioligand used. The concentration causing 50% inhibition of specific binding *(*IC_{50}) values was determined from competition binding assays with one-site competition binding analysis using the equation, Specific binding = Bottom + (Top - Bottom) / (1+10 $^{([A] - Log\ IC_{50})}$), where [A] was the log concentration of competitor. K_i values were calculated from the IC_{50} values using the Cheng and Prusoff (1973) equation, K_i = IC_{50} / (1 + [L] / K_d), where [L] was the concentration of radioligand used.

The quality of the assays was evaluated using *Z′* factor according to the equation: 1-(3SD_{TB}+3SD_{NSB}) / ($Mean_{TB}$ - $Mean_{NSB}$) as described by Zhang *et al.*, (1999), where SD_{TB} is the standard deviation of total binding, SD_{NSB} is the standard deviation of non-specific binding, $Mean_{TB}$ is the mean of total binding, and $Mean_{NSB}$ is the mean of non-specific binding.

4.5 Results and Discussion

4.5.1 Protein determination

The protein concentration of dopamine (D_1 and D_2) and 5-hydroxytryptamine (5-HT_{1A} and 5-HT_{2A}) receptors was determined by extrapolation of the absorbance value using the standard curve **(Figure 4.2)** developed with the BSA standards. The estimated protein concentration of the receptors was used to prepare the membranes required for subsequent experiments.

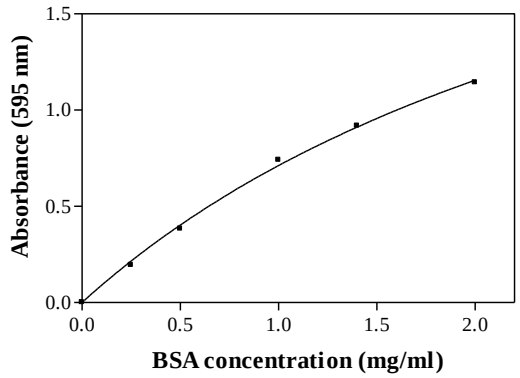


Figure 4.2: Standard curve of BSA concentration against absorbance for use in protein determination.

4.5.2 Effects of Different Physico-Chemical Properties on Dopamine (D_1 and D_2) and 5-Hydroxytryptamine (5-HT_{1A} and 5-HT_{2A}) Receptor Binding Assays

Initial optimisation of various parameters was carried out, particularly radioligand, membrane and unlabelled ligand concentration, buffer pH, filter plate type, incubation time and temperature, washing times and solvent interference to maximize total binding and minimise non-specific binding of the receptors.

4.5.2.1 Selection of Membrane Concentration

The choice of membrane concentration in incubation media is critical for receptor binding assays. Generally, the total amount of radioligand bound should be less than 10% of the total amount of radioligand added to avoid ligand depletion. Bylund and Toews (1993) reported that as the concentration of the membrane increased, the ratio of specific to nonspecific binding increased because a larger portion of the nonspecific binding is to glass fiber filters. The membrane concentration should be high enough so that at least 1000 cpm are bound when all receptors are occupied with radioligand, as this allows reasonable accuracy in counting the radioactivity at the lowest concentration of radioligand. Conversely, the membrane concentration also should not be too high because elevated membrane concentration could cause filter clogging and prolonged aspiration time (Harms *et al.*, 2000).

The effect of increasing the membrane concentration in the $D_{1,}$ D_2, 5-HT_{1A} and 5-HT_{2A} binding assays is shown in **Figure 4.3(a)**, **4.4(a)**, **4.5(a) and 4.6(a**) respectively. The specific binding of D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors observed at 30 µg of protein/well, 50 µg of protein/well, 100 µg of protein/well and 100 µg of protein/well each yielded 90%, 89%, 90% and 92% of the total binding respectively. Under saturating conditions, the observed nonspecific binding was about 8–11%, suggesting that there is no significant depletion of the radioligand during the course of the assay. Thus, the membrane concentration of 30 µg of protein/well for D_1, 50 µg of protein/well for D_2 and 100 µg of protein/well for 5-HT_{1A} and 5-HT_{2A} were selected for subsequent assays to achieve maximal signal to background ratio binding.

4.5.2.2 Selection of Unlabelled Ligand and its Concentration

The choice of the unlabelled ligand and its concentration to determine nonspecific binding is critical in receptor binding assays. Usually, the unlabelled ligand should be chemically different from the radioligand to avoid the labelling of specific nonreceptor sites (Bylund and Toews, 1993). The chosen unlabelled ligand should possess high specificity and selectivity towards the receptor being studied. As a general rule, the concentration of the unlabelled ligand should be 100 to 1000 fold in excess of its K_d value (Hulme and Trevethick, 2010).

The amount of radioligand bound to the D_1**(Figure 4.3(b))**, D_2**(Figure 4.4(b))**, 5-HT_{1A} **(Figure 4.5(b))** and 5-HT_{2A} **(Figure 4.6(b))** receptors decreased with increasing concentration of unlabelled ligand until a point where no matter how much unlabelled ligand is added, the amount of radioligand bound remains constant. This remaining binding of radioligand represents the nonspecific binding. In the D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptor binding assays, this plateau was reached at 1 µM of *R*-(+)-SCH23390 hydrochloride, 100 µM of butaclamol hydrochloride, 100 µM of serotonin hydrochloride and 100 µM of spiperone respectively. These concentrations were used in subsequent assays to obtain minimal background binding.

4.5.2.3 Selection of pH

The pH is critical for receptor binding assays. Commonly, tris(hydroxymethyl) aminomethane (tris-HCl) is often used as the homogenization or assay buffer that yields maximum binding. The pH of the buffer should be in physiological range, between pH 7 and 8, so that the *in vitro* binding assays are conducted in an isotonic medium and to

be comparable with what is seen *in vivo* (Bylund and Toews, 1993). Furthermore, a wide range of buffer pH could affect the ligand receptor interaction due to changing of the degree of ionisation of groups within the receptor or ligand, which may influence both the equilibrium binding constant and the kinetic rate constant.

In general, pH variations from 7.4 to 9.0 caused a minimal variation in binding of the radioligand to the D_1 **(Figure 4.3(c))**, D_2 **(Figure 4.4(c))**, 5-HT_{1A} **(Figure 4.5(c))** and 5-HT_{2A} **(Figure 4.6(c))** receptors. Nevertheless, the highest level of total binding and lowest level of nonspecific binding were observed at pH 7.4 followed by pH 8.0 and pH 9.0. The signal to background ratio of pH 7.4 was slightly higher as compared to pH 8.0 and pH 9.0. Thus, subsequent assays for the D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptor were performed at physiological pH, 7.4 as it ensured the maximal signal binding and minimal background binding.

4.5.2.4 Selection of Filter Plate Type

Vacuum filtration is a widely used method in separating bound and free radioligand by rapid filtration through filter plates (Bylund and Toews, 1993). As a general rule, filtration is unsuitable for radioligands with K_d $>10^{-8}$ M (Hulme and Trevethick, 2010). The most commonly used filter plates are GF/B (1.0 µm), GF/C (1.2 µm) and Durapore (0.22, 0.65, 1.0 µm). In some cases, pre-treatment of filter plates with polyethylenimine (PEI) gives them a positive surface charge that helps to capture negatively charged receptors and substantially improve the specific to nonspecific binding ratio (Hulme and Trevethick, 2010).

For the D_1 **(Figure 4.3(d))**, D_2 **(Figure 4.4(d))**, 5-HT_{1A} **(Figure 4.5(d))** and 5-HT_{2A} **(Figure 4.6(d))** receptors, total binding reached its highest value using the GF/C filters at all concentrations of radioligand. The nonspecific binding was also lower using the GF/C filters as compared to GF/B. Thus, subsequent assays were performed using GF/C filters for the D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors.

4.5.2.5 Selection of Incubation Temperature

It is important to control the temperature to minimise the variability in receptor binding assays. Bylund and Toews (1993) suggested that room temperature (~ 22°C) is most suitable to perform the assays. However, some assays work better at physiological temperature (37°C), as this allow the results to be compared to *in vivo* ligand receptor behaviour. In some instances, receptor binding assays are carried out below room temperature, especially on ice (4°C) to prevent degradation of peptide ligands by proteolytic enzyme. Such temperature variation can produce conformational changes in receptors and other proteins in lipid bilayer and provide insight into the molecular nature of ligand receptor interaction.

Maximum total binding for the D_1 **(Figure 4.3(e))**, D_2 **(Figure 4.4(e))**, and 5-HT_{2A} **(Figure 4.6(e))** receptors were achieved at 22°C and for 5-HT_{1A} **(Figure 4.5(e))** achieved at 25°C with all concentrations of radioligand. In addition, the estimated level of nonspecific binding was also minimal for all the receptors. Decreases in total binding were measured at 4 and 37°C in all assays. Thus, the remaining assays were conducted at 22°C for the D_1, D_2, and 5-HT_{2A} receptors and 25°C for the 5-HT_{1A} receptor.

4.5.2.6 Selection of Incubation Time

In receptor binding assays, the assay medium must be incubated for sufficient time to ensure that equilibrium has been reached, where the rate of association of the ligand receptor complex is equal to the rate of dissociation. The time required for complexes to achieve equilibrium depends on the temperature of the radioligand (Bylund and Toews, 1993). Most commonly used radioligands reach steady state conditions within 20–60 minutes.

Equilibrium was obtained after approximately 90 minutes for the D_1 **(Figure 4.3(f))**, 5-HT_{1A} **(Figure 4.5(f))**, and 5-HT_{2A} **(Figure 4.6(f))** receptors and 80 min for D_2 **(Figure 4.4(f))** receptor. After these times the total and specific binding observed for each receptor was constant as a function of incubation time, suggesting that a steady state has been attained. Thus, all further assays were carried out with an incubation period of time of 90 minutes for the D_1, 5-HT_{1A} and 5-HT_{2A} receptors and 80 minutes for the D_2 receptor.

4.5.2.7 Selection of Washing Times

The method to separate bound and unbound radioligand after the incubation medium has attained equilibrium involves aspiration on to filters where the receptor and bound radioligand are trapped, while unbound radioligand passes through the filter plates. The filters are then washed repeatedly with ice cold buffer using a FilterMate Cell Harvester to remove unbound radioligand, as this reduces nonspecific binding (Bylund and Toews, 1993). As a rule of thumb, ice cold wash buffer is recommended as it can extend the usable range by up to 100 fold ((K_d = 10^{-7}–10^{-6} M) (Hulme and Trevethick, 2010).

For the D_1 **(Figure 4.3(g))**, D_2 **(Figure 4.4(g))**, 5-HT_{1A} **(Figure 4.5(g))** and 5-HT_{2A} **(Figure 4.6(g))** receptors, as the number of washing times increased, the counting for total binding and nonspecific binding decreased. At a certain point, further washings had only negligible effects on the nonspecific binding. Thus, 3 washings for the 5-HT_{1A}, 4 washings for the D_1 and 5-HT_{2A} and 5 washings for the D_2 receptor were adequate for efficient removal of any free radioligand without disruption of the bound complexes.

4.5.2.8 Solvent Interference

Due to its high solvent power, low chemical reactivity and toxicity and high flash point and boiling point, dimethyl sulfoxide (DMSO) has been used extensively to dissolve a great variety of chemical classes to evaluate their biological activities (Balakin, 2003). The range of concentrations in binding assays is prepared by serially diluting a compound from the initial DMSO stock solution, which is the highest concentration of compound. Incomplete solubility of the compounds at the highest concentration results in a shift of the concentration curve and the IC_{50} will be estimated based on this 'apparent' concentration curve. The false, higher IC_{50} will classify the compound as less active (Di and Kerns, 2006).

As the concentration of DMSO increased from 0.5–10% v/v, the percentage of inhibition increased for the D_1 receptor **(Figure 4.3(h))**. For the 5-HT_{1A} receptor **(Figure 4.5(h))**, the percentage of inhibition increased from 0.5–4% v/v and there were no significant trend from 4–10% v/v. Whereas, concentrations of DMSO as high as 10% had no effect for the D_2 **(Figure 4.4(h))** and 5-HT_{2A} receptor **(Figure 4.6(h))** binding assays. Thus, the concentration of DMSO chosen for the D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptor assays was up to 3% which gave less than 20% of inhibition.

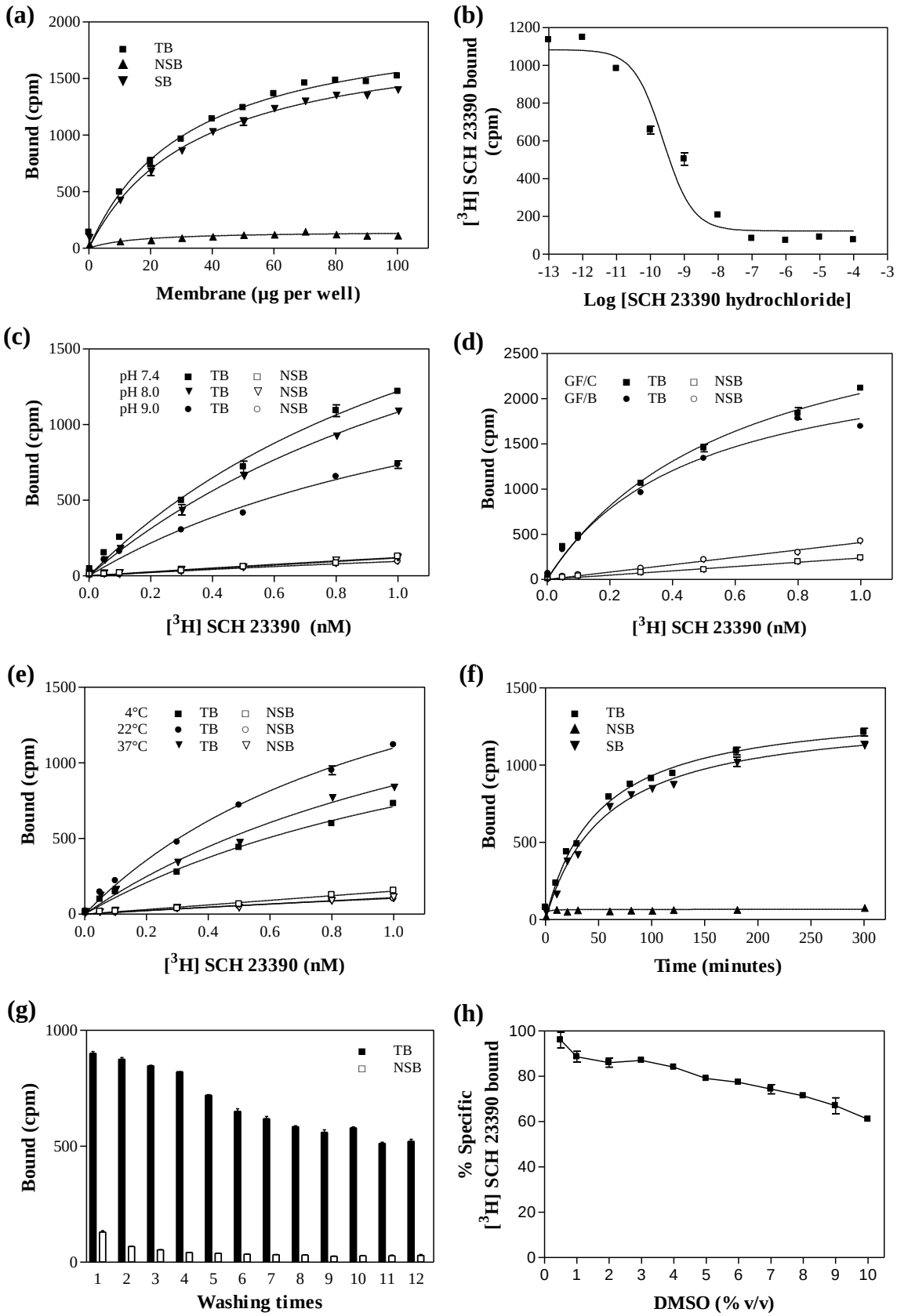


Figure 4.3: Optimisation of D_1 receptor binding assay. Selection of **(a)** membrane concentration, **(b)** unlabelled ligand concentration, **(c)** pH, **(d)** filter plate type, **(e)** incubation temperature, **(f)** incubation time, **(g)** washing times and **(h)** DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM.

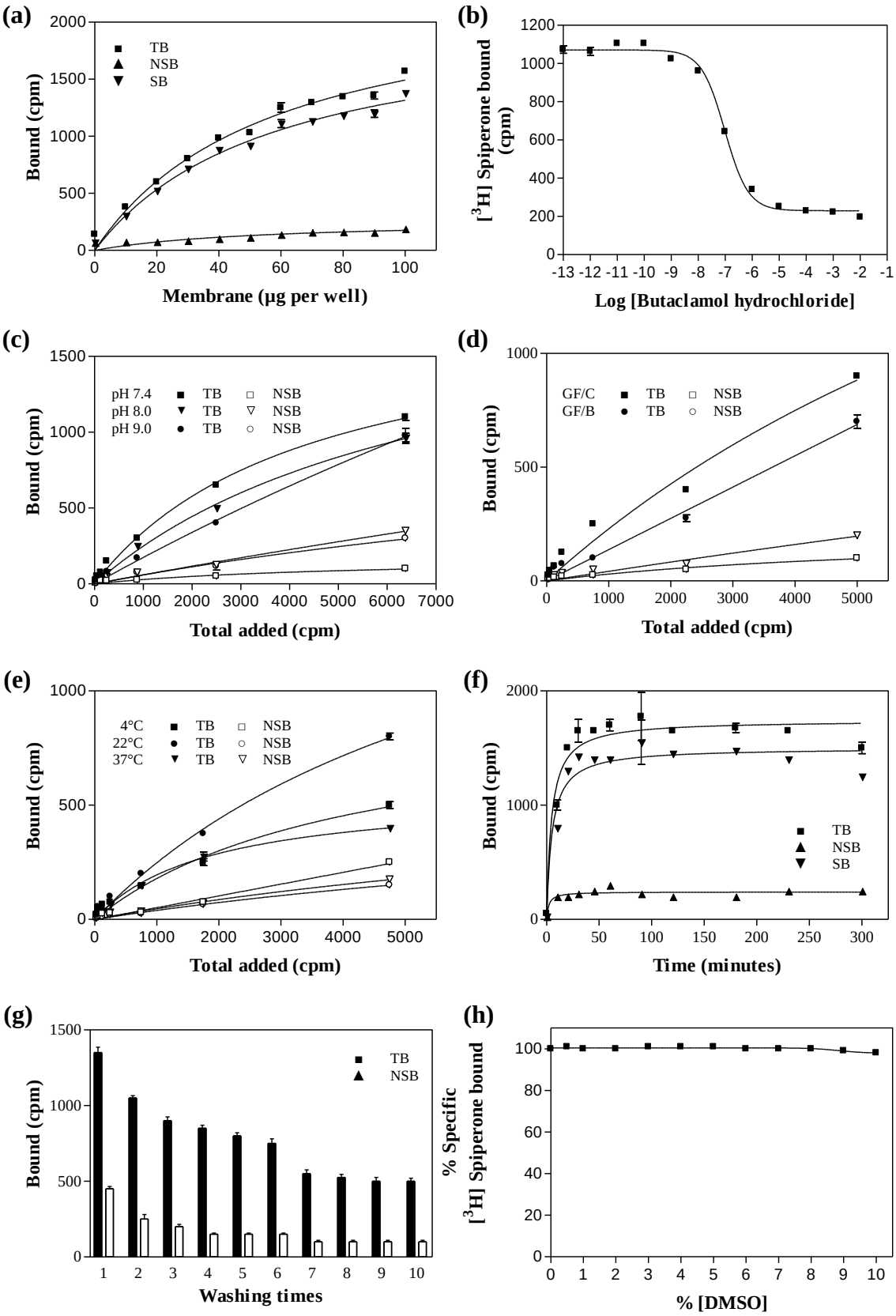


Figure 4.4: Optimisation of D_2 receptor binding assay. Selection of **(a)** membrane concentration, **(b)** unlabelled ligand concentration, **(c)** pH, **(d)** filter plate type, **(e)** incubation temperature, **(f)** incubation time, **(g)** washing times and **(h)** DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM. **(c)** – **(h)** adapted from Lo (2007).

