The contribution of $His^{7.36(305)}$ of the GnRH receptor to ligand binding and receptor activation

By

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DECLARATION

I, NKATEKO I.M. MASEVU, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Dedication

For my best friend Ginger, Thank you for holding my hand and taking the walk with me especially when the going wasn't going.

Table of Contents

List of Abbreviations.

CPM	counts per minute
DAG	diacylglycerol
EC	extracellular loop
Emax	maximal agonist-stimulated IP production
EC_{50}	peptide concentration required to half-maximally stimulate IP production.
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HPLC	High performance liquid chromatography
IC_{50}	peptide concentration required to half-maximally inhibit binding of radioactively labelled GnRH peptides.
IC	intracellular loop
IP	inositol phosphate
IP_3	inositol 1, 4, 5, -triphosphate
LH	luteinizing hormone
PIP_2	phosphatidylinositol 1, 4 bisphosphate
PLC	phospholipase C
Q	coupling efficiency
SEM	standard error of the mean
TM	Transmembrane

Abstract

The gonadotropin releasing hormone (GnRH) is responsible for all reproductive function in vertebrate organisms. GnRH acts on the anterior pituitary via its receptor (GnRH receptor) to activate the release of gonadotropin hormones, luteinizing (LH) and follicle stimulating (FSH) hormones. A functional GnRH receptor is critical for normal LH/FSH secretion, pubertal development and reproductive function. Understanding the interaction between the receptor and the hormone and the activation mechanism of intracellular signalling in the cell is vital for understanding the regulation of reproductive function. The current study was performed to give insights into the role of individual amino acid residues of both the GnRH receptor and the GnRH ligand in receptor function. A $His^{7.36(305)}$ residue on transmembrane helix seven of the GnRH receptor was investigated for its contribution to the overall function of the receptor.

The role of $His^{7.36(305)}$ in the GnRH receptor was studied by mutating His and testing the function of the mutant receptors. Site-directed mutagenesis was employed to generate GnRH receptor mutants in which His was replaced with alanine, the π-electron donor phenylalanine, hydrophilic glutamine and asparagine, basic arginine and a large aromatic tryptophan. These mutant receptors were tested for their response and binding affinity to GnRH and its analogs in functional assays.

The results obtained suggested that $His^{7.36(305)}$ had a role in receptor expression, ligand binding affinity and receptor activation. There was a consistent decrease in maximum binding of the tracer by the mutants. These decreases suggested that $His^{7.36(305)}$ may have a role in expression of the GnRH receptor. However, the results obtained from the homologous competition binding assays used to estimate the receptor expression were inconclusive. The positive charge of $His^{7.36(305)}$ may be important for GnRH receptor expression. A screen for high affinity radioligand binding revealed that only the alanine ($His^{7.36(305)}$Ala) and phenylalanine ($His^{7.36(305)}$Phe) showed a decrease in affinity for GnRH. The lack of the hydrogen

bonding groups of $His^{7.36(305)}$ in these mutants and the retention of binding affinity by the mutants (Asn, Gln, Arg) with the hydrogen bonding groups indicated the importance of these groups in ligand binding. Either the Nδ1 or Nε2 hydrogen bonding groups is required for ligand binding affinity. The Ala and Phe mutants were studied further using different GnRH analogs to determine whether there is an interaction between GnRH and the receptor through $His^{7.36(305)}$. The mutants had similar affinity for [2-Nal^3]-GnRH compared to the wildtype receptor. This indicated that there may be a hydrogen bond interaction between $His^{7.36(305)}$ and NH of tryptophan at position 3 of GnRH. IP assays with $His^{7.36(305)}$Asn and $His^{7.36(305)}$Gln revealed that both the Nδ1 and Nε2 hydrogen bonding groups of $His^{7.36(305)}$ are essential for receptor activation. The $His^{7.36(305)}$Arg mutant retained all the activity of the wildtype receptor indicating that the protonated form of $His^{7.36(305)}$ is required for wildtype signalling efficiency. The results show that the $His^{7.36(305)}$ side chain has multiple roles in GnRH receptor structure and function.

CHAPTER ONE: THE GnRH RECEPTOR AS A G PROTEIN-COUPLED RECEPTOR.

1.1. Introduction

The decapeptide hormone, gonadotropin releasing hormone receptor (GnRH) regulates reproductive function via binding to the GnRH receptor. By activating the GnRH receptor on the pituitary gonadotrope cells, GnRH regulates the synthesis and release of gonadotropins LH and FSH, which in turn regulates the synthesis of gonadal steroid hormones (Homburg *et al,* 1976). The GnRH receptors are part of the large family of membrane receptors, the G protein-coupled receptors (GPCRs) (Tsutsumi *et al,* 1992). GPCRs have been extensively studied and have proven to be highly successful as targets for therapeutic intervention. More than 50% of the currently marketed drugs are targeted as modulators of GPCR function (Wise *et al,* 2002). More than 1000 GPCRs have been identified since the sequencing of the human genome some with known ligands and some are orphan, receptors unknown ligands (Takeda *et al,* 2002). As the GnRH receptor is important clinically, its structure and function have been widely studied and several models have been proposed (Millar *et al,* 2004).

This thesis aims to characterise and define the role of the $His^{7.36(305)}$ residue in ligand binding, receptor activation and the overall maintenance of the GnRH receptor structure. The photoreceptor rhodopsin and β-adrenergic receptor were the first GPCRs to be cloned and are the most widely studied among GPCRs (Schertler *et al,* 1993; Strader *et al,* 1995). Rhodopsin is used widely as a model for many GPCRs as it is the only GPCR for which the 3D structure has been resolved (Palczewski *et al,* 2000). It is used as a reference to understand the different individual GPCRs and to define their special structural differences and similarities.

This chapter discusses GPCR structure and function and the GnRH receptor structure, ligand binding and activation as a rhodopsin-like GPCR. Several amino acid residues defining the ligand binding pocket for GnRH have been identified by different researchers and different models attempting to define the tertiary

structure of the GnRH receptor have been created. The ligand binding pocket of the GnRH receptor involves residues in different transmembrane helices and also the extracellular loops. Upon ligand binding, depending on the type of ligand, the GnRH receptor undergoes a conformational change within the transmembrane (TM) domains which results in the activation of intracellular signalling. Residues that are important for GnRH receptor activation have also been identified and are located on the intracellular loops of the receptor. This literature review will discuss these aspects of receptor function focusing initially on GPCRs as a whole and then the GnRH receptor as an example and the focus in this study. In this study the histidine residue ($His^{7.36(305)}$) on the seventh transmembrane helix of the GnRH receptor was investigated for its role in receptor function. The current definition of the role of the $His^{7.36(305)}$ residue of the GnRH receptor will assist in refining models of the structure and function of the receptor

1.2. G protein-coupled receptors.

GPCRs are membrane receptors, which transmit extracellular signals across the membrane to cause intracellular changes. Many GPCRs from different species have been discovered and they have been found to make up a large percentage of membrane proteins in the human genome (Takeda *et al,* 2002). Ligands for GPCRs have wide variations: ions, peptides, hormones, biogenic amines, glycoproteins, nucleotides, lipids, etc. Understanding the mechanism of how these receptors interact with their respective ligands and induce specific responses provides the opportunity for the rational design of highly specific drugs and approaches to treatment and prevention of disorders that are regulated by the specific GPCRs (Millar *et al,* 2004).

GPCRs have an extracellular amino terminus, seven stretches of 20-30 hydrophobic amino acids, which form membrane spanning α-helices connected by alternating intracellular and extracellular loops and an intracellular carboxy terminal. The highest homology among GPCRs is in the transmembrane regions

with some residues found in virtually all GPCRs (Probst *et al,* 1992). The receptors bind an agonist molecule on the extracellular side of the membrane and following activation, bind and activate a heterotrimeric G protein (hence the name G protein-coupled receptors) on the intracellular side of the membrane. The activated G protein subsequently initiates an intracellular signal (Adelman *et al,* 1986). Different GPCRs activate different G proteins that in turn elicit different responses that are specific for the activating ligand. This is a remarkable process as one receptor is able to activate several G proteins and initiate several cellular responses. High conservation of certain residues among these receptors suggests that these residues are important for conserved aspects in the structure and function of the receptors. These similar features among GPCRs have been used to construct structural models for the different individual GPCRs (Baldwin 1993; Konvicka *et al,* 1998; Lu *et al,* 2002).

1.2.1. G Protein-coupled receptor families

A proper system is essential to classify the different types of GPCRs. Several classification systems have been proposed to classify the GPCR superfamily based on their ligands and/or amino acid sequences. The most widely used system groups the receptors into classes termed A through F (Kolakowski 1994). Receptors with the closest sequence homology have been classified in the same subfamily. The GnRH receptor belongs to the class A family. This class of receptors includes the most studied receptors: the vision protein rhodopsin and the β-adrenergic receptors, while family B receptors are related to the glucagon, parathyroid and calcitonin-related receptors. The family C is related to metabotropic neurotransmitter receptors. The family D consists of the STE2 yeast pheromones, STE3 yeast pheromones make up the family E, while the slime mold cAMP receptor fall under the family F (Kolakowski 1994).

Recently Fredriksson et al provided an overall map of the GPCR ancestry (Figure 1.1), proposing one ancestral gene, which through gene duplication and exon

shuffling turns into thousands of different proteins which transduce light, odours, taste, lipids and many other ligands. (Fredriksson *et al,* 2003). Their results formed the GRAFS classification system: Glutamate, Rhodopsin, Adhesion, Frizzled/taste and Secretin. The only feature that is conserved among all the GPCRs is the disulphide bond, which links transmembrane helix 3(TM 3) and extracellular loop 2 (EC 2). These correspond to the $Cys^{3.25(110)}$-$Cys^{4.65(187)}$ disulphide bond in rhodopsin (Gether 2000) and $Cys^{3.29(114)}$/$Cys^{5.23(196)}$in the GnRH receptor (Sealfon *et al,* 1997).

The first family in the GRAFS classification system is the glutamate receptors. This group of receptors corresponds to what has been called the family C. This group includes the metabotropic glutamate receptors, γ-amino butyric acid (GABA) receptors, calcium-sensing receptors (CASR), taste receptors and mammalian pheromone receptors (Gether 2000; Fredriksson *et al,* 2003). The N-terminus of these receptors is very large (500-600 amino acids long) and acts as the ligand binding domain. The N-terminus forms two lobes separated by a cavity in which the glutamate binds, forming a "Venus fly trap" where glutamate causes the lobes to close around the ligand (Bockaert and Pin 1999; Fredriksson *et al,* 2003). A short highly conserved extracellular loop 3 is a unique feature of these receptors (Gomeza *et al,* 1996).

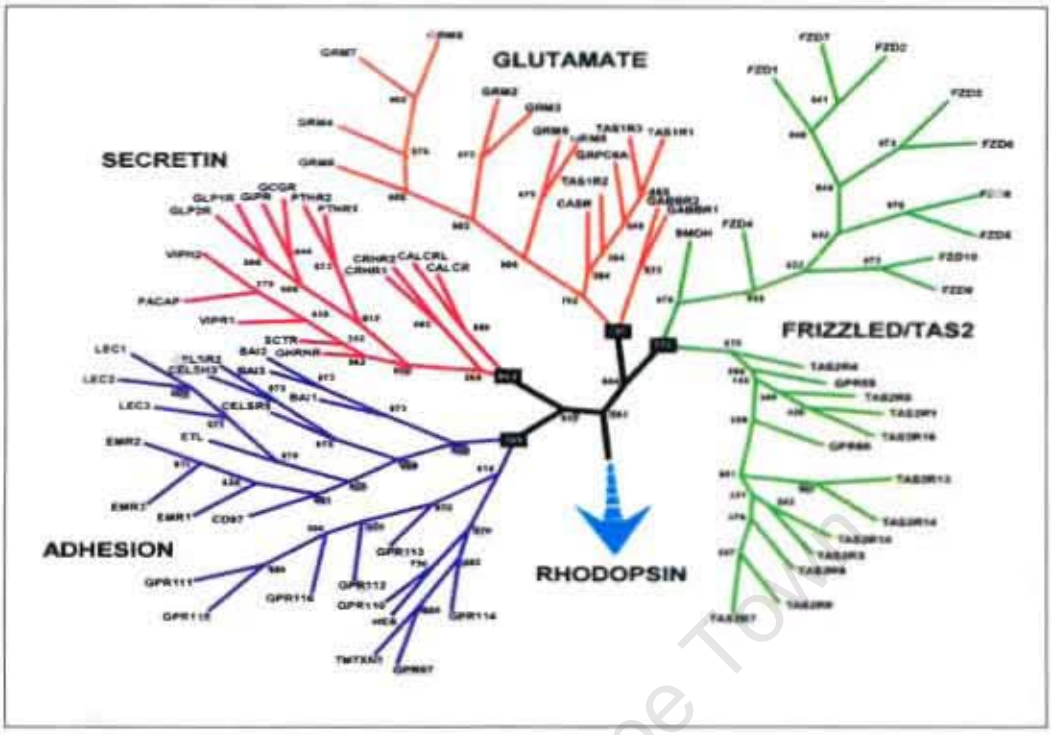


Figure 1.1. The phylogenetic relationship between GPCRs (TM 1 - TM 7) in the human genome. Approximately 200 GPCRs known from literature were downloaded from the Genbank database. Phylogenetic analyses were performed and the rhodopsin-like GPCRs and non-rhodopsins were identified. The position of the rhodopsin family was established by including twenty random receptors from the rhodopsin family. Rhodopsin family shown with arrow and continued in Figure 1.2. Figure taken from Fredriksson *et al*, 2003.

The GnRH receptor belongs to the rhodopsin family. This is the largest, and the most studied of all the receptor classes. Rhodopsin family receptors have at least one highly conserved amino acid residue in each transmembrane helix. To facilitate the comparison of equivalent residues between the large numbers of receptors belonging to the rhodopsin family three different numbering systems were formulated. In principle the Schwartz (Schwartz 1994) and Baldwin (Baldwin *et al*, 1997) numbering systems are identical. Helices are identified by aligning the most conserved residues in each helix with 26 residues. A given residue is then described by the helix it belongs to followed by a number indicating its position in the helix. These numbering systems are complex because

of the pre-determined lengths of the helices. Throughout this thesis the Ballesteros and Weinstein numbering system (Ballesteros and Weinstein 1995) will be used. In this system the most conserved residue in each helix is assigned the number 50 and each residue is numbered according to its position relative to this conserved residue. For example, the Histidine residue that was investigated in this study is given the number 7.36(305). This indicates that the His is located in TM helix 7, 14 residues amino terminal from the most conserved $Pro^{7.50(319)}$ in TM 7. Its amino acid position on the mouse GnRH receptor is indicated in brackets. The His residue is designated $His^{7.36(306)}$ on the human GnRH receptor because of an additional residue in EC 2 (Kakar *et al,* 1992).

The overall homology between the rhodopsin family receptors is relatively low, but there is high homology, in the domains that have highly conserved motifs and residues. These residues are proposed to be important for the conserved structure and function (i.e. receptor activation) of the receptors. The conserved features of the rhodopsin family include the DRY motif in TM 3 and the NPXXY motif in TM 7. Most of the ligands for the family A receptors bind within the TM core of the receptor with the exception of glycoprotein receptors (LH, FSH and TSH) where the ligand binding sites were believed to be in the N-terminus (Baldwin 1994). Most peptide receptors bind partially to the TM core and EC loops (Kobayashi *et al,* 1997). The rhodopsin family of receptors is divided into four different groups: α-, β-, γ- and δ-groups that are also divided into subgroups according to their ligands (Fredriksson *et al,* 2003). The α-group is made up of five main subgroups: receptors for prostaglandins, amines (serotonin receptors, dopamine receptors, muscarinic receptors, and histamine receptor), opsins (rod visual pigments, cone visual pigments, peropsins), melatonins and melanocortins (melanocortin receptors, cannabinoid receptors, adenosine-binding receptors). The β-group of which the GnRH receptor belongs to, has no main branches and contains receptors for tachykinins, cholecystokinin, endothelin, vasopressin and neuropeptide Y. The γ-group of rhodopsin receptors has three main branches: SOG receptors (somatostatin receptors and opioid receptor), MCH receptors (melanin-concentrating hormone) and the chemokine receptors (CCRs, CXCRs,

and angiotensin/bradykinin). The δ group of rhodopsin receptors consists of four main branches: the MAS-related receptors (MAS1 oncogene receptors), glycoprotein receptors (follicle-stimulating hormone receptors, luteinizing hormone receptors and thyroid-stimulating hormone), purin receptors (formyl peptide receptors, thrombin receptors and nucleotide receptors) and lastly the olfactory receptors (Figure 1.2).

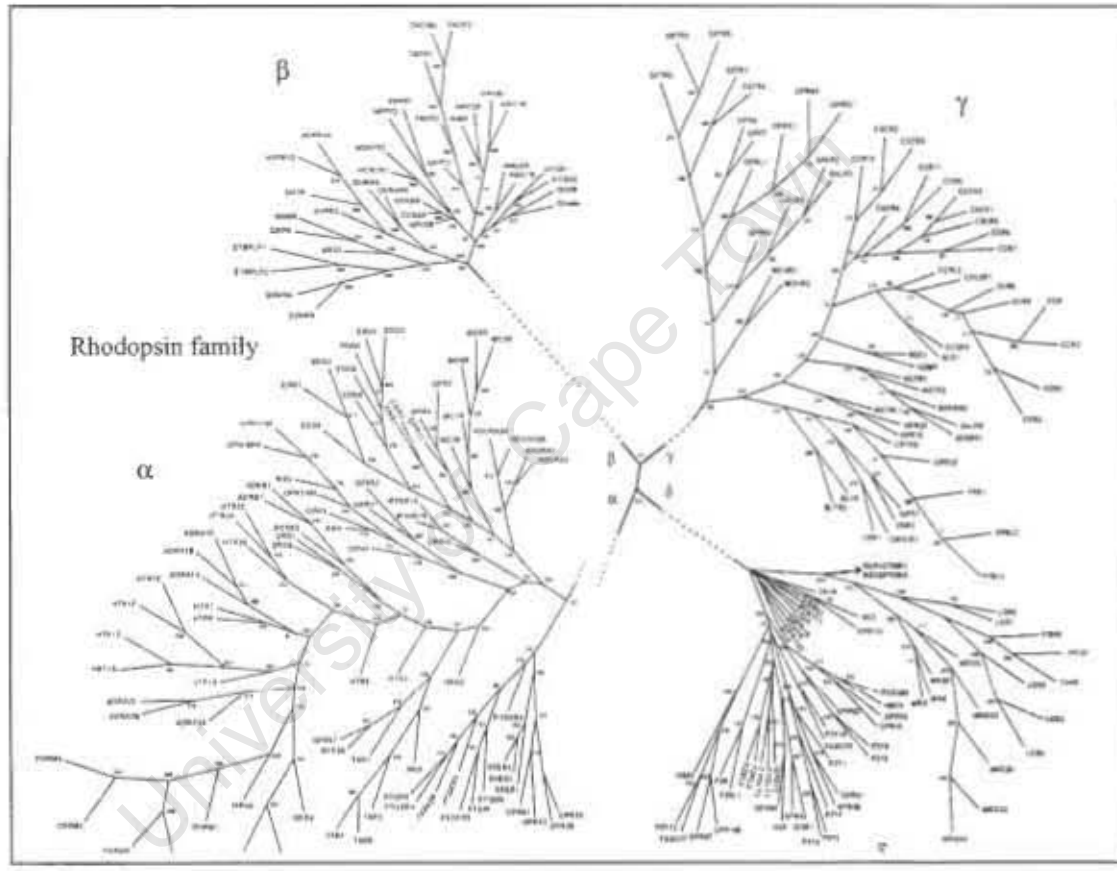


Figure 1.2. The phylogenetic relationship between rhodopsin family of GPCRs. The four major branches of rhodopsin family represent the arrow in Figure1.1 (Fredriksson *et al.* 2003).

The adhesion receptor family consists of receptors with GPCR-like transmembrane spanning regions fused together with one or several functional domains with adhesion-like motifs on the N-terminus. The N termini vary in length from 200 to 2800 amino acids long and are rich in glycosylation sites and proline residues. The long N-termini contain motifs proposed to participate in cell adhesion, hence the name adhesion receptors (Stacey M et al.2000).

The Frizzled/Taste2 receptor family has two distinct branches, frizzled and taste2 (TAS2) receptors. Although there is no obvious similarity between the TAS2 receptors, they seem to be clustered together in the phylogenetic analyses. There are several features common between the two groups which may explain why they clustered together, consensus sequences IFL in TM two, SFLL in TM five and SxKTL in TM seven. None of these motifs was found in the other four families of GPCRs. The role and function of the TAS2 receptors are not known except that they are expressed in the tongue and palate epithelium and they are thought to function as bitter taste receptors. Frizzled receptors control cell fate, proliferation and polarity during metazoan development (Flower DR et al.1999).

The secretin family of receptors binds large peptides that share a high amino acid homology. This group of receptors corresponds to the family B of the A-F system. Receptors for various peptide hormones and neuropeptides (e.g. Calcitonin, corticotropins, glucagons, etc.) belong to this family of GPCRs. A common feature of these receptors is a large (~ 80-100 residues) extracellular amino terminus containing several cysteines presumed to form disulphide bridges. The N-terminus of these receptors is particularly important for ligand binding. In the vasoactive intestinal peptide receptor (VIPR) and pituitary adenylyl cyclase-activating protein (PACAP) receptors the N-terminus is the only binding site (Tan *et al*, 2003).

1.2.2. The rhodopsin structure: A G protein-coupled receptor

The rhodopsin group of GPCRs includes opsins, neurotransmitter receptors (adrenergic, serotonergic, muscarinic, etc.), and glycoprotein receptors (FSH,

LH/CG, etc.). Rhodopsin is a special GPCR because its ligand (retinal) is tethered and this makes it possible to perform certain experiments to characterise the role and function of rhodopsin that cannot be done with other GPCRs. The rhodopsin chromophore retinal is attached through a schiff base to $Lys^{7.43(296)}$. Post-transcriptional modifications of rhodopsin include acetylation, palmitoylation, phosphorylation and glycosylation (Bockaert and Pin 1999).

