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Structure and function of 5-HT_3 receptors unveiled by computational and experimental studies

Marta Del Cadia

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Summary

5-hydroxytryptamine type-3 receptor (5-HT_3) is a cation selective transmembrane pentamer whose functional stoichiometries and subunit arrangements are still debated, due to the extreme complexity of the system. Five different 5-HT_3 receptor subunits, 5-HT_3A to E, have been identified. Only the subunit 5-HT_3A is capable of forming functional homopentameric receptors, while all the other subunits are apparently functional only if co-expressed with 5-HT_3A. The three-dimensional structure of any of the 5-HT_3R subunits has not been solved as yet. Moreover, most of the available structural and functional data are related to the extracellular ligand-binding domain, whereas the transmembrane and the intracellular receptor domains are far less characterised, although they are known to be crucial for receptor functionality.

In addition, the 5-HT_3 receptor is an important target of many neuroactive drugs, among which palonosetron is a potent new generation 5-HT_3 receptor antagonist, whose mechanism of action is not yet fully understood. Palonosetron acts at the 5-HT_3 receptor binding site, however, recent computational studies suggest a possible second site of action in the extracellular domain, hence stimulating further interest towards multivalency in the 5-HT_3 receptors.

This thesis is aimed at getting new insights into the structural and functional features of the 5-HT_3 system to deepen the knowledge of key aspects, still largely debated in the literature, such as the binding interface, the receptor multivalency and its functional stoichiometric composition. To this aim, a combined computational-experimental approach was envisaged. Computational techniques, such as sequence alignments, homology modelling, receptor-ligand docking, calculations of physicochemical parameters, are fundamental to get a detailed molecular description of the highly complex receptor system and make hypotheses about its functional mechanisms. Experiments, i.e. mutagenesis and radioligand binding techniques, are required to test/check the computational hypotheses.

First, the attention was focused on the characterization of the ligand binding interface, exploiting a previously modelled extracellular portion of the 5-HT_3R structure. **Docking studies** of new antagonists into the three-dimensional models of homomeric 5-HT_3R_{A-A} binding interface were performed to check whether the receptor could accommodate bivalent arylpiperazine-derivate ligands. The analysis of the binding modes of the bivalent ligands identifies potential putative additional binding pockets and suggests the presence of three potential accessory binding sites (for the arylpiperazine moiety) located on the receptor surface. This result, together with the putative allosteric binding site found for palonosetron, might be a key issue in the comprehension of 5-HT_3R functioning, since it further suggests that multivalency in 5-HT_3 receptor could involve receptor domains different from the main binding site.

In order to verify the presence of the putative allosteric binding site, previously found for palonosetron, mutagenesis experiments were needed. These studies have been carried out in the Biochemistry laboratories of Dr. Sarah Lummis at the University of Cambridge. A series of experimental **site-directed mutagenesis** were performed in both the 5-HT_3A and 5-HT_3B receptor subunits: Tyr^{68}, Phe^{125}, Ser^{158}, and Asp^{160} in the 5-HT_3A subunit and His^{66}, Phe^{123}, Glu^{158}, Tyr^{136} and Ile^{176} in the 5-HT_3B subunit. Homomeric (A only) and heteromeric (AB) receptors were then expressed in HEK293 cells to determine the potency of palonosetron by both functional studies and radioligand binding. The data show that mutating Tyr^{68} has a small effect on both palonosetron binding and inhibition of 5-HT induced responses, and thus this residue may contribute to an alternative palonosetron binding site. The other residues, although they do not directly affect binding, may be involved either in the conformational or in the structural changes of the putative binding site, modifying the receptor sensitivity.

Finally, a **physicochemical analysis** of both the extracellular and transmembrane/intracellular domains was performed with the aim of identifying factors which could determine the receptor stoichiometry. Being the binding site located at the extracellular interface between two adjacent subunits, the large variability of the 5-HT_3R composition becomes a crucial issue, since it can originate many different interfaces

providing non equivalent ligand binding sites and complicating the pharmacological modulation. Hence, the different extracellular 5-HT_3R interfaces were analysed, on the basis of the structural conformations of previously built 3D homology models, by addressing their physicochemical characterisation, in particular by calculating the molecular electrostatic potential and the hydrophobic potential. The results confirm the presence of an aromatic cluster located at the core of the A-A interface as a key determinant for having an interface both stable and functional. This is used as a discriminant to make hypotheses about the capability of all the other possible interfaces constituted by the known 5-HT_3R sequences A, B, C, D, and E to build active receptors.

Moreover, for the first time, three-dimensional homology models of the transmembrane and the intracellular receptor domains of all the known human 5-HT_3 subunits were built and assembled into homopentameric and heteropentameric receptors. The models have been built by **homology modelling** on the basis of the known three-dimensional structures of the nicotinic-acetylcholine receptor and of the ligand-gated ion channel from *Erwinia chrysanthemi*. The comparative analyses of sequences, modelled structures and computed electrostatic properties of single domain subunits and of assembled domain pentamers shed new light both on the stoichiometric composition and on the physicochemical requirements of the functional receptors. In particular, it emerges that the favourable environment that the Ca^{2+} ion encounters, when passing through the pore at the TM and IC domain levels, is granted by the fact that no more than two B subunits should be co-expressed in the 5-HT_3 pentamer.

In this thesis, a combined computational and experimental approach is described, that focuses on the study of the 5-HT_3R, a biological target. The computational techniques allow the study of biological complex systems at the molecular level, analysing structural properties and their influence on function. Understanding and predicting the behaviour of a protein with *in silico* methods can be very important in the pharmaceutical field, as they allow a dramatic reduction in terms of time and costs in the drug development process with respect to the experimental approach.

In particular, the computational techniques help in analysing protein-ligand interactions or interactions among proteins, in rationalising the structural requirements for specific binding, and in highlighting structural and electrostatic features which are important for protein function. Although the computational approaches provide a detailed description of biomolecular systems, these techniques provides 3D models that have to be considered working hypotheses, thus the results obtained need to be verified by comparison with experimental data. In addition to the proposed models validation, the knowledge acquired with the computational analyses is useful for suggesting further experiments aimed at obtaining additional information on the biological systems studied.