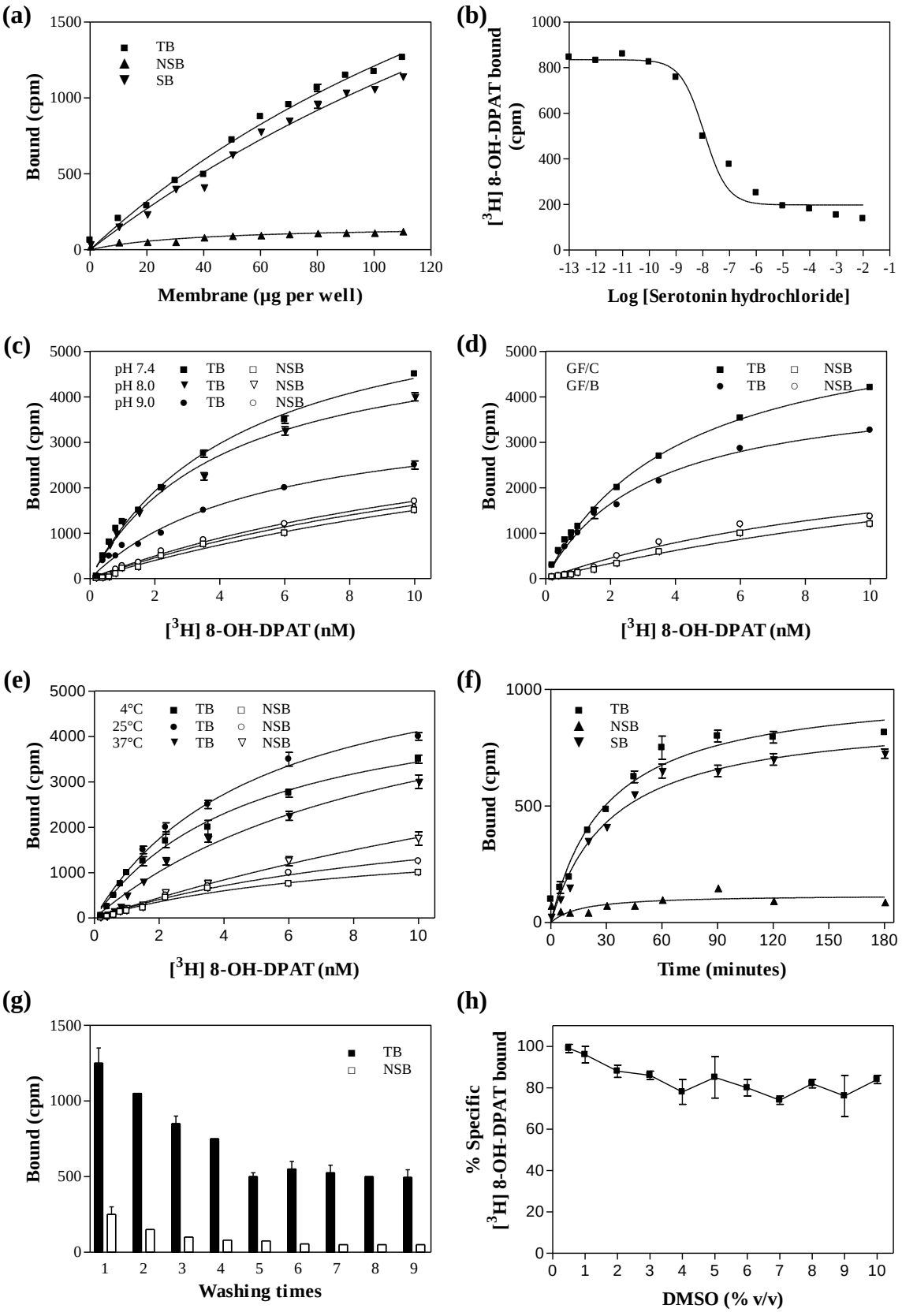


Figure 4.5: Optimisation of 5-HT_{1A} receptor binding assay. Selection of **(a)** membrane concentration, **(b)** unlabelled ligand concentration, **(c)** pH, **(d)** filter plate type, **(e)** incubation temperature, **(f)** incubation time, **(g)** washing times and **(h)** DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM. **(c)**, **(e)** – **(h)** adapted from Soo (2006).

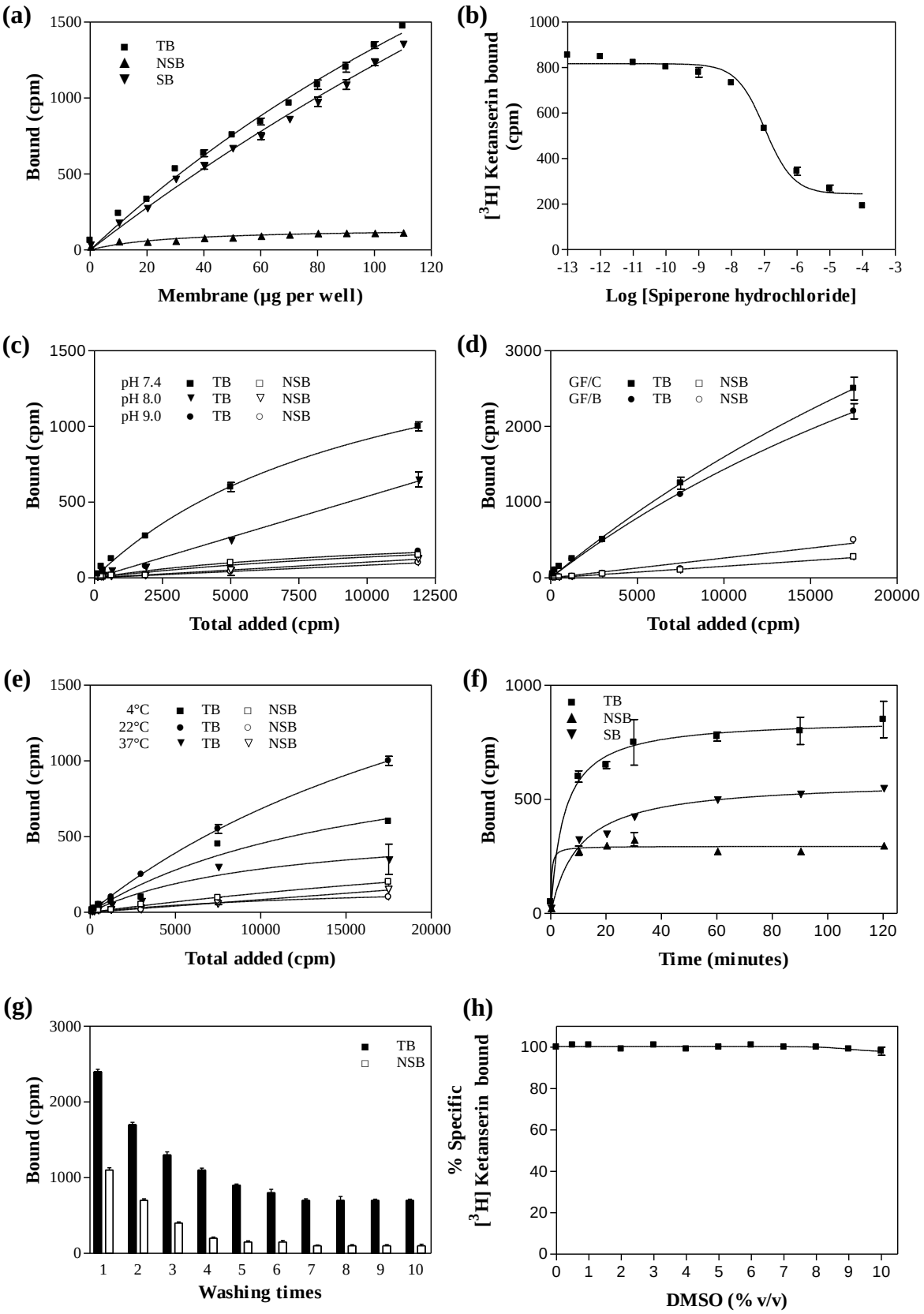


Figure 4.6: Optimisation of 5-HT_{2A} receptor binding assay. Selection of **(a)** membrane concentration, **(b)** unlabelled ligand concentration, **(c)** pH, **(d)** filter plate type, **(e)** incubation temperature, **(f)** incubation time, **(g)** washing times and **(h)** DMSO effect. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM. **(c)** – **(h)** adapted from Lo (2007).

4.5.2.9 Saturation Experiments

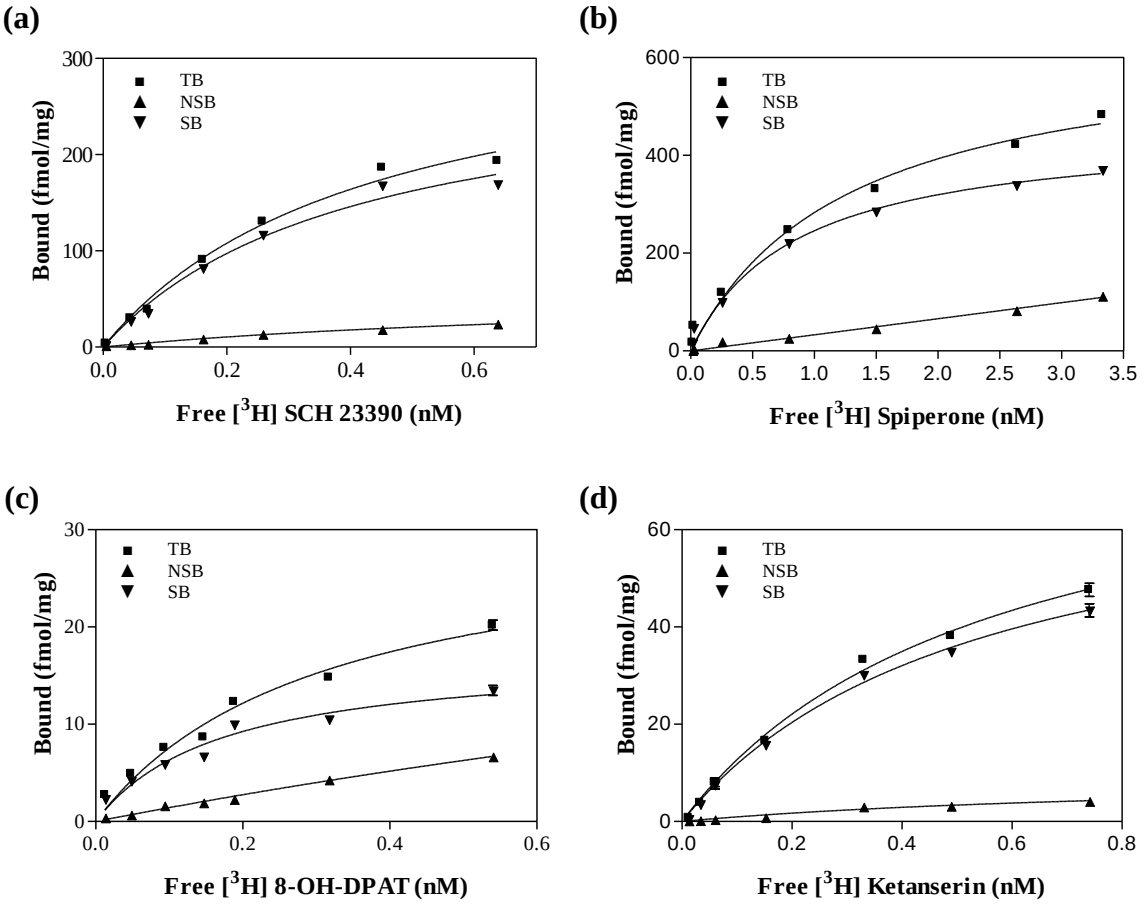


Figure 4.7: Saturation binding curve of **(a)** [^{3}H] SCH23390 for D_1 receptor, **(b)** [^{3}H] Spiperone for D_2 receptor, **(c)** [^{3}H] 8-OH-DPAT for 5-HT_{1A} receptor and **(d)** [^{3}H] Ketanserin for 5-HT_{2A} receptor. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=3) ± SEM.

The respective binding of [^{3}H] SCH23390 **(Figure 4.7(a))**, [^{3}H] Spiperone **(Figure 4.7(b))**, [^{3}H] 8-OH-DPAT **(Figure 4.7(c))** and [^{3}H] Ketanserin **(Figure 4.7(d))** to D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors was saturable, specific and of high affinity. The equilibrium dissociation constants (K_d) were estimated to be 0.40 nM ([^{3}H] SCH23390; D_1), 0.86 nM ([^{3}H] Spiperone; D_2), 0.17 nM ([^{3}H] 8-OH-DPAT; 5-HT_{1A}), and 0.54 nM ([^{3}H] Ketanserin; 5-HT_{2A}). The maximal binding capacities (B_{max}) were estimated to be 292.80 fmol/mg, 456.20 fmol/mg, 17.23 fmol/mg, and 74.98 fmol/mg for D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors, respectively. These values were similar to those of (Billard *et al.*, 1984 ; Chu *et al.*, 2001) (D_1), (Chu *et al.*, 2001; Cresee *et al.*, 1977) (D_2), (Hoyer *et al.*,

1985; Peroutka *et al.*, 1986) (5-HT_{1A}) and (Chung *et al.*, 2009b; Leysen *et al.*, 1982) (5-HT_{2A}).

4.5.2.10 Competition Experiments

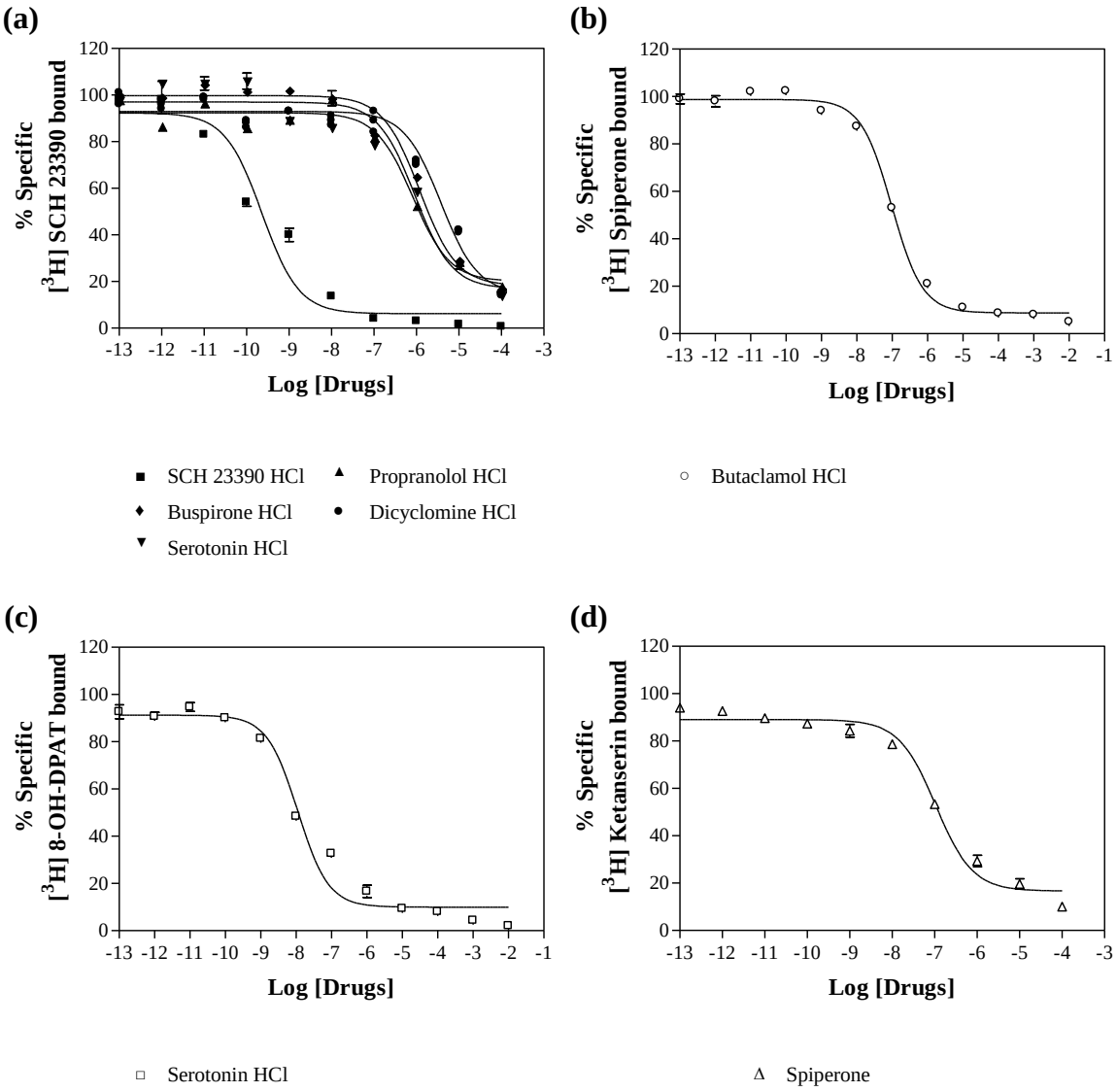


Figure 4.8: Competition binding curve of **(a)** [^{3}H] SCH23390 for D_1 receptor, **(b)** [^{3}H] spiperone for D_2 receptor, **(c)** [^{3}H] 8-OH-DPAT for 5-HT_{1A} receptor and **(d)** [^{3}H] ketanserin for 5-HT_{2A} receptor. Results are represented as mean (n=3) ± SEM.

Table 4.2 shows the K_i values obtained from the competition experiments with standard unlabelled ligands on the D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors. For comparison, **Table 4.2** also includes previously reported K_i values for the D_2, 5-HT_{1A} and 5-HT_{2A} receptors tested using a similar methodological approach.

For the D_1 receptor binding assay, the relative potency for displacing [^{3}H] SCH23390 was *R*-(+)-SCH23390 HCl > propranolol HCl > serotonin HCl > buspirone HCl > dicyclomine HCl **(Table 4.2)**. Thus, *R*-(+)-SCH23390 HCl with a K_i value of 1.03×10^{-10} M is a suitable ligand for defining [^{3}H] SCH23390 binding to the D_1 receptor **(Figure 4.8(a))**. This value is very similar to K_i value reported by Andersen and Braestrup (1986b).

For the D_2 receptor binding assay, the relative potency for displacing [^{3}H] Spiperone was butaclamol HCl > serotonin HCl > propranolol HCl > buspirone HCl > (±)-8-OH-DPAT > dicyclomine HCl **(Table 4.2)**. Thus, butaclamol HCl with a K_i value of 5.06×10^{-8} M is a suitable ligand for defining [^{3}H] spiperone binding to the D_2 receptor **(Figure 4.8(b))**. This value is very similar to K_i value reported by Lo (2007).

For the 5-HT_{1A} receptor binding assay, the relative potency for displacing [^{3}H] 8-OH-DPAT was (±)-8-OH-DPAT > serotonin HCl > buspirone HCl > propranolol HCl > scopolamine **(Table 4.2)**. Thus, serotonin HCl with a K_i value of 3.33×10^{-9} M is a suitable ligand for defining [^{3}H] 8-OH-DPAT binding to 5-HT_{1A} receptor **(Figure 4.8(c))** (Chung *et al.*, 2009a; Hoyer *et al.*, 1985; Peroutka *et al.*, 1986; Schlegel and Peroutka, 1986).

For the 5-HT_{2A} receptor binding assay, the relative potency for displacing [^{3}H] ketanserin was spiperone > phentolamine HCl > buspirone HCl > serotonin HCl > (±)-8-OH-DPAT > (±)-propranolol HCl **(Table 4.2)**. Thus, spiperone with a K_i value of 5.51×10^{-8} M is a suitable ligand for defining [^{3}H] ketanserin binding to the 5-HT_{2A} receptor **(Figure 4.8(d))**. This value is very similar to K_i value reported by Chung *et al*., (2009b).