Since GPCRs are integral membrane proteins obtaining crystals to study their structure by x-ray diffraction has been difficult to perform. The only high resolution structure available to date is that of rhodopsin and this structure can be used as a tool to model the tertiary structures of other GPCRs (Palczewski *et al*, 2000). The primary structure of GPCRs indicated the presence of seven transmembrane helices and had a similar topology to another opsin, bacteriorhodopsin although it is not a GPCR. This similarity provided a platform for deriving initial models for GPCRs from bacteriorhodopsin (Trumpp-Kallmeyer *et al*, 1992). Rhodopsin is a G protein-coupled light receptor with a covalently bound 11-*cis*-retinal, whereas bacteriorhodopsin is a light-driven proton pump that uses all-*trans*-retinal (Pettei *et al*, 1977). Differences in the structures of bacteriorhodopsin and rhodopsin were shown from diffraction studies performed on two-dimensional structures (Schertler and Hargrave 1995). The structure of rhodopsin is less elongated and slightly wider and the helices are tilted differently from bacteriorhodopsin. The highly conserved proline residues that are located on different helices in bacteriorhodopsin and rhodopsin could be associated with the difference in the helical arrangements (Baldwin 1993).

Sequence information combined with the low resolution data were used to derive a model that described the arrangement of the TM helices of GPCRs (Baldwin 1993; Schertler *et al*, 1993). The model proposed the arrangement of the helices with respect to the centre of the helical bundle and also assigned helices to the peaks in the projection map, suggesting how they may be orientated in a 3D model (Baldwin 1993) (Figure 1.3). An arc-shaped feature was interpreted as tilted helices and four peaks were interpreted as the three remaining helices from

projection maps (Baldwin 1993; Schertler *et al.* 1993). The four peaks observed indicated four helices that were perpendicular to the membrane and one curved, elongated peak arising from the superposition of three helices. TMs 1, 2, 3 and 5 were tilted whereas helix 4, 6 and 7 were oriented perpendicular to membrane plane (Schertler *et al.* 1993). TM 1, 2, 3 and 5 were shown to be tilted 28, 27, 30 and 23 degrees respectively (Unger *et al.* 1997). TM 2 interacted with TM 1, 3 and 7. TM 3 was shown as the most tilted of all and buried deeply inside the molecule. (Baldwin 1993; Schertler *et al.* 1993). Its central portion forms extensive interactions with TM 2, 4, 5 and 6 on the extracellular side and with TM 2, 4 and 7 on the intracellular side (Unger *et al.* 1997). TM 4 was the least tilted and shortest helix forming contacts with TM 2 and 3, forming an additional interaction with TM 3 and 5 from extracellular side. The TM 5 was found to be more tilted that originally thought to be in the earlier models (Schertler and Hargrave 1995). TM 6 was oriented nearly perpendicular to the membrane plane. TM 6 was proposed to bend towards and form contact with TM 5. TM 7 lies perpendicular to the plane on the membrane and was proposed to be in contact with TM 1, 2 and 6 (Unger *et al.* 1997).

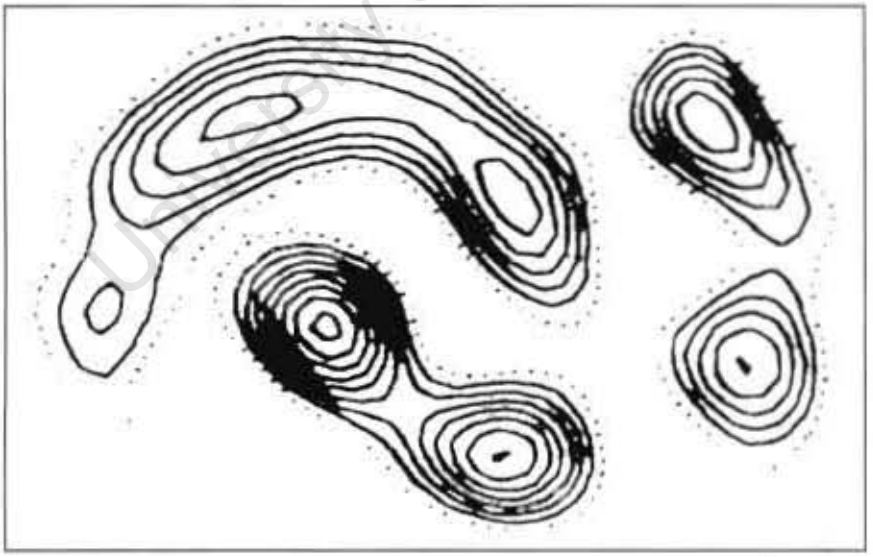


Figure 1.3. Projection map of bovine rhodopsin at 9Å resolution. Model predicted that helices 4, 6, and 7 are tilted perpendicularly to the place of the membrane. Figure from Schertler *et al.* 1993.

The information gathered from the cryo-electron microscopy studies of the low resolution structure of the rhodopsin led to increased understanding of the organisation of the seven transmembrane helices of rhodopsin. The 3D-structure of rhodopsin confirms and provides further information on the arrangement of the transmembrane helices, ligand binding and G protein sites and mechanisms of receptor activation (Palczewski *et al,* 2000) (Figure 1.4). Currently the rhodopsin crystal model includes more than 95% if its amino acid residues. In addition to the seven helices, the high resolution structure revealed a cytoplasmic helix (TM 8) running parallel to the membrane. This is due to the palmitoylation of Cys residues at the C-terminal tail. In general the helices are tilted at various angles with respect to each other and the membrane, the calculations of the various angles are in good agreement with the low resolution structures previously reported (Schertler *et al,* 1993; Unger and Schertler 1995; Palczewski *et al,* 2000).

The helix ends of rhodopsin inferred from electron paramagnetic resonance spectroscopy studies of spin-labelled Cys substituted residues (Altenbach *et al,* 1996; Altenbach *et al,* 1999) are consistent with the seven TM helix ends in the high resolution structure of rhodopsin (Palczewski *et al,* 2000). The glycosylated extracellular loop 2 displays a compact structure with four distorted short β-strands. These strands form a plug that restricts access to the chromophore binding site from the extracellular side. The cytoplasmic side contains three loops between helix one and two, three and four and five and six. Unfortunately, the current model for intracellular loop three is incomplete. This region of the receptor is known to be flexible and variable among the different GPCR because of its involvement in G protein activation.

Several potential interhelical interactions formed by some of the most conserved residues have been revealed by the rhodopsin crystal structure e.g. interactions between $Asn^{1.50(55)}$ and $Asp^{2.50(83)}$ and the carbonyl backbone of $Ala^{7.46(299)}$ (Palczewski *et al,* 2000). The C-terminal end of TM 3 contains the E(D)RY motif located in an extremely hydrophobic environment formed among residues from helices 2, 4, 5 and 6. The E(D)RY residues have been implicated in the regulation

of the receptor's interaction with its G protein. In an inactive receptor $Glu^{3.49(134)}$ and $Arg^{3.50(135)}$ of the DRY motif form an interaction that is thought to stabilise the inactive conformation and disruption of the interaction is thought to be involved in receptor activation for rhodopsin (Alewijnse *et al,* 2000). This interaction has also been proposed for other GPCRs including the GnRH receptor, where the equivalent residue for tyrosine is a serine (Ballesteros *et al,* 1998).

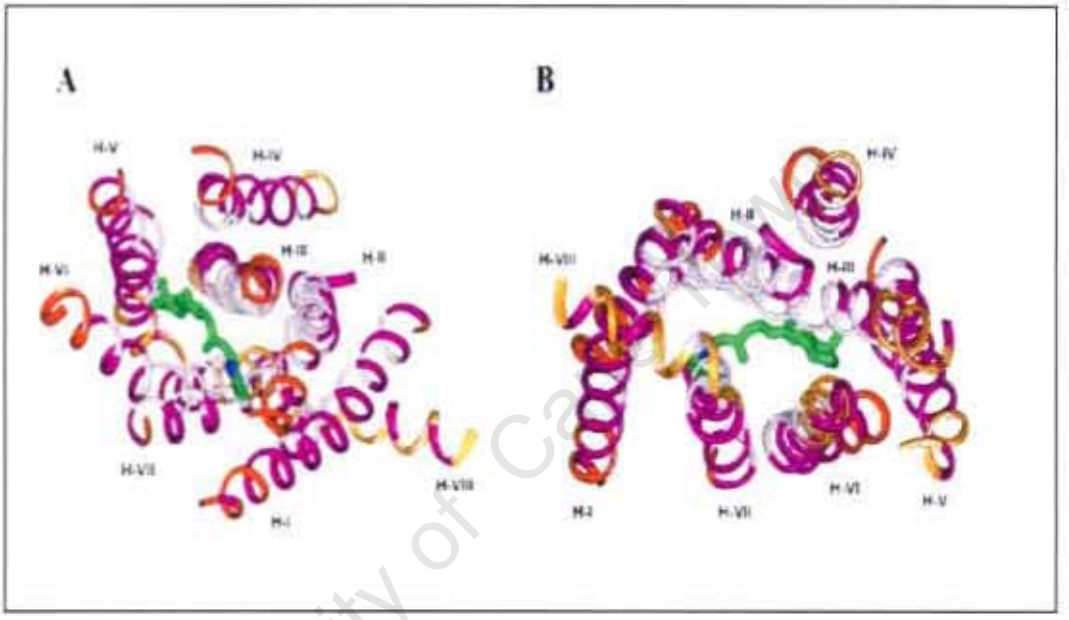


Figure 1.4. A ribbon diagram of the 3D structure of rhodopsin (taken from Filipek *et al.*, 2003). Helices are shown in different colours and retinal is shown in green from the extracellular side (panel A) and cytoplasmic side (panel B). Residues in contact with the surface (purple), upper charged region of membrane (orange), lower part of membrane (red), and internal residues of rhodopsin (grey). The interaction between retinal and $Lys^{7.43(296)}$ is shown as stick and ball model.

During receptor activation $Arg^{3.50(135)}$ has been proposed to interact with residues in other helices (Ballesteros *et al,* 1998). Another characteristic motif in rhodopsin and other GPCRs is the Asn-Pro-X-X-Tyr motif in helix 7 (Fritze *et al,* 2003). The side chain of $Asn^{7.49(302)}$ in the motif projects towards $Asp^{2.50(83)}$. This interaction appears to involve some water molecules. $Tyr^{7.53(306)}$ also projects inside rhodopsin and interacts with the conserved $Asn^{2.60(73)}$ in TM 2. The helices

in rhodopsin are regular α-helices except that residues $Lys^{7.43(296)}$ to $Ala^{7.46(299)}$ form a helix in a 3_{10} helix conformation in TM 7. This part of the helix gives more freedom to $Lys^{7.43(296)}$ because this residue has to have the appropriate area to induce the several changes involved in retinal structure during isomerisation (Filipek *et al,* 2003). Generally the high resolution crystal structure of rhodopsin has confirmed what was predicted with the models based on the projection maps and experimental data (Palczewski *et al,* 2000; Filipek *et al,* 2003). To have the complete representation of GPCR activation high resolution structures of the various intermediates of rhodopsin activation will be essential to understand the changes in receptor conformations that are associated with activation as the present model represents only the inactive form of rhodopsin.

1.2.3. Does the crystal structure of rhodopsin provide an accurate template for the prediction of the 3D structure of other GPCRs?

Transmembrane proteins play an important role in conveying information across the hydrophobic cell membrane. GPCRs represent one of the largest groups of membrane proteins (Takeda *et al,* 2002) and are involved in many biological and physiological processes. This poses a great challenge in understanding the mechanism of action and structure of GPCRs. Rhodopsin is by far the most extensively studied GPCR and the significance of understanding its mechanism extends far beyond just vision. Molecular models based on the rhodopsin low resolution structures were used to predict structures for other GPCRs. These models were used in conjunction with experimental data and data from sequence alignments read in terms of conservation (Baldwin 1993; Ballesteros and Weinstein 1995). The crystal structure of rhodopsin has been envisaged to be an advance for predicting the tertiary structures of other GPCRs. Whether or not this assumption is true will emerge as more studies are done to compare the currently known experimental and modelling studies of GPCRs with that of rhodopsin. It is likely that the rhodopsin structure will provide the most detailed insights

regarding the structure/activity relations of the rhodopsin receptors, rather than all the GPCR families (Sakmar 1998).

Comparison of the crystal structure with experimental data revealed that the overall structure of the transmembrane domains of rhodopsin and other GPCRs are likely to be similar (Ballesteros *et al,* 2001a). Compared with previous results from cryomicroscopy the high-resolution structure of rhodopsin showed a great degree of accuracy in predicting the helix ends and protein-protein interface of GPCRs (Ballesteros *et al,* 2001a). The rhodopsin template has been applied to several GPCRs, including the CCK_1 cholecystokinin (Archer *et al,* 2003), D_2 Dopamine (Ballesteros *et al,* 2001a), prostacyclin (Stitham *et al,* 2003) and M_1 muscarinic (Lu *et al,* 2002) receptors to interpret the structure/function data from the three receptors. Several highly unusual features of rhodopsin were also found to be present in other GPCRs, although the amino acids involved in these features are not conserved (e.g. Proline residues). It has been suggested that different amino acids and alternative micro-domains could form slight changes in the α-helices that could result in the same tertiary structure. Several differences resulted in the modelling of the CCK 1 receptor using rhodopsin as a template being unsuccessful. In CCK1, TM 3 is predicted to have two kinks instead of an α-helix as in rhodopsin, TM 1 is a regular α-helix because of the absence of a Pro whereas in rhodopsin the $Pro^{1.52(53)}$ bends the helix inwards (Archer *et al,* 2003).

The overall structure of rhodopsin and amine receptors was proposed to be similar, but certain regions of the receptor can cause some structural divergence (Ballesteros *et al,* 2001a). For example, the Pro residue at different positions in TM 2 bends the helix differently. The conserved Pro-kinks in TM 5, 6 and 7 could form different conformations and result in changes of the binding pocket of different GPCRs (Ballesteros *et al,* 2001a). Intramolecular interaction within the different transmembrane helices and the ligand binding site of the ground-state M_1 muscarinic acetylcholine receptor were successfully predicted from the rhodopsin high-resolution structure (Lu *et al,* 2002). The human prostacyclin receptor was modelled on a rhodopsin template to determine its ligand binding

pocket (Stitham *et al,* 2003). A unique ligand binding pocket for prostacyclin that was distinct from that of rhodopsin was postulated. There are many factors to consider before a conclusion can be made to say whether rhodopsin is a suitable template for other GPCRs. The fact that the rhodopsin crystal structure corresponds to an inactive conformation of the protein structure could account for the failure in directly applying it to the CCK1 receptor. The arrangement and position of the helices relative to each other is most likely to be different in an active or agonist-occupied receptor. The rhodopsin crystal structure can potentially be very useful in validating the different ligand binding pockets of the various GPCRs. This would reveal all the important differences and similarities between the GPCRs. In conclusion, the rhodopsin crystals do appear to provide significant information on the 3D structures of other receptors, but different factors will need to be considered to come up with viable 3D structures for the individual receptors. In addition to the rhodopsin crystal structure, it would be a breakthrough to have high resolution structures of other GPCRs to confirm the overall GPCR structure and to identify the unique features of each GPCR.

1.2.4. Rhodopsin-like GPCR ligand binding pocket.

The binding of an agonist to the binding pocket of the receptor activates the receptor by causing conformational changes in the receptor (Gether and Kobilka 1998). Studies have been conducted to determine the mechanism of ligand-receptor interactions in GPCRs, which lead to the activation of the receptor. The ligand binding pocket differs in different GPCRs because of the various ligands that are specific for their different classes of GPCRs. Binding can occur in the transmembrane helices, EC loops and N-terminal regions (Ji *et al,* 1998; Gether 2000). Hydrogen bonds, ionic interactions and hydrophobic contacts are proposed to be involved in ligand-receptor interactions (Ji *et al,* 1998) (Figure 1.5).

The next section will focus on the ligand binding pocket of rhodopsin-like GPCR family since the GnRH receptor falls under this class of GPCRs. There are conserved motifs among GPCRs that are unique for the rhodopsin-like family and

these motifs make it easier to compare the different receptors in this family. Rhodopsin is unique among GPCRs because its ligand, 11-cis-retinal is covalently linked to the amino group of $Lys^{7.43(296)}$ by formation of a schiff base.

The 11-cis-retinal acts as an inverse agonist, upon illumination the retinal isomerises to the all-trans form and drives the protein through a series of transient photo intermediates. The protonated schiff base is ion-paired to the conserved Glu^{113} in boundary between TM 3 and EC 1, bringing TM 3 and TM 7 close to each other (Ji *et al,* 1998). Different studies show that the β-ionone ring of the retinal associates with residues in TM 3, 5 and 6 (Han *et al,* 1997; Palczewski *et al,* 2000). From the β-ionone ring to the C^{11} methyl group the retinylidene group runs almost parallel to TM 3 (Palczewski *et al,* 2000). Gly^{121} in TM 3 associates with the C^{9} methyl group of retinal (Han *et al,* 1997).

Amino acids from TM 1, 2 and 7 form part of the region around the schiff base (Palczewski *et al,* 2000). The extracellular side of the polyene chain is shielded partly by EC 2 and the β-sheet β4 (Palczewski *et al,* 2000). Isomerisation causes part of the TM backbone to be exposed and results in a formation of at least one water molecule hydrogen bond (Rath *et al,* 1998) and the salt bridge between $Lys^{7.43(296)}$ and $Glu^{3.32(113)}$ breaks. These changes rearrangement of TM 3, 6 and 7, thereby generating signal (Han *et al,* 1997).

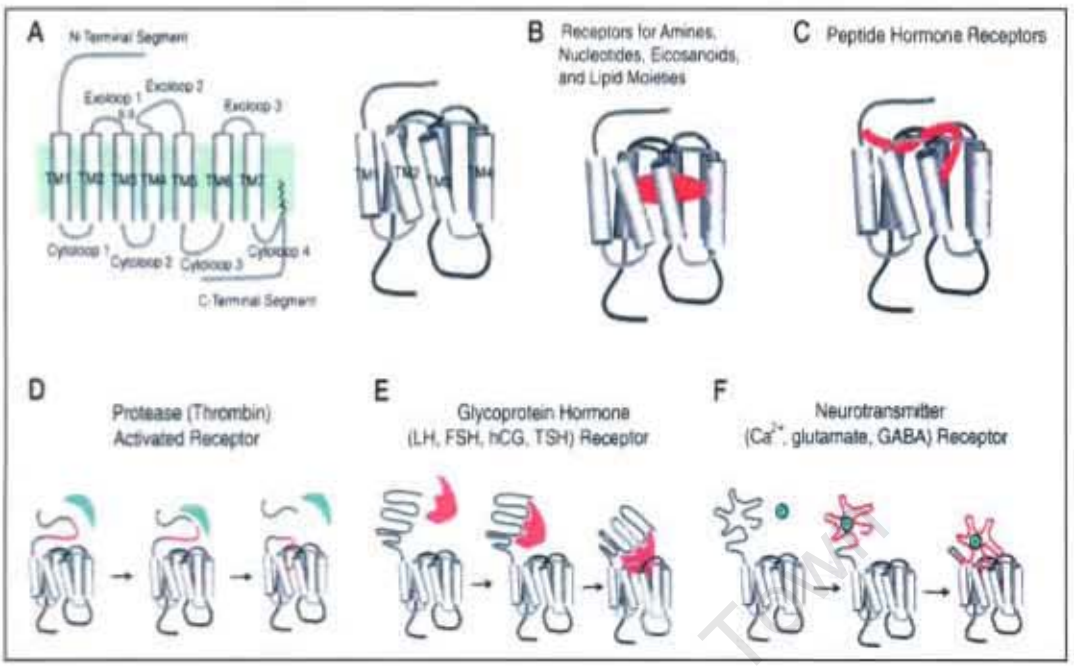


Figure 1.5. Schematic presentation of the general structure of GPCRs and receptor-ligand interactions. A, general structure of GPCRs. Several distinct modes have been observed for ligand binding and signal generation at exclusively the TM core for photons, biogenic amines, nucleotides, eicosanoids, (B), and the core, extracellular loops, and N-terminal segment for peptide ligands of 40 amino acids (C). Protease ligands like thrombin bind to and cleave the N-terminal segment, resulting in a shorter N-terminal segment that interacts with extracellular loops to generate a signal, whereas the released peptide binds to platelet to stimulate platelet aggregation (D). Glycoprotein hormones, LH, FSH, human CG, and TSH, bind to the long N-terminal domain and the liganded N-terminal segment interacts with extracellular loops to generate a signal (E). Small neurotransmitters, Ca2+, glutamate, and GABA, bind to the ~ 600-amino acid N-terminal segment and the liganded N-terminal segment interacts with the membrane-associated domain, thus generating a signal (F). (Ji TH et al. 1998)

Biogenic amines (epinephrine, norepinephrine, dopamine and histamine) bind the transmembrane interior transmembrane core of their receptors (Strader *et al.*, 1994; Gether and Kobilka 1998). The helices contain a predominance of

hydrophobic residues on one side and hydrophilic on the other. The hydrophilic sides face inwards to form a polar ligand binding pocket (Probst *et al,* 1992; Baldwin 1993). All adrenergic agonists and antagonists are basic amines, suggesting the presence of an acidic residue in the receptor that provides a counter ion for the protonated amine in the ligand binding pocket (Strader *et al,* 1994; Strader *et al,* 1995). In the β_2-adrenergic receptor, an $Asp^{3.32(113)}$ residue in TM 3 was found through mutagenesis studies to be critical for binding monoamines and not for signal generation (Probst *et al,* 1992; Strader *et al,* 1995). Sequence alignments of other biogenic amine GPCRs revealed that an Asp residue was found at position 3.32 corresponding to that of Asp^{113} of the β_2-adrenergic receptor in all GPCRs that bind biogenic amines. This discovery strongly suggests that the main function of the $Asp^{3.32}$ is to interact with the amines. Subsequent mutagenesis of other biogenic amine receptors confirmed that the Asp is crucial for ligand binding in the α_{2A}-adrenergic (Wang *et al,* 1993), β-adrenergic (Strader *et al,* 1995), H_2 histamine (Gantz *et al,* 1992), M_1 muscarinic (Fraser *et al,* 1989) and D_2 dopamine (Mansour *et al,* 1992) receptors. $Lys^{3.32(121)}$ in the GnRH receptor was also found to be important in ligand binding (Zhou *et al,* 1995). The aromatic catechol moiety of the β-adrenergic receptor agonists docks to the binding pocket involving residues in TM 5 and TM 6. Mutation of $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$ in TM 5 to Ala resulted in a decrease in affinity of the catecholamine for the receptor and agonist efficacy (Strader *et al,* 1989). The two Ser residues hydrogen bond with the meta- and para-hydroxyl groups of the catechol ring and $Phe^{6.52(290)}$ in TM 6 interacts with the aromatic catechol-containing ring. These Ser residues lie one helix turn apart from each other; therefore there is simultaneous binding to the ligand that would constrain the receptor conformation resulting in receptor activation (Strader *et al,* 1995; Ji *et al,* 1998). The conservation pattern of these Ser residues among other receptors strongly suggests that the TM 5-6 region binds the polar aromatic portion of the biogenic amine.