Contents

CHAPTER I

INTRODUCTION

1.1 The 5-hydroxytryptamine 3 receptor

The serotonin receptors, also known as 5-hydroxytryptamine receptors or 5-HT receptors, are a group of membrane proteins found in the central and peripheral nervous systems that mediate both excitatory and inhibitory neurotransmission. They are activated by the neurotransmitter serotonin, which acts as their natural ligand.

The serotonin receptors influence various biological and neurological processes such as anxiety, cognition, memory, mood, nausea, sleep, and so they are target of a variety of pharmaceutical drugs, including many antidepressants, antipsychotics, antiemetics and gastroprokinetic agents.

Most of the receptors for serotonin are part of the G-protein coupled family with the exception of the 5-HT type-3 receptor (5-HT_3R), which is part of the ligand-gated ion channel (LGIC) family. In particular, the 5-HT_3R belongs to the Cys-loop LGIC superfamily [Thompson & Lummis 2006 & 2007; Hannon & Hoyer 2008], which also includes receptors for nicotinic Acetylcholine (nACh), ɣ-aminobutyric acid ($GABA_A$) and glycine (Gly), all responsible for fast synaptic transmission at chemical synapses [Le & Changeux, 2001; Peters *et al*., 2005].

They are known as Cys-loop receptors because all family subunits contain two invariant cysteine residues, separated by 13 amino acids and linked by a disulphide bridge (Figure 1), which form a loop, the “cys-loop”, located at the interface between extracellular and transmembrane domain.

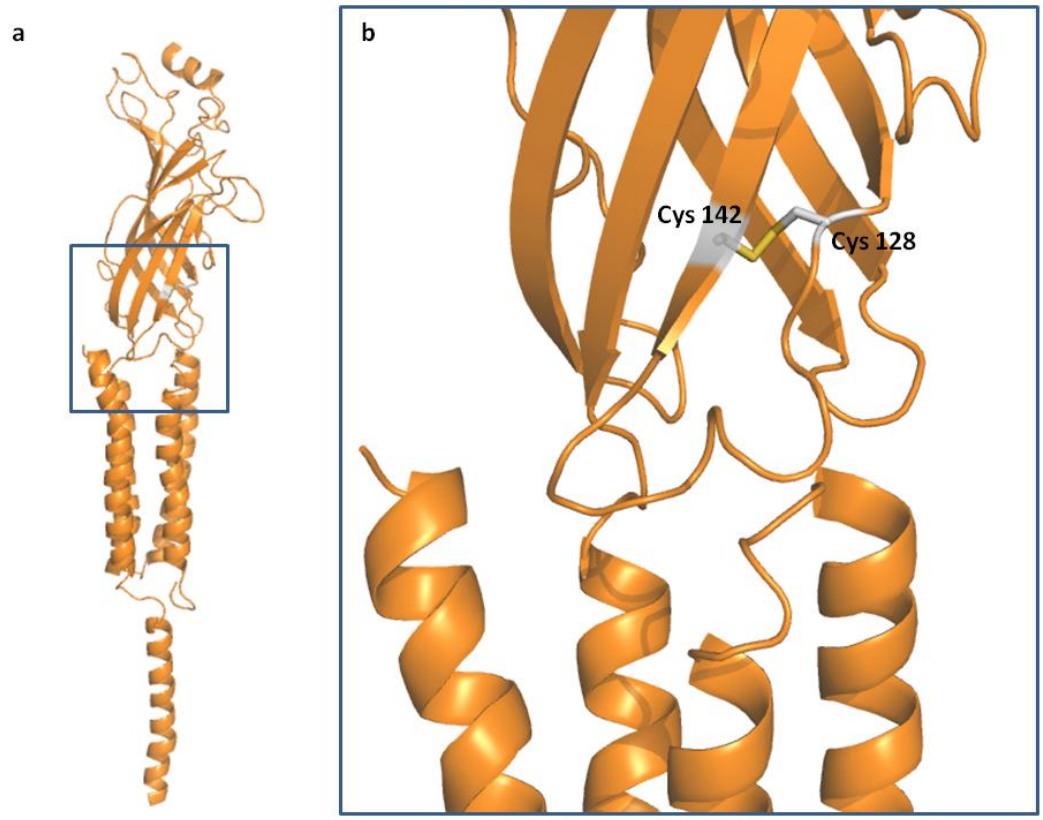

Figure 1- a) Side view of an α-subunit from the *Torpedo* acetylcholine receptor (Protein Data Bank code 2BG9); **b)** Close-up view of the binding–channel interface with the Cys-loop (Cys 142 and 128) highlighted in grey.

Cys-loop receptors play an important role in neurotransmission, converting chemical recognition into an electrical impulse. At chemical synapses, the presynaptic neuron releases neurotransmitter molecules into the synaptic cleft, which bind selectively to their receptors on the postsynaptic neuron. The binding of a selective ligand results in a conformational change of the receptor protein. This triggers the opening of the ion channel causing a flux of ions across the membrane. As a result, an excitatory or inhibitory response in the postsynaptic neuron will be generated. Within milliseconds the channel closes and the receptor desensitises. Subsequently, the neurotransmitter dissociates and the receptor re-sensitises ending the synaptic event (Figure 2).

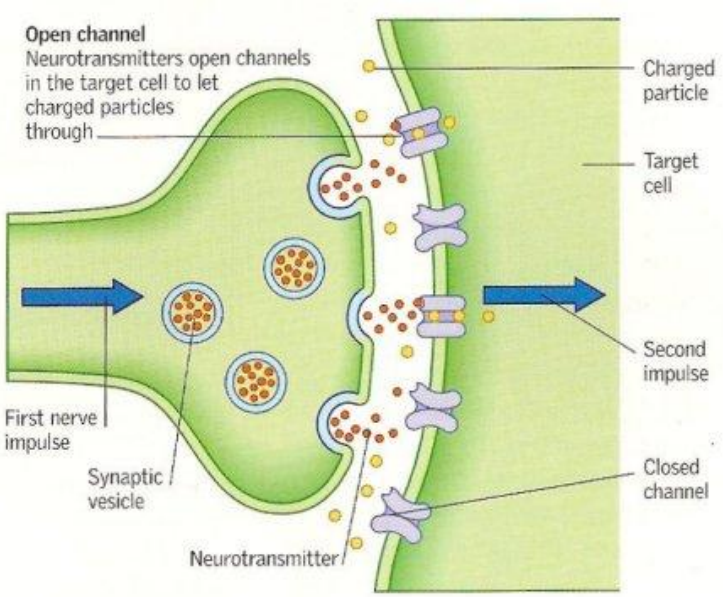

Figure 2- In neuronal signaling, the neurotransmitter is released from the synaptic vesicles in the presynaptic cell, diffuses across the synaptic cleft and binds to the receptors on the post-synaptic membrane. *This image is courtesy of David Darling/Encyclopedia of Science.*