Table 4.2: K_i values obtained from competition experiments with standard unlabelled ligands on D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors.

Standard unlabelled ligands	Physiological mechanisms	K_i (M)			
		D_1	**D2**	**5-HT_{1A}**	**5-HT_{2A}**
8-OH-DPAT	5-HT_{1A} receptor agonism	-	1.43×10^{-6} [b]	1.0×10^{-9} [c]	6.32×10^{-5} [b]
spiperone	Selective D_2 receptor antagonism and mixed 5-HT_1/ 5-HT_2 antagonism	-	-	-	5.51×10^{-8}
(+)-butaclamol HCl	D_2 receptor antagonism	-	5.06×10^{-8}	-	-
R-(+)-SCH23390 HCl	D_1 receptor antagonism	1.03×10^{-10}	-	-	-
propranolol HCl	β-adrenergic receptor antagonism and mixed 5-HT_1/ 5-HT_2 antagonism	3.67×10^{-7}	0.36×10^{-6} [b]	1.37×10^{-7} [a]	1.23×10^{-4} [b]
serotonin HCl	5-HT receptor agonism	4.11×10^{-7}	0.13×10^{-6} [b]	3.33×10^{-9}	2.39×10^{-5} [b]
buspirone HCl	5-HT_{1A} receptor agonism	5.34×10^{-7}	0.86×10^{-6} [b]	1.5×10^{-8} [c]	3.24×10^{-6} [b]
dicyclomine HCl	M_1 muscarinic receptor antagonism	1.68×10^{-6}	12.2×10^{-6} [b]	-	-
phentolamine HCl	α-adrenergic receptor antagonism and 5-HT_{1A} receptor antagonism	-	-	-	2.16×10^{-6} [b]
scopolamine	Muscarinic receptor antagonism	-	-	1.1×10^{7} [a]	-

Data were taken from Soo, 2006[a]; Lo, 2007[b]; Peroutka, 1986[c]

4.5.2.11 Z′ factor

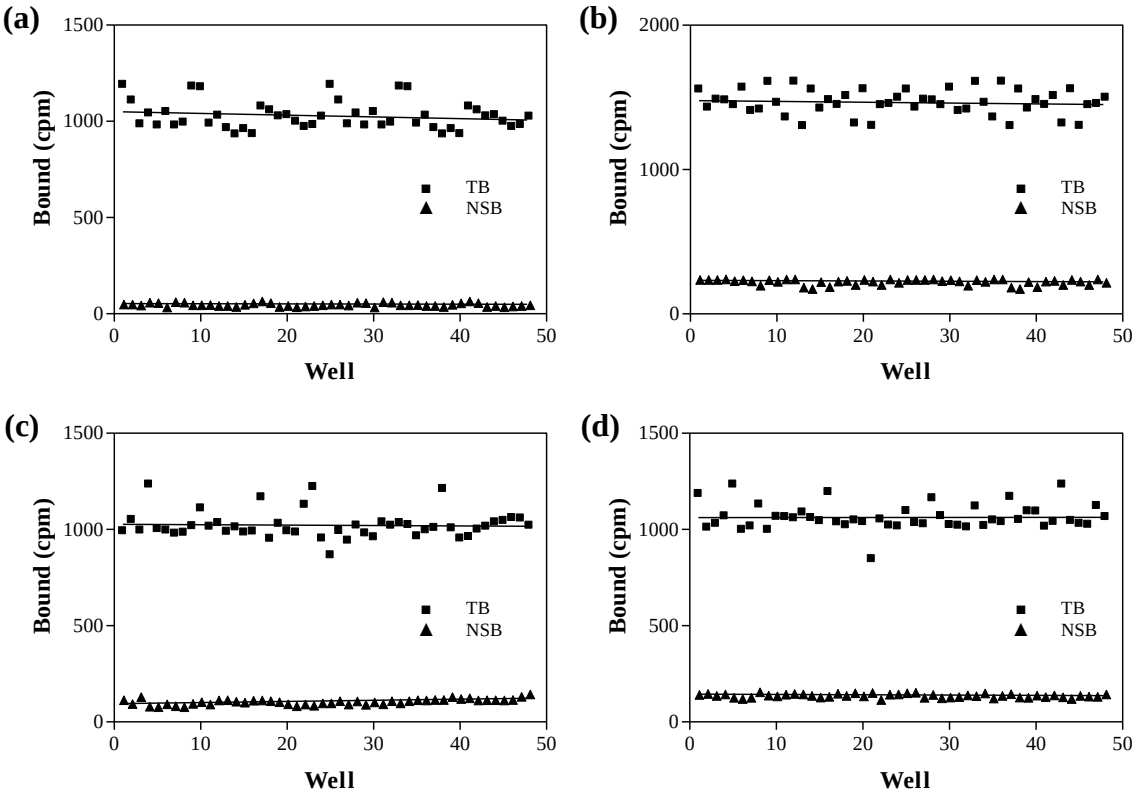


Figure 4.9: Z′ analysis for **(a)** D_1, **(b)** D_2, **(c)** 5-HT_{1A} and **(d)** 5-HT_{2A} receptors. TB=Total binding, NSB=Nonspecific binding, SB=Specific binding. Results are represented as mean (n=48) ± SEM.

To confirm the robustness of the assays, a Z′ analysis was performed for the D_1 **(Figure 4.9(a))**, D_2 **(Figure 4.9(b))**, 5-HT_{1A} **(Figure 4.9(c))** and 5-HT_{2A} **(Figure 4.9(d))** receptors each using 48 replicate values for both total binding and nonspecific binding. For each binding assay, the Z′ value yielded more than 0.5, which was within the acceptable range (0.5–1.0) and confirmed the robustness of the assay **(Table 4.3)**. The percentage of coefficient of variability (CV) for total binding and nonspecific binding for each assay was less than 20, suggesting minimal variation and good reproducibility of the assay

Table 4.3: Data from Z' factor analysis for the D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors.

Receptor	Z′factor	S/B	Mean		SD		CV (%)	
			TB	NSB	TB	NSB	TB	NSB
D_1	0.75	20.11	1028.08	51.13	72.86	8.83	7.09	17.27
D_2	0.74	6.44	1464.38	227.25	86.31	19.83	5.89	8.73
5-HT_{1A}	0.72	9.36	1021.92	109.17	71.08	14.73	6.96	13.49
5-HT_{2A}	0.75	7.56	1062.67	140.56	67.21	9.55	6.32	6.80

TB=Total binding, NSB=Nonspecific binding, S/B= Signal to background ratio; SD= Standard deviation; CV= Coefficient of variation

4.6 Conclusion

The optimised assay conditions for the dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptor binding assays were summarized in **Table 4.4**. Saturation binding assays of each receptor revealed a K_d and B_{max} of 0.40 nM and 292.80 fmol/mg (D_1), 0.86 nM and 456.20 fmol/mg (D_2), 0.17 nM and 17.23 fmol/mg (5-HT_{1A}), and 0.54 nM and 74.98 fmol/mg (5-HT_{2A}), respectively. The inhibition constant (K_i) for the respective competitive/reference ligands were 0.10 nM (SCH23390 HCl; D_1), 50.60 nM (butaclamol HCl; D_2, 3.33 nM (serotonin HCl; 5-HT_{1A}) or 5.10 nM (spiperone; 5-HT_{2A}). The respective Z' values were 0.75, 0.74, 0.72 and 0.75 for the D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors, indicated that the adopted assays were suitable, robust and reliable for medium throughput screening.

Table 4.4: Optimised assay conditions for D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors.

Parameters	**Receptors**			
	D_1	**D_2**	**$5HT_{1A}$**	**$5HT_{2A}$**
Receptor source (SD rat)	striatum	striatum	cerebral cortex	cerebral cortex
Membrane (µg protein/well)	30	50	100	100
Radioligand	[^{3}H] SCH 23390	[^{3}H] Spiperone	[^{3}H] 8-OH-DPAT	[^{3}H] Ketanserin
Radioligand (nM)	0.5	0.8	0.4	0.5
Reference ligand	*R*-(+)-SCH23390 HCl	butaclamol HCl	serotonin HCl	spiperone
Reference ligand (µM)	1	100	100	100
pH buffer (50 mM Tris-HCl)	7.4	7.4	7.4	7.4
Filter plate type	GF/C	GF/C	GF/C	GF/C
Incubation time (min)	90	80	90	90
Incubation temperature (°C)	22	22	25	22
Washing times	4	5	3	4

5.0 Virtual and Biomolecular Screening of Potential Ligands for 5-HT_{2A} Receptor

5.1 Introduction

5.1.1 Structure Activity Relationship (SAR) of Aporphines

In the realm of central nervous system (CNS) activity, naturally occurring aporphines and their synthetic derivatives are known to have affinity and selectivity for adrenergic, dopaminergic and serotonergic receptors (Cabedo *et al.*, 2009; Rios *et al.*, 2000; Zhang *et al.*, 2007). The structural modifications on the tetracyclic aporphine skeleton with varying substituents can lead to major changes in its pharmacological activity. Since there is no 3D X-ray structures for any of dopamine and serotonin receptors, the information gained from structure activity relationship (SAR) studies are important for the development of structure-based drug design.

For example, structure activity relationships of 2-substituted aporphines with aryl (Sondergaard *et al.*, 2005), *O*-alkyl (Sipos *et al.*, 2008), methylthio (Toth *et al.*, 2006), or halogen (Neumeyer *et al.*, 1990; Ramsby *et al.*, 1989; Zhang *et al.*, 2005) substituents suggest their potential to increase selectivity and affinity at D_2 receptors due to the existence of a lipophilic cleft or region on the surface of D_2 receptor proteins. 11-hydroxyaporphines, and especially their esters, have more prolonged behavioural arousal-inducing activity with far superior oral bioavailability (Csutoras *et al.*, 2004). Aporphines containing a single hydroxyl group at C-11 have some affinity for the D_1 dopamine receptor and properties as D_1 antagonists (Schaus *et al.*, 1990).

High affinities to 5-HT_{1A} receptors are displayed by a number of aporphines carrying sterically and electronically different C-11 substituents (Hedberg *et al.*, 1996).

The *N*-substituted 11-hydroxy-10-hydroxylmethyl aporphine and 11-hydroxy-2-methoxy-10-methyl congeners displayed selective and potent affinity for the 5-HT_{1A} receptor but low affinity at D_1 and D_2. These findings support that ortho-dihydroxy substitution in the aporphine D ring enhances affinity to dopamine receptors, whereas analogous methyl or hydroxymethyl substitution enhances interactions with serotonin 5-HT_{1A} receptor (Si *et al.*, 2007).

Removal of the 11-hydroxy group from the 2-methoxy-11-hydroxy-*N*-alkylaporphines decreased affinity at dopamine receptors, and afforded low affinity for the serotonin 5-HT_{1A} receptor (Si *et al.*, 2008). Alkylation of the 11-hydroxy group with an alkyl, but adding a hydroxy substituent on the 2-position also decreased the affinities at dopamine and serotonin receptors, supporting the assumption that 11-hydroxy substitution in aporphines is required for dopaminergic and serotonergic activity.

5.2 Materials

The test compounds were purchased from MolPort Ltd (Riga, Latvia). Details of all the other chemicals used are given in **Section 4.2.1**.

5.3 Methodology

5.3.1 Receptor Binding Assays

The compounds selected from the docking studies on the 5-HT_{2A} receptor model were tested experimentally against the dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptors according to protocol described previously **(see Section 4.3.3.3)**.

5.4 Results and Discussion

5.4.1 Receptor Binding Assays

The compounds selected from the docking studies on the 5-HT_{2A} receptor model were assayed *in vitro* for their ability to displace [^{3}H] SCH 23390, [^{3}H] spiperone, [^{3}H] 8-OH-DPAT, and [^{3}H] ketanserin binding to rat D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors, respectively. The results are summarized in **Table 5.1**. The results indicate that (*R*)-roemerine has the most favourable affinity-selectivity profiles for the 5-HT_{2A} receptor and has 20–400-fold higher affinity towards the 5-HT_{2A} receptor than towards D_1, D_2 and 5-HT_{1A} receptors **(Figure 5.1(a))**. This finding confirms previous work by Chen and co-workers which showed that (*R*)-roemerine has selectivity for 5-HT_2 receptors over D_1, D_2 and 5-HT_1 receptors (Chen *et al.*, 1987). Compared to (*R*)-roemerine, all the compounds evaluated including the standard, (*S*)-boldine, have reduced affinity for the 5-HT_{2A} receptor by 20- to 300-fold.

(*S*)-bulbocapnine is particularly selective for the D_1 receptor and has a 60–6000-fold greater affinity at the D_1 receptor than at the D_2, 5-HT_{1A} and 5-HT_{2A} receptors **(Figure 5.1(b))**. The observed results are in agreement with the work by Schaus and colleagues who reported only moderate to low affinity of (*S*)-bulbocapnine to D_2, 5-HT_{1A} and 5-HT_{2A} receptors due to the presence of the hydrophobic binding site at the D_1 receptor which accounted for the high antagonistic activity of (*S*)-bulbocapnine (Schaus *et al.*, 1990). Likewise, (*S*)-boldine also showed higher affinity for the D_1 over D_2 receptor by 20-fold **(Figure 5.1(c))**, in agreement with the findings of a previous study (Asencio *et al.*, 2005).

All compounds with the dihydroxy substitutions at position 10 and 11 including (*R*)-apomorphine **(Figure 5.1(d))**, (*S*)-apomorphine **(Figure 5.1(e))** and (*R*)-*N*-propylapomorphine **(Figure 5.1(f))** displayed potent affinity for the dopamine receptors (D_1 and D_2) than the serotonin (5-HT_{1A} and 5-HT_{2A}) receptors. (*R*)-apomorphine exhibited a 12-fold higher affinity at the D_1 receptor and 11-fold higher affinity at the D_2 receptor than its (*S*)-enantiomer. This observation is consistent with the previous report which demonstrated that the (*R*)-configuration was more favourable for high affinity at dopamine receptors, especially of the D_2 type (Csutoras *et al.*, 2004; Gao *et al.*, 1990). Replacement of the *N*-propyl to *N*-methyl group as in (*R*)-*N*-propylapomorphine showed the higher selectivity by 19-fold for D_2 over D_1 receptor. This is in agreement with the previous study which proposed that the presence of the propyl cleft would accommodate a low energy conformation of the *N*-propyl substituent and enhanced the affinity at D_2 receptor (Csutoras *et al.*, 2004; Hedberg *et al.*, 1996).

On the other hand, there is no significant difference in the binding of (±)-nuciferine, (±)-laureline and (*R*)-2-hydroxy-*N*-propylapomorphine for the D_1, D2, 5-HT_{1A} and 5-HT_{2A} receptors. Lastly, (*S*)-isocorydine, (±)-isothebaine, (*S*)-corydine and (*S*)-corytuberine showed little or no affinity towards all four receptors.

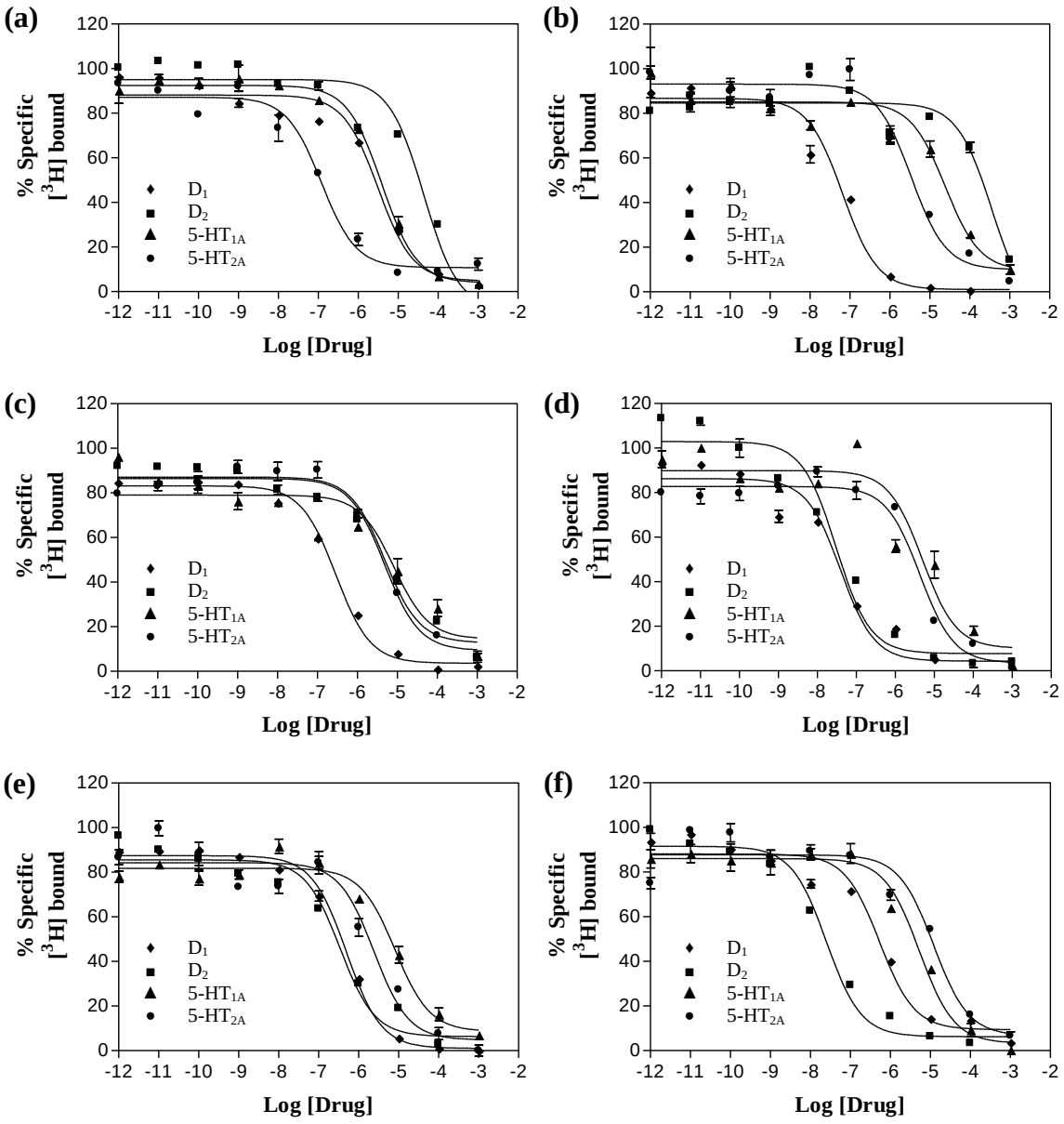


Figure 5.1: Competition binding curves of **(a)** (*R*)-roemerine, **(b)** (*S*)-bulbocapnine **(c)** (*S*)-boldine, **(d)** (*R*)-apomorphine, **(e)** (*S*)-apomorphine and **(f)** *N*-propylapomorphine towards [^{3}H] SCH23390 for D_1 receptor (♦), [^{3}H] spiperone for D_2 receptor (■), [^{3}H] 8-OH-DPAT for 5-HT_{1A} receptor (▲) and [^{3}H] ketanserin for 5-HT_{2A} receptor (●). Results are represented as mean (n=3) ± SEM.

Table 5.1: Binding affinities of the selected ligand compounds towards rat D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors.