Another group of receptors eicosanoid receptors, also in the class A of GPCRs, interact with their ligands (prostaglandins and prostacyclins) directly in the transmembrane core (Kobayashi *et al,* 1997; Kobayashi *et al,* 2000; Stitham *et al,*

2003). The binding pocket of the human A2a adenosine receptor receptors is also in the transmembrane core of the receptor. These receptors bind ligands like uridine nucleotides and adenine nucleotides (Kim *et al,* 1995; Jiang *et al,* 1997).

Extracellular loops together with other residues in the transmembrane region of the neurokinin (NK) receptors form the binding sites for tachykinins, substance P, neurokinin A and neurokinin B (Yokota *et al,* 1992; Gether *et al,* 1993; Maggi *et al,* 1993). Although these receptors are homologous, there are structural differences that contribute to different binding sites. These differences enable subtype selectivity of the different peptides (Yokota *et al,* 1992; Gether *et al,* 1993). The tops of TM 3 and 7 and some residues in the N-terminus are necessary for the binding of substance P (Fong *et al,* 1992a; Huang *et al,* 1994).

The angiotensin receptors bind to their ligands partially in the extracellular loops and the transmembrane regions. Angiotensin II interacts with the receptor on TM 5 ($Lys^{5.42(199)}$ and $His^{6.51(256)}$ (Noda *et al,* 1995a; Noda *et al,* 1995b). The binding site for the GnRH also involves both the extracellular loops and transmembrane region of its GnRH receptor (Sealfon *et al,* 1997; Millar *et al,* 2004). The N-terminus of GnRH was shown to interact with the transmembrane core of the receptor (Davidson *et al,* 1996a). The residues $Asp^{2.61(98)}$, $Trp^{2.64(101)}$, $Asn^{2.65(102)}$, $Lys^{3.32(121)}$ and $Asn^{5.61(212)}$ have been shown to be vital in ligand binding (Flanagan *et al,* 1994; Zhou *et al,* 1995; Davidson *et al,* 1996a; Hoffmann *et al,* 2000). $Glu^{7.32(301)}$ in the mouse GnRH receptor was shown to have a role in recognizing the Arg^{8} residue in GnRH (Flanagan *et al,* 1994). Subsequent studies in the human GnRH receptor suggested that the interaction between $Asp^{7.32(302)}$ and Arg^{8} induces GnRH to interact with the binding pocket in a high affinity conformation (Fromme *et al,* 2001) (See detailed discussion under "The ligand binding site for GnRH" below.

1.2.4.1. Residues in EC 3 and Top of TM 7.

The locus (top of TM 7) of the $His^{7.36(305)}$ residue on the GnRH receptor is one of the reasons this residue was chosen for investigation in receptor ligand

interactions and receptor activation. Residues on EC 3 and TM 7 have been investigated in a number of GPCRs and have been implicated in a variety of functions on the specific receptors. This section describes examples of the different roles played by residues close to the 7.36 locus of GPCRs. Ligand selectivity among different receptor subtypes in many GPCRs requires the presence of particular residues in TM 7 for interaction. In GnRH receptors, an acidic residue ($Asp^{7.32(302)}$ or $Glu^{7.32(301)}$) interacts with Arg^8 of mammalian GnRH conferring specificity for the Arg^8-containing GnRH (Flanagan *et al,* 1994; Fromme *et al,* 2001). Additionally the position of $Pro^{7.33}$ and $Ser^{7.31}$ residues relative to the $Asp/Glu^{7.32}$ is also important for ligand selectivity (Wang *et al,* 2003; Fromme *et al,* 2004). Because the fully ionized form of nucleotides has been shown to be important for interactions with the P_{2u} and P_{2y} purinoceptors it was suggested that the negative phosphate group could be interacting with one of the conserved positively charged residues on the receptors. These positively charged residues of the P_{2u} purinoceptors were mutated to uncharged residues leucine and isoleucine. $Lys^{7.36(289)}$ and $Arg^{7.39(292)}$ of the P_{2u} purinoceptors were shown to be important for determining agonist potency and for nucleotide selectivity (Erb *et al,* 1995).

In the Lutropin/choriogonadotropin receptor $Lys^{7.35(583)}$ was shown to be essential for full ligand-mediated signalling and not involved in hormone binding (Fernandez and Puett 1996). Conservative segment exchange on the AT_1 angiotensinII receptor, where 6 – 17 residues were mutated to chemically similar residues, showed regions on the receptor responsible for peptide binding. These were located on top of TM 1 and in EC 3, close to the top of TM 7. Mutant receptors with substitutions at the regions had decreases in affinity of 5000 – 20 000 fold magnitude for angiotensin II (AII) and peptide antagonist [Sar^1, Leu^8] AII without affecting binding of nonpeptide antagonists (Hjorth *et al,* 1994). Alanine scanning revealed that agonist binding was dependent on $Asp^{7.29(278)}$ and $Asp^{7.39(281)}$ in ECL3 (Hjorth *et al,* 1994; Perlman *et al,* 1995). $Phe^{7.38(411)}$ of the D_2 dopamine receptor is essential for receptor activation and not for ligand binding. A series of hydrophobic residues was mutated to alanine and the affinity and stimulation with different dopamine ligands was measured. The data showed that

Phe$^{7.38(411)}$ may be responsible for inducing the active conformation required for G protein binding and not essential for ligand binding (Cho *et al,* 1995). A lot more residues deep in the hydrophobic core of TM 7 have also been investigated in various GPCRs for different functions, for example Trp$^{7.44(299)}$ of the A_2 thromboxane receptor discriminates between the agonist and antagonist binding sites (Funk *et al,* 1993).

1.2.5. Activation of rhodopsin-like GPCRs.

A ligand binds to a receptor and causes a conformational change in the receptor, the activated receptor then interacts with a heterotrimeric G protein. This interaction causes an exchange of GTP for GDP on the α-subunit of the G protein. The GTP-bound α-subunit dissociates from the βγ-dimer. Both the GTP-bound α-subunit and the βγ-dimer can act on several cellular signalling pathways. These pathways can be stimulation or inhibition of adenylate cyclase, activation of phospholipase C and regulation of potassium and calcium channels. Structural rearrangement of the receptor involving TM helix movements is a vital step in receptor activation. Different ligands stabilise different conformations in the receptor, and certain of these conformations facilitate interactions with G protein for signal transduction (i.e. antagonists and inverse agonists stabilise conformations that destabilise G protein interaction (Kenakin 1997; Christopoulos and Kenakin 2002).

1.2.5.1. Models of receptor activation.

Changes in receptor conformation upon ligand binding is a critical requirement in receptor activation (Christopoulos and Kenakin 2002). The receptor has to change between distinct binding sites, one for the agonist and the other for the G protein.

Different models for receptor activation exist but the ternary complex (De Lean *et al,* 1980) and extended ternary complex models (Samama *et al,* 1993) are the most widely accepted models for receptor activation. The De Lean model of GPCR activation proposed that a ternary complex exists, which includes the

hormone, receptor and G protein. This is a simple model of receptor activation where the binding of a ligand promotes a conformational change that couples to and activated a G protein (De Lean *et al,* 1980). Different receptor conformations had to be included in the development towards the present models of GPCR activation. This model was later extended with the demonstration of constitutively active receptors, receptors that can activate G proteins in the absence of agonists and that certain mutations can enhance agonist-independent activity (Samama *et al,* 1993). The extended model also accounts for different classes of ligands, e.g. Full agonists, partial agonists, neutral antagonists, and inverse agonists. The extended ternary complex model allows the formation of a receptor-G protein complex. The model proposes that the receptors exist in equilibrium of two states, inactive R and active R* (Samama *et al,* 1993). Receptors are thought to change between R and R* conformations. In the absence of agonists the equilibrium favours the inactive R state which cannot interact with the G protein. Agonists bind with a high affinity to the R* and in this way shift the equilibrium to the R* state. Inverse agonists (which can inhibit agonist-independent activity) are predicted to stabilize the R state, shifting the equilibrium to R state. Neutral antagonists bind both R and R* state with the same affinity (Samama *et al,* 1993; Black and Shankley 1995; Gether and Kobilka 1998). Although this model delineated some of the complex structures of receptor activation, it failed to accommodate the thermodynamics of activation. A more complex cubic ternary complex model was proposed (Weiss JM et al.1996)

A wide variety of complexes in GPCRs could not be accommodated by a two state model (Gether and Kobilka 1998). Another model was formulated to accommodate the different complexes. This model predicts that the free receptor exists in an R^0 conformation that can shift to other states like R and R* (Kenakin 1997). The R^0 state can be stabilised by inverse agonists while the R* is stabilised by agonists. It was proposed that there is a sequential binding of the agonists which result in a series of conformational changes between the R and R* states. The R* may include different conformations of the receptor that can be induced by different agonists (Kenakin 1997). Inverse agonist may also stabilise the

inactive conformation of the receptor by inducing various sequential structural changes (Gether 2000).

A multistate model of GPCR activation was proposed after the discovery that different sites exist for peptide agonist and non-peptide antagonists (Wiens *et al,* 1998). In this model the receptor alternates between multiple active and inactive conformations. An agonist binds a conformation that activates the receptor while an inverse agonist binds a conformation that inactivates the receptor (Gether 2000). Two agonists with same receptor but different binding sites can be accommodated by this model and both agonists can stabilise the active conformation.

1.2.5.2. Role of "Asp/Glu-Arg-Tyr" motif in receptor activation.

During activation of rhodopsin, the isomerisation of retinal is a trigger that forces $Lys^{7.43(296)}$ in TM 7 away from $Glu^{3.32(113)}$, disrupting the TM 3-TM 7 interhelical salt bridge and causes hydrolysis of the schiff base (Palczewski *et al,* 2000; Struthers *et al,* 2000). Protonation of $Glu^{3.49(134)}$, the acidic residue that is part of the highly conserved "Asp/Glu-Arg-Tyr" (DRY) motif at the base of TM 3 is important to attain a switch to a fully activated receptor. A $Glu^{3.49(134)}$Gln mutant of rhodopsin showed increased coupling efficiency, suggesting that protonation of $Glu^{3.49(134)}$ was important in the formation of the active metarhodopsin state (Arnis *et al,* 1994). Mutation of an equivalent highly conserved $Asp^{3.49(130)}$ in the β-adrenergic receptor gives rise to a receptor with high affinity ligand binding, but reduced G protein coupling (Dixon *et al,* 1987). Similar results were also obtained in the M_1 muscarinic and the α_{2A}-adrernergic receptors (Fraser *et al,* 1989; Wang *et al,* 1991). Mutation to Asn or Gln, mimicking the protonated form of Asp or Glu at locus 3.49 caused constitutive activation in the α_{1B}-adrenergic receptor (Kjelsberg *et al,* 1992; Scheer *et al,* 1997) suggesting that protonation is important for receptor activation. $Asp^{3.49(130)}$ of the α_{2A}-adrenergic receptor appears to influence receptor-G protein coupling (Wang *et al,* 1991). The $Asp^{3.49}$ has also been implicated in ligand binding in human AT_2 angiotensin II (Black and

Shankley 1995). Mutational analysis of the DRY motif in the AT_2 angiotensin II revealed more insights into the hormone binding, stability and G protein coupling. $Arg^{3.50}$ may be important for interacting with G proteins, whilst $Asp^{3.49}$ and $Ala^{6.34}$ may be important for constraining the receptor in an inactive conformation. The most conserved residue $Arg^{3.50}$ in the DRY motif on TM 3 is also critical for signal transduction in CB_2 cannainoid-2 (Rhee *et al,* 2000; Feng and Song 2003), N-formyl peptide (Bennett *et al,* 2000), H_2 histamine (Alewijnse *et al,* 2000), GnRH (Arora *et al,* 1995; Ballesteros *et al,* 1998) and β_{2A}-adrenergic (Rasmussen *et al,* 1999) receptors. A model showed that unprotonated $Asp^{3.49(142)}$ forms an interaction with $Arg^{3.50(143)}$ and $Asp^{2.50(91)}$ and maintains the Arg residue and other amino acids in IC 2 and IC 3 buried with respect to the cytosol. When the receptor is activated, with $Asp^{3.49(142)}$ protonated, the Arg shifts out of the polar pocket (Scheer *et al,* 1997; Scheer *et al,* 2000). Computational studies on the GnRH receptor suggested that $Arg^{3.50}$ formed an interaction with $Asp^{3.49}$ and not $Asp^{2.50}$ in inactive state (Ballesteros *et al,* 1998). During receptor activation, $Asp^{3.49}$ is protonated and $Asp^{2.50}$ substitutes for $Asp^{3.49}$ in interacting with $Arg^{3.50}$ (Ballesteros *et al,* 1998). The GnRH receptor contains serine rather than tyrosine in the DRY sequence. The effects of mutating the unique Ser^{140} to the conserved Tyr on agonist binding, internalization, and signal transduction were investigated. The $S^{140}Y$ mutant showed an increase in agonist binding affinity and its internalization above that of the wild-type receptor. These showed that substitution of Ser^{140} by Tyr does not affect G protein coupling but significantly increases receptor affinity and internalization rate (Arora *et al,* 1995).

1.2.5.3. The role of (Asn/Asp-)-Pro-X-X-Tyr motif in receptor activation.

The seventh transmembrane helix plays an important role in GPCR activation. The highly conserved (Asn/Asp-)-Pro-X-X-Tyr (N/DPXXY) motif in TM seven of all GPCRs introduces a perturbation in the α-helix and forms a part of a flexible hinge region (Konvicka *et al,* 1998). $Asn^{7.49(302)}$ and $Tyr^{7.53(306)}$ in rhodopsin project towards the inside of the helical bundle (Palczewski *et al,* 2000). There is a 3_{10} helix around $Pro^{7.50}$ at position 7.43-7.46 proposed to

stabilise the attachment of retinal to Lys$^{7.43(296)}$(Palczewski *et al,* 2000). TM seven in many GPCRs plays a major role in receptor function (see previous section 1.2.4.1). Most of the proposed interactions between TM 7 and other TMs are inconsistent with the TM 7 being an ideal α-helix. The N/DPXXY motif has special features that allow flexibility and also satisfy the required hydrogen bonding network between the different helices (Konvicka *et al,* 1998). The NP sequence forms α-helix from nonhelical structures, the Pro residue forms a Pro-kink that causes a bend and perturbation of the helix. During receptor activation the structure of the N/DPXXY motif is proposed to transform between the Pro kink in rhodopsin and a characteristic Pro-induced hinge conformation in the N/DPXXY (Konvicka *et al,* 1998; Ballesteros *et al,* 2001a). Twisting and kinking of a helix can result from a transmembrane based hinge formed by a Pro within a given motif (Sansom and Weinstein 2000). These hinges and swivels in the helices may have an important role in receptor activation. Crystal structures of rhodopsin and other GPCRs in different states of activation would provide more information onto conformational changes during receptor activation.

In other GPCRs the N/DPXXY motif is thought to be an internalization sequence, but the mechanism of how the N/DPXXY motif contributes to internalization is still ambiguous (Hausdorff *et al,* 1991). Receptor internalization regulation by N/DPXXY motif is different among GPCRs. The Tyr residue is suggested to be important for agonist-mediated receptor sequestration of the β_2-adrenergic receptor (Barak *et al,* 1995) and the neurokinin 1 receptor (Bohm *et al,* 1997). Internalization of the angiotensin II receptor does not require the presence of the NPLFY motif, but is essential for ligand binding and activation (Hunyady *et al,* 1995; Hunyady *et al,* 1995a). The residues within the N/DPXXY motif of the formyl peptide receptor regulate various functions. The Tyr residue is important for maintaining receptor conformation required for efficient ligand-induced internalisation and chemotaxis (He *et al,* 2001). A hydrophobic interaction between aromatic side chains of Tyr$^{7.53(306)}$ and Phe$^{7.60(313)}$ is responsible for stabilising the helix-turn-helix fold of the NPXXY(X)F region in rhodopsin (Fritze *et al,* 2003). The release of the constraint imposed by the Tyr$^{7.53(306)}$-

Phe$^{7.60(313)}$ interaction occurs during a transitions from Meta I to the Meta II state suggesting that these residues are critical for proper conformation of the NPXXY(X)F region of rhodopsin (Fritze *et al,* 2003). Interplay between NPXXY(X)F and the D(E)RY motifs are essential for receptor activation.

1.3. What is gonadotropin releasing hormone?

The hypothalamic decapeptide GnRH interacts with receptors on the gonadotrope cells in the anterior pituitary. GnRH plays a vital role in the reproductive cascade. GnRH is released into the portal system in a pulsatile manner and interacts with the GnRH receptors. This stimulation leads to the release of gonadotropes LH and FSH by the gonadotrope cells. LH and FSH then regulate steroidogenesis and gametogenesis in male and female vertebrates (Ying *et al,* 1981).

1.3.1. The structure and different variants of GnRH.

Different structural variants of GnRH have been cloned from various species, vertebrates and non-vertebrates and have been named from the species in which they were first identified (Matsuo *et al,* 1971; Sherwood *et al,* 1993; King and Millar 1995). The different variants of GnRH are widely distributed in vertebrate tissues and are important in different functions. There is evidence of 2 or 3 additional forms of GnRH in vertebrate species that have been studied, (King and Millar 1982; Miyamoto *et al,* 1984; Millar *et al,* 1987; Sherwood *et al,* 1993; King and Millar 1995; Millar *et al,* 2001; Millar 2002). GnRH II was first isolated from chicken brain by Miyamoto and colleagues. It is hypothesised that GnRH II is the earliest evolved form of GnRH because of its conservation from fish to man (Millar *et al,* 2004). GnRH III is the third conserved form of GnRH (salmon GnRH) and occurs in the forebrain in fish (White *et al,* 1998).

The amino and carboxy terminal of the GnRH are highly conserved among all the different forms. The type I GnRH forms have the most variable region at positions five, seven and eight. Position 8, which is important in mammalian reproductive

system (Millar *et al.* 1989) is the most variable followed by positions 5 and 7. The high variability of position 8 suggests that any residue can be substituted in this position, yet the mammalian receptor requires a positively charged Arg at position 8 (Millar *et al.* 1989; Illing *et al.* 1999). GnRHs from non-mammalian vertebrates contain uncharged residues at this position (Table 1.1).

Table 1.1. Primary amino acid sequences of naturally occurring GnRHs. Conserved residues, most of which play a role in receptor binding and activation, are shaded. Nonconserved residues are not important or confer species selectivity. Table reproduced from Sealfon S, et al., 1997.

	1	2	3	4	5	6	7	8	9	10
Mammalian	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Guinea Pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH_2
[Gln^8]-GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
Rana	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly-NH_2
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH_2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Leu	Pro	Gly-NH_2
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Trp	Ser	Pro	Gly-NH_2
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly-NH_2
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH_2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Leu	Leu	Pro	Gly-NH_2
GnRH II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH_2
Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH_2
Tunicate I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH_2
Tunicate II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH_2

1.3.2. Individual amino acids contribute to GnRH activity.

How the individual amino acids of GnRH contribute to ligand binding and activation may be explored by substituting individual amino acids and studying the function of the modified peptides compared to the natural ligand. Conservation of the N- and C-terminus indicates that these motifs are important for ligand binding and receptor activation. The lack of conservation of amino acids 5-8 suggests that these residues are not important for ligand binding, but for receptor selectivity among different subtypes and classes of receptors. Monitoring

the effect of amino acid substitutions on LH release and inositol phosphate production identified residues necessary for ligand interaction and receptor activation. Most of the studies on the different GnRH analogs in the past were tested using *in vivo* systems as there were no methods in place to perform *in vitro* assays (Sandow 1978). This present study attempted to test some of the analogs in the current *in vitro* assays used. These studies revealed that caution should be observed when extrapolating results from *in vivo* systems, as they may not necessarily reflect *in vitro* conditions. Some of the analogs were tested in this study using ligand binding assays.

Okada synthesised and investigated the biological activity of GnRH analogs lacking the N-terminal pGlu ring structure (Gly-, formyl-, acetyl-, propionyl-, palmitoyl-, and acetyl-GnRH). The studies showed that the –CO-NHCHCO- group of acetylated Gly^1 analogs was the minimum structure required for activity and that the pyrrolidone ring is not always essential (Okada *et al,* 1973). It was suggested that the –CO-NHCHCO- group is fixed into a desirable conformation by cyclic structures to result in receptor activation by the hormone. Substitution of the pGlu with formyl-sarcosine and N-Me-pyroglutamic acid retained half of the activity (Okada *et al,* 1973; Sandow 1978). GnRH antagonist studies revealed that pGlu is important for receptor activation (Karten and Rivier 1986). A peptide with a substitution with acetylated glycine at position one could not displace the labelled [His^5, D-Tyr^6]-GnRH analog in the $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe in the current study.

The multiple functional properties of the His^2 side chain render it appropriate for ligand-receptor interactions. Most substitutions of His^2 resulted in almost total loss of activity (Fujino *et al,* 1972b; Yanaihara 1973; Sandow 1978). The necessity of the aromatic ring was demonstrated by the substantial activity of [Phe^2]-GnRH, [3-Me-His^2]-GnRH and [Trp^2]-GnRH (Fujino *et al,* 1972a; Monahan *et al,* 1973; Yanaihara *et al,* 1973; Sealfon *et al,* 1997). His^2 has been proposed to interact with $Lys^{3.32(121)}$ (Zhou *et al,* 1995) and $Asp^{2.61(98)}$ (Flanagan *et al,* 2000) of the GnRH receptor.

An aromatic residue is critical at position 3 of GnRH. Substitutions with nonaromatic amino acids gives very low activity, while Tyr, Phe, 2napthyl-Ala and pentamethyl-Phe substitutions have some activity (between 1% and 50% activity) (Yanaihara *et al,* 1973; Coy *et al,* 1974; Sandow 1978). A π-π interaction has been proposed between Trp^3 and an aromatic residue in the receptor (Sealfon *et al,* 1997). Unaltered stereochemistry is critical for agonist activity since [D-Trp^3]-GnRH has poor activity (0.1%) (Hirotsu *et al,* 1974; Karten and Rivier 1986). Incorporation of NMe in Trp^3 converts the peptide into an antagonist, suggesting that this residue may be important in receptor activation (Haviv *et al,* 1993).