Stimulation of $GABA_A$ receptors or glycine receptors allows influx of chloride ions and the hyperpolarisation of the membrane, which produces an inhibitory response. Activation of 5-HT_3 or nACh receptors, on the other hand, produces an excitatory postsynaptic potential (EPSP) as cations enter and cause depolarization of the membrane. The nAChR has been well-characterised, using pharmacological and molecular biological approaches, and consequently is often used as a prototype for this family.

1.2 Location and function

Radioligand binding and *in situ* hybridization studies have shown that 5-HT_3Rs are present throughout the central nervous system (CNS) and peripheral nervous system (PNS). A high density of 5-HT_3 receptors (although still low compared to other serotonin receptors) has been found at the following CNS localizations (mostly on GABAnergic neurons): area postrema, nucleus tractus solitarii, nucleus dorsalis nervi vagi, nucleus caudatus, nucleus accumbens, amygdala, hippocampus, entorhinal cortex, frontal cortex, cingulate cortex,

and dorsal horn ganglia [Tecott *et al.*, 1993]. The receptors are thus concentrated in regions that are involved, among other processes, in integration of the vomiting reflex, pain processing, the reward system and anxiety control. Furthermore, 5-HT_3 receptors are found predominantly in presynaptic regions associated with axons and nerve terminals, localization which is consistent with a physiological role of the receptors in the control of a variety of neurotransmitters release.

5-HT_3 receptors have also been characterised in peripheral tissues, such as enteric, sympathetic and parasympathetic autonomic and primary sensory neurons, where they have roles in evoking neurotransmitter release, controlling intestinal contraction [Gaddum & Picarelli, 1957], reflex bradycardia and hypotension [Fozard & Host, 1982] and pain transmission [Richardson *et al.*, 1985].

At molecular level, activation of presynaptic 5-HT_3 receptors is followed by rapid depolarization of the peripheral or central neuron. The depolarization causes a rapid rise in cytosolic Ca^{2+} concentration by inducing calcium influx and mobilization of intracellular calcium stores, as well as the release of various neurotransmitters and neuropeptides such as dopamine, cholecystokinin, glutamate, acetylcholine, GABA, substance P or serotonin itself [Greenshaw & Silverstone, 1997; Funahashi *et al.*, 2004]. Postsynaptic activation leads to depolarization by Na^+- and K^+-influx [Ronde & Nichols, 1998]. The activation status of the 5-HT_3 receptor is influenced by multiple factors which are probably only in part identified at present.

1.3 The 5-HT_3 heterogeneity

To date, five different 5-HT_3 receptor subunits have been identified: subunits A and B have been largely investigated, while subunits C, D, and E have been sequenced but not yet fully characterised [Barnes *et al.*, 2009; Karnovsky *et al.*, 2003].

The subunit 5-HT_3A, cloned in 1991 [Maricq *et al.,* 1991], is capable of forming functional homopentameric receptors *in vitro*; however, expression of this subunit does not fully

reproduce the biophysical characteristics of some native 5-HT_3 receptors [Hussy *et al.,* 1994]. This suggests that *in vivo* the 5-HT_3R is sometimes composed of more than one subunit type. A heteromeric receptor would be more consistent with other members of the Cys-loop LGIC family, which are often composed of 2-4 subunit types.

The 5-HT_3B subunit, discovered in 1999 [Davies *et al.,* 1999; Dubin *et al.,* 1999], is present in the same chromosomal location as the A subunit and shares 45% sequence identity with it. The B subunit is unable to express as homopentamer but co-expresses rather as AB heteropentamer [Davies *et al.*, 1999; Boyd *et al.*, 2002]. In the absence of the A subunit, the B subunit is retained in the endoplasmatic reticulum [Boyd *et al.*, 2002]. The presence of the B subunit in the 5-HT_3AB heteromeric receptor seems to modulate the biophysical and the pharmacological properties of the receptor [Dubin *et al.,* 1999; Brady *et al.,* 2001; Hapfelmeier *et al.,* 2003], with the most notable alteration being a significantly larger single-channel conductance [Davies *et al.,* 1999; Hanna *et al.,* 2000].

The stoichiometry of the 5-HT_3AB heteromeric receptor has been studied for the first time by Barrera and co-workers [Barrera *et al*., 2005] using atomic force microscopy (AFM) and suggesting a 3B:2A ratio, with a BBABA arrangement. Recently, this stoichioimetry of the receptor has been brought into question because site-directed mutagenesis experiments showed the presence of at least one AA interface in the 5-HT_3AB heteromeric receptor [Lochner & Lummis, 2010, Thompson *et al.*, 2011]. Functional stoichiometries and subunit arrangements of the heteromeric receptors are still debated.

The cloning of additional subunits, 5-HT_3C, 5-HT_3D, and 5-HT_3E is reported in literature, but information supporting their specific characteristics either as receptor or accessory protein is not available. In recent times, Niesler and co-workers re-investigated whether these novel subunits are able to form functional 5-HT_3 receptor complexes [Niesler *et al.*, 2007]. Using immunofluorescence and immunoprecipitation, it was demonstrated that each of 5-HT_3C, 5-HT_3D, and 5-HT_3E co-assembles with 5-HT_3A. Radioligand binding and calcium-influx studies in HEK 293 cells indicated that 5-HT_3C, 5-HT_3D, and 5-HT_3E subunits expressed alone are non-functional, like the 5-HT_3B [Nielser *et al*. 2007]. Co-expression

with 5-HT_3A, however, resulted in the formation of functional heteropentameric complexes showing quantitatively different functional properties compared to homopentameric 5-HT_3A receptors. However, further work is required to confirm a contribution of these subunits to differences in pharmacology of 5-HT_3 receptors [Barnes *et al.*, 2009].