Compounds	6a	R_1	R_2	R_6	R_{10}	R_{11}	K_i ± SEM (nM)			
							D_1	D_2	5-HT_{1A}	5-HT_{2A}
Boldine	*S*	OMe	OH	Me	OH	OMe	128 ± 10	2530 ± 350	2450 ± 390	2500 ± 250
Roemerine	*R*	O-CH_2-O		Me			1400 ± 100	22600 ± 2600	1170 ± 60	62.1 ± 5.0
Bulbocapnine	*S*	O-CH_2-O		Me	OMe	OH	29.3 ± 5.0	185000 ± 2000	7260 ± 710	1780 ± 130
Laureline	±	O-CH_2-O		Me	OMe		1420 ± 360	8500 ± 320	520 ± 150	3130 ± 660
Nuciferine	±	OMe	OMe	Me			820 ± 120	2050 ± 790	275 ± 37	139 ± 45
Apomorphine	*R*			Me	OH	OH	17.5 ± 1.0	15.9 ± 2.0	1440 ± 850	2230 ± 120
Apomorphine	*S*			Me	OH	OH	221 ± 13	178 ± 7	2440 ± 620	1260 ± 500
NPA	*R*			Pr	OH	OH	239 ± 11	12.4 ± 0.2	1590 ± 220	6300 ± 500
2-OH-NPA	*R*		OH	Pr	OH	OH	1320 ± 20	1220 ± 50	3750 ± 200	5400 ± 290
Isocorydine	*S*	OMe	OMe	Me	OMe	OH	1250 ± 200	140000 ± 7500	3290 ± 990	3040 ± 530
Isothebaine	±	OH	OMe	Me		OMe	5030 ± 370	NE	19400 ± 6200	7020 ± 390
Corydine	*S*	OH	OMe	Me	OMe	OMe	1800 ± 70	41300 ± 3500	27400 ± 400	21700 ± 2000
Corytuberin	*S*	OH	OMe	Me	OMe	OH	NE	NE	NE	NE

The values shown for K_i were the mean (n = 3) ± SEM.; NE = could not be estimated (10^{-4} or higher); NPA=*N*-propylapomorphine; 2-OH-NPA=2-hydroxy- *N*-propylapomorphine

To gain a further insight into the binding characteristics of the highly selective 5-HT_{2A} receptor compound (*R*)-roemerine, the ligand-receptor interactions were investigated using the generated 5-HT_{2A} receptor model. The predicted binding pose for (*R*)-roemerine is shown in **Figure 5.2** with the ligand bound in the site bordered by transmembrane regions 3, 5, 6 and 7 (**Table 5.2**). In particular, (*R*)-roemerine was found to bind to the 5-HT_{2A} receptor through a combination of a hydrogen bonding and electrostatic interaction formed between the protonated amino group at position R_6 of the ligand and the carboxylate group of the conserved residue Asp155 of the 5-HT_{2A} receptor, a polar interaction involving side chain of amino acid residue Tyr370, a pi-cation interaction at Phe339 and additional van der Waals contacts between the aporphine rings and the aromatic residues (Phe234, Trp336, Phe339, Phe340 and Tyr370) and non-aromatic residues (Ser159, Thr160 and Val366). These observations were in agreement with site-directed mutagenesis experiments which have reported the importance of these amino acid residues for 5-HT_{2A} receptor ligand binding (Choudhary *et al.*, 1993; Kristiansen *et al.*, 2000; Roth *et al.*, 1997; Shapiro *et al.*, 2000; Wang *et al.*, 1993).

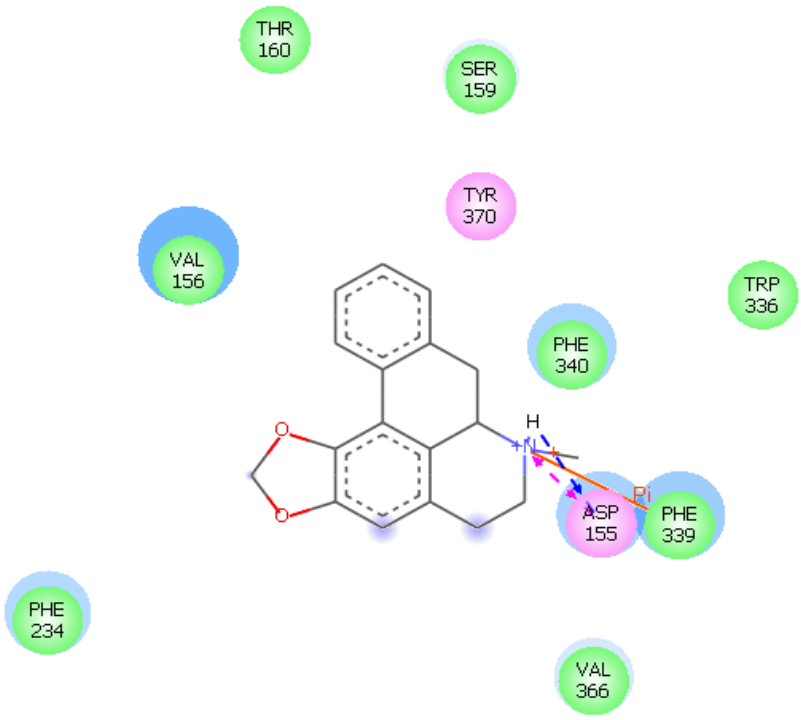


Figure 5.2: Proposed binding mode of (*R*)-roemerine in the 5-HT_{2A} receptor. The residues involved in hydrogen bonding, electrostatic or polar interactions were represented in pink circles with their hydrogen bonds with their main chains (blue dashed arrow), charge interactions (pink dashed double-ended arrow), pi interaction (orange line) and residues involved in van der Waals interactions (green circles).

Investigation into the ligand-receptor interactions involving the selected compounds revealed a significant difference in the binding affinity of (*R*)-roemerine towards 5-HT_{2A} receptor versus D_1, D_2 and 5-HT_{1A} receptors. **Table 5.2** shows the complete list of residues and interactions involved in the binding of the selected aporphines with the 5-HT_{2A} receptor. In case of (±)-laureline, (±)-nuciferine and (±)-isothebaine, the (*S*)-enantiomer was selected over the (*R*)-enantiomer because better binding affinities were observed towards 5-HT_{2A} receptor in computational studies.

The results from this study showed that the (*S*)-boldine (**Figure 3.2)**, (*S*)-bulbocapnine (**Figure 8.8(a) in Appendix E)**, (*S*)-laureline (**Figure 8.8(b) in Appendix E)**, (*S*)-apomorphine (**Figure 8.8(e) in Appendix E)**, (*S*)-corydine (**Figure 8.8(j) in Appendix E)** and (*S*)-corytuberine (**Figure 8.8(k) in Appendix E)** also formed

hydrogen bonding and electrostatic interactions with the conserved Asp155 in 5-HT_{2A} receptor. However, apart from (*S*)-laureline, they failed to interact via a pi-cation interaction as observed in (*R*)-roemerine. This may explain the unfavourable affinities of these compounds at the 5-HT_{2A} receptor and the higher affinity-selectivity profile of (*R*)-roemerine at the 5-HT_{2A} receptor. The 50-fold reduction in affinity at the 5-HT_{2A} receptor observed for (*S*)-laureline seems to be due to the additional methoxy substitution at position 10.

On the other hand, (*R*)-apomorphine (**Figure 8.8(d) in Appendix E)**, (R)-*N*-propylnorapomorphine (**Figure 8.8(f) in Appendix E)** and (*R*)-2-hydroxy-*N*-propylapomorphine (**Figure 8.8(g) in Appendix E)** were only able to form hydrogen bonds between the conserved residue Asp155 and substituents in the aporphine D-ring, particularly the hydroxyl group. Pi-cation interactions were also observed at Phe339 for (*R*)-apomorphine and at Phe234 for (R)-*N*-propylnorapomorphine and (*R*)-2-hydroxy-*N*-propylapomorphine. Similarly, the hydroxyl substituent in the aporphine D-ring of (*S*)-isocorydine (**Figure 8.8(h) in Appendix E)** was also hydrogen bonded with the Asp155, but without any pi-cation interaction. However, the lack of electrostatic interaction of the Asp155 with the protonated amino group of these ligands compared to (*R*)-roemerine led to poor binding and selectivity towards the 5-HT_{2A} receptor.

(*S*)-nuciferine (**Figure 8.8(c) in Appendix E)** was only able to interact with Asp155 via an electrostatic interaction. Hydrogen bonding of the Asp155 with hydrogen from the protonated nitrogen and pi-cation interaction was not observed in (*S*)-nuciferine. Likewise, the conserved residue Asp155 failed to form electrostatic and hydrogen bond with the protonated amino group of the (*S*)-isothebaine (**Figure 8.8(i) in Appendix E)**. Instead of a pi-cation interaction, (*S*)-isothebaine displayed a pi-pi

interaction at Phe234 together with hydrogen bonding between Gly238 and the hydroxyl group in the aporphine D-ring. The observed binding differs from (*R*)-roemerine which may suggest the lower affinity and selectivity for the 5-HT_{2A} receptor.

Table 5.2: 5-HT_{2A} residues found from the docking studies to interact with the selected aporphines

Interactions / Compounds	Electrostatic	H-bond	Polar	pi-cation	pi-pi	van der Waals Residues	
						Aromatic	Non-aromatic
(*S*)-boldine	Asp155	Asp155, Val156	Ser159, Thr160, Phe234	-	-	Trp336,Phe339, Phe340,Tyr370	Leu229, Val235, Ser239,Ala242, Val366
(*R*)-roemerine	Asp155	Asp155	Tyr370	Phe339	-	Phe234,Trp336, Phe339,Phe340	Ser159,Val156, Val366,Tyr370
(*S*)-bulbocapnine	Asp155	Asp155	Val156,Ser159, Thr160, Phe234	-	-	Trp336,Phe339, Phe340, Tyr370	Val235,Ser239, Val366
(*S*)-laureline	Asp155	Asp155	Thr160, Gly238, Tyr370	Phe339	-	Phe234,Trp336, Phe339, Phe340	Val156, Ser159, Ser239, Ala242, Val366
(*S*)-nuciferine	Asp155	-	Val156, Thr160	-	-	Trp336, Phe339, Phe340	Ser159,Gly238, Ser239, Ala242, Asn343
(*R*)-apomorphine	-	Asp155	Ser159, Ser239, Tyr370	Phe339	-	TRP336,Phe339, Phe340	Val156, Ala242, Gly238, Val366
(*S*)-apomorphine	Asp155	Asp155, Ser239	Phe234,Val235, Asn343	-	-	TRP336,Phe339, Phe340	Val156, Ser159, Thr160, Val235, Gly238

(continued)

Interactions / Compounds	Electrostatic	H-bond	Polar	pi-cation	pi-pi	van der Waals	
						Residues	
						Aromatic	Non-aromatic
(*R*)-NPA	-	Asp155	Ser159, Asn343,Tyr370	Phe234	-	Phe234, Trp336, Phe339, Phe340	Val156, Thr160, Val235,Ser239, Gly238,Ala242, Val366
(*R*)- 2-OH-NPA	-	Asp155, Val156, Thr160	Leu157, Ser159, Asn343,Tyr370	Phe234	-	Phe234, Trp336, Phe339, Phe340	Val235,Ser239, Gly238,Ala242, Val366
(*S*)-isocorydine	-	Asp155, Tyr370	Ser159, Asn343, Asn363,	-	-	Trp336, Phe339	Leu229,Val156, Val366
(*S*)-isothebaine	-	Gly238	Thr160, Ser239, Ala242,	-	Phe234	Phe234,Phe243, Phe339, Phe340	Asp155,Val156, Ser159, Ile163, Asn343
(*S*)-corydine	Asp155	Asp155	Tyr370	-	-	Phe234, Trp336, Phe339, Phe340	Val156,Ser159, Thr160,Leu229, Asp231, Ser239, Val235, Asn343, Val366,
(*S*)-corytuberine	Asp155	Asp155 Ser239	Val156, Thr160, Ser239, Asn343	-	-	Phe234, Trp336, Phe339, Phe340, Tyr370	Ser159, Val235, Val366

(*R*)-NPA =*N*-propylapomorphine; (*R*)- 2-OH-NPA =2-hydroxy-*N*-propylapomorphine

5.5 Conclusion

(*R*)-roemerine had been identified as a potential 5-HT_{2A} receptor-selective compound. This suggest that preferential affinity for the 5-HT_{2A} receptor results from that substitution at position 1 and 2, particularly with methylenedioxy group, non-substitution at position 10 and 11 and *N*-methyl substitution at position 6. Further investigation into the ligand-receptor interactions of (*R*)-roemerine also revealed that high 5-HT_{2A} selectivity over $D_{1,}$ D_2 and 5-HT_{1A} receptors were probably due to the strong hydrogen bonding and electrostatic interactions involving the protonated amino group and a pi-cation interaction with Asp155 and Phe339, two of the conserved residues in the binding pocket of the 5-HT_{2A} receptor.

6.0 General Conclusion, Limitations and Future Perspectives

A combination of structure based *in silico* and *in vitro* experiments were implemented in this study to identify potential ligands for dopaminergic and serotonergic receptors. A 3D homology model of the rat 5-HT_{2A} receptor was first generated based on the crystal structure of the human β_2-adrenergic receptor as a structural template and further validated with standard 5-HT_{2A} receptor ligands. Thereafter, a filtered set of aporphines using (*S*)-boldine as the backbone structure was docked into the generated 5-HT_{2A} receptor model. A set of 13 aporphines with higher or comparable activity to (*S*)-boldine were selected for experimental testing.

A medium throughput screening assay for dopamine (D_1 and D_2) and serotonin (5-HT_{1A} and 5-HT_{2A}) receptors based on radioligand receptor binding experiments was used to evaluate the affinity of the selected compounds towards these receptors. (*R*)-roemerine was found to have potent 5-HT_{2A} affinity **(K_i = 62.1 nM)** and selectivity **(20–400-fold higher)** versus D_1, D_2 and 5-HT_{1A} receptors. Investigation into the binding mode of (*R*)-roemerine revealed the importance of the hydrogen bonding and electrostatic interactions with the conserved residue Asp155 and a pi-cation interaction at Phe339. These observations suggest that (*R*)-roemerine could form the basis of the design of aporphinoid compounds with high affinity and selectivity towards the 5-HT_{2A} receptor.

There were however, several limitations to this project. The application of structure based *in silico* methods using a homology model for the identification of new drugs is more challenging and inaccurate than docking to 3D X-ray structures. Potential reasons of error may be due to structural differences between the template and the

target, incorrect sequence alignment and difficulties in obtaining variable loop regions. Additionally, only a small number of compounds were screened due to limited availability.

On the other hand, although the *in vitro* radioligand receptor binding assays used were simple and rapid, errors may be introduced due to the limited time allowed to reach steady state and through the process of separating the bound and free ligand, drying the harvested filter plates and counting the filter plates.

In order to improve the *in silico* approach, the constructed homology model should be further refined using simulation techniques such as constrained molecular mechanics or molecular dynamics simulations and validated with known 5-HT_{2A} receptor active ligands. Additionally, in an attempt to rationalize the observed affinity data and to predict ligand-receptor interactions at the molecular level, homology models of D_1, D_2 and 5-HT_{1A} receptors also should be built. A larger set of screening compounds should be obtained from the database to dock into the newly refined model. A library could also be obtained through use of synthetic chemistry.

The receptor binding assays could be improved by using purified or cloned receptor in order to minimise the nonspecific binding and ultimately increase the sensitivity of the assay.

7.0 References

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8.0 Appendices

A: Sequence Analysis

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CLUSTAL 2.0.12 multiple sequence alignment


uniprot|P28564|5HT1B_RAT      ------------------MEEQGIQCAPPPPATSQTGVPLAN-------- 24
uniprot|P28565|5HT1D_RAT      ----------------------------MSLPNQSLEGLPQEA-------- 15
uniprot|P30940|5HT1F_RAT      ----------------------------MDFLNSSDQNLTSEE-------- 15
uniprot|P19327|5HT1A_RAT      --------------------------MDVFSFGQGNNTTAS-------- 15
uniprot|P35364|5HT5A_RAT      ------------------MDLP--------INLTSFSLSTPS-------- 16
uniprot|P35365|5HT5B_RAT      ------------------MEVSNLSGATPGIAFPPGPESCSD-------- 24
uniprot|P32305|5HT7R_RAT      MMDVNSSGRPDLYGHLRSLILPEVGRGLQDLSPDGGAHPVVSSWMPHLLS 50
uniprot|P14842|5HT2A_RAT      ----------MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTS 40
uniprot|P08909|5HT2C_RAT      -----------------MVNLGNAVRSLLMHLIGLLVWQFDIS-----IS 28
uniprot|P30994|5HT2B_RAT      ------------MASSYKMSEQSTISEHILQKTCDHLILTDRSG-LKAES 37
uniprot|Q62758|5HT4R_RAT      ----------------------------MDRLDANVSSNE-------- 12
uniprot|P31388|5HT6R_RAT      ----------------------------MVPEPGPVNSSTPAWG------ 16



uniprot|P28564|5HT1B_RAT      -------------LSHNCSADDYIYQDSIALPWKVLLVALLALITLATTL 61
uniprot|P28565|5HT1D_RAT      -------------SNRSLNATG-AWDPEVLQALRISLVVVLSIITLATVL 51
uniprot|P30940|5HT1F_RAT      -------------LLN-------------RMPSKILVSLTLSGLALMTTT 39
uniprot|P19327|5HT1A_RAT      -------------QEPFGTGGNVTSISDVTFSYQVITSLLLGTLIFCAVL 52
uniprot|P35364|5HT5A_RAT      -----TLEPNRSLDTEA----LRTSQSFLS-AFRVLVLTLLGFLAAATFT 56
uniprot|P35365|5HT5B_RAT      -----SPSSGRSMGSTPGGLILSGREPPFS-AFTVLVVTLLVLLIAATFL 68
uniprot|P32305|5HT7R_RAT      GFLEVTASPAPTWDAPPDNVSGCGEQINYGRVEKVVIGSILTLITLLTIA 100
uniprot|P14842|5HT2A_RAT      EASNWTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIA 90
uniprot|P08909|5HT2C_RAT      PVAAIVTDTFN----SSDGGR----LFQFPDGVQNWPALSIVVIIIMTIG 70
uniprot|P30994|5HT2B_RAT      AAEEMKQTAEN-----------------QGNTVHWAALLIFAVIIPTIG 69
uniprot|Q62758|5HT4R_RAT      --------------------------GFGSVEKVVLLTFFAMVILMAIL 35
uniprot|P31388|5HT6R_RAT      ------------------------PGPPPAPGGSGWVAAALCVVIVLTAA 42
                                                         :   :

uniprot|P28564|5HT1B_RAT      SNAFVIATVYRTRKLHTP-ANYLIASLAVTDLLVSILVMPISTMYTVT-- 108
uniprot|P28565|5HT1D_RAT      SNAFVLTTILLTKKLHTP-ANYLIGSLATTDLLVSILVMPISIAYTTT-- 98
uniprot|P30940|5HT1F_RAT      INCLVITAIIVTRKLHHP-ANYLICSLAVTDFLVAVLVMPFSIVYIVR-- 86
uniprot|P19327|5HT1A_RAT      GNACVVAAIALERSLQNV-ANYLIGSLAVTDLMVSVLVLPMAALYQVL-- 99
uniprot|P35364|5HT5A_RAT      WNLLVLATILRVRTFHRV-PHNLVASMAISDVLVAVLVMPLSLVHELS-G 104
uniprot|P35365|5HT5B_RAT      WNLLVLVTILRVRAFHRV-PHNLVASTAVSDVLVAALVMPLSLVSELSAG 117
uniprot|P32305|5HT7R_RAT      GNCLVVISVCFVKKLRQP-SNYLIVSLALADLSVAVAVMPFVSVTDLI-G 148
uniprot|P14842|5HT2A_RAT      GNILVIMAVSLEKKLQNA-TNYFLMSLAIADMLLGFLVMPVSMLTILY-G 138
uniprot|P08909|5HT2C_RAT      GNILVIMAVSMEKKLHNA-TNYFLMSLAIADMLVGLLVMPLSLLAILY-D 118
uniprot|P30994|5HT2B_RAT      GNILVILAVSLEKRLQYA-TNYFLMSLAVADLLVGLFVMPIALLTIMF-E 117
uniprot|Q62758|5HT4R_RAT      GNLLVMVAVCRDRQLRKIKTNYFIVSLAFADLLVSVLVNAFGAIELVQ-- 83
uniprot|P31388|5HT6R_RAT      ANSLLIVLICTQPALRNT-SNFFLVSLFTSDLMVGLVVMPPAMLNALY-- 89
                               *  ::  :     ::   .: :: *   :*. :.  * .
                              TM I                      TM II

uniprot|P28564|5HT1B_RAT      GRWTLGQVVCDFWLSSDITCCTASIMHLCVIALDRYWAIT-DAVDYSAKR 157
uniprot|P28565|5HT1D_RAT      RTWNFGQILCDIWVSSDITCCTASILHLCVIALDRYWAIT-DALEYSKRR 147
uniprot|P30940|5HT1F_RAT      ESWIMGQGLCDLWLSVDIICCTCSILHLSAIALDRYRAIT-DAVEYARKR 135
uniprot|P19327|5HT1A_RAT      NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDRYWAIT-DPIDYVNKR 148
uniprot|P35364|5HT5A_RAT      RRWQLGRRLCQLWIACDVLCCTASIWNVTAIALDRYWSIT-RHLEYTLRA 153
uniprot|P35365|5HT5B_RAT      RRWQLGRSLCHVWISFDVLCCTASIWNVAAIALDRYWTIT-RHLQYTLRT 166
uniprot|P32305|5HT7R_RAT      GKWIFGHFFCNVFIAMDVMCCTASIMTLCVISIDRYLGIT-RPLTYPVRQ 197
uniprot|P14842|5HT2A_RAT      YRWPLPSKLCAIWIYLDVLFSTASIMHLCAISLDRYVAIQ-NPIHHSRFN 187
uniprot|P08909|5HT2C_RAT      YVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIR-NPIEHSRFN 167
uniprot|P30994|5HT2B_RAT      ATWPLPLALCPAWLFLDVLFSTASIMHLCAISLDRYIAIK-KPIQANQCN 166
uniprot|Q62758|5HT4R_RAT      DIWFYGEMFCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVYRNKM 133
uniprot|P31388|5HT6R_RAT      GRWVLARGLCLLWTAFDVMCCSASILNLCLISLDRYLLIL-SPLRYKLRM 138
                                *      *      *:   :.**  :  *::***  *    :
                                                 TM III