Through evolution Ser^4 has been 100% conserved among all known forms of GnRH, but its role has not been established. Ser^4 can be substituted with a number of amino acids and still retain some activity (Fujino *et al,* 1972b; Sandow 1978); (Fujino *et al,* 1972a; Coy *et al,* 1973; Yanaihara *et al,* 1973). The significant activity of [Ala^4]-GnRH showed that the hydroxyl group of serine is not essential for biological activity (Coy *et al,* 1973). However, [Ala^4]-GnRH could not displace the labelled ligand in membrane binding assays performed in the current study with $His^{7.36(305)}$Ala and the $His^{7.36(305)}$Phe mutant receptors. Very low activity is observed when Ser^4 is substituted with larger side chains Ser (But) and Leu, suggesting that spatial constraints are introduced by these substitutions of Ser^4 (Sandow 1978). This was confirmed when an inactive biotinylated Ser was introduced in the place of Ser^4 (Miller *et al,* 1992).

The lack of conservation of Tyr^5 in vertebrate GnRHs, suggests that different substitutions can be tolerated at this position. Phe may substitute Tyr while retaining half of the biological activity (Coy *et al,* 1974). Substitution with His conserves high affinity for mammalian GnRH receptors (Millar *et al,* 1989). This showed that the hydroxyl group of Tyr is not required for hormone action. An aromatic ring at position 5 is required for high activity in mammalian GnRH receptors. The results suggest that Tyr^5 is involved only in ligand interaction and it may have an indirect role in receptor activation (Millar *et al,* 1989). It was

suggested that His^5 substitutions might form a basis for the development new series of antagonists (Millar *et al,* 1989).

The Gly^6 residue is highly conserved in vertebrate GnRH isoforms, except the ancient lamprey and it is also absent in the tunicate GnRHs (Powell *et al,* 1996). The lack of side chain of Gly allows the peptide to assume a β-II type bend involving Tyr^5-Gly^6-Leu^7-Arg^8 residues (Monahan *et al,* 1973). Substitution with L-amino acids is unfavourable and led to a low biological activity (Coy *et al,* 1973; Monahan *et al,* 1973). Substitution with D-Ala^6 increased the activity to 400% and this led to the exploration of several substitutions with D-amino acids at this position (Coy *et al,* 1973; Monahan *et al,* 1973; Karten and Rivier 1986). Substitution with bulky hydrophobic D-amino acids increased activity and enhanced the formation of a β-II type bend (Monahan *et al,* 1973; Freidinger *et al,* 1980; Millar *et al,* 1986a; Millar *et al,* 1989). Replacing the Gly^6 with D-Tyr^6 is favourable for radio-iodination as the side chain is thought to face away from the receptor during binding. This side chain has been exploited for iodinations. Incorporating the large iodine atom doesn't interfere with binding of the GnRH analog [His^5,D-Tyr^6]-GnRH (Flanagan *et al,* 1998).

Substituting Leu^7 with uncharged L-amino acids with varying size side chains is well tolerated (Millar *et al,* 1989). Trp^7-substituted analogs resulted in relatively lower binding affinity in sheep pituitary, suggesting enhanced receptor activation by these analogs (Millar *et al,* 1989). Substitutions with amino acids that have small side chains and charged residues decreased activity (Fujino *et al,* 1972a).
Arg^8 is the most variable residue in the known forms of GnRH and it is required for high affinity binding of mammalian receptors (Millar *et al,* 1989). This basic residue has been proposed to have an important role in ligand selectivity of mammalian GnRH receptors (Sealfon *et al,* 1997). The role of Arg^8 in ligand selectivity was shown when $Glu^{7.32(301)}$ in the mouse GnRH receptor and $Asp^{7.32(302)}$ in human GnRH receptor were shown to confer selectivity for Arg^8 (Flanagan *et al,* 1994; Fromme *et al,* 2001). Nonmammalian receptors do not have selectivity for Arg^8-containing GnRH (Troskie *et al,* 2000; Sun *et al,* 2001;

Wang *et al,* 2001). Substituting Tyr for Gln^8 in the chicken I GnRH increases its activity by 3-fold. There is a severe decrease in activity when Tyr^8 in GnRH II is replaced with Arg or Gln (Millar *et al,* 1989). Position 8 has a role in GnRH of determining the selectivity for their equivalent receptors.

The conservation of Pro^9 suggests a conformational limitation caused by Pro on the peptide. Substitutions at this position are not being easily tolerated. Replacing Pro with N-Me-Gly^9 had a 10% activity compared to less than 1% of sarcosine^9 GnRH and Ala^9 (Sandow 1978). Naturally occurring [hydroxyproline]-GnRH was found to be 24 times (in vitro) and 5 times (in vivo) less potent than GnRH (Gautron *et al,* 1992). Its higher resistance to enzymatic degradation compensates the lesser affinity of the hydroxylated compound (Gautron *et al,* 1992).

The C-terminal glycine amide was found not to be essential for the high level of hormonal activity for LH release. The total chain length of GnRH is required for ligand-receptor interaction (Fujino *et al,* 1972a). Replacing Gly-NH_2 with Ala or Gly-NMe_2 caused a reduction in activity (Fujino *et al,* 1972b; Coy *et al,* 1975). Replacing Gly-NH_2 with alkylamides resulted in an increase of activity up to 500% (Fujino *et al,* 1972b). $Asn^{2.65(102)}$ was proposed to form a hydrogen bond with the carbonyl group of Gly^{10}-amide. Mutating $Asn^{2.65(102)}$ to Ala decreased binding affinity more for Gly-NH_2 ligands than N-ethylamides. This suggests that $Asn^{2.65(102)}$ is essential for determining potency for GnRH analogs with C-terminal glycinamide, while GnRH analogs with a C-terminal ethylamide are less dependent on $Asn^{2.65\,(102)}$ (Davidson *et al,* 1996a).

Conformational energy analysis proposed that different conformers exist for GnRH (Momany 1976; Momany 1978). Low energy GnRH conformers have a β-type turn at the Gly^6 residue (Guarnieri 1996). 2D-NMR studies showed that both GnRH and [Gln^8]-GnRH exist in a mixture of conformers. In GnRH it was proposed that the N and C-termini are closely opposed, whereas in [Gln^8]-GnRH the termini are far apart (Maliekal 1997). The Arg^8 residue of GnRH was proposed to stabilise an active conformation of GnRH which consists of a β-II-

bend involving the Tyr^5-Gly^6-Leu^7-Arg^8 residues (Shinitzky *et al,* 1976); (Monahan *et al,* 1973). Substitutions with a D-amino acid or γ-lactam in position six and seven are proposed to stabilise the active conformation of GnRH (Monahan *et al,* 1973; Momany 1976; Freidinger *et al,* 1980) (Figure 1.6).

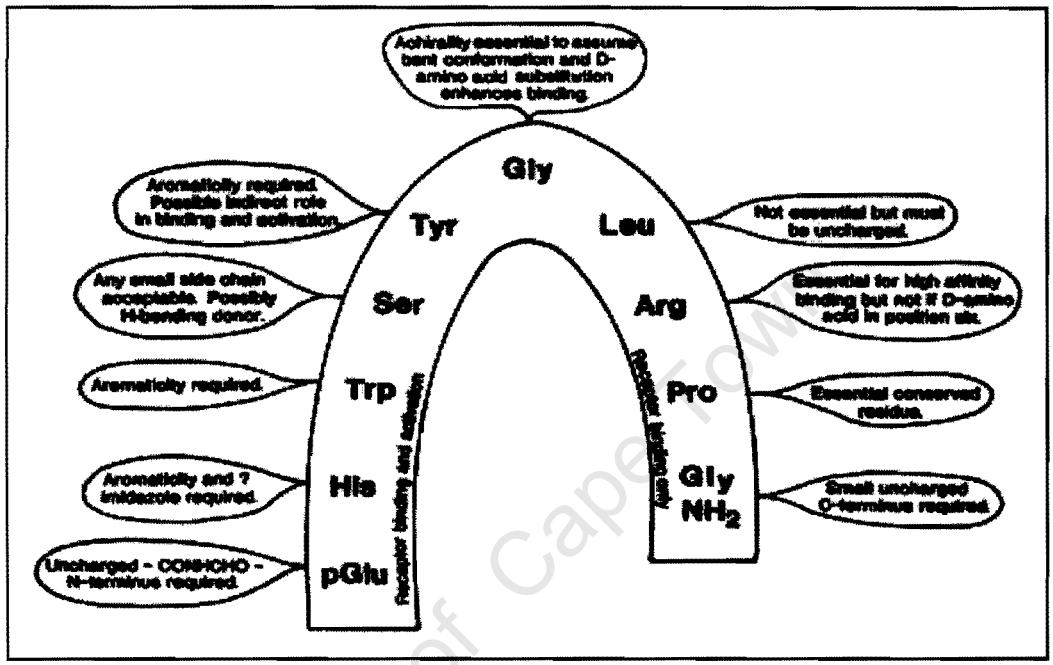


Figure 1.6. Schematic representations of the functional properties of GnRH amino acids. This figure summarises the functional properties of individual amino acid residues. Taken from Sealfon SC et al. 1997

1.4. The GnRH receptor

The GnRH receptor was first cloned from the cDNA of the αT3 mouse gonadotrope cell line (Tsutsumi *et al,* 1992). Several mammalian GnRH receptors have been cloned from the pituitary of rat (Kaiser *et al,* 1992), human (Kakar *et al,* 1992), sheep (Illing *et al,* 1993) and share 80% overall amino acid homology with each other (Sealfon *et al,* 1997; Millar *et al,* 2004). Other GnRH receptors homologous to the mammalian receptors were cloned from non-mammalian species (Troskie *et al,* 2000), chicken (Sun *et al,* 2001) and two forms of goldfish (Illing *et al,* 1999). Receptors have also been cloned from non-mammalian species and these receptors share 42-47% homology with the mammalian receptors and a 58-67% homology to each other. (Illing *et al,* 1999; Troskie *et al,* 2000; Okubo *et al,* 2000b;

Wang *et al,* 2001) (Figure1.7). Because of the three GnRH forms that exist it is thought that more than one type of GnRH receptor may exist. Using the extracellular loop 3 of the receptor as a determinant of receptor selectivity, a type II receptor was discovered. This GnRH receptor subtype was used to clone the non-functional human type II GnRH receptor (Millar *et al,* 1999; Faurholm *et al,* 2001) and bullfrog (Wang *et al,* 2001), marmoset monkey (Millar *et al,* 2001). A type III GnRH receptor subtype was also cloned using the same approach (Wang *et al,* 2001). The conservation of amino acids among the different GnRH receptors may reveal residues that are essential in ligand-receptor interactions and receptor activation in all the GnRH receptors.

1.4.1. The structure of GnRH receptor

The structure of the GnRH receptors is characteristics of G protein-coupled receptors (Figure 1.6). Seven helical transmembrane domains connected by alternating extracellular and intracellular loops follow the N-terminal. The seven domains are arranged in a bundle with the hydrophilic side enclosed and the hydrophobic side on the outside (Donnelly *et al,* 1989; Sealfon *et al,* 1997). The extracellular domains and exterior regions of the TMs are usually involved in binding of GnRH, the TMs are believed to be involved in receptor arrangement and conformational change associated with receptor activation. The conformational change associated with receptor activation involves the arrangement of intracellular loops, which interact with G proteins for signal transduction (Sealfon *et al,* 1997; Soderhall *et al,* 2002). The rhodopsin projection map was used to construct a model for the GnRH receptor using the alignment of TM helices (Sealfon *et al,* 1997). The GnRH receptor model suggested some interhelical interaction. These interactions were further confirmed by mutagenesis studies. The highly conserved Asp in TM 2 and Asn in TM 7 in GPCRs, appear to have undergone reciprocal mutation to $Asn^{2.50(87)}$ and $Asp^{7.49(318)}$ in the mouse GnRH receptor. This suggests that the two residues interact with each other. Mutation of $Asn^{2.50(87)}$ in TM 2 to Asp abolished receptor function, but a second mutation in TM 7 recreating the arrangement in other GPCRs restored ligand binding (Zhou *et al,* 1994; Flanagan *et al,* 1999).

These results indicate that the two TM helices are close to each other and have equivalent roles in the structure of the receptor. The crystal structure of inactive rhodopsin indicates that these residues interact with water in the inactive conformation, thus mediating a contact between TM 2, 3 and 7 (Palczewski *et al,* 2000; Okada *et al,* 2002). It has been proposed that an interaction between $Asn^{2.50}$-$Asp^{7.49}$ exists in the active form of GnRH receptor (Flanagan *et al,* 1999). Homology modelling of the TM domains of the GnRH receptor and the crystal structure of rhodopsin were compared (Soderhall *et al,* 2002). Additional to the TM 2/TM 7 mutations and the disulphide bridges a hydrogen bond web was noted between Glu^{90}-Lys^{121}-Asp^{98}. The formation of a disulphide bridge between $Cys^{3.25(114)}$ in EC 1 and $Cys^{5.23(196)}$ in EC 2 is responsible for the stability of TM 3 and TM 4 (Davidson *et al,* 1997). The position of the N-terminal and the extracellular loop is demonstrated by the formation of a second disulphide bridge between $Cys^{1.11(14)}$ and $Cys^{5.27(200)}$. Great effort has been made to define the structure of the intracellular and extracellular loops of the GPCRs and this is difficult because of the variations in lengths. It has been proposed that both intracellular and extracellular loops form distinct conformations different from the TM domains (Abdulaev *et al,* 2000; Yeagle *et al,* 2000). The crystal structure of rhodopsin revealed the lack of an α-helix in EC 3, whilst the mammalian GnRH receptor can easily accommodate the formation of two α-helices (Sealfon *et al,* 1997; Palczewski *et al,* 2000). Synthetic peptides of the EC 3 of the mouse GnRH receptor were used to study the conformation of this loop (Petry *et al,* 2002). Synthetic peptides with and without structural constraints were used to measure the ability of EC 3 to inhibit GnRH stimulated signalling in cells expressing the GnRH receptor. EC 3 of the mammalian GnRH receptor was proposed to exist as a random coil with a central β-hairpin and might be responsible for the orientation of the acidic residue ($Asp^{7.32(301)}$) in selecting Arg^{8}-containing GnRH (Petry *et al,* 2002).

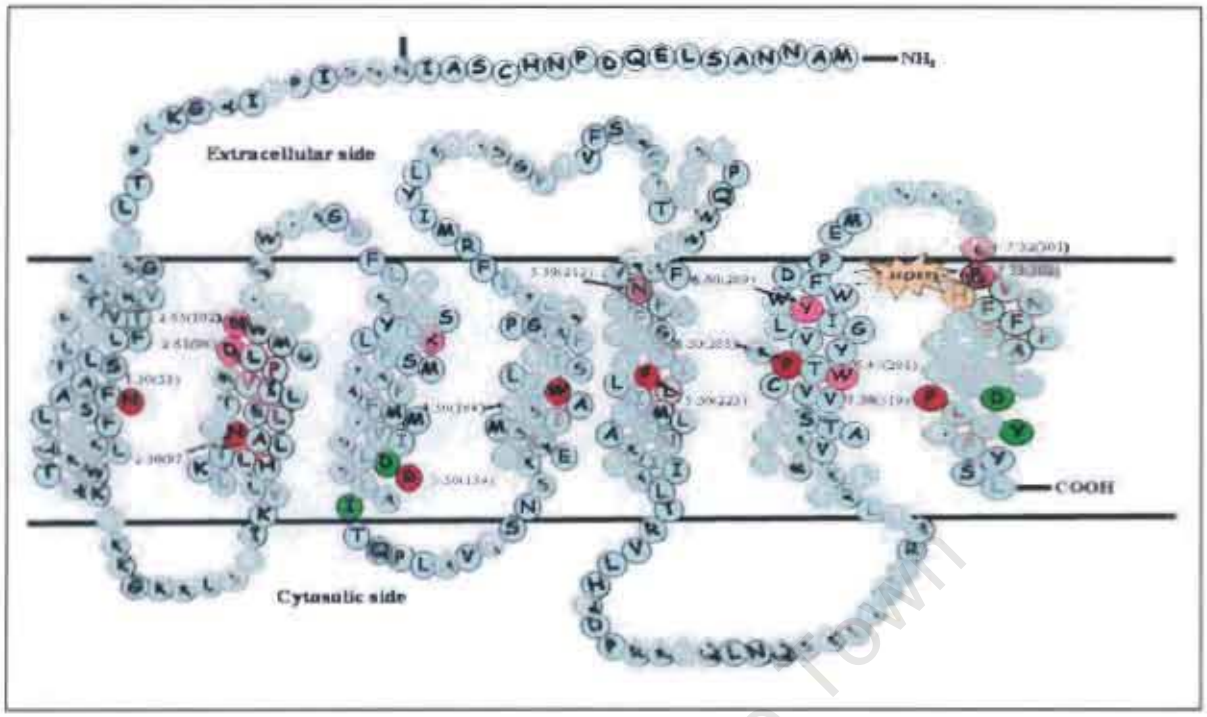


Figure 1.6. A two-dimensional structure of the mouse GnRH receptor. The most conserved residues in the rhodopsin family of GPCRs are shown in red. Residues in pink are ligand binding sites. Residues shown in green are known to be responsible for receptor activation.

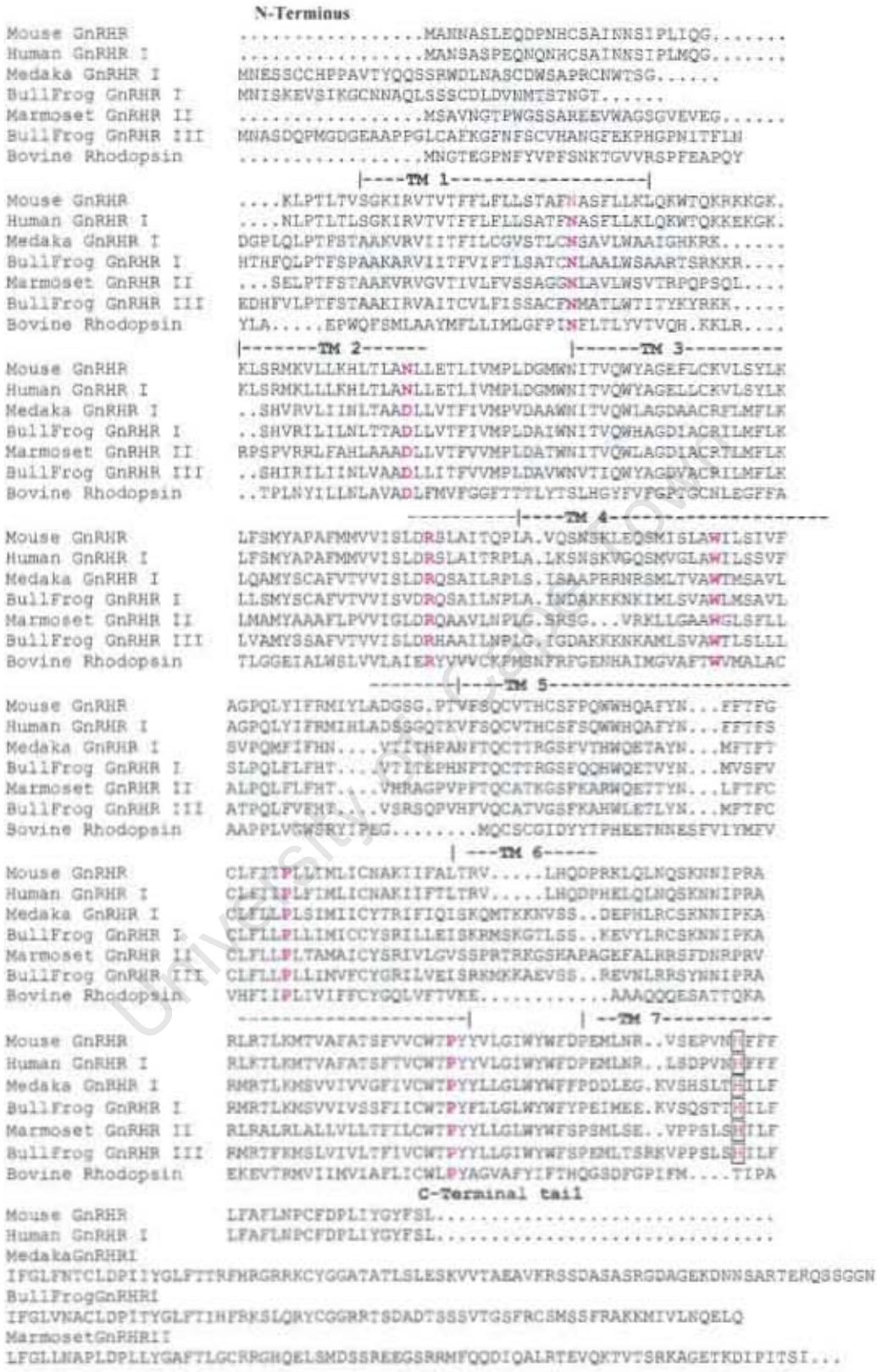


Figure 1.7. Sequence alignment of some of the cloned GnRH receptors with Bovine rhodopsin. Most conserved residues in each helix are shown in purple. His$^{7.36(305)}$ in TM seven is shown in red.

1.4.2. The ligand binding site for GnRH.

Ligand binding is the first step in receptor activation. Considerable research has been done to define the ligand binding site for GnRH and it was found to be located in the extracellular and TM domains of the GnRH receptor. Specific residues have been identified as interaction points for residues in GnRH. This thesis will attempt to further explain the ligand binding domain of GnRH.