The expression of C, D and E subunits as homopentamer is equivocal: Niesler and co-workers [Niesler *et al.*, 2007] indicate that subunits C, D and E do not reach the plasma membrane in HEK 293 cells unless co-expressed with the A subunit, while Holbrook and co-workers [Holbrook *et al*., 2009] suggest that these subunits can independently reach the plasma membrane in CHO cells. However, both the studies agree on the non-functionality of these three homopentamers.

The C, D, and E subunits are expressed in numerous mammalian species [Holbrook et al., 2009], but are absent in mice and rats [Karnovsky *et al*., 2003; Niesler *et al*., 2003; Holbrook *et al*., 2009]. Unlike the other subunits that are expressed in both the central nervous system and in the periphery, 5-HT_3D is predominantly and 5-HT_3E is exclusively expressed in the gastrointestinal tract [Nielser *et al*., 2003].

1.4 The 5-HT_3 structure

Despite the physiological and pharmacological relevance of membrane proteins, less than 1% of their structures have been resolved. The difficulty in purification and crystallisation of membrane proteins account for the difficulty in obtaining high-resolution X-ray images of these proteins.

Although the 3D structure of 5-HT_3R is not yet known, a large amount of experiments, mainly mutagenesis, have been conducted in the last decade, with the aim of getting information on the structure and function of the 5-HT_3 receptor [Thompson & Lummis, 2006, 2007]. The results, together with the comparison with the closely related nAChR crystal structure, support the idea that the 5-HT_3 receptor is formed by the pentameric

assembly of subunits, which could be identical (homomeric) or different (heteromeric), surrounding a central ion channel (Figure 3a) permeable to small ions (Na^+, K^+) [Barnes *et al*., 2009; Boess *et al*. 1992, 1995]. Each subunit is composed of an extracellular N-terminal domain (ECD), a transmembrane domain (TMD) and an intracellular C-terminal domain (ICD) (Figure 3b).

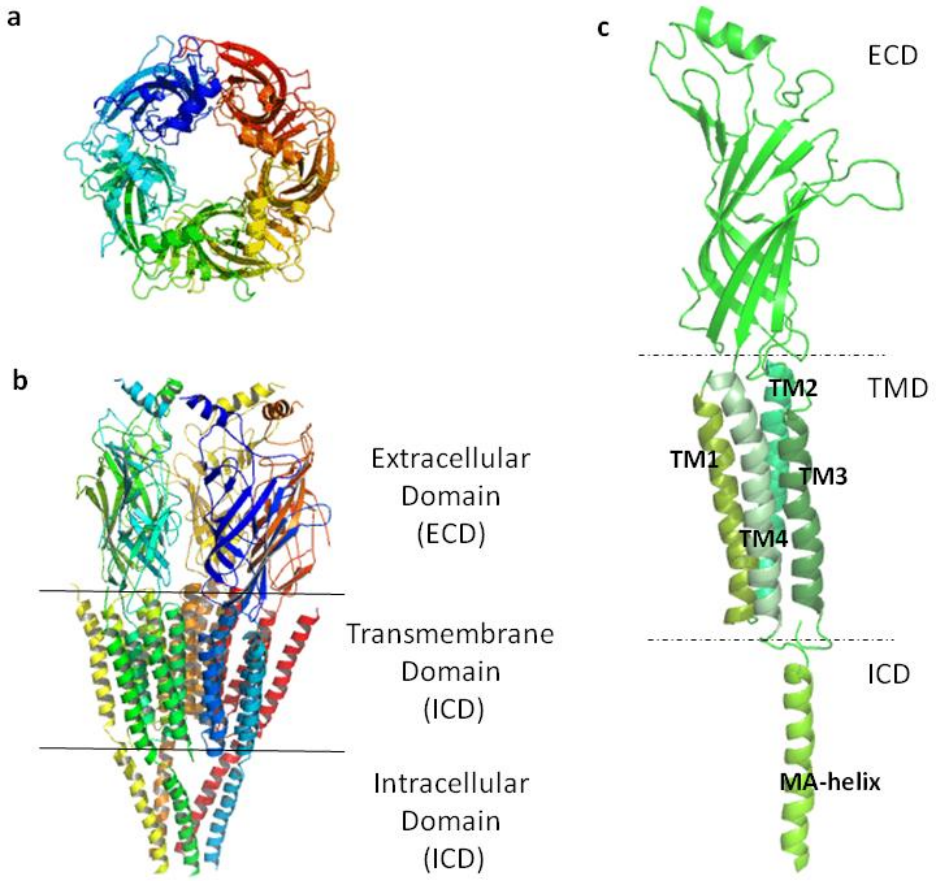

Figure 3- a) Top view of the nACh receptor from *Torpedo marmorata* (PDB: 2BG9): the five subunits (each subunit in a different colour) surround a central ion-conducting pore; **b)** side view of the receptor highlighting the three main functional domains; **c)** side view of a receptor subunit showing the three domains, the four transmembrane α-helices (TM1-TM4) and the MA-helix .

The ECD contains the ligand binding region which lies at the interface between two subunits, and for this reason it is the major therapeutic target in 5-HT_3 receptor [Reeves & Lummis 2002; Thompson and Lummis, 2007]. The TMD is composed of four α-helices TM1-TM4 connected by an extracellular loop (TM2-TM3) and two intracellular linkers

TM1-TM2 and TM3-TM4). The pore-lining domain consists of the TM2 transmembrane α-helix, and it is responsible for the ionic selectivity of the receptor, while the TM1, TM3 and TM4 helices keep TM2 apart from the membrane [Miyazawa *et al.*, 2003; Unwin, 2005; Peters *et al*., 2005]. The ICD is formed by the long TM3-TM4 linker, consisting of a large unstructured loop and a α-helical segment, called the MA-helix [Karlin, 2002; Unwin, 2005]; it modulates channel function by secondary messengers, regulates receptor trafficking and sorting with intracellular proteins and modulates ion flow and conductance [reviewed in Thompson *et al.,* 2010].