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uniprot|P19327|5HT1A_RAT      TPRRAAALISLTWLIGFLISIPPMLGWRTPEDRSDPDACTISKDHG---- 194
uniprot|P35364|5HT5A_RAT      RKRVSNVMILLTWALSAVISLAPLLFGWGETYSELSEECQVSREPS---- 199
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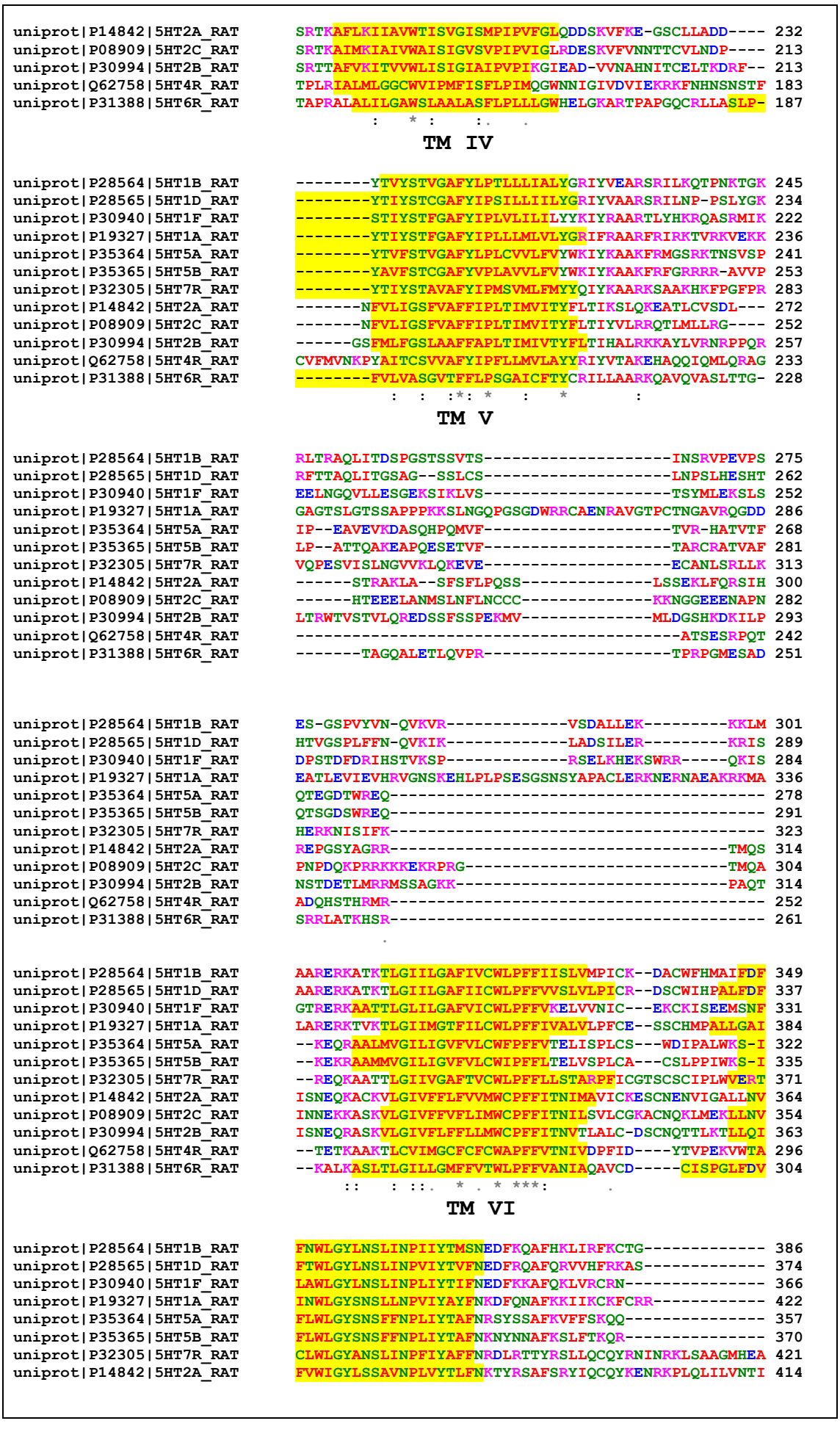
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uniprot|P35365|5HT5B_RAT --------YAVFSTCGAFYVPLAVVLFVYWKIYKAAKFRFGRRRR-AVVP 253
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TM V
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uniprot|P35365|5HT5B_RAT QTSGDSWREQ-- 291
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uniprot|P08909|5HT2C_RAT PNPDQKPRRKKKEKRPRG--------------------------TMQA 304
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TM VI
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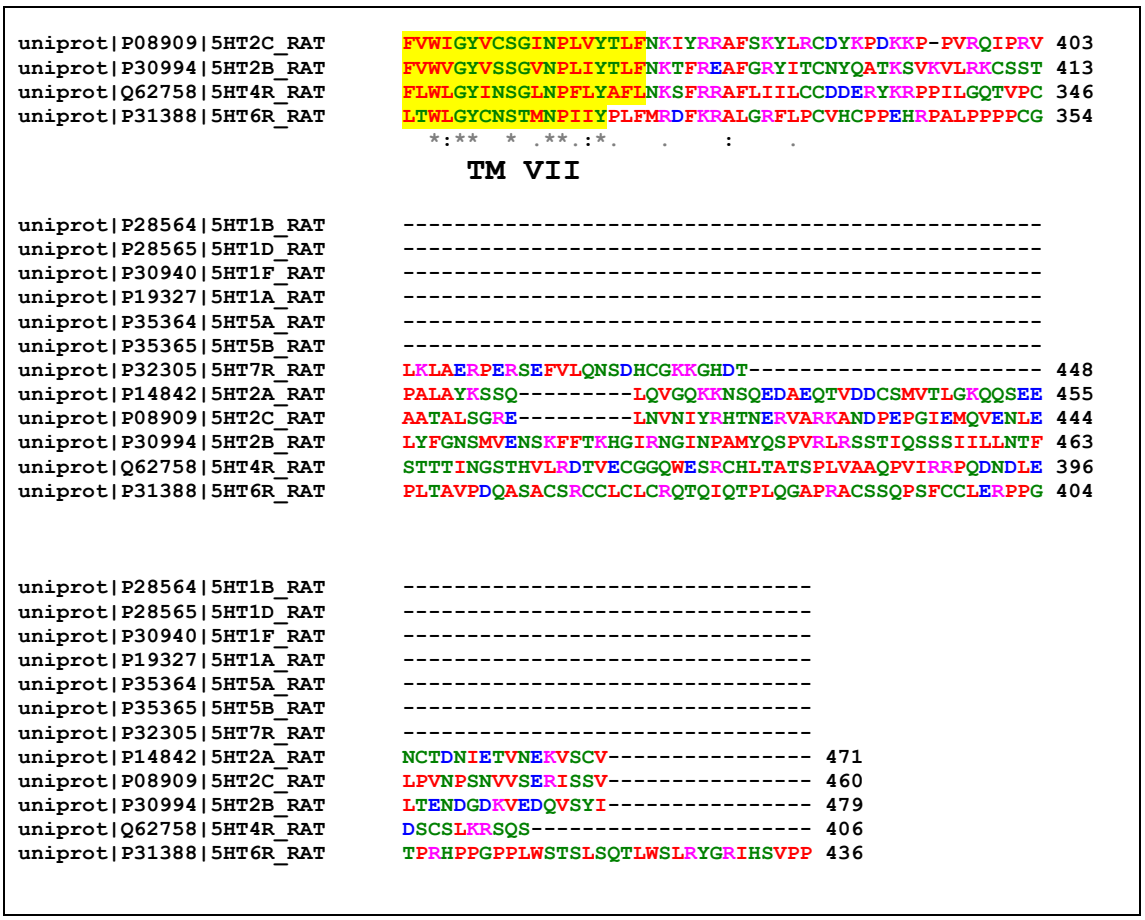
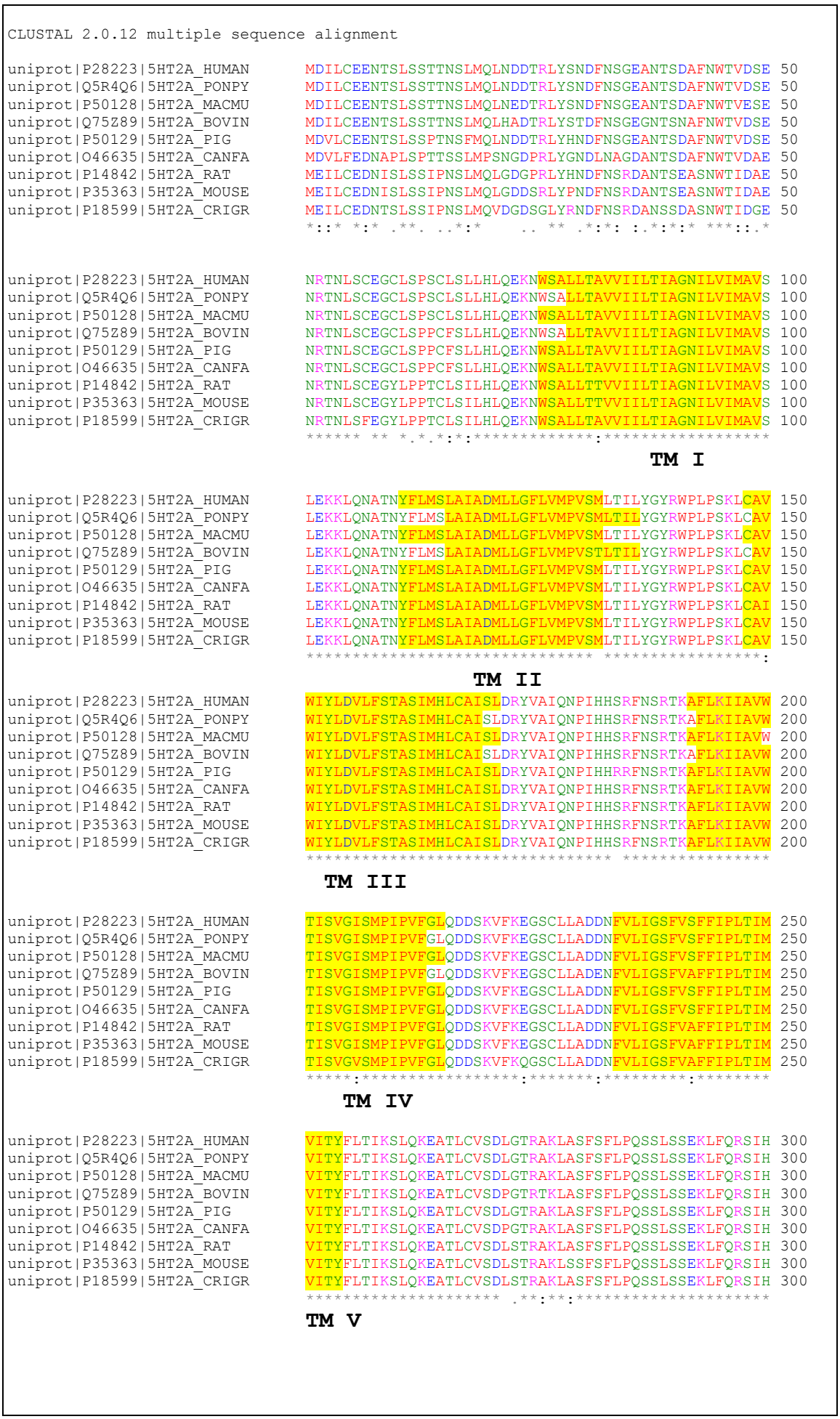


Figure 8.1: Alignment of rat 5-HT family receptor. The lowest and highest sequence identity between any two sequences was between 19% and 68%, respectively.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TM V

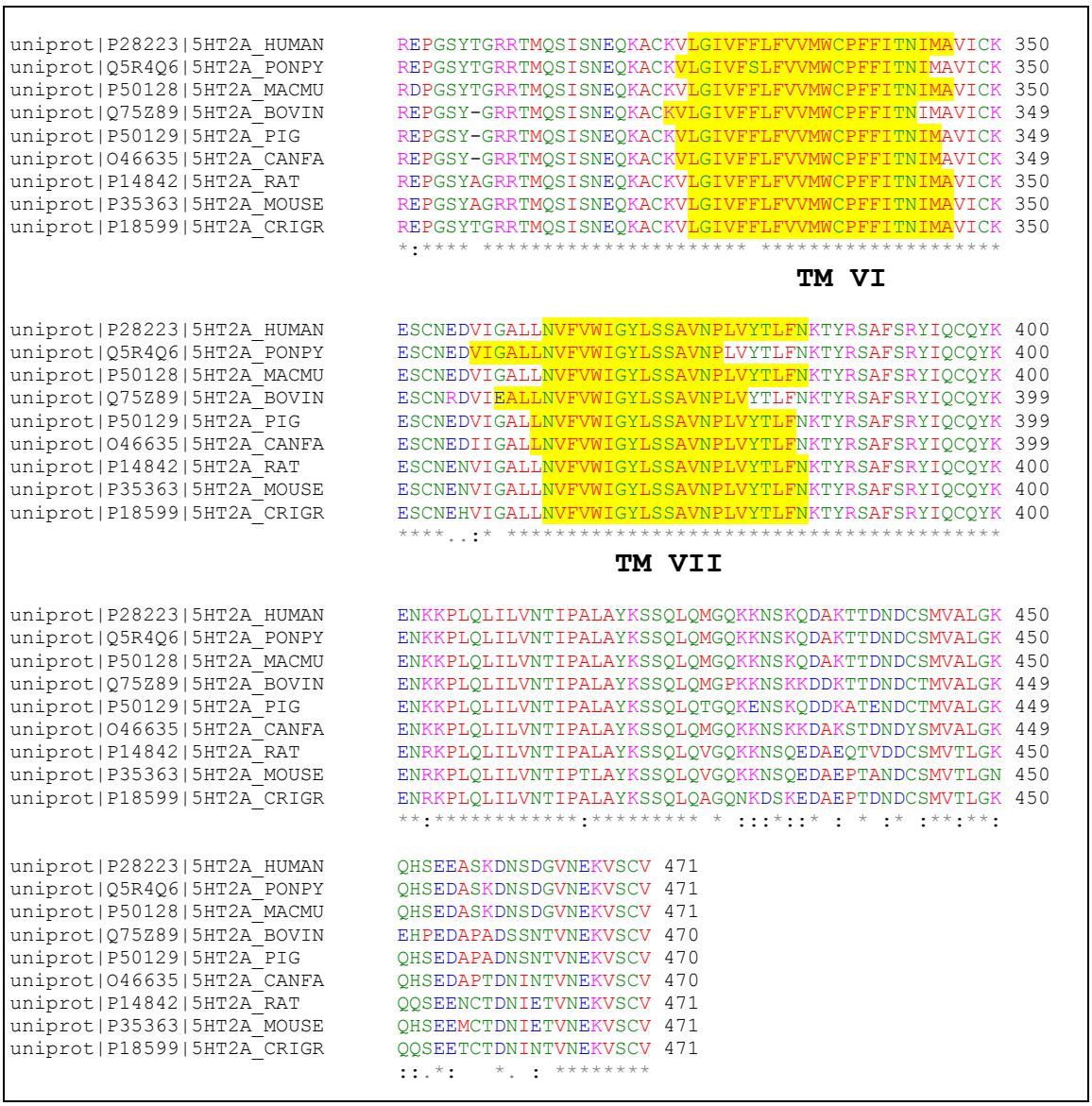


Figure 8.2: Sequence alignment of 5-HT_{2A} multiple species. A multiple sequence alignment indicates that the rat 5-HT_{2A} receptor shares the lowest and highest sequence identity between 88% and 97% between any of two sequences. The major differences among the multiple species sequences were found at the N- and C-terminus ends.

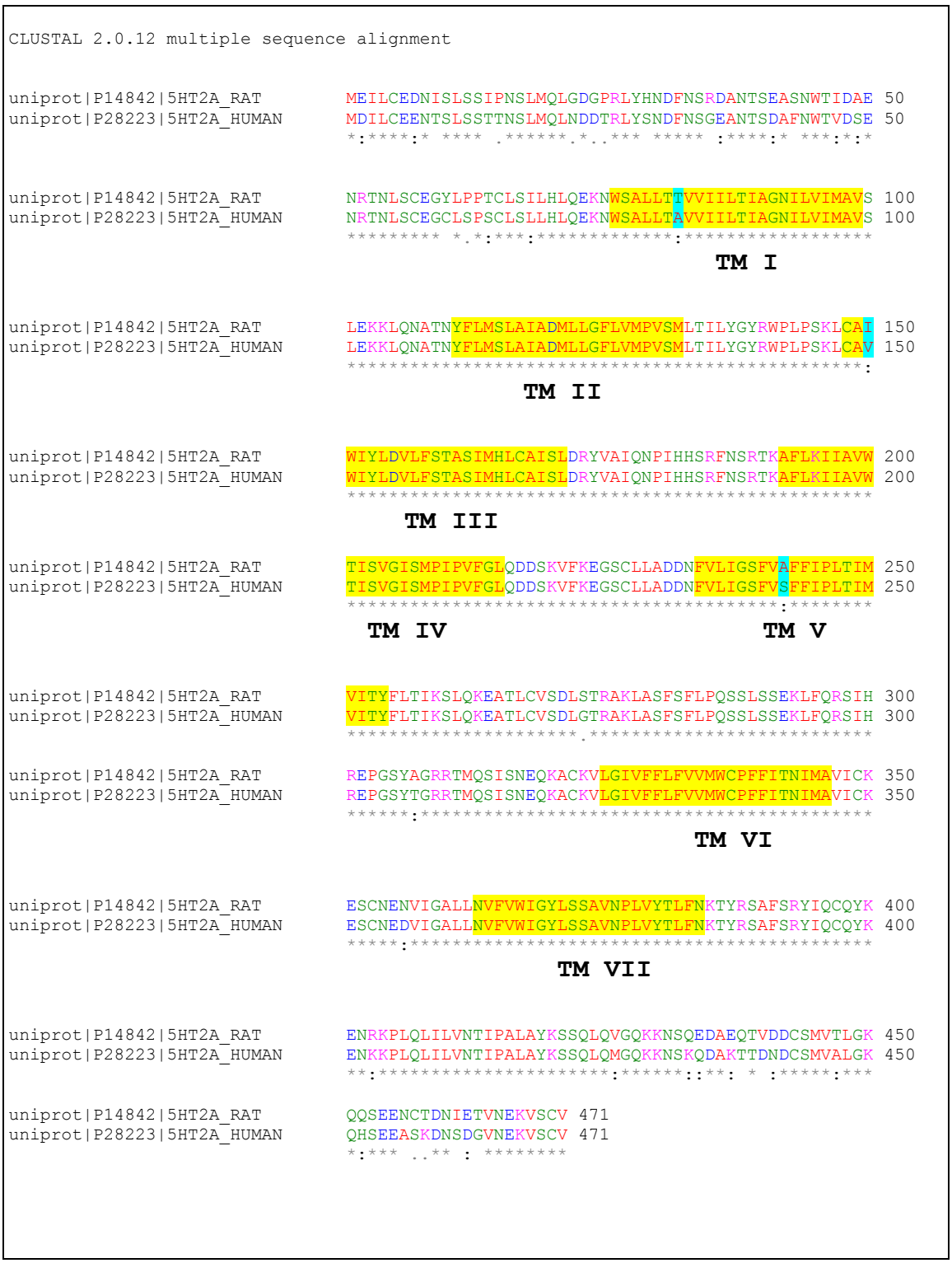


Figure 8.3: Sequence comparison of rat 5-HT$_{2A}$ against human 5-HT$_{2A}$. A close comparison of sequences between the rat and human 5-HT$_{2A}$ indicated the species specific residues were found to be distributed in the loop regions (except three residues, Ala 82, Val 150 and Ser 242 (shaded in cyan), that were found in transmembrane 1, 4 and 5, respectively). The sequence identity between the rat and human 5-HT$_{2A}$ receptor was 91%.