Residues in EC 3 and transmembrane helix seven on the GPCRs are important for ligand binding as reviewed earlier and other residues have been identified in GnRH receptors (Asp/Glu$^{7.32}$ and Pro$^{7.33}$). Residues in this locus have been found in many receptors to be important in ligand binding and subtype selectivity. The current study of His$^{7.36(305)}$ on TM 7 revealed that this residue is required for ligand recognition. The location (TM 7) of this residue on the GnRH receptor and its multi-functional side chain of this residue suggested that it may be important in the function of the GnRH receptors. Systematic mutations of extracellular acidic residues to Gln or Asn revealed that substituting Glu$^{7.32(301)}$ in the mouse receptor resulted in a decrease in affinity for GnRH. Substituting Arg^{8} of GnRH greatly decreased GnRH affinity for mammalian type I receptors (Millar *et al,* 1989). An interaction between Glu$^{7.32(301)}$ and Arg^{8} was proposed to have a role in ligand selectivity (Flanagan *et al,* 1994). The Glu$^{7.32(301)}$Gln mutant receptor had a loss in affinity for mammalian GnRH, but unchanged affinity for analogs with substitutions for Arg^{8}, showing that Glu$^{7.32(301)}$confers selectivity for Arg^{8}-containing GnRH (Flanagan *et al,* 1994). Fromme et al. mutated the equivalent residue (Asp$^{7.32(302)}$) in the human GnRH receptor to Asn, the Asp residue replaces Glu in the human GnRH receptor. The human Asp$^{7.32(302)}$Asn mutant receptor showed that Asp$^{7.32(302)}$ also confers selectivity of the human receptor for Arg^{8} (Fromme *et al,* 2001). The high affinity binding of conformationally-constrained GnRH analogs was not dependent on either positive Arg^{8} or the negative Glu/Asp (Flanagan *et al,* 1994; Fromme *et al,* 2001). It is proposed that once the ligand is in the high affinity conformation, the electrostatic interaction of Arg^{8} with the acidic

residue is not essential for the final ligand-receptor complex. It is proposed that the acidic residue selects a β-II conformation of the ligand and interacts initially with the Arg residue to induce a high affinity conformation of the ligand. The high affinity conformation ligand then interacts with the final binding pocket which does not involve Asp or Glu (Fromme *et al,* 2001).

The role of the $Asn^{2.65(102)}$ residue, located near the extracellular end of TM 2 in high affinity binding with GnRH analogs has been identified (Davidson *et al,* 1996a). Mutation of $Asn^{2.65(102)}$ to Gln results in an increased affinity for GnRH (Davidson *et al,* 1996a). A loss of GnRH potency is observed when $Asn^{2.65(102)}$ is mutated to Ala. The $Asn^{2.65(102)}$Ala mutant had a smaller decrease in potency for analogs with C-terminal N-ethylamide. It was concluded that $Asn^{2.65(102)}$ forms a hydrogen bond via the carbonyl group with Gly^{10}-NH_2 (Davidson *et al,* 1996a; Sealfon *et al,* 1997). The $Asn^{2.65(102)}$Ala mutant showed a decrease in affinity for of an antagonist which has D-Ala^{10}-NH_2 substituted for Gly^{10}-NH_2 showing that there is a possible disruption of a hydrogen bond with the carbonyl group of D-Ala^{10}-NH_2 (Hoffmann *et al,* 2000).

Mutating $Asp^{2.61(98)}$ to Asn markedly decreased inositol phosphate production (Flanagan *et al,* 1994). Site-directed mutagenesis of $Asp^{2.61(98)}$ and ligand modification studies were performed to investigate the role of $Asp^{2.61(98)}$ in high affinity binding. Multiple interactions of the Asp side chain in the receptor with GnRH (His^2, Trp^3 and Ser^4) have been identified and characterised. Mutating $Asp^{2.61(98)}$ to Glu decreased affinity for GnRH analogs with His^2 amino acid and caused a smaller decrease for GnRH analogs with substitutions at position 2. This indicated that $Asp^{2.61(98)}$ confers specificity for His^2 of GnRH. A series of analogs with different His^2 substitutions has shown that $Asp^{2.61(98)}$ probably forms a hydrogen bond with the δ-NH group of His^2 (Flanagan *et al,* 2000). Mutating $Asp^{2.61(98)}$ to a neutral residue led to a decrease in affinity for GnRH that did not involve His^2. These results indicated that the $Asp^{2.61(98)}$ side chain has one or more charge-dependent interactions with that are important for high affinity binding, but different from the interaction with His^2. The computational modelling shows an interhelical ionic interaction of $Asp^{2.61(98)}$ in TM 2 with $Lys^{3.32(121)}$ in TM 3. The

model also showed $Lys^{3.32(121)}$ might form a hydrogen bond with the backbone C=0 group of Ser^4 in GnRH. $Asp^{2.61(98)}$ also seems to form an interaction with the backbone NH group of Trp^3 (Flanagan *et al,* 2000).

The highly conserved $Asp^{3.32}$ residue in TM 3 of the biogenic amine receptors interacts with the positively charged amine head group of biogenic amine ligands (Strader *et al,* 1994). The GnRH receptor has a Lys residue in the 3.32 locus. $Lys^{3.32(121)}$ of the GnRH receptor was investigated for its possibility in interacting with GnRH. Mutation of $Lys^{3.32(121)}$ to Arg had little effect on ligand binding and inositol phosphate production, while mutation to Asp, Ala or Leu led to a total loss of agonist binding and inositol phosphate production. The $Lys^{3.32(121)}$Gln mutant exhibits in a 2000-fold loss in GnRH potency but has no significant effect on peptide antagonists (Zhou *et al,* 1995). GnRH receptor antagonists have modifications in the N-terminus, thus it was suggested that $Lys^{3.32(121)}$ interacts with this part of the GnRH molecule. This interaction is not a salt bridge, as GnRH does not have any negatively charged amino acid side chains. $Lys^{3.32(121)}$ was proposed to interact with His^2 of GnRH by a charge-strengthened hydrogen bond (Zhou *et al,* 1995). From computational modelling and mutagenesis studies it was suggested that $Lys^{3.32(121)}$ also forms an interaction with $Glu^{2.53(90)}$ (Hoffmann *et al,* 2000). It was proposed that either the His^2 or Trp^3 rings may form a hydrogen bond with $Lys^{3.32(121)}$ (Hoffmann *et al,* 2000).

Mutation of $Asn^{5.39(212)}$ to Ala decreased affinity for agonists and antagonists. The $Asn^{5.39(212)}$Gln mutant had decreased potency and little effect on antagonist binding (Hoffmann *et al,* 2000). $Asn^{5.39(212)}$ was suggested to be important for ligand binding, forming an interaction with the backbone C=O group of His^2 of GnRH and not with an antagonist (Hoffmann *et al,* 2000). Subsequently, $Asn^{5.39(212)}$ was shown in computational modelling to interact with $pGlu^1$ residue which points towards TM 5 (Chauvin *et al,* 2000; Hovelmann *et al,* 2002). To further, define the role of $Asn^{5.39(212)}$ in antagonist binding and receptor structure systematic substitutions of GnRH analogs and additional $Asn^{5.39(212}$ mutants will need to be tested thoroughly.

A mutation with Phe$^{6.60(292)}$Ala in TM 6 retained activity, while Tyr$^{6.51(283)}$Ala, Tyr$^{6.52(284)}$Ala and Trp$^{6.59(291)}$Ala had a complete loss of activity. A reduced expression was observed with the Trp$^{6.57(289)}$Ala and Tyr$^{6.58(290)}$Ala mutants while retaining antagonist binding affinity. Tyr$^{6.58(290)}$Ala showed a great decrease in potency of agonists. Tyr$^{6.58(290)}$ is thought to play an important role in agonist and not antagonist binding. Tyr^{5} was proposed to interact with Tyr$^{6.58(290)}$ (Hovelmann *et al,* 2002). Trp$^{6.57(289)}$ is proposed to interact with D-Trp^{6} of the high affinity [D-Trp^{6}]-GnRH analog (Hovelmann *et al,* 2002).

Interactions between the GnRH receptors and antagonist have been less extensively studied compared to agonist binding. GnRH antagonists have 50-70% amino acid substitutions. In most cases, the agonist and antagonist binding sites are overlapped(Samama *et al,* 1993). The interaction of GnRH agonists and antagonists was studied by using ^{125}I-labeled agonist, [D-Ser(t-Bu)^{6}, Gly^{10}NHet]-GnRH, and antagonist [D-pGlu^{1}, D-Phe^{2}, Trp^{3,6}]-GnRH with pituitary membranes. Binding of antagonists was decreased more than agonist indicating that both ligands bind the receptor but may occupy different sites when pituitary membranes were pre-treated with proteolytic enzymes (Hazum 1981a). GnRH mutations with different effects on antagonist interactions have been shown. These mutagenesis studies have been used to distinguish between agonist and antagonist binding sites. A mutation of Asp$^{2.61(98)}$ to Glu disrupted the interaction with His^{2} of GnRH, but Asp$^{2.61(98)}$ is probably not involved in antagonist binding (Flanagan *et al,* 2000). Minimal effect of antagonist was observed for the Asp$^{7.32(302)}$Asn mutant receptor. Antagonists with Arg^{8} also showed no effect for the Asp$^{7.32(302)}$Asn mutant (Fromme *et al,* 2001). A mutation of Asn$^{5.39(212)}$ to Ala had a decrease in antagonist affinity while a mutation to Gln had a slight decrease in affinity (Hoffmann *et al,* 2000). A mutation of Lys$^{3.32(121)}$ showed to a certain extent different effects on agonist and antagonist interactions. The mutations had a marked effect on agonist potency and its failure to affect antagonist affinity. This suggested that, Lys$^{3.32(121)}$ may be important for agonist binding and it is evidently not an antagonist contact site (Zhou *et al,* 1995).

1.4.3. GnRH receptor activation.

1.4.3.1. The DRX motif.

The $Arg^{3.50}$ residue in TM 3 has been shown to be important for receptor activation in many GPCRs (Scheer *et al,* 1997; Hulme *et al,* 1999; Rhee *et al,* 2000; Feng and Song 2003). Ballesteros et al. suggested that the $Arg^{3.50(139)}$ forms an ionic interaction with $Asp^{3.49(138)}$ when the receptor is in an inactive form. (Ballesteros *et al,* 1998). This interaction was confirmed from the crystal structure of the inactive rhodopsin where $Glu^{3.49(134)}$ forms a salt bridge with $Arg^{3.50(135)}$ (Palczewski *et al,* 2000). During GnRH binding, the $Asp^{3.49(138)}$ is proposed to be protonated and the interaction with $Arg^{3.50(139)}$ is broken to release the $Arg^{3.50(139)}$ side chain. $Ile^{3.54(143)}$ located one helical turn below the $Arg^{3.50(139)}$ was proposed to have a role in preventing the movement of $Arg^{3.50(139)}$into the aqueous intracellular domain acting as a "cage". The Ile residue located one helical turn below the Arg forms a cage around the Arg side chain limiting its movement. The $Arg^{3.50(139)}$ is proposed to then interact with $Asp^{7.49(319)}$ in TM 7 (Ballesteros *et al,* 1998). Mutating the $Asn^{2.50(87)}$ and $Asp^{7.49(318)}$ in the mouse GnRH receptor revealed that $Asp^{7.49(318)}$ is involved in receptor activation. The reciprocal mutant retained good binding but had poor IP stimulation. The $Asp^{7.49(319)}$ residue is part of conserved residues involved in GnRH receptor activation (Zhou *et al,* 1994). Helix movements during activation may involve an interaction of $Arg^{3.50(139)}$ with $Tyr^{7.53(323)}$ (conserved NPxxY motif) in TM 7 (Arora *et al,* 1996). This residue had been found in many GPCRs to be important in receptor activation (He *et al,* 2001; Prioleau *et al,* 2002; Fritze *et al,* 2003). $Arg^{3.50(139)}$ probably forms multiple interactions with both $Tyr^{7.53(323)}$ and $Asp^{7.49(319)}$ in the active conformation.

1.4.3.2. Asn/Asp in transmembrane helices two and seven in GnRH receptor activation.

In the GnRH receptor the conserved motif, $Asp^{(2.50)}$ in TM 2 and $Asn^{(7.49)}$ in TM 7 appear to been interchanged compared to other rhodopsin-like GPCRs (Sealfon *et al,* 1995b; Flanagan *et al,* 1999; Xu *et al,* 1999). Reciprocal mutagenesis studies on several GPCRs have shown that mutating the $Asp^{2.50}$ side chain disrupts receptor activation. This disruption can be restored by a second mutation in TM 7 interchanging the two conserved residues. These residues were shown to have a role in receptor expression and signalling of GnRH receptors. Mutating $Asn^{2.50(87)}$ and $Asp^{7.49(318)}$ in the mouse GnRH receptor showed that receptor activation requires the presence of these residues. Ligand binding of $Asn^{2.50(87)}Asn^{7.49(318)}$ and $Asp^{2.50(87)}Asp^{7.49(318)}$ was similar to that of wildtype but had poor IP production (Zhou *et al,* 1994). The peculiar arrangement of the $Asn^{2.50(87)}$ and $Asp^{7.49(318)}$ residues in the GnRH receptor is important for receptor activation. Additionally to its requirement in receptor activation, $Asn^{2.50(87)}$ is essential for the expression of GnRH receptor on the cell surface. $Asn^{2.50(87)}$ has been suggested to form an interaction with $Asn^{1.50(53)}$ on TM 1, which is also required for receptor expression. Coupling to phospholipase D (PLD) was prevented by the presence of $Asp^{7.49(318)}$. Mutating this residue recovers the PLD coupling in GPCRs. $Asn^{2.50}$ and $Asp^{7.49}$ are required for PLC activation and not for PLD activation (Flanagan *et al,* 1999).

1.4.3.4. Intracellular loop three in GnRH receptor activation.

For several GPCRs the C-terminal domain of IC 3 is essential for G protein activation as mutations to this locus results in constitutively active receptors. Mutating the $Ala^{6.34(293)}$ of the α1B-adrenergic receptor to any other amino acid results in constitutive activation of the receptor (Kjelsberg *et al,* 1992). Mutations of the somatic and germ line of the thyrotropin (TSH) receptor gene can cause hyperfunctioning thyroid adenomas and e toxic thyroid hyperplasia by constitutive activation of the TSH receptor. Several adenomas of the thyroid were screened for constitutively activating mutations and two mutations were found, changing alanine

in position 623 to valine (Ala$^{6.34(623)}$Val) and threonine in position 632 to isoleucine (Thr$^{6.43(632)}$Ile). Both the mutations constitutively activated cAMP when expressed in COS cells. The Thr$^{6.43(632)}$Ile mutant showed that TM 6 is important for the activation of the TSH receptor and distinguishes the region as a ideal locus for constitutively activating mutations. In the GnRH receptor, Ala$^{6.29(261)}$ was found to be important for $G_{q/11}$ activation. Mutation of Ala$^{6.29(261)}$ to several amino acid residues resulted in a complete loss of coupling but retained the ability to bind the ligand (Myburgh *et al,* 1998). Coupling is also dependent on the size of the residue at position 261. As the size of the substituted residue increase, the more the receptors were uncoupled.

1.5. Possible Histidine interactions.

The side chain of histidine has a complex structure and therefore has a potential to form numerous interactions with other amino acids. It can form ionic interactions, hydrogen bonds and π-π interactions with the different atoms. Cation-π interactions are recognised as one of the major noncovalent interactions that play a crucial role in determining structures of proteins and for recognition of ligands by proteins. In the M2 proton channel of influenza A has a cation-π interactions between a His and Trp residue on different helices (Takeuchi *et al,* 2003). His and Phe rings are both capable of π-π and CH/π interactions, and π/OH interactions with Tyr, and π/NH interactions with Trp and His. Only His can be a hydrogen donor in an NH/π interaction with any aromatic side chain through its Nδ1 H-group and its protonated Nε2 atom. (Meurisse *et al,* 2003). Hydrogen bonds have been identified to be very important in maintaining the structure of proteins. A number of amino acids can form hydrogen bonds with their side chains in addition to their peptide bond. For example, Asn and Gln through their amide groups, the charged groups, Lys, Arg, Glu and Asp, and the aromatic His, Tyr and Trp. The unprotonated Nε2 atom can also form H-bonds with either the hydroxyl group of Tyr and Ser, NH group of Trp and Nδ1 or protonated Nε2 of His. Until recently most focus had been on the ability of these amino acids to form conventional H-bonds of OH/O or NH/O type. Phe, Tyr, Trp and His were

compared for their abilities to form three different types of H-bonds. His was found to form the strongest OH/O and OH/N bonds, followed by Tyr and then Trp (Scheiner *et al,* 2002). The protonated His side chain makes a very potent proton donor, even its CH/O bonds are stronger than the conventional H-bonds. Among the aromatic residues in protein structures, His is unique, as it can exist in the neutral or positively charged form at the physiological pH. The neutral imidazole side chain, generally found in high pH exists in tautomeric forms: the Nε2 and the Nδ1. Aromatic-aromatic interactions were suggested to be a stabilizing force in determining globular protein structures (Bhattacharyya *et al,* 2003). An average of about 60% of aromatic side chains in proteins are involved in aromatic pairs, 80% of which form networks of three or more interacting aromatic side chains. In the α-helix a particular combination of aromatic residues was found to interact more than expected. Depending on the number of residues between the aromatic residues, different aromatic-aromatic pairs are preferred. The highest pairing occurs when aromatic residues are next to or 2 residues apart. The His-Trp pair was shown to be important for stabilising an α-helix when His is protonated (Fernandez-Recio *et al,* 1997).

Concluding remarks

The GnRH receptor is a typical GPCR with many of the conserved motifs responsible for different functions in the receptor. Different models have been proposed to define ligand binding domain and structure of the GnRH receptor. However, more investigations still need to be done to understand the mechanisms of ligand-receptor interactions and receptor activation. This thesis will describe the role of the $His^{7.36(305)}$ residue, located at the top of TM 7 in multiple functins of the GnRH receptor.

CHAPTER TWO: EXPERIMENTAL APPROACH

2.1 GnRH Analogs

Mammalian GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) and GnRH II [His^5,Trp^7,Tyr^8]-GnRH were purchased from Bachem AG (Bubendorf, Switzerland), [His^5, D-Tyr^6]-GnRH, [Ala^4]-GnRH [AcGly^1]-GnRH, [2-Nal^3]-GnRH were specially synthesised for this project by Dr Roger Roeske (Indiana University School of medicine, Indianapolis, Indiana, USA. [D-Trp^6, Pro^9NHet]-GnRH and Antagonist 27 [Ac-D-Nal(2)^1, D-Me-4-Cl-Phe^2, D-Trp^3, Ipr-Lys^5, D-Tyr^6, D-Ala^{10}]-GnRH were synthesised by R. Milton in our laboratory. [Trp^2]-GnRH and [Hyp^9]-GnRH were synthesised by Dr Jean Rivier in our laboratory

2.2. Site-directed mutagenesis

The Dpn I method of polymerase chain reaction-based site-directed mutagenesis was used to replace the $His^{7.36(305)}$with different amino acids (Ala, Phe, Asn, Gln, Arg, Trp) in the mouse GnRH receptor using the deep vent polymerase . The wild-type mouse GnRH receptor cloned into the Eco RI and Xho I sites of the pcDNAI/Amp vector (with ampicillin resistance gene) (Invitrogen, San Diego, USA) was used as a template, (New England Biolabs, England, UK). To prevent contamination, pcr reactions were set up in a designated fume hood using filter tips (Quality Scientific Plastics, Midwest Scientific, St. Louis, USA). Sense and antisense primers were designed to have the desired mutations and a silent restriction site to confirm the mutation (see appendix for primers sequences). For each mutation, a final concentration of 10ng/µl of template and 10µM of primers were used. 16 cycles of the pcr were run to amplify the whole plasmid (94^0C for 30 seconds, 55^0C for 30 seconds, 72^0C for 12 minutes and an extension at 72^0C for 10 minutes in 50µl final volume, because the whole plasmid is amplified)(Perkin-Elmer-Cetus thermal cycler). After the pcr reaction the products were treated with 2µl (40 units) of Dpn I restriction enzyme (New England Biolabs, Inc, Beverly, USA) added directly into the pcr tube. Dpn I digests the methylated template only and not the pcr products because it is specific for methylated DNA. The DH10B strain of E.coli bacteria, in which the

DNA is prepared, methylates the template. 10ng of mouse GnRH receptor cDNA was amplified in a Perkin-Elmer-Cetus thermal cycler in 50µl; 55µl of the Dpn I-treated PCR product was used to transform DH10B bacterial cells and cultured on ampicillin agar plates overnight. Colony pcr and restriction digests with silent-site specific enzymes on minipreps DNA were performed to confirm the presence of the mutations.

Large-scale DNA preparations were done to make large quantities of quality mutant DNA for automated sequencing. Mutant DNA constructs were prepared from cultures grown overnight in 500ml of 2 x YT medium (10g/l NaCl, 10g/l Yeast extract, 16g/l Tryptone) containing Ampicillin (100µg/ml). Plasmid DNA was extracted with the PC100 and PC500 kits (Machery-Nagel, Düren, Germany). The bacteria are lysed and the chromosomal DNA is precipitated and centrifuged. The plasmid DNA is adsorbed onto a cartridge and eluted. Lastly, the mutant DNA was precipitated with cold 70% ethanol. The DNA constructs were digested with the specific endonucleases for the silent sites to confirm the mutations. Because the whole plasmid is pcr generated and even though the Deep vent polymerase that was used was a high fidelity polymerase it may have generated potential pcr errors. The mutant receptors containing appropriate restriction sites were subcloned into pcDNAI/Amp expression vector (for selection in ampicillin-containing medium) using the Fast link DNA ligation kit (Epicentre Technologies, Madison, USA) to get rid of any pcr-generated errors in the vector. The ligation reaction contained the two fragments of DNA, a fast-link ligation buffer, 10mM ATP and a fast-link DNA ligase. The ligation products were used to transform competent DHI0B bacteria.

All the mutant receptors were sequenced (automated) to confirm the mutations (UCT specialist sequencing services, MegaBACE 500) and to ensure that there were no PCR-generated mutations.

2.3. Cell Culture

COS-1 cells (American Type Culture Collection) were maintained in antibiotic-free Dulbecco's modified eagle's medium (DMEM) (Gibco, Paisley, Scotland) containing 10% fetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) at 37°C in a 10% CO_2 humidified incubator.

2.3.1. Transient transfection of COS-1 cells

3 x10^6 COS- 1 cells were plated on 10cm dishes (Corning, Cambridge, USA) for transfection in DMEM containing 10% FCS (Delta Bioproducts, Kempton Park, South Africa), 2mg/ml streptomycin sulphate and 4000U/ml sodium benzylpenicilin. COS-1 cells were transiently transfected using the DEAE-Dextran method as described previously (Keown *et al,* 1990; Millar *et al,* 1995). 15µg of the plasmid DNA and DEAE-Dextran (3mg/ml in HEPES Buffered Saline buffer) (Sigma, Bellefonte, and USA) (Millar *et al,* 1995) was used to transfect each dish. After 4-5 hours at 37^0C in a CO_2 incubator, the cells were treated with 200µM chloroquine (raises pH in lysosomes, which then inhibits degradation of the transfected DNA) (50 minutes), and then treated for 2 minutes with 10% dimethylsulfoxide (the shock step increases the expression level of the transfected gene) in DMEM with penicillin/streptomycin. The cells were incubated overnight in DMEM with 10% FCS (Luthman and Magnusson 1983; Lopata *et al,* 1984). Cells were grown for 48 hours before each experiment. The transfected cells were harvested, homogenised, and crude membrane preparations concentrated (1ml/dish) then frozen at -70°C for future use in assays. The membrane preparations could be frozen for up to 1 month.