1.5 5-HT_3 Receptor models

Despite the fact that the research on the 5-HT_3 receptor is thriving, the absence of a resolved structure for this receptor represents a significantly deficit. With the aim to fill this gap, rationalise and interpret the experimental data, different computer-aided 3D models of the 5-HT_3 receptor have been created throughout the years [Menziani *et al.*, 2001; Maksay *et al.,* 2003; Reeves *et al.,* 2003; Yan & White 2005; Joshi *et al*. 2006; Moura Barbosa *et al*., 2010]. First models were built using as template a model of the nACh receptor [Tsigenly *et al*. 1997], as in the case of the rat 5-HT_3 receptor model built by Menziani and co-workers [Menziani *et al.*, 2001]. Then, with the crystallisation of the Acetylcholine Binding Protein structure (AChBP, PDB 1IB9, [Brejc *et al.*, 2001]), which is highly homologous to the nACh receptor and shows 20-25% sequence identity with the nACh receptor ECD and 19% with the 5-HT_3A receptor ECD, and later, with the production of the whole nACh *Torpedo* receptor by electron microscopy at 4 Å resolution (PDB 2BG9, [Unwin, 2005]), new and more accurate 3D models were built [Maksay *et al.,* 2003; Reeves *et al.,* 2003; Yan & White 2005; Joshi *et al*., 2006; Moura Barbosa *et al*., 2010]. In particular, the availability of the 3D nAChR structure, which is likely to be structurally representative of the LGIC receptors, has been invaluable in the interpretation of functional and pharmacological data about the 5-HT_3 receptor, opening also the possibility of acquiring direct information on the LGICs.

In addition to these structural models, more recently high resolution structural information has become available from studies of proteins which show close sequence similarity to nAChR: the X-ray pentameric structures of the prokaryotic ligand gated ion channels from the bacterium *Erwinia chrysanthemi* (ELIC, 3.3 Å; PDB 2VL0, [Hilf & Dutzler, 2008]) and from *Gloeobacter violaceus* (GLIC, 3.1 Å; PDB 3EHZ, [Bocquet *et al*., 2009]); the X-ray eukaryotic homopentameric glutamate-gated chloride channel α from *Caenorhabditis elegans* (GluCl, 3.26 Å; PDB 3RHW, [Hibbs & Gauaux, 2011]).

The vast protein modelling activity, combined with experimental studies, has permitted to obtain a vast and reliable mapping of several functional residues on the receptor model, in particular within the receptor binding site. In fact, the extensive studies performed on the 5-HT_3R extracellular domain led to quite a vast and deep knowledge of this portion of the receptor. On the contrary, the TMD and the ICD are far less characterised from both the experimental and computational points of view.

1.6 The ligand binding site

The ligand binding site lies at the interface of two adjacent subunits in the extracellular, N-terminal, domain and is formed by the convergence of six loops [Lummis, 2012; Thompson & Lummis 2006, 2007; Barnes *et al.*, 2009]: *A*, *B* and *C* from one of the two subunits, named the *principal* subunit, and loops *D*, *E* and *F* in the second subunit, the *complementary* subunit (Figure 4).

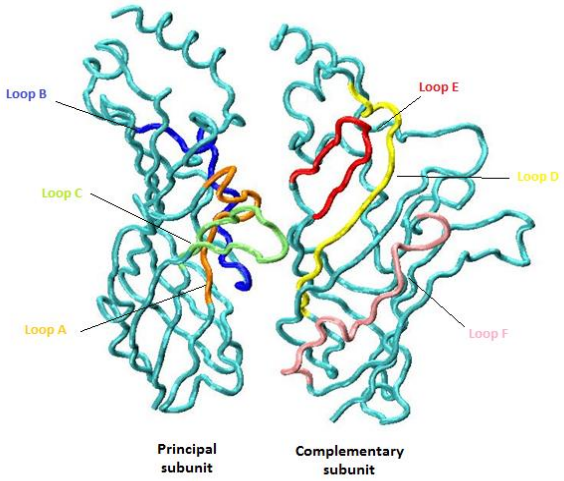

Figure 4- Homology model of the mouse 5-HT_3 receptor homodimer A, showing 2 adjacent subunits with their loops in evidence: loop A in orange, loop B in blue, loop C in green, loop D in yellow, loop E in red and loop F in pink.

Only some of the amino acids within

each loop face into the binding pocket, resulting as key residues for the binding of agonists and/or antagonists; the other residues have roles in maintaining the structure of the binding pocket and/or contributing to the receptor conformational changes [Lummis, 2012;Thompson & Lummis 2006, 2007].

Briefly, the results of single point mutagenesis studies (performed on mouse 5-HT_3R) suggest that various residues from the principal and the complementary subunits of the homopentamer are involved in important interactions with the ligands, both serotonin and antagonists (see Table 1 and Figure 5).