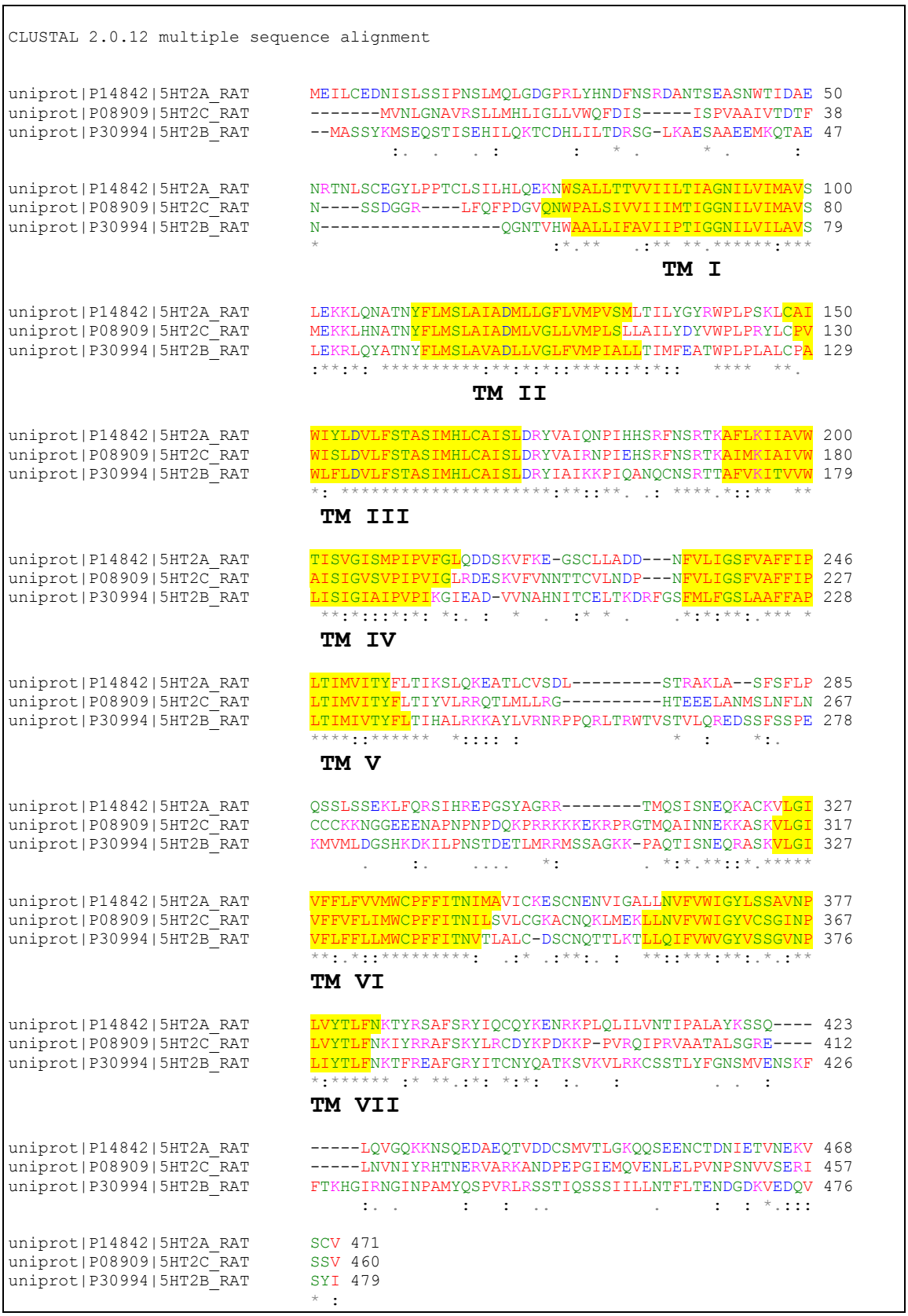


Figure 8.4: Sequence comparison of rat 5-HT_{2A} against rat 5-HT_{2B} and 5-HT_{2C} sequences. The sequence identity of rat 5-HT_{2A} with rat 5-HT_{2B} and 5-HT_{2C} is 38% and 48% respectively suggesting that the rat 5-HT_{2A} is closer to 5-HT_{2C} than 5-HT_{2B}.

B: Ramachandran plot

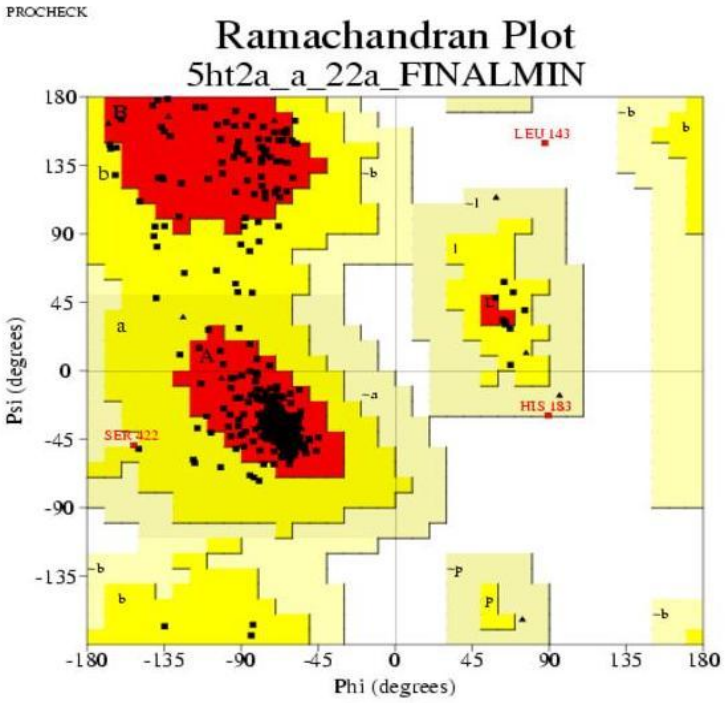
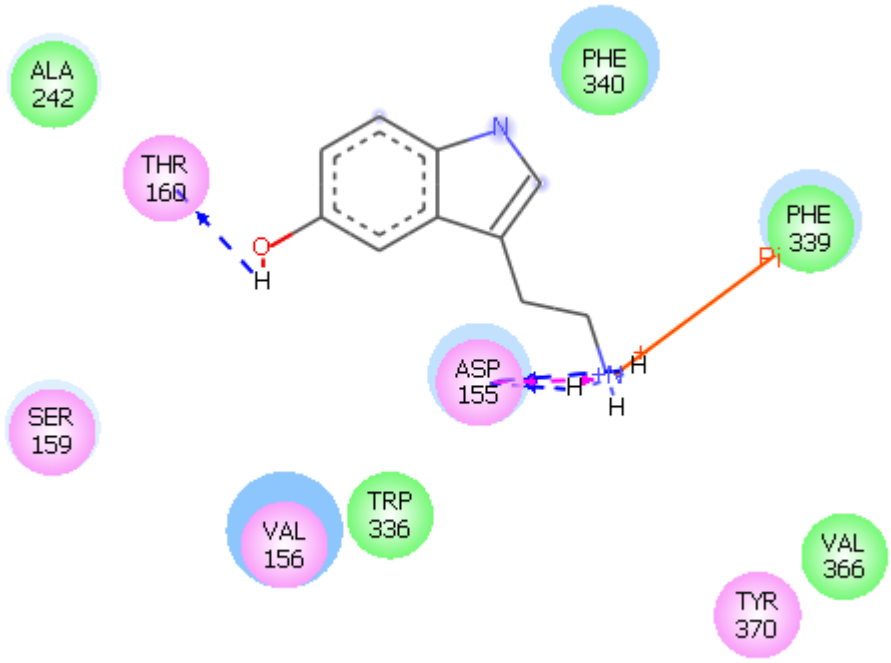
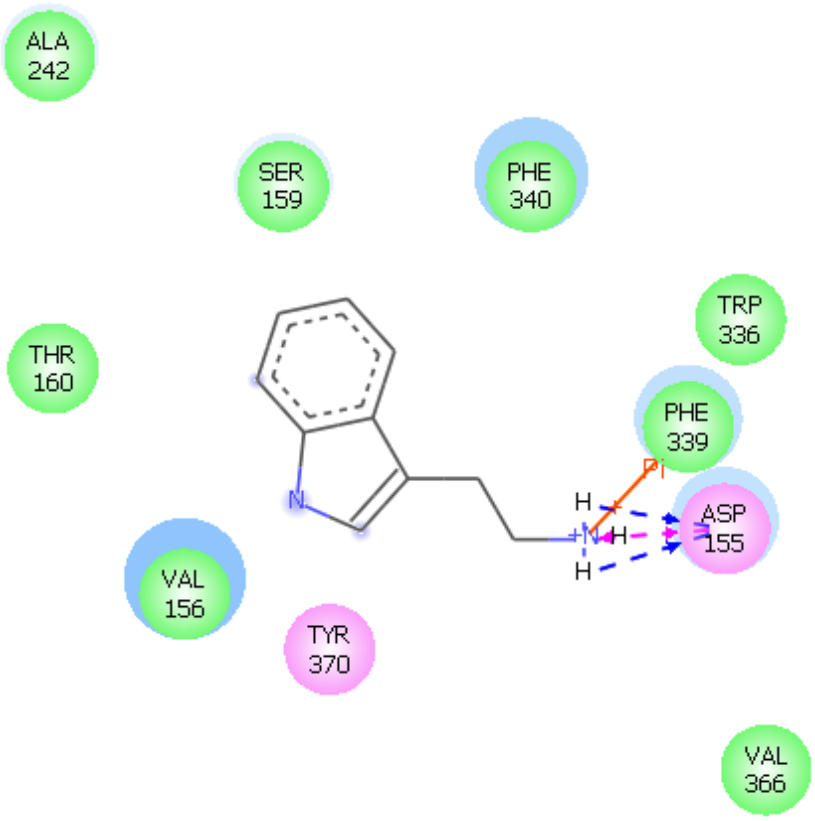


Figure 8.5: Structure validation of the model using Ramachandran plot computed with the PROCHECK program. The result shows 89.4% in the most favourable region, 9.9% in the additionally allowed region, 0.5% in the generously allowed region and 0.2 % in the disallowed region.

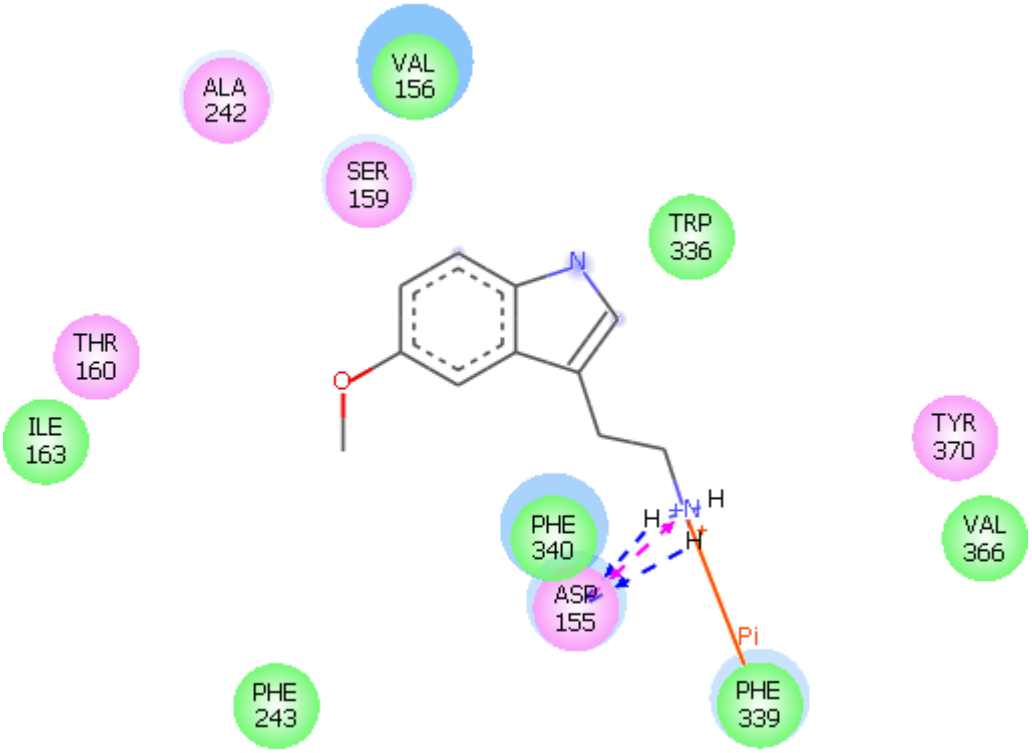
C: Binding Mode of Standard Ligands in the 5-HT$_{2A}$ Receptor Model



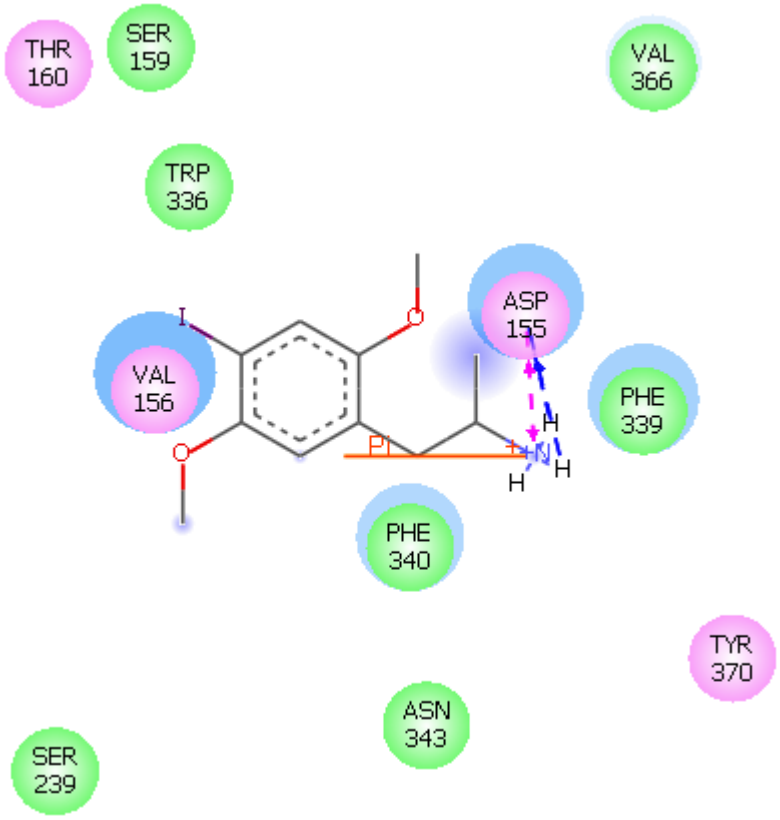
(a) The 5-HT2$_A$ receptor model in complex with endogenous ligand, serotonin



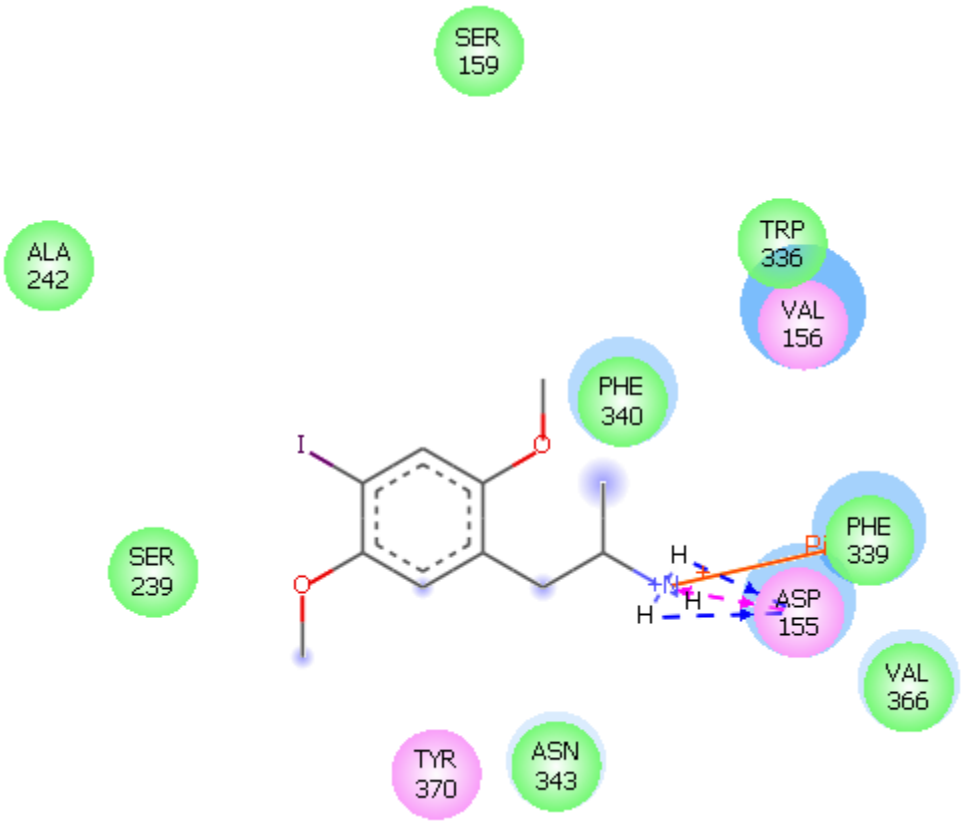
(b) The 5-HT2$_A$ receptor model in complex with tryptamine