2.4. Ligand Binding Assays

2.4.1. Radio-iodination of [His^5, D-Tyr^6]-GnRH.

A radioactively labelled analog, [His^5, D-Tyr^6]-GnRH was used as a tracer in ligand binding assays. The method of iodination has been previously described (Flanagan *et al,* 1998). The chloramine T method was used with variations. 5μg of [His^5, D-Tyr^6]-GnRH peptide in 40μl of 0.5M NaH_2PO_4 (pH 7.6) was added to 10μl of ^{125}INa (1mCi; AEC-Amersham) and 10μl chloramine T (85nmoles) as an oxidising agent for about 10 seconds. Sodium metabisulfite (84nmoles) as a reducing agent was added to stop the reaction. The iodinated peptide was purified on a reverse phase HPLC (to remove the unincorporated ^{125}I and for removal of damaged peptide) with an 80% acetonitrile/0.01M ammonium acetate (pH 4.6) gradient. 45-50 fractions were collected every 30 seconds, at a flow rate of 1.5ml/30seconds. The specific activity of iodinated [His^5,D-Tyr^6]-GnRH ranged between 900 and 1800μCi/μg (Flanagan *et al,* 1998). Different fractions with high radioactivity from the eluent were tested for their specific binding and fractions with the highest specific binding were used in competition binding assays (Figure 2.1).

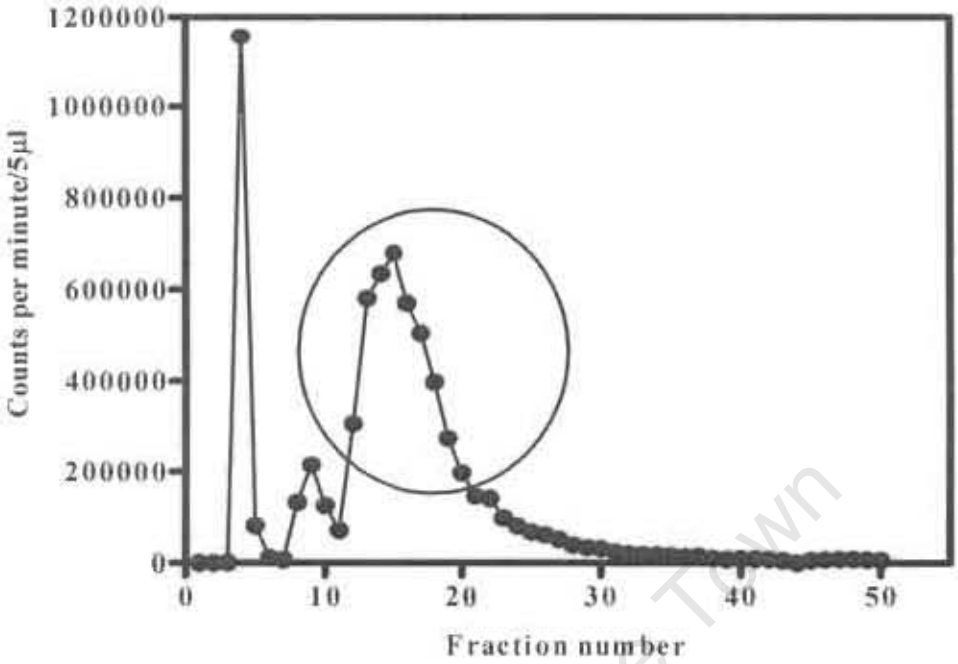


Figure 2.1. The iodination profile of [His^5, D-Tyr^6]-GnRH. 50 fraction were collected from the purification. The circled fractions were tested for specific binding and the fractions with the highest specific binding were used in competition binding assays. Data is representative of 6 independent experiments.

2.4.2. Whole Cell Binding Assay

A day after transfection cells were trypsinised and re-plated in 12-well plates. One day later the attached cells were washed with 1ml cold Buffer I (140mM NaCl; 4mM KCl; 20mM HEPES; 8.6mM glucose; 1mM $CaCl_2$; 1mM $MgCl_2$; 0.1% BSA, fatty acid free; pH 7.4). The cells were then incubated with 100 000 cpm of ^{125}I-[His^5, D-Tyr^6]-GnRH and appropriate amount of unlabelled GnRH agonist for 4-5 hours at 4°C in 500µl final volume. After incubation the cells were washed 3 times with PBS to remove the unbound peptides, then lifted from plates with 0.1M NaOH and the bound radioactivity was counted in a gamma counter.

2.4.3. Membrane Binding Assay

Varying the amount of membranes in this assay system makes it possible to optimise receptor concentrations as desired. The low total binding of some mutant receptors was compensated by adjusting the concentration of membranes. Cells were scraped with a cell scraper (Corning, Cambridge, USA) and harvested in detaching buffer (1mM EDTA; 10mM HEPES, pH 7.4), and homogenised with a dounce homogeniser (Kontes, Vineland, USA). The homogenates were centrifuged at 15000 x g for 20 minutes at 4°C. The pellet of crude membranes was resuspended in detaching buffer with 0.1% BSA to various concentrations. Membrane suspensions (400µl/tube) were incubated overnight at 4°C with 50µl (50 000 CPM, 50pM) of ^{125}I-[His^{5}, D-Tyr^{6}]-GnRH and 50µl of varying concentrations of unlabelled GnRH analogs. After incubation the suspension was washed with 3ml of 0.01% polyethylenimine (PEI) and quickly filtered (Brandel Cell Harvester) through glass fibre filters (GF/C) (Whatman) pre-soaked for 30 minutes in 1% PEI (to reduce non-specific binding). Filters were washed twice with 3ml 0.01% PEI. The retained radioactivity was counted. 1µM antagonist 27 was used to measure non-specific binding (Millar *et al,* 1995).

2.5 Inositol Phosphate Assay

Transfected cells were transferred to 12-well plates and incubated overnight with *myo*- [2-^{3}H] inositol (1µCi/well) (Amersham, Arlington Heights, England) in 0.5ml of inositol-free Medium 199 (Gibco, Paisley, Scotland) with antibiotics. The cells were not re-counted an treated the same to avoid inconsistency. The cells were washed twice with 1ml/well Buffer I containing 10mM LiCl. (LiCl prevents IP degradation) (Huckle and Conn 1987). The cells were then pre-incubated for 15 minutes in Buffer I at 37°C, followed by incubation with varying concentrations of ligand for 45 minutes. The reaction was terminated by removal of the medium and addition of 10mM formic acid (1ml/well) and which was left on the cells for 30 minutes at 4°C to release the inositol phosphates. Inositol phosphates extracts were separated on 1ml 1X8-200 Dowex-1 ion exchange

columns (Sigma-Aldrich, South Africa) (Millar *et al,* 1995). The columns were soaked in 3ml of 3M ammonium formate with 0.1M formic acid, then washed with 10ml of distilled water. 1ml of sample extract was loaded on the column and washed with 10ml distilled water, followed by 5ml of 5mM myoinositol with 1.0M formic acid. The total inositol phosphates were eluted with 3ml of 1M ammonium formate with 0.1M formic acid into 18ml scintillation fluid (Zinsser Analytical, Frankfurt, Germany). The radioactivity was counted in a beta counter.

2.6 Data Analysis

IP assays and whole cell binding were performed on 12 well plates in duplicate for at least four times. A non-linear regression curve fitting was used to calculate the EC_{50} (sigmoidal dose-response curve) and IC_{50} (one site competition curve) values. Maximum binding (Btotal) of GnRH calculated as % of total counts in the absence of ligand relative to the initial total amount of ^{125}I-[His^5, D-Tyr6]-GnRH. Homologous membrane binding assays were used to estimate the Bmax and IC_{50} values that were used to calculate the binding sites per cell of the mutant receptors. See appendix for equations used to calculate Bmax and receptor coupling efficiency values. P values were calculated using unpaired two tailed T-tests performed on the negative log of Bmax, IC_{50} and EC_{50}. (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA).

CHAPTER THREE: RESULTS

In this thesis, the functional role of the $His^{7.36(305)}$ residue of the mouse GnRH receptor in receptor expression, ligand binding and coupling to intracellular signalling was investigated. This investigation was based on the fact that the histidine imidazole side chain has multiple chemical properties and the position of histidine 305 residue at the extracellular end of TM 7 of the GnRH receptor. The roles of the different properties of histidine residues have been studied in various enzymes (Kubiak *et al,* 2001; Montero-Moran *et al,* 2001; Tuganova *et al,* 2001). In the current study, site-directed mutagenesis was used to investigate the roles of the different functional groups of $His^{7.36(305)}$ in GnRH receptor function. The $His^{7.36(305)}$ was mutated to alanine which removes all the functional groups of histidine, a π-electron donor phenylalanine which mimics the aromatic ring of histidine but removes the hydrophilic groups and potential positive charge, the hydrophilic residues asparagine and glutamine, which can donate or accept a hydrogen bond, the basic arginine, or an aromatic tryptophan. The different mutant receptors were tested for their response to GnRH ligands, binding affinity and expression levels estimated from functional assays. The results show that the imidazole side chain contributes in both ligand binding and receptor activation.

3.1. IP Production

GnRH stimulation of IP accumulation was used as a measure of functional activity of the wildtype and mutant receptors. The wildtype receptor showed a dose-dependent increase in IP production with an EC_{50} value of 0.162nM ± 0.04 (Figure 3.1, Table 3.1). The $His^{7.36(305)}$Trp mutant exhibited almost no response to the GnRH stimulation, with the lowest maximum stimulation (Emax value) (Figure 3.1). All the mutants showed varying decreases in Emax values compared to the wildtype receptor. Substituting the histidine side chain with the small alanine side chain resulted in a large decrease in GnRH -stimulated IP production (EC_{50} 24.6nM ± 3.7) (Figure 3.1, Table 3.1) indicating the importance of the histidine side chain in this position. The $His^{7.36(305)}$Phe mutant also showed a large

decrease in GnRH-induced IP production (EC_{50} 26nM ± 3.9) (Figure 3.1, Table 3.1) compared to the wildtype receptor. GnRH showed a smaller loss of potency at the $His^{7.36(305)}$Asn (EC_{50} 1.07nM ± 0.36) and $His^{7.36(305)}$Gln (EC_{50} 0.92nM ± 1.194) mutants, showing a recovery of the loss of potency that was seen in the $His^{7.36(305)}$Phe and $His^{7.36(305)}$ Ala mutants (Figure 3.1, Table 3.1). The Asn and Gln mutations mimic the δ– and ε-nitrogen atoms respectively of the histidine side chain. The enhanced activity of the Asn and Gln mutants suggests that the δ- and ε-nitrogen atoms mimicked by the Asn and Gln recovered some of the activity of histidine that was lost with the $His^{7.36(305)}$Phe and $His^{7.36(305)}$ Ala mutations. The $His^{7.36(305)}$Arg mutant had a similar response to GnRH compared with the wildtype receptor (EC_{50} 0.24nM ± 0.04) (Figure 3.1, Table 3.1). This suggests that Arg is able to mimic His and that the protonation of the histidine side chain is required for full receptor activation with response to GnRH. All the mutant receptors showed some varying decreases in coupling to PLC indicating that His is important for signalling efficiency.

The decrease in potency of GnRH at the mutant receptors could be a result of a decrease in the binding affinity of the mutant receptors for GnRH or a decrease in the expression levels of the mutant receptor compared to the wildtype. To distinguish whether decreased IP production resulted from decreased receptor expression, decreased ligand affinity or decreased coupling efficiency, ligand binding assays were used to determine affinity and expression of the mutant receptors (see below) and used to determine relative receptor coupling efficiency (Table 3.1).

3.2. Ligand Binding Assays

To define the molecular basis of the decreases in potency in GnRH and Emax of the different mutants, competition binding assays were performed on transiently transfected COS-1. The well established ^{125}I-[His^5, D-Tyr^6]-GnRH (Flanagan *et al,* 1998) was used as a high affinity tracer in the competition ligand binding assays. Initially whole cell binding assays were performed using live COS-1 cells transiently transfected with the wildtype and the different $His^{7.36(305)}$ mutant

receptors. The cells were incubated in the presence of ^{125}I-[His^5, D-Tyr^6]-GnRH as a tracer and varying concentrations of unlabelled GnRH or [His^5, D-Tyr^6]-GnRH as described in experimental approach.

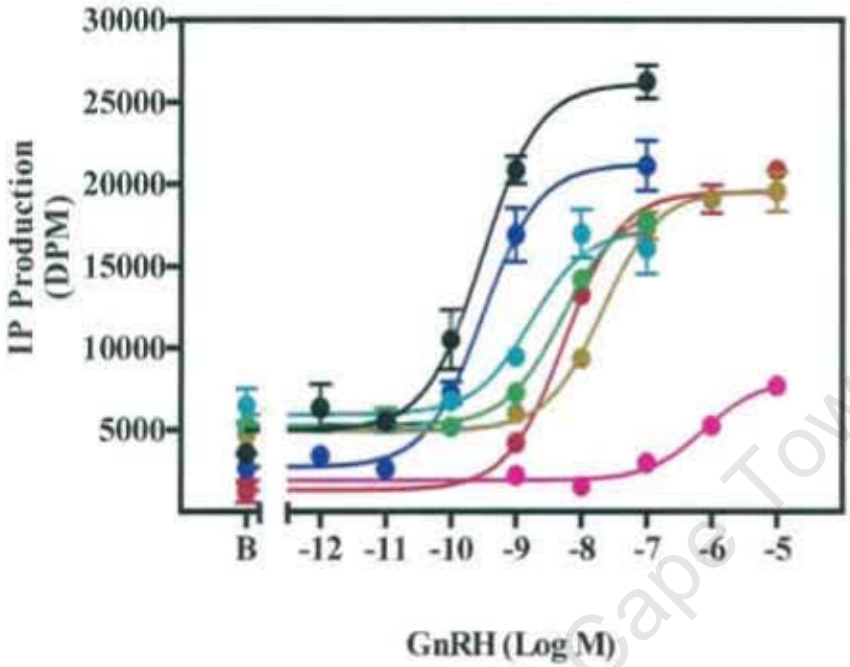


Figure 3.1. GnRH receptors response to GnRH stimulation in IP assays . COS-1 cells expressing mouse GnRH receptor (•), His$^{7.36(305)}$Ala(•), His $^{7.36(305)}$ Phe(•), His$^{7.36(305)}$Asn(•), His$^{7.36(305)}$Gln(•), His$^{7.36(305)}$Arg(•) and His$^{7.36(305)}$Trp(•) were labelled with ^{3}H-myoinositol and stimulated with increasing concentrations of GnRH. Data are representative of at least 4 independent experiments performed in duplicate. Basal level with no added ligand is indicated with B.

Table 3.1. Summary of IP assay results. Results are the mean ± standard error of the mean. pEC_{50} is the negative log of EC_{50}.T-tests were performed on the pEC_{50}Fold changes were calculated as the ratio of EC_{50} values in the mutant and wildtype (wt) receptor. Emax of each mutant receptor calculated as percentage of wildtype. Undefined (U).

Receptor	EC_{50} (nM)	pEC_{50}	Fold Change	Emax (% wt)	Coupling Efficiency (% wt)
Wildtype	0.162 ± 0.04 n = 14	9.96 ± 0.13		100	100
$His^{7.36(305)}Ala$	24.6 ± 3.7 n = 8	7.65 ± 0.069**	152	70	6
$His^{7.36(305)}Phe$	26 ± 3.9 n = 4	7.6 ± 0.067**	161	61.7	8
$His^{7.36(305)}Asn$	1.07 ± 0.36 n = 9	9.19 ± 0.165*	6.6	56.9	20
$His^{7.36(305)}Gln$	0.92 ± 0.194 n = 10	9.21 ± 0.194*	5.7	58.9	22
$His^{7.36(305)}Arg$	0.24 ± 0.04 n = 9	9.41 ± 0.27	1.5	79.3	52
$His^{7.36(305)}Trp$	770 ± 267 n = 8	6.59 ± 0.38	4745	29.4	U

* Significantly different from the wildtype receptor $p < 0.05$

**Significantly different from the wildtype receptor $p < 0.0001$

3.3.1 Whole cell competition binding.

The wildtype receptor exhibited a typical competition binding curve, with a decrease in the amount of tracer bound as the concentration of the agonists was increased. The receptor bound the [His^5, D-Tyr^6]-GnRH analog with a lower IC_{50} value than (IC_{50} 8.18nM ± 4.8) the native GnRH (IC_{50} 27nM ± 4.64) (Figure 3.2

and 3.3, Table 3.2), consistent with the reports that [His^5, D-Tyr^6]-GnRH is a high affinity GnRH analog (Flanagan *et al,* 1998). No apparent binding of the ligands could be seen with the $His^{7.36(305)}$Trp mutant receptor, while the rest of the mutants showed some degree of binding. Although the mutants bound the labelled ligand, there was an indication of ligand depletion (more than 10% of tracer bound) with the wildtype and the Arg mutant (Figure 3.2, Table 3.2). Ligand depletion prevents accurate calculations of affinity. The mutant receptors showed decreases in maximum binding (Btotal in table 3.2) compared to the wildtype receptor (Figure 3.2 and 3.3). The total binding of tracer to the mutants as a percentage of the total counts were, 13.5 % for the wildtype, 3.5 for the Ala, 5 % for the Phe, 6 % for the Asn, 5.5 % for the Gln and 10.8 % for the Arg mutants. These decreases in maximum binding could be a result of a decrease in expression levels of the mutant receptors or a decrease in affinity for the ligand. There were differences in B0 of the mutant receptors relative to the wildtype receptor with both GnRH and [His^5, D-Tyr^6]-GnRH. These differences could be a result of variations in specific activity of the tracer ligand or interassay variations (Figures 3.2 and 3.3).The $His^{7.36(305)}$Phe mutant had significant increase in IC_{50} values for both GnRH (1052nM ± 23.8) and [His^5, D-Tyr^6]-GnRH (145.9nM ± 119) compared to the wildtype (Figure 3.2 and 3.3, Table 3.2). The $His^{7.36(305)}$Ala mutant showed a significant increase in IC_{50} values for [His^5, D-Tyr^6]-GnRH (54.7nM ± 22) and GnRH (119.7nM ± 99.7) compared to wildtype. There was no significant change in IC_{50} values observed in the $His^{7.36(305)}$Gln, $His^{7.36(305)}$Asn and $His^{7.36(305)}$Arg mutant receptors (Figure 3.2 and 3.3) for both ligands relative to the wildtype receptor. The different mutations of $His^{7.36(305)}$ showed that this residue may have a role in receptor function.

Table 3.2. Summary of whole cell competition ligand binding results. The competition binding assays were performed on COS-1 cells expressing the wildtype GnRH receptor or mutant receptors, incubated with ^{125}I-[His^5, D-Tyr6]-GnRH and varying concentrations of unlabelled ligands (See Experimental approach). Data are reported as IC_{50} (nM) and negative log of the IC_{50} (pIC_{50}) ± SEM. Fold change is the ratio of wildtype EC_{50} values and mutant in parentheses. Maximum binding (Btotal) of GnRH calculated as % of total counts in the absence of ligand relative to the initial total amount of ^{125}I-[His^5, D-Tyr6]-GnRH.

Receptors	**GnRH**	**GnRH**	**[His^5,D-Tyr^6]-GnRH**	**[His^5,D-Tyr^6]-GnRH**	**Btotal**
	IC_{50} (nM)	pIC_{50} (Fold Change)	IC_{50} (nM)	pIC_{50} (Fold Change)	(%TC)
Wildtype	27 ± 4.64 n = 8	7. 61 ± 0. 08	8.18 ± 4.8 n = 7	8.94 ± 0.31	13.5 ± 5.5
His$^{7.36(305)}$Ala	119.7±99.7 n = 5	7.3 ± 0.29 (4.425)	54.7 ± 22 n = 3	7.34 ± 0.18* (6.69)	3.5 ± 1.6
His$^{7.36(305)}$Phe	1052±23.8 n = 3	6.14±0.26** (38.94)	145.9 ± 119 n = 3	7.19 ± 0.39* (17.9)	5 ± 2.5
His$^{7.36(305)}$Asn	46.9 ± 11.7 n = 5	7.39± 0.13 (1.73)	5.6 ± 2.64 n = 4	8.56 ± 0.36 (0.102)	6 ± 2.7
His$^{7.36(305)}$Gln	18 ± 5.7 n = 4	7.94 ± 0.31 (0.67)	7.91 ± 3.19 n = 3	8.212 ± 0.25 (0.054)	5.5 ± 2.5
His$^{7.36(305)}$Arg	25.7 ± 5.1 n = 6	7.67 ± 0.14 (0.96)	5.4 ± 4.1 n = 3	8.67 ± 0.47 (0.6)	10.8 ± 4.4
His$^{7.36(305)}$Trp	NMB	NMB	NMB	NMB	NMB

* Significantly different from the wildtype receptor $p < 0.05$

**Significantly different from the wildtype receptor $p < 0.0001$

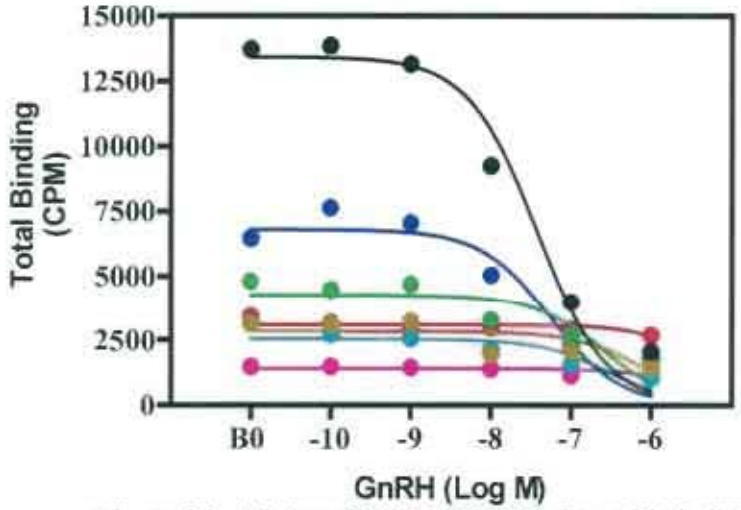


Figure 3.2. Whole cell competition binding with GnRH. Figure represents data fitted on nonlinear curve. COS-1 cells expressing the mouse GnRH receptor •, $His^{7.36(305)}$Ala (•), $His^{7.36(305)}$Asn(•), $His^{7.36(305)}$Gln(•), $His^{7.36(305)}$Phe(•), $His^{7.36(305)}$Arg(•) and $His^{7.36(305)}$Trp(•) incubated with ^{125}I-[His^5, D-Tyr^6]-GnRH in the presence of increasing concentrations of GnRH. Data are representative of at least three experimantes performed in duplicate.