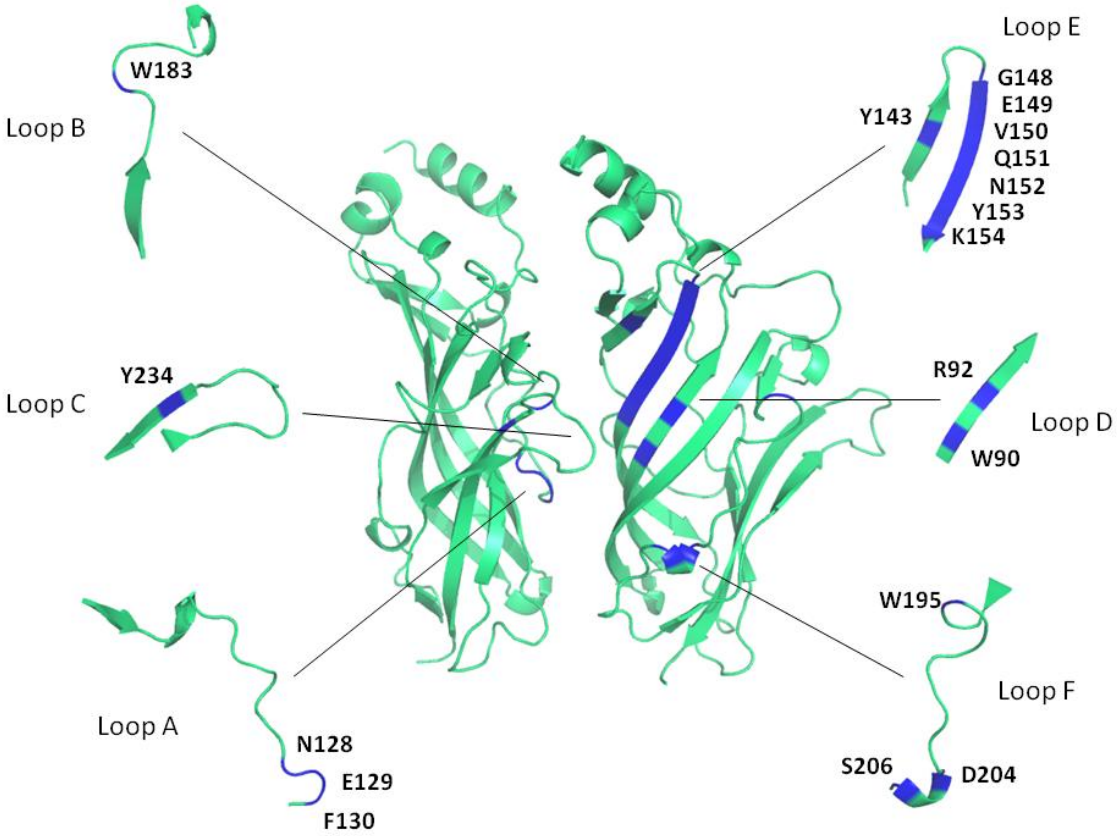

Figure 5- Homology model of the mouse 5-HT_3 receptor homodimer (AA interface), showing the residues into the binding site that were demonstrated to cause significant changes in the binding of agonists or antagonists [Beene *et al*., 2002; Boess *et al.*, 1997; Lester *et al*., 2004; Price *et al*., 2008; Price & Lummis, 2004; Spier & Lummis, 2000; Steward *et al.*, 2000; Sullivan *et al.*, 2006; Thompson *et al*., 2006, 2008; Venkataraman *et al*., 2002, Yan & White 2005].

Mutations in loop A were focussed on the sequence ^{128}AsnGluPhe^{130}, even though only Glu^{129} is involved in ligand binding, by forming a hydrogen bond with the hydroxyl of 5-HT [Price *et al*., 2008]. The substitution of the other two residues has large effects on receptor function, playing a role in receptor gating without being directly involved in binding ligands [Boess *et al*., 1997; Steward *et al*. 2000; Sullivan *et al*., 2006; Yan *et al*., 1999]. In loop B many residues are important for receptor function, and among these Trp^{183} plays an especially critical role, forming a cation-π interaction with the primary amine of 5-HT [Spier & Lummis, 2000; Thompson *et al*., 2008; Venkataraman *et al*., 2002; Beene *et al*., 2002]. Loop C, whose residues are the most divergent between species, is important in determining the species specificity of various drugs [Suryanarayanan *et al*., 2005]. Point mutations throughout the loop C region did not identify any residues essential for binding of agonist or antagonist, except Tyr^{234}, which forms part of the aromatic box found in all Cys loop receptors [Lester *et al*., 2004; Price & Lummis, 2004], resulting critical for both agonist and antagonist binding.

In loop D the aromatic residue Trp^{90} is critical for ligand binding [Spier & Lummis, 2000], and double-mutant cycle analysis at Trp^{90} and Arg^{92} has indicated that antagonists may directly contact these two residues [Yan & White, 2005]. Loop E residues Tyr^{143}, Gly^{148}, Glu^{149}, Val^{150}, Gln^{151}, Asn^{152}, Tyr^{153} and Lys^{154} may all be important for antagonist binding, and perhaps function, although it is not clear if some of these effects are due to alterations in the binding site structure [Yan & White, 2005; Price & Lummis, 2004; Venkataraman *et al.*, 2002]. The structure of loop F is not well defined yet, nevertheless a study of antagonist binding has implicated Trp^{195}, Asp^{204} and Ser^{206} as potentially important residues for ligand binding [Thompson *et al*., 2006]; alternatively these residues may influence conformational changes in or close to the binding pocket.

Table 1- Residues of the mouse 5-HT_3 homomeric receptor involved in important interactions with the ligands.

Residues	Loop	References
E129	A	Price *et al.*, 2008
W183	B	Spier and Lummis, 2000; Thompson *et al*., 2008; Venkataraman *et al*., 2002; Beene *et al*., 2002
Y234	C	Lester *et al*., 2004; Price & Lummis, 2004
W90, R92	D	Spier & Lummis, 2000; Yan & White, 2005
Y143, G148, E149, V150, Q151, N152, Y153, L154	E	Yan & White, 2005; Price & Lummis, 2004; Venkataraman *et al*., 2002
W195, D204, S206	F	Thompson *et al.,* 2006

Despite the physiological and potential therapeutic significance of the 5-HT_3AB heteromeric receptor, little is known about its architecture and it is not clear which residues are responsible for ligand binding. In 2005, Barrera and co-workers used atomic force microscopy to study the 5-HT_3AB receptor and suggested that the subunit stoichiometry is 2A:3B with the A and B subunit arrangement around the receptor rosette being BBABA. Thus, while in the homopentamer there are only equivalent AA subunit interfaces, in the heteropentamer, there are two AB, two BA and one BB interfaces. These different interfaces provide non equivalent ligand binding sites, as suggested by the different values of the Hill coefficient observed in the heteropentamer and in the homopentamer for the binding of the natural ligand serotonin [Bower *et al.,* 2008]. However, the stoichiometry found out by Barrera and co-workers has been recently brought into question by Lochner and Lummis [Lochner & Lummis, 2010]. In their work,

binding-site residues in the mouse 5-HT_3A subunit were mutated to the corresponding mouse 5-HT_3B subunit residues, demonstrating that the mutant receptors reduced significantly antagonist-binding affinity and increased 5-HT EC_{50}. On the contrary, no significant changes were observed when A-like mutations were introduced in the mouse 5-HT_3B subunit. Thus, the experimental data suggested that the 5-HT_3B subunit does not contribute to the binding site and 5-HT_3AB receptors presumably contain at least one AA interface.

1.7 Gating in 5-HT_3 Receptor

The 5-HT_3Rs, similar to other LGICs, are gated, i.e. they switch between an activated (functional) open conformation, when the ligand binds to the receptor and the ions flow through the channel, and a non-activated closed conformation, in the absence of ligand binding, which precludes the ion flux [Barry & Lynch, 2005]. When the ligand is continuously present in high concentrations, LGICs can also go into a desensitised and non-conducting state: the ligand is still bound, but the receptor is unable to conduct [Barry & Lynch, 2005]. The channel opening-closing mechanism is still far from being completely understood.