(c) The 5-HT2$_A$ receptor model in complex with 5-MeOT

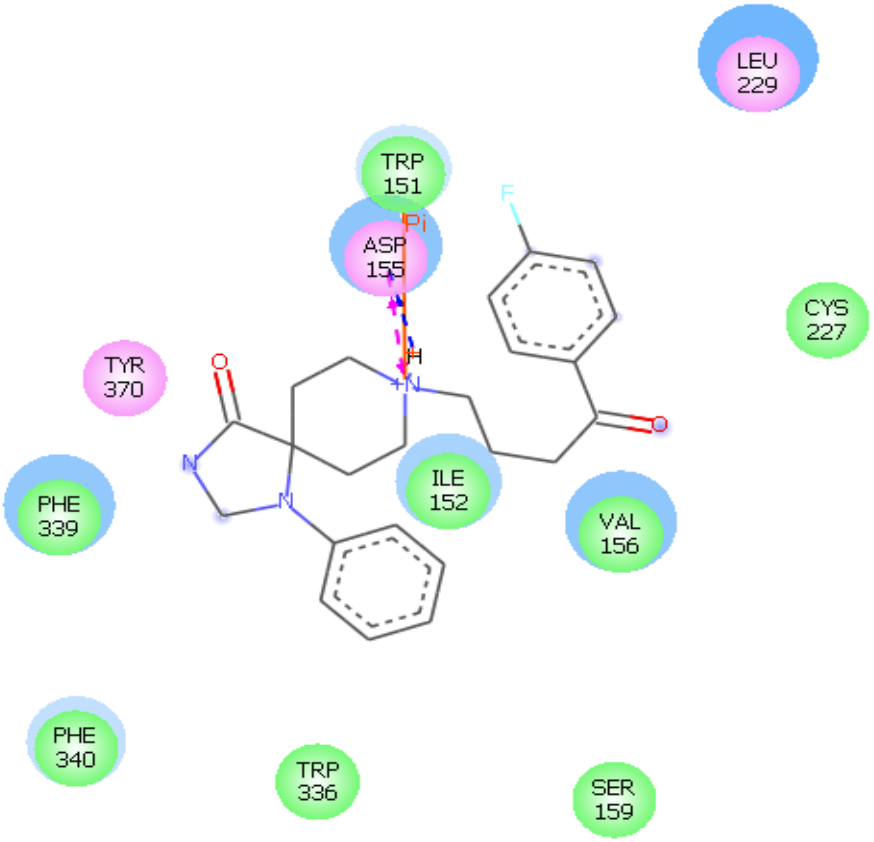


(d) The 5-HT2$_A$ receptor model in complex with (*R*)-(-)-DOI

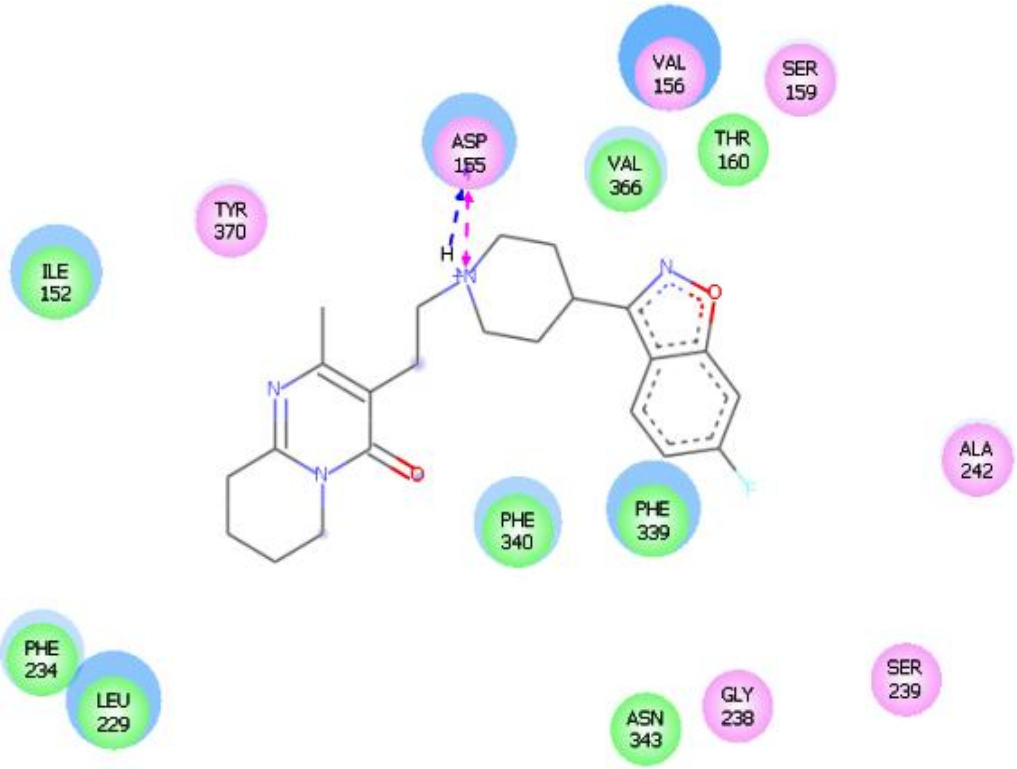


(e) The 5-HT2$_A$ receptor model in complex with (*S*)-(-)-DOI

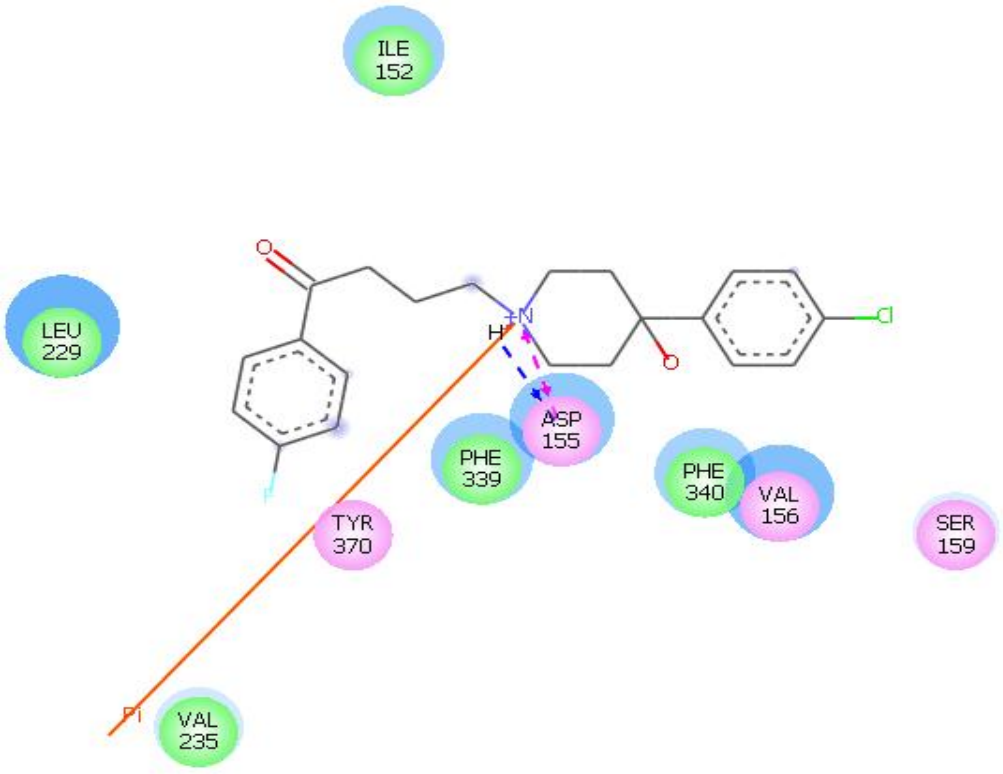
Figure 8.6: The 5-HT$_{2A}$ receptor model in complex with endogenous ligand and agonists **(a)** serotonin **(b)** tryptamine **(c)** 5-MeOT **(d)** (*R*)-(-)-DOI **(e)** (*S*)-(+)-DOI. The residues involved in hydrogen bonding, electrostatic or polar interactions were depicted in pink circle with their hydrogen bonds (blue dashed arrow), charge interactions (pink dashed double-ended arrow) and residues involved in van der Waals interactions (green circle).



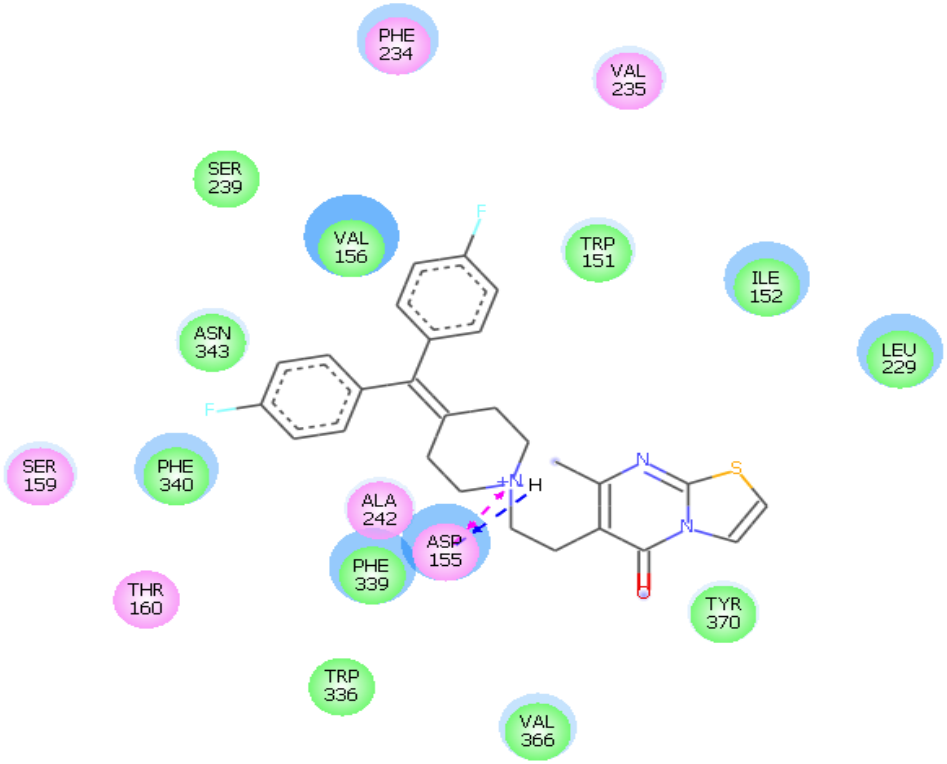
(a) The 5-HT2$_A$ receptor model in complex with spiperone



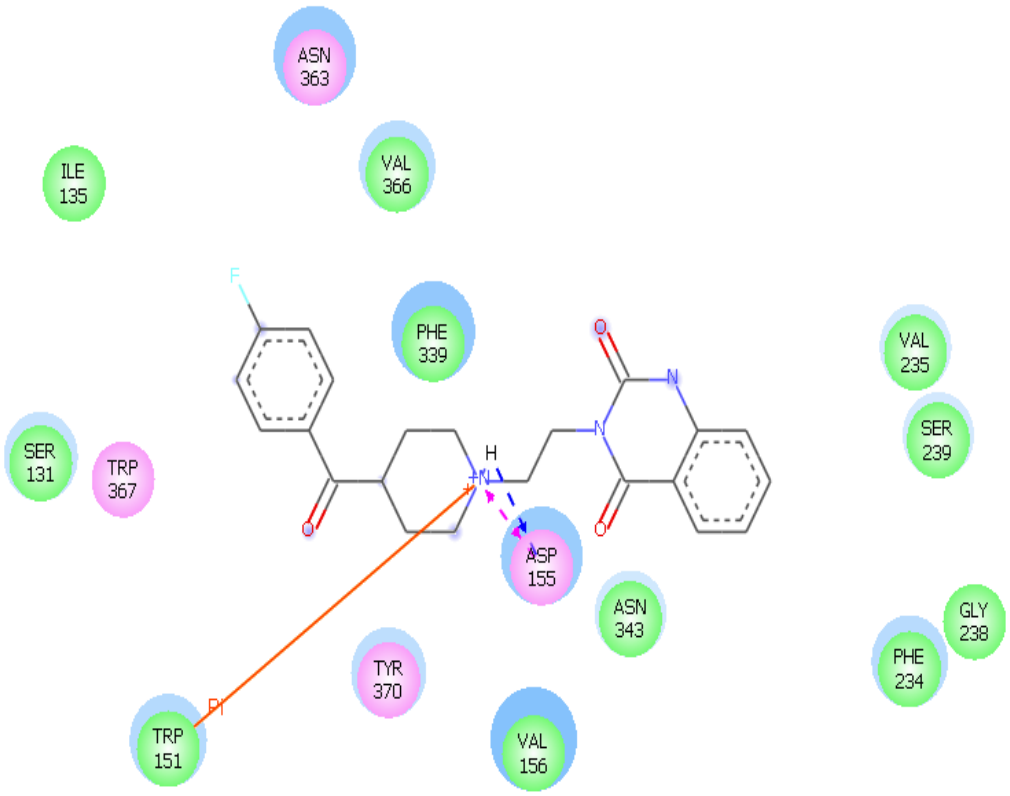
(b) The 5-HT2$_A$ receptor model in complex with risperidone



(c) The 5-HT2$_A$ receptor model in complex with haloperidol



(d) The 5-HT2$_A$ receptor model in complex with ritanserin



(e) The 5-HT2$_A$ receptor model in complex with ketanserin

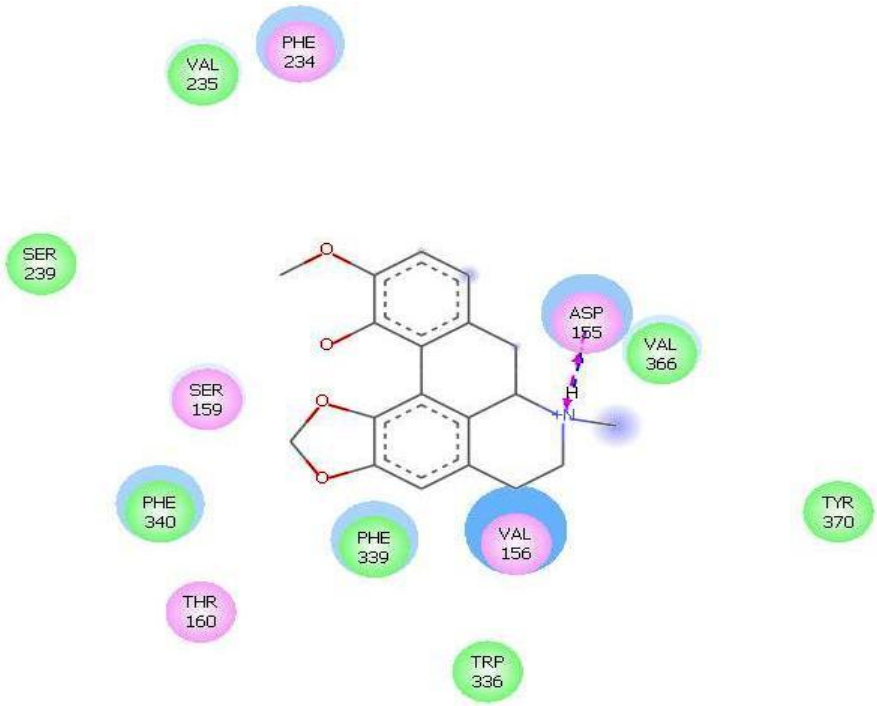
Figure 8.7: The 5-HT$_{2A}$ receptor model in complex with antagonists **(a)** spiperone **(b)** risperidone **(c)** haloperidol **(d)** ritanserin **(e)** ketanserin. The residues involved in hydrogen bonding, electrostatic or polar interactions were depicted in pink circle with their hydrogen bonds (blue dashed arrow), charge interactions (pink dashed double-ended arrow) and residues involved in van der Waals interactions (green circle).

D: ZINC IDs and the Corresponding Compounds Names

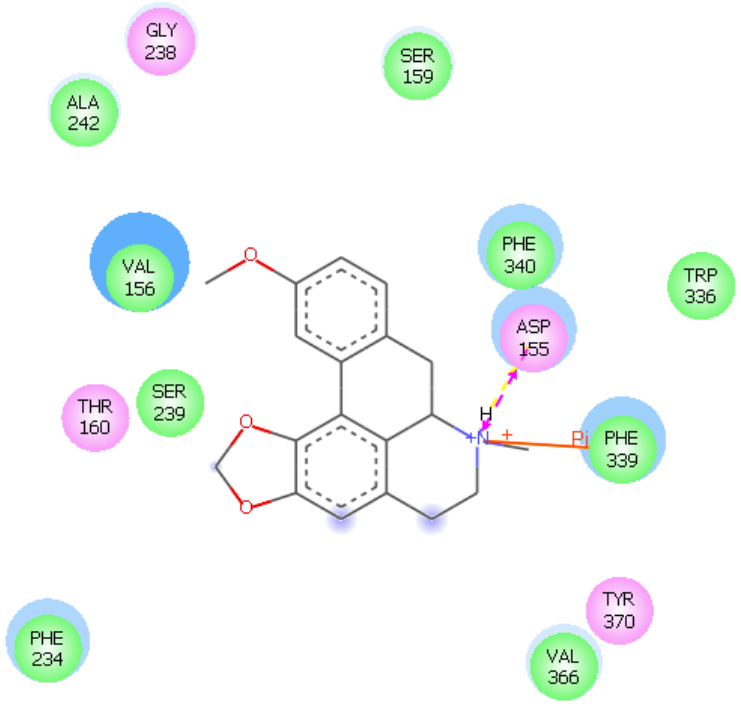
Table 8.1: The ZINC IDs of the compounds selected from virtual screening.