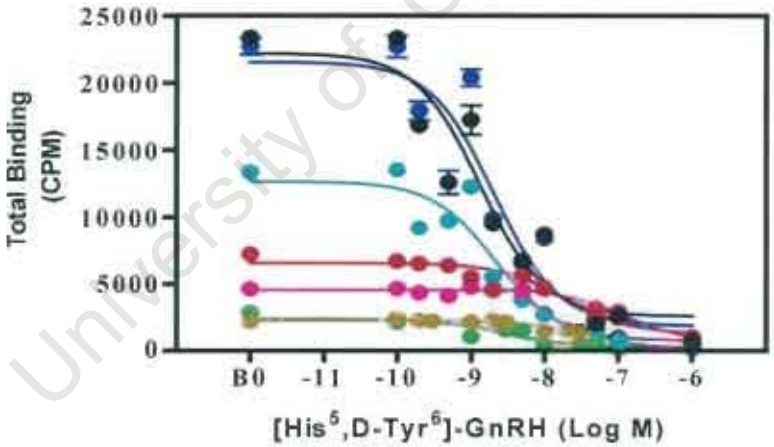


Figure 3.3. Whole cell competition binding with [His^5,D-Tyr^6]-GnRH. Figure represents data fitted on nonlinear curve. COS-1 cells expressing the mouse GnRH receptor •, $His^{7.36(305)}$Ala (•), $His^{7.36(305)}$Asn (•), $His^{7.36(305)}$Gln(•), $His^{7.36(305)}$Phe (•), $His^{7.36(305)}$Arg(•) and $His^{7.36(305)}$Trp(•) incubated with ^{125}I-[His^5, D-Tyr^6]-GnRH in the presence of increasing concentrations of unlabelled [His^5,D-Tyr^6]-GnRH. Data are representative of at least three experimantes performed in duplicate.

3.3.2 Membrane competition binding assays.

Performing membrane binding assays was essential as it is very complex to manipulate the number of cells in whole cell binding assays to achieve the desired conditions for ligand binding assessment. It was important to avoid ligand depletion by decreasing the concentration of receptor membranes that have a very high maximum binding as this will give rise to erroneous estimations of binding affinity and expression levels of the receptor constructs. Adjusting the concentration of the membranes to give measurable binding in the low-binding mutant receptors can be more easily achieved in membrane binding assays compared to the whole cell binding assays.

Different concentrations of crude membrane preparations were used for the different mutant receptors to obtain the optimum binding conditions. $1/10^{th}$ of 10cm dish (~300 000 cells) was used per reaction tube for the $His^{7.36(305)}Ala$, $His^{7.36(305)}Phe$ and $His^{7.36(305)}Trp$, $1/50^{th}$ dish per reaction tube for $His^{7.36(305)}Asn$ and $His^{7.36(305)}Gln$, $1/100^{th}$ dish per tube for the $His^{7.36(305)}Arg$ and the wildtype. These concentrations were determined by testing various crude membrane concentrations to determine one that does not cause ligand depletion, but gives adequate measurable binding (Hulme 1992).

There was a consistent decrease in the maximum ^{125}I-[His^5, D-Tyr^6]-GnRH binding of the mutant receptors compared with wildtype in both whole cell binding and membrane binding assays indicating that the expression was affected by the introduced mutations (Figures 3.2 and 3.3, Table 3.2). This suggests that the lower Emax values of the mutant receptor in IP assays may result from the lower maximum binding (B0) observed in binding assays.

Homologous competition binding assays were used to assess expression of the wildtype and mutant receptors. In spite of the decreased total binding, no statistically significant differences were observed in binding sites per cell (Table 3.3). The wildtype receptor has a higher affinity for the [His^5, D-Tyr^6]-GnRH (IC_{50} 3.15nM ± 0.40) compared to the GnRH (IC_{50} 54.8nM ± 3.7) (Fig 3.3, Table

3.3). IC_{50} values are used to estimate the affinity of the mutant receptors. Sinnce the values of IC50 and Kd are similar, it is acceptable to estimate ligand binding affinity using IC50 values (Hulme 1992).The affinity for GnRH was slightly lower in the membrane binding assay compared to the whole cell binding. The different conditions of the assay systems are most likely to be the reason for this slight variation. There was no significant difference in the affinity of the wildtype receptor for [His^5, D-Tyr^6]-GnRH in whole cell binding and membrane binding assays. The $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe mutant receptors had significant decreases in affinity for GnRH and [His^5, D-Tyr^6]-GnRH. IC_{50} values of 213nM ± 39 for GnRH and 26nM ± 7.5 for [His^5, D-Tyr^6]-GnRH were observed for the $His^{7.36(305)}$Ala mutant receptor (Figure 3.2 and 3.3, Table 3.3). The $His^{7.36(305)}$Phe mutant receptor had an increased IC_{50} values of 541nM ± 111 for GnRH and 19nM ± 9.4 for [His^5, D-Tyr^6]-GnRH (Figure 3.2 and 3.3, Table 3.3). The change in affinity of the $His^{7.36(305)}$Phe and $His^{7.36(305)}$Ala may account for the decrease in potency observed in IP assays for the same mutant receptors. The decrease in potency of the [His^5, D-Tyr^6]-GnRH analog was similar to that observed with GnRH. There was no significant difference in affinity between GnRH and [His^5, D-Tyr^6]-GnRH with the $His^{7.36(305)}$Asn, $His^{7.36(305)}$Gln and $His^{7.36(305)}$Arg mutant receptors (Fig 3.3, Table 3.3). As in the whole cell binding assays, the $His^{7.36(305)}$Trp mutant receptor had no binding to the agonist. Steric hindrance introduced by the large tryptophan side chain could be resulting in improper folding of the receptor hence the lack of binding and stimulation. The decrease in affinity for the $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe mutant receptors could also be an indication of a loss of an interaction between the ligand and the receptor or a loss of an interaction between residues in the receptor that disrupts the interaction of another functional group with the ligand. A series of GnRH analogs with substitutions at different positions were tested to explore the possibility of an important interaction between the $His^{7.36(305)}$ and a side chain functional group of GnRH.

Table 3.3. Summary of membrane competition binding assays The competition binding assays were performed on crude membranes from COS-1 cells expressing the wildtype GnRH receptor and mutant receptors, incubated with ^{125}I-[His^5, D-Tyr^6]-GnRH and varying concentrations of unlabelled ligands (See experimental approach). Data are reported as pIC_{50} ± SEM (number of experiments). The number of replicates is indicated in parentheses. No measurable binding (NMB).

Receptor	**GnRH**	**GnRH**	**[His^5,D-Tyr^6]-GnRH**	**[His^5,D-Tyr^6]-GnRH**	**Binding Sites/cell**
	IC_{50} (nM)(n)	pIC_{50}	IC_{50}(nM) (n)	pIC_{50}	**(x 10^5)**
Wildtype	54.8 ± 3.7(16)	7.28 ± .036	3.15 ± 0.40 (29)	8.6 ± 0.06	46.7 ± 6.7
His$^{7.36(305)}$Ala	213 ± 39(8)	6.73 ± 0.09**	26 ± 7.5 (11)	7.95 ± 0.17**	54 ± 13.8
His$^{7.36(305)}$Phe	541±111(5)	6.3 ± 0.09	19 ± 9.4 (7)	7.88 ± 0.15**	34.2 ± 8.2
His$^{7.36(305)}$Asn	57 ± 12.3(5)	7.29 ± 0.10	3.9 ± 1.09 (15)	8.6 ± 0.14	37.5± 21.6
His$^{7.36(305)}$Gln	41.8 ± 8.7(4)	7.41 ± 0.09	2.7 ± 0.59 (8)	8.6 ± 0.08	27.8 ± 9.7
His$^{7.36(305)}$Arg	86.4 ± 31.7(3)	7.13 ± 0.16	2.2 ± 0.63 (13)	8.8 ± 0.08	34.7 ± 7.6
His$^{7.36(305)}$Trp	NMB	NMB	NMB	NMB	NMB

* Significantly different from the wildtype receptor $p < 0.05$

**Significantly different from the wildtype receptor $p < 0.0001$

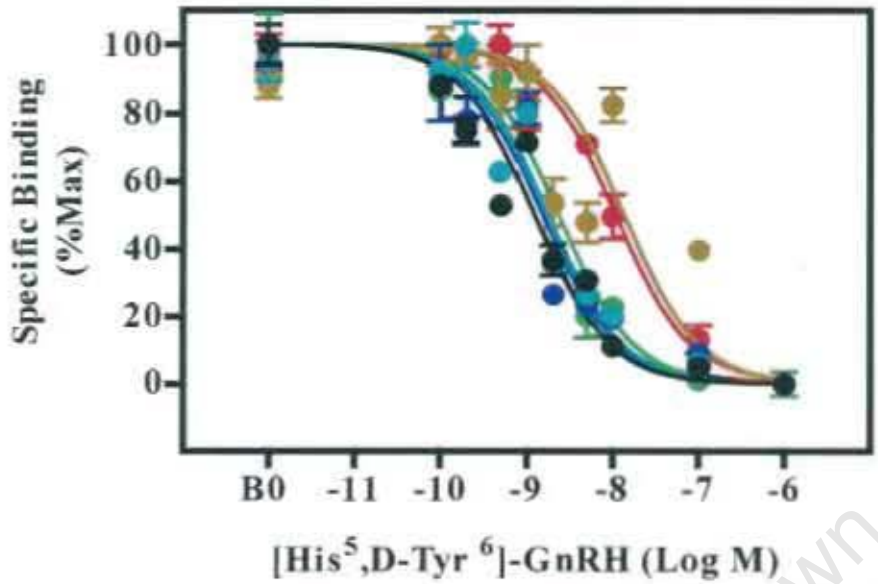


Figure 3.4. Homologous competition membrane binding. COS-1 cells transiently transfected with mouse GnRH receptor, His$^{7.36(305)}$Ala (•), His $^{7.36(305)}$ Phe (•), His$^{7.36(305)}$Asn (•), His$^{7.36(305)}$Gln (•) and His$^{7.36(305)}$Arg (•) were incubated with ^{125}I-[His^5, D-Tyr^6]-GnRH in the presence of varying concentrations of unlabelled [His^5, D-Tyr^6]-GnRH analog. Data representative of at least three independent experiments performed in triplicate. Data is expressed as a percentage of specific binding in the absence of unlabelled ligand. Total binding indicated as B0.

3.3.2.1 Binding of GnRH analogs with substitutions at different positions.

To investigate if the decreases in affinity of the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutants are a result of a loss of interaction between the mutant receptors and the ligands, GnRH analogs were tested in membrane competition binding assays. These GnRH analogs had different substitutions of amino acid residues in positions one to ten. If a receptor mutation decreases binding affinity for the ligand by interrupting an interaction between the receptor and the ligand, then a ligand that lacks the interacting group should have similar affinities for both the wildtype and mutant receptors.

Since the structure of GnRH was determined, many analogs have been synthesized to study the structure-activity relationships of the hormone (Karten and Rivier 1986). In vivo studies investigating different GnRH analogs revealed

that analogs with acylated residues at position one had a little but significant activity. Replacing the cyclic pyro-glutamine of GnRH with an acetylated glycine at position one yielded a ligand that displaced the ^{125}I-[His^5, D-Tyr^6]-GnRH tracer at very high concentrations (Figure 3.5. A), with the wildtype, His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe receptors. A similar poor displacement was observed with GnRH analog where serine is substituted with an alanine residue at position four of the GnRH peptide (Figure 3.5.D) and the naturally occurring analog with hydroxyproline at position nine (Figure 3.5.G). The poor displacement indicates very low affinity of the three GnRH analogs. The low affinity for the wildtype receptor makes it difficult to determine whether the mutant receptors have similar of lower affinity for these analogs. An analog in which histidine at position 2 is substituted with a tryptophan was also tested for affinity with the wildtype, His$^{7.36(305)}$Phe and His$^{7.36(305)}$Ala mutant receptors. The wildtype receptor had a slightly higher affinity for [Trp^2]-GnRH (IC_{50} 18.5nM ± 4.66) compared to GnRH (IC_{50} 54.5nM ± 3.8). [Trp^2]-GnRH displaced ^{125}I-[His^5, D-Tyr^6]-GnRH with an IC_{50} of 136nM ± 39.8 for His$^{7.36(305)}$Ala and 362nM ± 148 for His$^{7.36(305)}$Phe respectively (Figure 3.5.B, Table 3.4). This indicated that there is no direct interaction between the GnRH receptor and the hormone via the two imidazole rings.

GnRH II which has substitutions at positions 5, 7 and 8, displaced the tracer with an IC_{50} value of 116nM ± 10.7 for the wildtype receptor. The wildtype receptor has a decrease in affinity for GnRH II compared to GnRH. When the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutants were incubated in the presence of GnRH II there was a decrease in affinity of the mutant receptors for GnRH II (5.4 and 12.9 fold difference, respectively from wildtype) (Figure 3.5.F, Table 3.4). This decrease in affinity indicated that Tyr^5, Leu^7 and Arg^8 of GnRH does not interact with His$^{7.36(305)}$. [D-Trp^6, Pro^9-NHEt]-GnRH analog with a D-tryptophan at substitutions at position 6 and an ethylamide at position 9 exhibited a decreased affinity with the mutants (IC_{50} 1.01nM ± 0.23 for His$^{7.36(305)}$Ala and 6.8nM ± 0.63 His$^{7.36(305)}$Phe) compared to the wildtype (Figure 3.5.E, Table 3.4). GnRH analogs with D-amino acids at positions 6 have been reported to have a slightly higher

affinity than GnRH. These results from the mutants indicate that there is no direct interaction between Gly^6 and Pro^9 of GnRH and $His^{7.36(305)}$.

Compared to GnRH, an analog with a substitution of 2-naphthylalanine in the place of tryptophan 3 of GnRH had a higher affinity at the wildtype receptor (IC_{50} 1.54 ± 0.32). [2-Nal^3]-GnRH displaced the tracer with an of IC_{50} value 2.97nM ± 1.62 at the $His^{7.36(305)}$Ala mutant. There was no significant difference in affinity of $His^{7.36(305)}$Ala and wildtype receptor. The $His^{7.36(305)}$Phe mutant (IC_{50} 2.99 ± 0.43)also had no change in affinity for [2-Nal^3]-GnRH compared to the wildtype receptor (Figure 3.5. C). These results indicate the possibility of an interaction between $His^{7.36(305)}$ and Trp^3 of GnRH. This GnRH analog was tested in IP assays for its activity with the $His^{7.36(305)}$Ala, $His^{7.36(305)}$Phe and the $His^{7.36(305)}$Asn which had no decrease in affinity for GnRH, and compared with the wildtype to confirm these similar affinities. [2-Nal^3]-GnRH activated the wildtype receptor with a low potency (EC_{50} 0.49nM ± 0.07) similar to GnRH and [His^5, D-Tyr^6]-GnRH. The position 3-substituted analog had moderately high potency (EC_{50} 9.73nM ± 3) at the $His^{7.36(305)}$Ala mutant compared with GnRH (EC_{50} 0.152nM ± 0.032). There was a 20-fold decrease in potency at the Ala mutant, 162 fold for GnRH and 97 fold for [His^5, D-Tyr^6]-GnRH. This suggests that the component of the decreased potency of GnRH is loss of an interaction with Trp^3 of GnRH. [2-Nal^3]-GnRH showed a similar higher potency at the $His^{7.36(305)}$Phe (EC_{50} 2.98nM ± 0.4) mutant compared to GnRH (Figure 3.6). The [2-Nal^3]-GnRH analog had 6 fold change in potency at the Phe mutant, 60 fold for [His^5, D-Tyr^6]-GnRH and 169 fold for GnRH. The negative control, $His^{7.36(305)}$Asn which showed no change in affinity for GnRH but decreased potency was also included in the study . This mutant did not show any significant change in potency from the wildtype with [2-Nal^3]-GnRH (Figure 3.5).

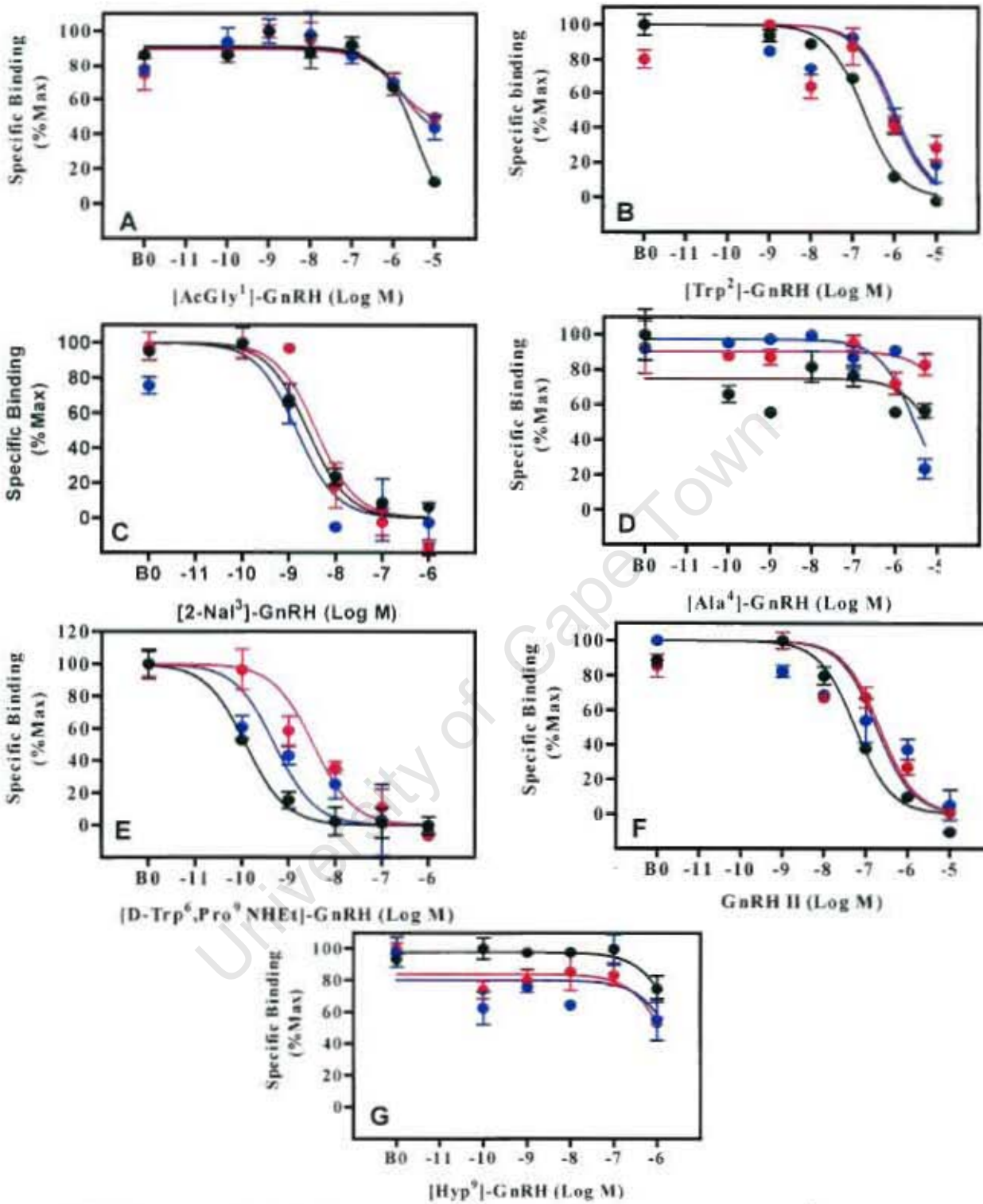


Figure 3.5. Competition Membrane binding with [AcGly^1]-GnRH (Panel A), [Trp^2]-GnRH (Panel B), [2-Nal^3]-GnRH (Panel C), [Ala^4]-GnRH (Panel D), [D-Trp^6,Pro^{9}NHet]-GnRH (Panel E), GnRH II (Panel F) and [Hyp^9]-GnRH (Panel G) on the wildtype receptor (black), His$^{7.36(305)}$Ala (blue)and His$^{7.36(305)}$Phe(red). Data are representative of 4 independent experiments performed in triplicate.

Table 3.4. Summary of competition binding of His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe with different GnRH analogs. The binding affinity of the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutants was tested using different GnRH analogs with different substitutions on the peptide. Data expressed as negative log of IC_{50} ± SEM. No of replicates as indicated.

GnRH Analog	Wildtype	His$^{7.36(305)}$Ala	His$^{7.36(305)}$Phe
	pIC_{50}	pIC_{50}	pIC_{50}
GnRH	7.29 ± 0.036 n = 16	6.73 ± 0.09** n = 8	6.31 ± 0.09** n <6
[AcGly^1]-GnRH	4.79 ± 0.32 n <6	6.04 ± 0.23* n <6	5.75 ± 0.09* n <6
[Trp^2]-GnRH	7.88 ± 0.16 n = 8	7.017 ± 0.15 * n = 8	6.57 ± 0.194* n <6
[2-Nal^3]-GnRH	8.84 ± 0.10 n <6	8.69 ± 0.27 n <6	8.5 ± 0.08 n <6
[Ala^4]-GnRH	5.066 ± 0.16 n <6	4.69 ± 0.159 n = 6	4.72 ± 0.65 n <6
[His^5,D-Tyr^6]-GnRH	8.601 ± 0.058 n = 29	7.95 ± 0.18** n = 11	7.74± 0.16** n = 7
GnRH II	6.96 ± 0.043 n = 10	6.33 ± 0.109** n = 7	6.03 ± 0.22** n <6
[D-Trp^6,Pro^{9}NHEt]-GnRH	9.77 ± 0.14 n = 7	9.07 ± 0.13* n = 7	8.17 ± 0.04* n <6
[Hyp^9]-GnRH	5.76 ± 0.075 n = 7	6.18 ± 0.23 n <6	5.76 ± 0.18 n <6

* Significantly different from the wildtype receptor $p < 0.05$

**Significantly different from the wildtype receptor $p < 0.0001$

Table 3.5. IP assay results using GnRH analogs, [His^5, D-Tyr^6]-GnRH and [2-Nal^3]-GnRH. Data are presented as EC_{50} and ± SEM. Assays repeated four times in duplicate. Fold change as ratio of wildtype EC_{50} and mutant in parentheses.