A peculiar activation/deactivation mechanism was proposed, involving conformational changes of the large loop C, which surrounds the binding cavity. Loop C of the Cys-loop LGIC family has been suggested to behave like a “lid”, opening and closing the access to the binding site [Karlin, 2002]. The “lid-shut” conformation seems to correspond to the activated ionophore, contrary to the “lid-open” conformation which corresponds to a ligand-free receptor state [Karlin, 2002].

In addition, on the basis of studies performed on nAChR, it is known that the ligand binding in the ECD induces conformational changes, transferred from the ECD to the TMD, that cause in particular the twist of the TM2 helix [Grosman *et al*., 2000]. Molecular dynamics simulations suggest that, after ligand binding, the ECD rotates around the pore axis, and this rotation propagates first to TM1 and then to TM2, and their global rotation

drives TM3 and TM4 to rotate [Beckstein & Sansom, 2006; Liu *et al*. 2008]. Movement of the TM2 leads to a separation of the helices, disrupting the hydrophobic interactions between the five TM2 helices, and to an increasing of the pore radius, moving and distancing the side chains of those residues involved in the hydrophobic gate [Hung *et al*., 2005]. The gate is formed by rings of hydrophobic residues located at the centre of the TM2 helix, in particular results of cryo-electron microscopy studies of the nAChR [Miyazawa *et al*., 2003] suggest that the 9' leucine residue and the structurally adjacent 13' valine residue form a hydrophobic girdle in the channel, which acts as an energetic barrier to ions. Several experiments proved that residues which are highly conserved across all LGICs have a critical role in channel function [Lester *et al*., 2004; Filatov & White, 1995; Corringer *et al*., 2000].

Comparison of the structures of prokaryotic pentameric ligand-gate ion channels has provided insights into the channel opening mechanism [Bouzat, 2012]. The two structures represent different conducting states, a closed channel in ELIC and opened in GLIC. The transmembrane pore of ELIC presents two hydrophobic rings carrying bulky side chains that interrupt the water-filled channel, probably preventing ion conduction and acting as a gate, similarly to the prediction for the nAChR. In contrast, the equivalent region of GLIC exhibits a funnel-shaped opening with an increase of the pore diameter, showing a clear difference between what could be a closed or an open state and suggesting that pore opening proceeds by a change of the tilt of the pore-forming helices [Hilf & Dutzler, 2009].

1.8 5-HT_3R and diseases: 5-HT_3 antagonists

Since its functional characterization [Rapport *et al.,* 1947], work on serotonin has shown its action in triggering activation or inhibition of neuronal, muscular, endocrine, haematopoietic and immune systems [Reeves & Lummis, 2002].

The identification of the 5-HT_3 receptor in the intestinal tract and the CNS in the late 1980s prompted an investigation into the role of this receptor in digestive and neurologic

diseases which quickly led to discovery of many very high affinity and selective antagonists [Glennon, 2006; Thompson *et al.*, 2005]. Due to the unfavourable effects of 5-HT_3 agonists (e.g., nausea and anxiety), no clinical use of them is likely in the near future.

Blocking the 5-HT_3R mechanism is a particularly important issue because of its involvement in chemotherapy inducing nausea and vomiting (CINV) [Herrstedt & Dombernowsky, 2007]. 5-HT_3R antagonists allow for higher doses of radiation to be used in chemotherapy, specifically inhibiting the large amounts of serotonin produced in response to the cancer treatment from stimulating intestinal 5-HT_3 receptors.

Based on the structure of serotonin (5-HT), bemesetron (MDL 72222, [Fozard, 1984]) and tropisetron (ICS 205-930, [Richardson *et al.,* 1985]) were formulated as the first selective high affinity 5-HT_3 antagonists. Further developments led to additional compounds belonging to the class of "setrons", among which ondansetron (GR38032F, [Butler *et al.,* 1988]) was the first 5-HT_3 antagonist approved in 1991 for the treatment of CINV. To date, several 5-HT_3 inhibitors are available for clinical use in addition to tropisetron (Novaban®) and ondansetron (Zofran®), such as granisetron (BRL 43694, [Sanger & Nelson, 1989]; Kytril®), dolasetron (MDL 73,147EF, [Sorensen *et al*., 1989]; Anzemet®) and palonosetron (RS 25259-197, [Wong *et al.*, 1995]; Aloxi®). They are especially used to treat the nausea and vomiting that occur as side effects of cytotoxic drugs in chemotherapy (CINV) and during post-operation (PONV), and as treatment of the Irritable Bowel Syndrome (IBS), depression and other mental and psychological illnesses [Thompson & Lummis 2007; Walstab *et al*., 2010; Machu, 2011].