ZINC IDs	Compound Names
ZINC00135449	(*S*)-boldine
ZINC00306698	(*R*)-roemerine
ZINC00000103	(*S*)-bulbocapnine
ZINC00338183	(*S*)-laureline
ZINC00320695	(*S*)-nuciferine
ZINC00009073	(*R*)-apomorphine
ZINC00155525	(*S*)-apomorphine
ZINC02539817	(*R*)-*N*-propylapomorphine
ZINC00011665	(*R*)-2-hydroxy-*N*-propylapomorphine
ZINC00056792	(*S*)-isocorydine
ZINC00174790	(*S*)-isothebaine
ZINC01651264	(*S*)-corydine
ZINC02554931	(*S*)-corytuberine

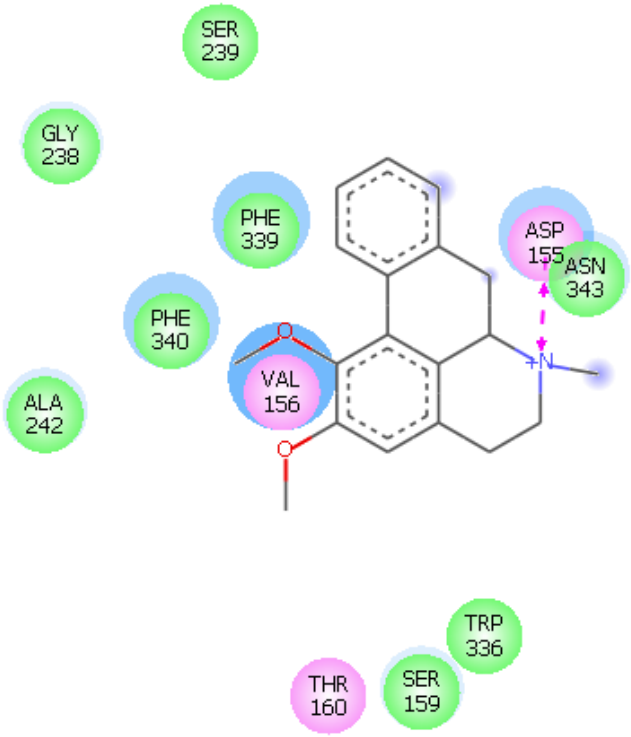
E: Binding Modes of the Selected Aporphines with the 5-HT_{2A} Receptor Model



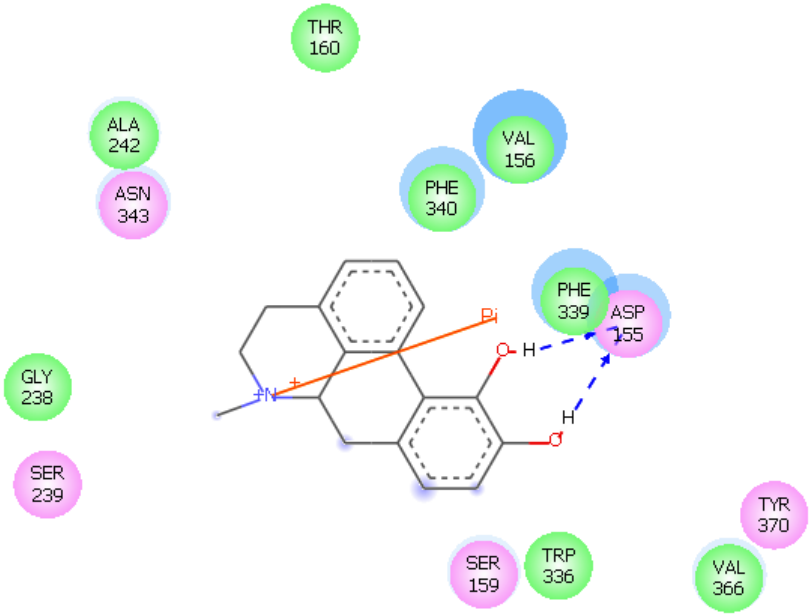
(a) Proposed binding mode of (*S*)-bulbocapnine in the 5-HT_{2A} receptor



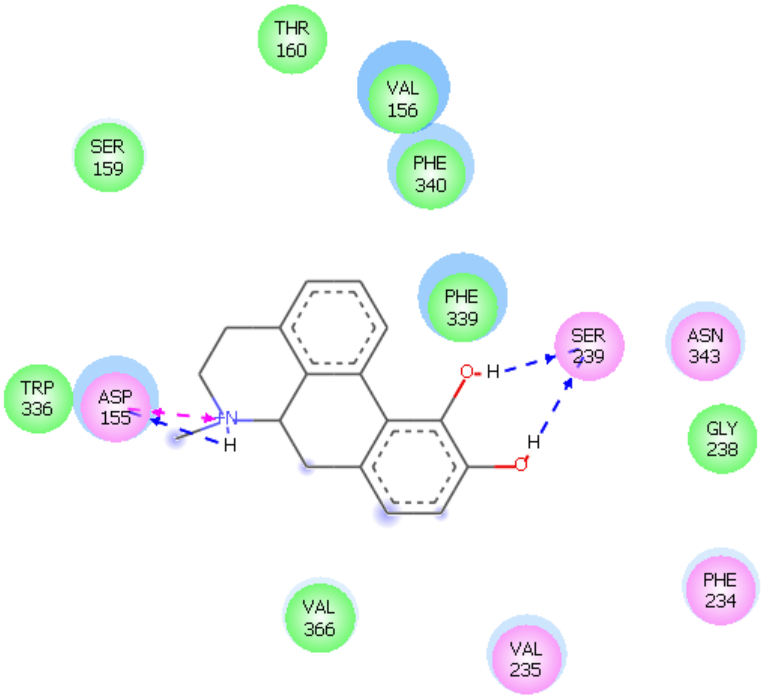
(b) Proposed binding mode of (*S*)-laureline in the 5-HT_{2A} receptor



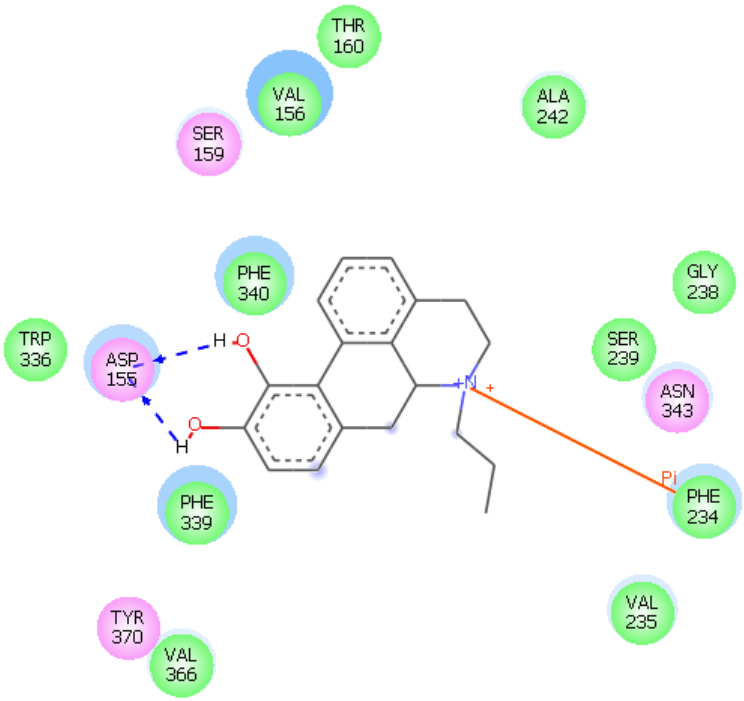
(c) Proposed binding mode of (*S*)-nuciferine in the 5-HT_{2A} receptor



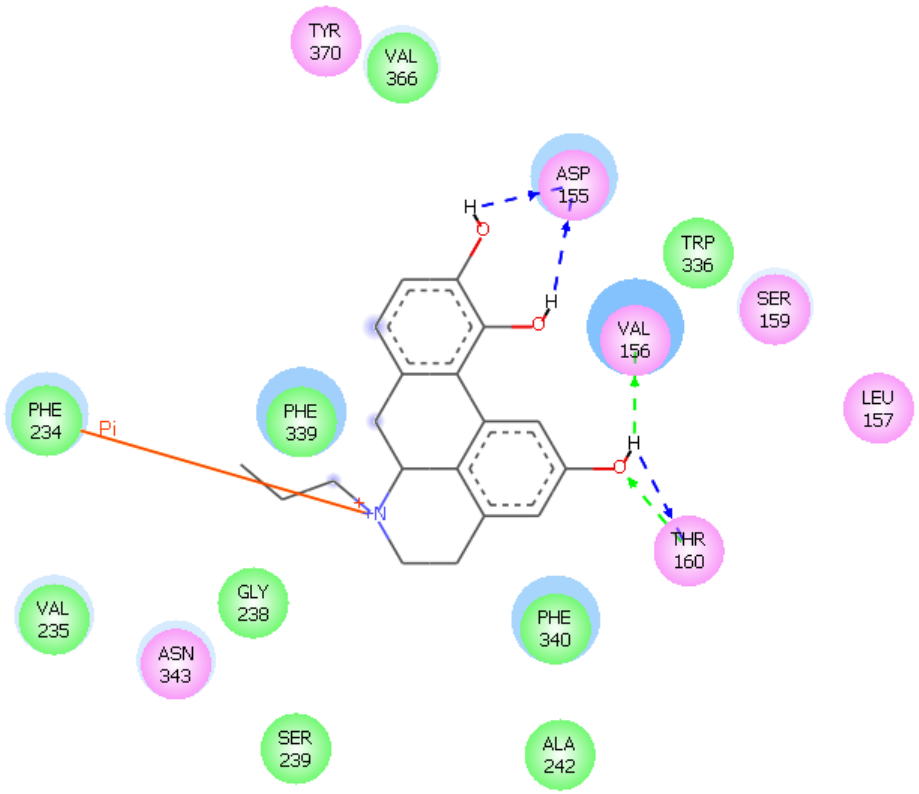
(d) Proposed binding mode (*R*)-apomorphine in the 5-HT_{2A} receptor



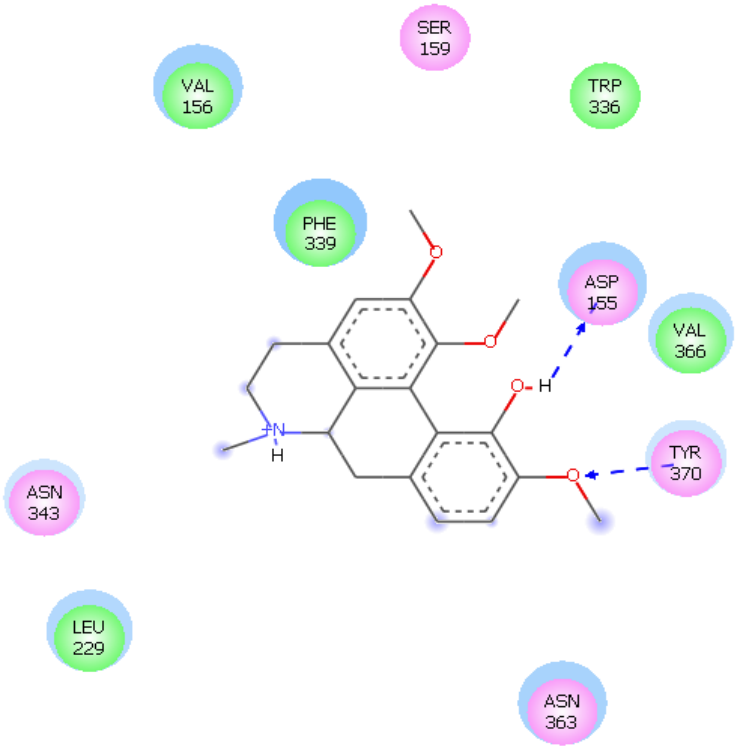
(e) Proposed binding mode (*S*)-apomorphine in the 5-HT_{2A} receptor



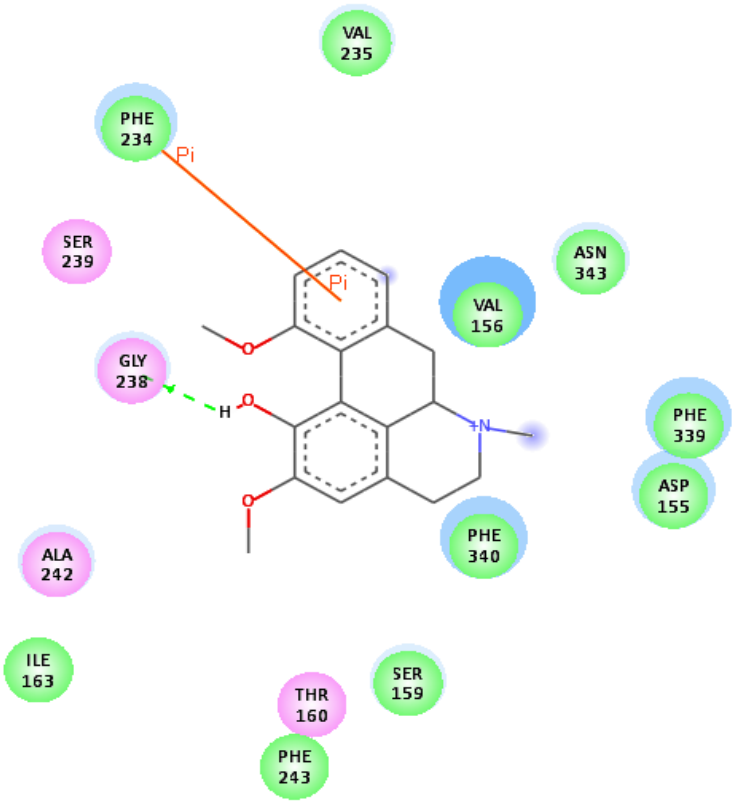
(f) Proposed binding mode of (*R*)-*N*-propylapomorphine in the 5-HT_{2A} receptor



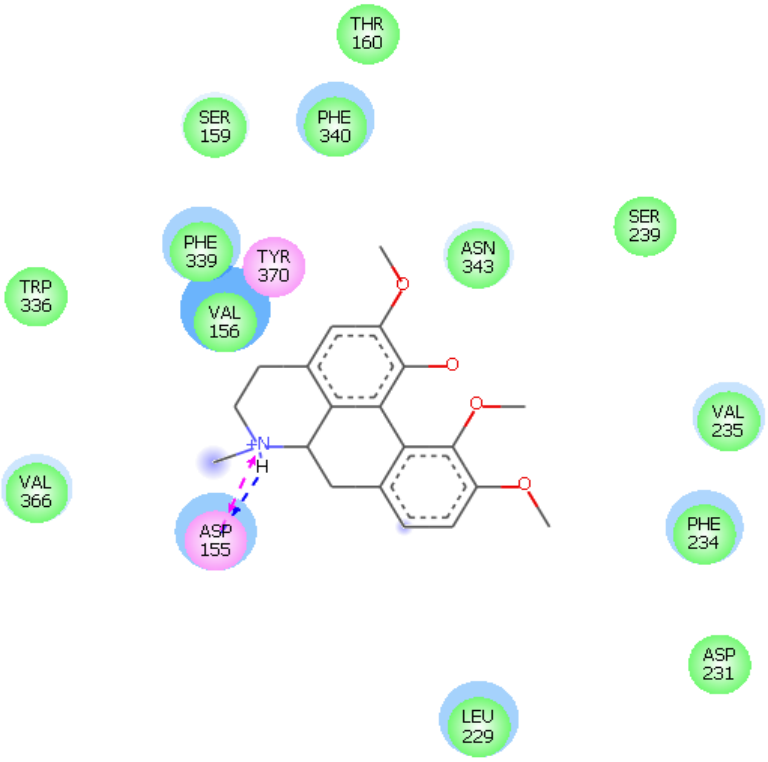
(g) Proposed binding mode of (*R*)-2-hydroxy-*N*-propylapomorphine in the 5-HT_{2A} receptor



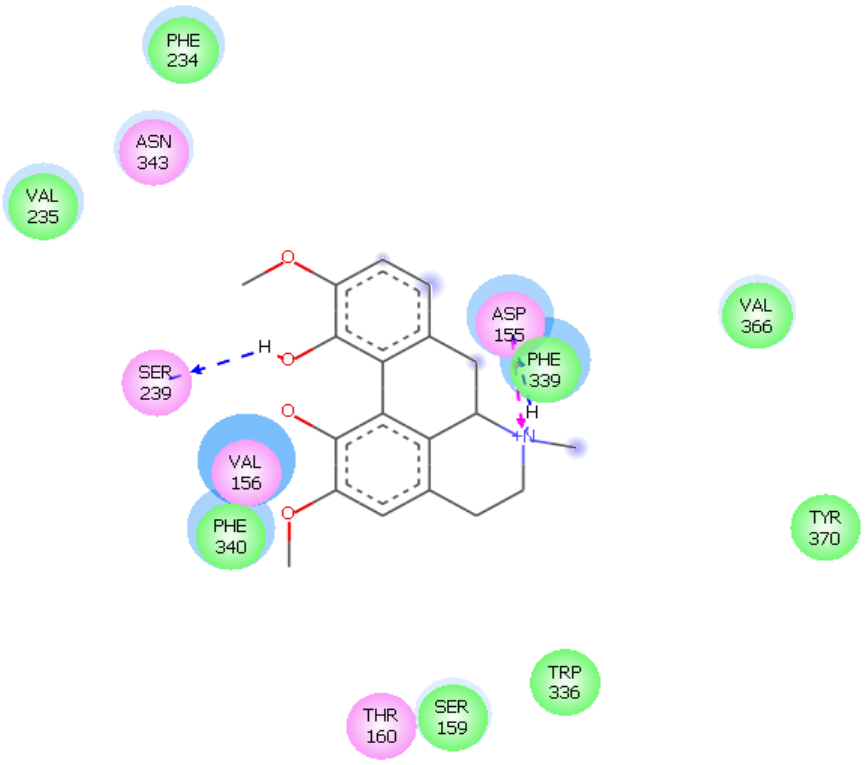
(h) Proposed binding mode of (*S*)-isocorydine in the 5-HT_{2A} receptor



(i) Proposed binding mode of (*S*)-isothebaine in the 5-HT_{2A} receptor



(j) Proposed binding mode of (*S*)-corydine in the 5-HT_{2A} receptor



(k) Proposed binding mode of (*S*)-corytuberine in the 5-HT_{2A} receptor

Figure 8.8: Proposed binding mode of **(a)** (*S*)-bulbocapnine **(b)** (*S*)-laureline **(c)** (*S*)-nuciferine **(d)** (*R*)-apomorphine **(e)** (*S*)-apomorphine **(f)** (*R*)-*N*-propylapomorphine **(g)** (*R*)-2-hydroxy-*N*-propylapomorphine **(h)** (*S*)-isocorydine **(i)** (*S*)-isothebaine **(j)** (*S*)-corydine **(k)** (*S*)-corytuberine in the 5-HT_{2A} receptor. The residues involved in hydrogen bonding, electrostatic or polar interactions were represented in pink circles with their hydrogen bonds with their main chains (blue dashed arrow), side chains (green dashed arrow), charge interactions (pink dashed double-ended arrow), pi interaction (orange line) and residues involved in van der Waals interactions (green circles).

F: Abstracts of Research Presentations

Following is the abstract for oral presentation at the 1st Pharmaceutical Sciences Conference & Exhibition (PSCE), Penang, Malaysia, 27th–28th September 2010

COMPUTATIONAL INVESTIGATION OF THE 5-HT_{2A} RECEPTOR BINDING INTERACTIONS OF STANDARD LIGANDS AND APORPHINES

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[2]*School of Pharmacy, University of Nottingham Malaysia Campus, 43500 Semenyih, Selangor*

The 5-hydroxytryptamine 2A (5-HT_{2A}) receptor has been implicated in numerous psychiatric disorders, including schizophrenia and depression. Several aporphines have been found to have 5-HT_{2A} receptor binding activity. The aim of this study was to investigate the ligand-receptor interactions of standard 5-HT_{2A} ligands and aporphines. A 3D molecular model of the rat 5-HT_{2A} receptor was constructed by homology modelling against the known crystal structure of the human β_2-adrenergic receptor (PDB ID: 2RH1). Docking studies were then conducted using Autodock4.0. The standard ligands and aporphines were found to interact with residues belonging to transmembrane domains 2, 3,5,6,7 and extracellular loop 2 (ECL2). The proposed binding interactions involve three sets of residues, (1) Asp155 (3.32) forming a hydrogen bond with the protonated amine of the ligands, (2) an aromatic network consisting of Trp151(3.28), Trp336(6.48), Phe339(6.51), Phe340(6.52), and Tyr370(7.43), and (3) Val156(3.33), Thr160(3.37), Leu229(ECL2), Gly238(5.42), Ser239 (5.43), Asn363(7.36) as possible hydrogen bond acceptors or donors. These findings are in agreement with currently available experimental data and suggest that the model has the potential to predict ligand-receptor interactions at the molecular level.

Following is the abstract for poster presentation at the 25th Scientific Meeting of The Malaysian Society of Pharmacology and Physiology (MSPP), University Putra Malaysia, Malaysia, 25th–26th of May 2011:

DEVELOPMENT OF FILTRATION BASED DOPAMINE$_1$ RECEPTOR BINDING ASSAY USING 96-WELL MICROPLATE

Vani Munusamy, Chung Lip Yong & Michael James Christopher Buckle

Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.

The dopamine$_1$ receptor plays a pivotal role in the treatment of neuropsychiatric and neurodegenerative disorders such as schizophrenia and Parkinson's disease. This study aims to develop a medium throughput screening assay for the search of potential dopaminergic ligands. A 96 well microplate was adopted in combination with Filterplate Cell Harvester and TopCount NXT. The optimised parameters consisted of 30 µg of protein/well of striatum in the presence of 0.5 nM of [^{3}H] SCH23390 and 1 µM of SCH 23390 HCl. The equilibration time of ligand receptor interaction was reached after 90 min of incubation at 22°C. GF/C filter plates gave lower nonspecific binding compared to GF/B filter plates after 4 times washing with Tris-HCl buffer (50mM, pH 7.4). Saturation analysis of [^{3}H] SCH23390 binding revealed mean B_{max} and K_d values of 293.7 fmol/mg protein and 0.41 nM respectively. Competition experiments with standard ligands include *R*-(+)-SCH23390 HCl (K_i = 1.05 x 10^{-10}), propranolol HCl (K_i = 3.66 x 10^{-7}), serotonin HCl (K_i = 4.06 x 10^{-7}), buspirone HCl (K_i = 5.38 x 10^{-7}) and dicyclomine HCl (K_i = 1.69 x 10^{-6}). A z′ factor of 0.74 demonstrates the suitability, reliability and robustness of the assay for screening purposes.

Keywords: Central nervous system, dopamine$_1$ receptor, receptor binding assay, saturation experiment, competition experiment