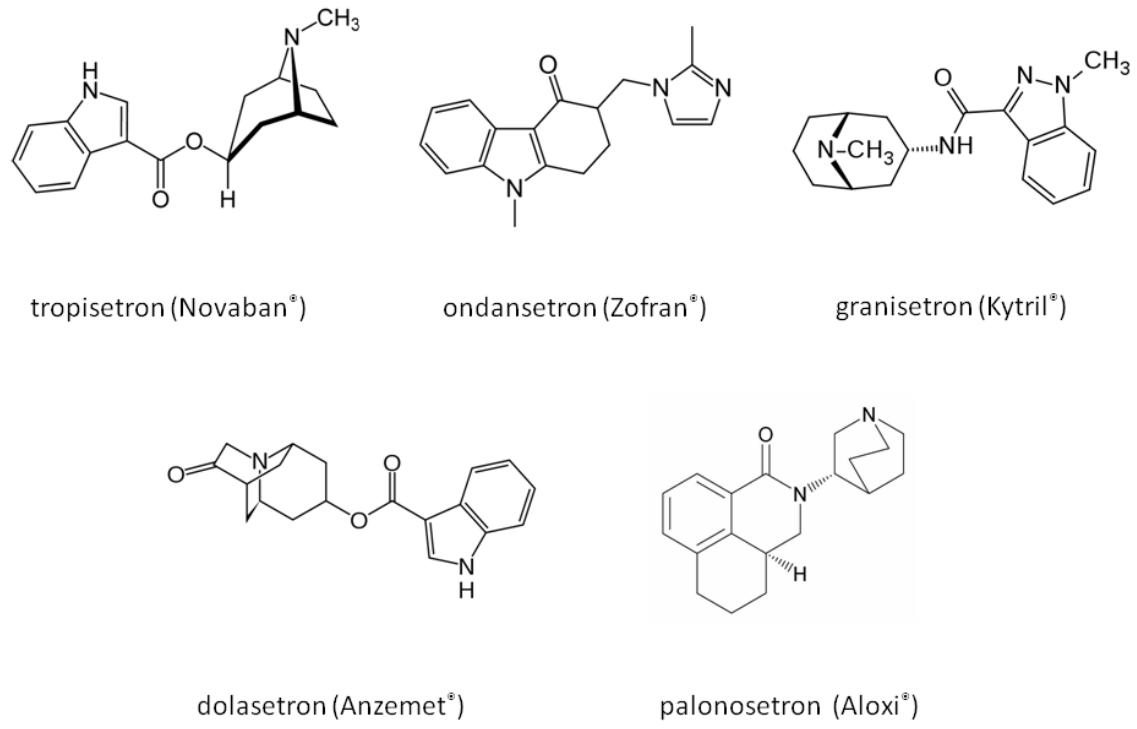

Figure 6- 5-HT_3 antagonists belonging to the class of "setrons".

The research activity performed in the development of 5-HT_3R antagonists has continued over the years, and it has produced drug molecules that show noticeable efficacy at the 5-HT_3R level. Cappelli and co-workers [Cappelli *et al.*, 2002, 2005, 2010], starting from the study of arylpiperazine derivatives related to quipazine, developed a tacrine-related heterobivalent ligand (Figure 7) that showed nanomolar potency for both 5-HT_3 receptor and human AChE and represented the first example of a rationally designed high affinity 5-HT_3R ligand showing nanomolar AChE inhibitory activity.

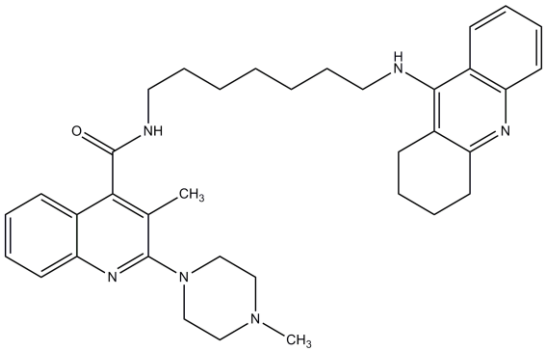

Figure 7- Heterobivalent ligand tacrine-related.

Important to note is that the 5-HT_3 receptor-related actions of all drugs have been determined using homomeric 5-HT_3A receptors. This may not prove to be the most useful testing protocol given that homomeric 5-HT_3A receptors do not fully reproduce the

characteristics of native 5-HT_3 receptors [Hussy *et al.,* 1994] and other subunits may play important roles. A better understanding of the roles of B, C, D and E subunits contained in heteromeric receptors may allow a wide range of other diseases to be treated with 5-HT_3 receptor selective drugs, potentially including addiction, pruritis, emesis, fibromyalgia, migraine, chronic heart pain, bulimia, and neurological phenomena such as anxiety, psychosis, nociception and cognitive function [Lummis, 2012; Walstab *et al*., 2010; Machu, 2011].

A range of studies, such as heterologous expression and information on receptor stoichiometry, molecular modelling and the study of the binding modes of drugs molecules to the 5-HT_3 receptor, may open new pathways and provide new insights at a molecular level in developing more selective and highly successful antagonists.

1.10 Aim of the thesis.

The 5-HT_3 receptor has been recognised to have important therapeutic effects and the research in this field is thriving. However, no three-dimensional structure has been reported for any of the 5-HT_3R subunits, as yet. Moreover, functional stoichiometries and subunit arrangements of the heteromeric receptors are still debated and the channel opening-closing mechanism is still largely unclear. At present, the research faces the ambitious challenge of obtaining new insights on these important data.

The aim of this thesis is to expand the knowledge about the 5-HT_3 family receptor, focusing on different features, such as the interaction with ligands and its physicochemical properties, by means of both computational and experimental methods.

The homology model of the human extracellular domain of the 5-HT_{3A}R, previously built in our laboratory [Moura Barbosa *et al*., 2010], is used as an instrument to check whether the receptor can accommodate new bivalent arylpiperazine-derivate ligands. Thus, **docking studies** are carried out and the binding modes of the bivalent ligands analysed on

the basis of three-dimensional models of ligand-receptor complexes in order to identify potential putative additional binding pockets.

Moreover, the homology model of the human extracellular domain of the 5-HT_{3A}R [Moura Barbosa *et al*., 2010] is used for further studies addressed to the **physicochemical characterization** (in particular, the hydrophobic and electrostatic properties) of the 5-HT_3R interfaces. Many different possible interfaces, which can form the 5-HT_3Rs, are analysed on atomistic bases with the aim to predict the role played by the extracellular moieties of the A and B subunits in the formation of functional or non functional receptors. The conclusions and hypotheses advanced are further extended to the additional C, D, and E subunits.

The previous computational study [Moura Barbosa *et al.,* 2010] identified a putative allosteric binding site in the homo- and heteromeric 5-HT_3 receptors for palonosetron, which will be also confirmed for the arylpiperazine bivalent ligands. **Experimental site-directed mutagenesis** studies are therefore performed in order to explore the role of the residues lining up this second site and to either validate or confute the computational hypothesis. These studies have been carried out in the Biochemistry laboratories of Dr. Sarah Lummis at the University of Cambridge (U.K.).

Finally, the **3D homology models** of the **TM and IC regions** of the human subunits 5-HT_3A to 5-HT_3E are built with the aim to analyse their structural features, and hereafter to get new insights into the possible stoichiometric composition of the functional receptors and into the structural and electrostatic requirements for the channel to be entered by ions. To our knowledge, no 3D structural model of the 5-HT_3 B, C, D and E subunit TM and IC domains has been built and reported in the literature, as yet.