Receptors	GnRH	[His^5,D-Tyr^6]-GnRH	[2-Nal^3]-GnRH
	EC_{50} (nM	EC_{50} (nM)	EC_{50} (nM)
Wildtype	0.162 ± 0.04	0.24 ± 0.14	0.49 ± 0.07
His$^{7.36(305)}$Ala	24.6 ± 3.7 (152)	35 ± 17.6 (145)	9.73 ± 3 (20)
His$^{7.36(305)}$Phe	26 ± 3.9 (160)	21.7 ±6.2 (90)	2.98 ± 0.4 (6)
His$^{7.36(305)}$Asn	1.07 ± 0.36 (6.6)	1.5 ± 0.27 (6.25)	0.81 ± 0.14 (1.7)

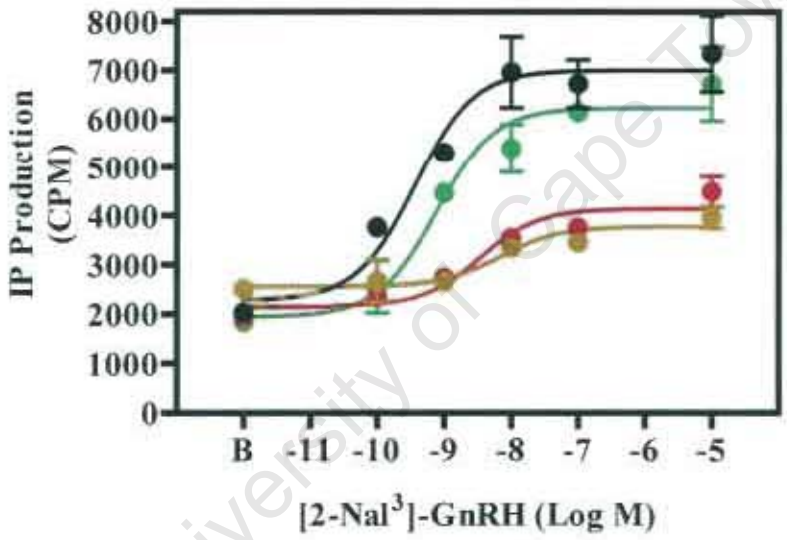


Figure 3.6. IP assay with [2-Nal^3]-GnRH on wildtype, His$^{7.36(305)}$Ala (•), His$^{7.36(305)}$Phe (•) and His$^{7.36(305)}$Asn (•) mutant receptors. Data represents 4 independent experiment performed in duplicate.

These results suggest the possibility of an interaction between His$^{7.36(305)}$ and tryptophan at position three of GnRH possibly through a hydrogen bond.

CHAPTER FOUR: DISCUSSION AND CONCLUSION

Studies on the ligand binding pocket of the GnRH receptors have revealed several amino acid residues that are important for maintaining and stabilising the binding pocket. Residues in EC 3 and TM 7 have also been shown to be important for ligand binding, ligand selectivity among receptor subtypes and receptor signalling in several GPCRs (Hjorth *et al,* 1994; Perlman *et al,* 1995; Fernandez and Puett 1996). A highly conserved residue among the GnRH receptors, $His^{7.36}$ is located at the extracellular end of TM 7 close to residues that have been shown to be important in ligand selectivity in the GnRH receptors ($Glu/Asp^{7.32}$ and $Pro^{7.33}$) (Flanagan *et al,* 1994; Fromme *et al,* 2001; Fromme *et al,* 2004). The high conservation of $His^{7.36}$ among GnRH receptors (Figure 1.8), its locus on the receptor and the complex structure of the histidine side chain suggested that it may have an important role in GnRH binding, receptor activation or in the three dimensional structure of the receptor. The histidine side chain can potentially form multiple interactions, including hydrogen bonds, ionic interactions, or π-π interactions with GnRH or with other residues within the GnRH receptor.

The current study explored the contribution of the $His^{7.36(305)}$ residue of the mouse GnRH receptor in distinct functions of the GnRH receptor. The residue was mutated to different amino acids that mimic the distinct functional groups of the histidine side chain. The expression of the mutant receptors, their ligand affinity and their response to GnRH and its analogs in signalling assays were tested. The experiments revealed that the $His^{7.36(305)}$ may be involved both in GnRH binding and efficient signal transduction.

The role of $His^{7.36(305)}$ in receptor expression is uncertain.

Homologous competition binding assays were used to determine the role of $His^{7.36(305)}$ side chain in receptor expression. The consistent decreases of the maximum binding observed in both whole cell and membrane binding assays at the Asn and Gln mutants, which showed no decrease in affinity, suggested that

the $His^{7.36(305)}$ may have a role in receptor expression, whereas the low total binding seen with the Ala and Phe mutants may be a result of the decrease in affinity of these mutants. This suggestion was not supported by the formal determination of receptor number using homologous competition binding assays. This may be due to the wide confidence intervals that were inherent in the determination of receptor number using competitions binding assays. Another method of estimating expression (e.g. quantitative immunoblotting or ELISA) may yield a more direct measure of expression of the mutant receptors. There was no measurable binding or IP production with the Trp mutant. Steric hindrance caused by the introduction of a Trp residue has been reported (Hannemann *et al,* 2002). The large side chain of Trp may be interfering with the maintenance of the structure of the receptor. This could lead to the poor delivery of the receptor to the membrane or a disruption of the proper folding of the receptor. These would ultimately have an indirect effect on ligand binding affinity or decrease receptor expression. Unfortunately, because of the lack of binding of this receptor, the expression levels of this mutant could not be estimated from the calculations used. Alternative methods that do not rely on ligand binding would be necessary to validate whether this mutant is poorly expressed or has low binding affinity or both.

Ligand affinity requires the presence of the hydrogen bonding groups of $His^{7.36(305)}$.

Ligand binding assays with the different mutant receptors using both GnRH and [His^5, D-Tyr^6]-GnRH revealed that there was a decrease in affinity of the $His^{7.36(305)}$Ala in both whole cell and membrane binding assays. The decrease in affinity of the mutant receptor suggested that the His side chain may be involved in ligand binding. The decrease in affinity of the Phe mutant showed that the hydrophilic groups of the His side chain may be important in ligand binding affinity. There was no significant difference in affinity for the $His^{7.36(305)}$Asn, $His^{7.36(305)}$Gln and the $His^{7.36(305)}$Arg mutant receptors in ligand binding assays. Replacing the His residue with Asn and Gln mimics the Nδ1 and Nε2 hydrogen bonding groups of His. The retention of wildtype receptor binding affinity by

these mutants showed that one of these H-bonding groups of His is important for ligand binding. The presence of either of the two hydrogen binding groups was sufficient to mimic the wildtype binding. At high pH, histidine forms two different tautomers upon proton dissociation, Nδ1 and Nε2 (Perez-Canadilllas *et al,* 2003). The pH and the environment surrounding the histidine residue as they have different pKs at different conditions determine the tautomeric states. Previous studies on the tautomeric states of histidine showed that the proton is preferred on the Nε2 (Perez-Canadilllas *et al,* 2003). The Arg mutant, showed that the protonation of $His^{7.36(305)}$ is not essential for ligand binding affinity. The decrease in affinity of the $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe suggest the loss of a potential ligand binding contact site between the receptor and the ligand or a loss of an interaction that affects ligand binding indirectly.

$His^{7.36(305)}$ may form an interaction with Trp^3 of GnRH.

The $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe mutant receptors with the lower binding affinity for GnRH, were further tested for ligand affinity to determine whether the $His^{7.36(305)}$ residue is an interaction site for GnRH using a series of GnRH analogs with substitutions at different positions of GnRH. Because many of the analogs used in this study have only been tested in biological assays, and no affinities have been reported, the effect of substitutions on affinity for the wildtype receptor is discussed before the potential interactions with $His^{7.36(305)}$ are considered. An analog with an acetylated glycine at position one instead of the N-terminal pGlu ring could only displace the tracer ligand from the wildtype receptor at very high concentrations indicating a low affinity for the [acyl-Gly^1]-GnRH ligand compared to GnRH. Like the [acyl-Gly^1]-GnRH membrane binding assays with [Ala^4]-GnRH displaced the labelled ligand very poorly in membrane binding assays at the wildtype receptor. A serine at position four is reported to be important in biological activity and all modification where the serine is substituted result in a severe loss of potency. The naturally occurring analog [Hyp^9]-GnRH (hydroxyproline) also showed very poor displacement of the labelled tracer at the wildtype receptor. [Hyp^9]-GnRH and [acyl-Gly^1]-GnRH have very low activity *in vitro* compared to GnRH and this could explain the poor

displacement in the membrane binding assays (Okada *et al,* 1973; Gautron *et al,* 1992). Membrane binding assays using [Trp^2]-GnRH with the wildtype receptor showed a slightly higher affinity compared to GnRH consistent with previous observations (Flanagan *et al,* 2000). A substitution of Trp^3 with 2-naphthyl alanine led to a higher affinity at the wildtype receptor. [2-Nal^3]-GnRH was previously shown to have 50% activity of GnRH *in vivo* (Sandow 1978; Sealfon *et al,* 1997). This could explain the higher binding affinity of [2-Nal^3]-GnRH compared to wildtype. The GnRH II peptide [His^5, Trp^7, Tyr^8]-GnRH, was used to assess the possible interaction between residues at position 5, 7 and 8 of GnRH with $His^{7.36(305)}$ of the receptor. The wildtype receptor had a decrease in affinity for GnRH II. [His^5, D-Tyr^6]-GnRH, which also has a substitution at position 5 had a higher affinity at the wildtype receptor. This analog has been reported as a high affinity ligand (Flanagan *et al,* 1998). The wildtype receptor had the highest affinity for the [D-Trp^6, Pro^9NHEt]-GnRH analog. Introducing a D-amino acid at position 6 increases the potency of the analog (350-400%) (Monahan *et al,* 1973; Hirotsu *et al,* 1974). [D-Trp^6, Pro^9NHEt]-GnRH and [His^5, D-Tyr^6]-GnRH, which both had a D-amino acid at position 6, had higher affinity for the wildtype GnRH receptor relative to GnRH. This increased affinity was attributed to the conformational stabilising effect of the D-amino acids.

Together with the wildtype receptor, $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe mutant receptors were tested for their affinity for analogs with substitutions at different positions. The mutants showed very poor displacement of the tracer with both [acyl-Gly^1]-GnRH, [Ala^4]-GnRH and [Hyp^9]-GnRH analogs. This shows that the mutant receptors had very low affinity for these analogs. The low affinity of both wildtype and mutant receptors for these ligands precludes any firm conclusion about a direct interaction between pGlu at position one, Ser at position four or Pro at position nine with the $His^{7.36(305)}$. Other ligands with higher affinity for the wildtype receptor could be used to clearly define the possibility of an interaction between the receptor and the hormone through amino acids residues at these positions. [Thr^4]-GnRH could have been a better analog to test for an interaction at position four as it had 19% potency *in vivo* (Coy *et al,* 1973). Finding suitable

analog for position one would be complex as the only reported analogs for position one have very little activity *in vivo* and have not been assayed *in vitro* (Sandow 1978).

The mutant receptors showed the same decrease in affinity for [Trp^2]-GnRH compared as for GnRH. This shows that there was no direct interaction between $His^{7.36(305)}$ and the His^2 of GnRH. The His^2 side chain has been previously reported to form a hydrogen bond with $Asp^{2.61(98)}$ of the GnRH receptor (Flanagan *et al,* 2000). Although, His can form multiple interactions with its heteroatoms, the results do not support simultaneous interaction of His^2 with $Asp^{2.61(98)}$ and $His^{7.36(305)}$. The wildtype receptor showed a decrease in affinity for GnRH II with substitutions for residues 5, 7 and 8 of GnRH. The native tyrosine at position 5 has been an important site for mono-iodination. This residue can be iodinated and still retain most of the GnRH activity (Sandow 1978). There was a similar decrease in affinity for [His^5, D-Tyr^6]-GnRH, which also has a substitution at position 5 at the mutant receptors. These results indicate that there is no interaction of $His^{7.36(305)}$ and GnRH via residues at these positions. The mutant receptors also showed a decrease in affinity for [D-Trp^6, Pro^9NHEt]-GnRH compared with wildtype receptor. The affinity for this analog was higher than that of GnRH, consistent with the reports that conformationally constraint analogs have higher affinity compared to wildtype GnRH. These results did not suggest the possibility of an interaction between $His^{7.36(305)}$ and either Gly^6 or Pro^9 of GnRH.

The only GnRH analog that gave any significant information on the ligand binding properties of $His^{7.36(305)}$ was the 2-naphthyl alanine 3 substituted analog. The $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe receptors showed no significant difference from wildtype in affinity for the [2-Nal^3]-GnRH analog. The lack of significant difference in affinity of the mutant receptors compared to the wildtype suggests that there might be an interaction between $His^{7.36(305)}$ and Trp^3 of GnRH. There are previous suggestions that Trp^3 of GnRH interacts with $Trp^{6.47(279)}$ on TM six of the GnRH receptor (Chauvin *et al,* 2001). The model suggested that Trp^3 is in contact

with a cluster of aromatic residues on TM 6 ($Trp^{6.47(279)}$ and $Tyr^{6.50(282)}$) and TM 7 ($Phe^{7.37(307)}$ and $Phe^{7.40(310)}$). The complex structures of the His and Trp side chains can allow multiple interactions. The histidine side chain can form salt bridges, π-π and CH/ π interactions with phenylalanine rings, π/OH interactions with tyrosine, π/NH interactions with tryptophan and histidine and also form a bond between the N of histidine and the NH of tryptophan (Bromme *et al,* 1996; Scheiner *et al,* 2002). There are several possible interactions between the histidine and tryptophan side chains that have been reported and these can vary depending on the conditions of the surroundings where these side chains are found. A cation-π interaction between the indole ring of Trp and the imidazole ring of His was reported in the M2 proton channel of influenza A and microbial ribonuclease, barnase (Loewenthal *et al,* 1992; Takeuchi *et al,* 2003). The stabilisation of a cation in the imidazole ring of His by the negative charge of π-electron of the Trp indole ring were accredited to the formation of these cation-π interactions. The five-membered ring of Trp interacts with the atoms of His favouring an Nδ1 - Nε1 interaction (Loewenthal *et al,* 1992; Bromme *et al,* 1996).

The extent of the decrease in affinity of the mutant receptors for the different GnRH analogs does not suggest the loss of an ionic interaction, but of a hydrogen bond. Firstly, Arg^{8} is the only positively charged functional group of GnRH under physiological pH and this residue has been shown to be essential for high-affinity binding of mammalian GnRH receptors (Flanagan *et al,* 1994; Fromme *et al,* 2001). Secondly, His is a positively charged amino acid and would need a negatively charged residue on GnRH for an interaction. Therefore these exclude the possibility of an ionic interaction through $His^{7.36(305)}$. The similar affinities of the wildtype and the mutant receptors with [2-Nal^{3}]-GnRH could be due to the differences between the structures of tryptophan and 2-naphthlyalanine (See appendix A.4). 2-naphthlyalanine lacks the hydrogen bonding group that is seen on Trp. The high affinity for the wildtype receptor of [2-Nal^{3}]-GnRH in the absence of the hydrogen bonding group indicates that there is an additional interaction occurring that enhances the affinity even in the absence of the

hydrogen bond. This could explain the lack of change in affinity of this ligand for the wildtype. The lack of change in affinity of the mutant receptors for this ligand which lacks a δNH group suggests that His in the wildtype receptor interacts with Trp^3 of GnRH. Mutants that are not unable to interact with the NH of Trp^3 could distinguish [2-Nal^3]-GnRH from GnRH.

$His^{7.36(305)}$ plays a role in GnRH receptor activation.

Inositol phosphate production assays were performed as an indicator of how well the wildtype and $His^{7.36(305)}$ mutant receptors are activated by GnRH. The Arg mutant was the only mutant receptor that did not show any significant difference from the wildtype receptor in IP assays indicating that the positive charge that is mimicked by Arg may be important for receptor activation. All of the $His^{7.36(305)}$ mutant receptors exhibited an agonist-stimulated increase in inositol phosphate production, except for the tryptophan mutant. The $His^{7.36(305)}$Trp mutant receptor showed no response when stimulated with GnRH. This lack of activation was postulated to be the result of the steric hindrance introduced by the large side chain of tryptophan at this position. All the mutant receptors showed decreased coupling to the effector PLC, indicating the effects of the mutation on activation. These results of the coupling efficiency should be interpreted cautiously because of the high interassay variation observed in receptor expression calculations. The $His^{7.36(305)}$Ala and the $His^{7.36(305)}$Phe mutant receptors showed the highest decrease in potency for both GnRH and the [His^5,D-Tyr^6]-GnRH analog. The Ala mutant shows that the imidazole side chain of $His^{7.36(305)}$ has an important role in the signalling of the GnRH receptor. The Phe mutant shows that the hydrophilic groups of $His^{7.36(305)}$ are required for ligand binding. The decreased ligand binding affinity of these mutants may partially account for the decreased GnRH potency, and low activity of the Phe mutant, which has an aromatic ring substituted for His suggests that the aromatic ring of His is not important. The $His^{7.36(305)}$Asn and $His^{7.36(305)}$Gln mutants showed lesser decrease in GnRH potency compared to that of the $His^{7.36(305)}$Phe and $His^{7.36(305)}$Ala mutants. This suggests that the ε-nitrogen of glutamine and the δ-nitrogen of asparagine were able to recover the loss of potency observed with the alanine and phenylalanine mutants. These mutant

receptors had no change in affinity for GnRH, whilst showing a decrease in Emax. This suggests that both the NH ε2 and NH δ1 of histidine contribute to the full activation of the receptor. The $His^{7.36(305)}$Arg mutant receptor showed no change in GnRH potency compared to the wildtype receptor indicating that the positive charge of the $His^{7.36(305)}$side chain contributes to the activation of the receptor. Different transfection efficiencies could also be responsible for the difference in the baseline IP production. The differences in both basal and Emax values were consistent in multiple independent assays. The cell densities were not re-calculated when seeding into 12-well plates. The number of all the mutant receptors was assumed to be 250 000/well in the 12-well plates.

Conclusion

$His^{7.36(305)}$ of the mouse GnRH receptor was studied to determine whether it contributes to the receptor structure and function. Mutations of the residue revealed that $His^{7.36(305)}$ plays an important role in both ligand binding and receptor activation. Estimations of receptor expression were inconclusive about the role of $His^{7.36(305)}$ in receptor expression. However, ligand binding assays showed decreases in maximum binding of the tracer ligand by the Asn and Gln mutants indicating that $His^{7.36(305)}$ may have a role in receptor expression.

The different mutagenesis studies revealed that the hydrogen bonding groups of the $His^{7.36(305)}$ residue are important for both ligand binding and receptor activation. Both hydrogen bonding groups of histidine are important for receptor activation, but only one is required for ligand binding. Both the Asn and Gln mutants had a decrease in potency for GnRH, but the decrease was less than that seen with the Ala and Phe mutants. These results showed that the hydrogen bonding groups of $His^{7.36(305)}$ mimicked by Asn and Gln contribute to the full activation of the GnRH receptor. In ligand binding assays, the Asn and Gln mutants had no change in affinity for GnRH indicating that the hydrogen bonding groups of His are sufficient for ligand binding affinity. The presence of either two Nδ1 or Nε2 hydrogen bonding groups of $His^{7.36(305)}$ are sufficient for GnRH binding affinity. The positive charge of the $His^{7.36(305)}$ residue mimicked by the

Arg mutant is essential for full GnRH receptor activation and expression but not required for ligand binding affinity.

Further ligand binding studies with the Ala and Phe mutants using GnRH analogs with substitutions at different positions were performed. These analogs were used to investigate the possibility of an interaction between GnRH and $His^{7.36(305)}$. Of the eight analogs tested, only [2-Nal^3]-GnRH showed no change in affinity between the wildtype and the two mutants, $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe. The results showed that the $His^{7.36(305)}$ may form a hydrogen bond with the NH of Trp^3 in GnRH. This work shows that $His^{7.36(305)}$ of the contributes to multiple GnRH receptor functions.

References

Abdulaev N. G., Ngo T., Chen R., Lu Z. and Ridge K. D. (2000). "Functionally Discrete Mimics of Light-activated Rhodopsin Identified through Expression of Soluble Cytoplasmic Domains." J Biol Chem **275**: 39354-39363.

Adelman J. P., Mason A. J., Hayflick J. S. and Seeburg P. H. (1986). "Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat." Proc Natl Acad Sci U S A **83**: 179-83.

Alewijnse A. E., Timmerman H., Jacobs E. H., Smit M. J., Roovers E., Cotecchia S. and Leurs R. (2000). "The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H(2) receptor." Mol Pharmacol **57**: 890-8.

Altenbach C., Klein-Seetharaman J., Hwa J., Khorana H. G. and Hubbell W. L. (1999). "Structural features and light-dependent changes in the sequence 59-75 connecting helices I and II in rhodopsin: a site-directed spin-labeling study." Biochemistry **38**: 7945-9.

Altenbach C., Yang K., Farrens D. L., Farahbakhsh Z. T., Khorana H. G. and Hubbell W. L. (1996). "Structural features and light-dependent changes in the cytoplasmic interhelical E-F loop region of rhodopsin: a site-directed spin- labeling study." Biochemistry **35**: 12470-8.

Archer E., Maigret B., Escrieut C., Pradayrol L. and Fourmy D. (2003). "Rhodopsin crystal: new template yielding realistic models of G-protein-coupled receptors?" Trends Pharmacol Sci **24**: 36-40.

Arnis S., Fahmy K., Hofmann K. P. and Sakmar T. P. (1994). "A conserved carboxylic acid group mediates light-dependent proton uptake and signaling by rhodopsin." J Biol Chem **269**: 23879-81.

Arora K. K., Sakai A. and Catt K. J. (1995). "Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor." J Biol Chem **270**: 22820-6.

Arora K. K., Cheng Z. and Catt K. J. (1996). "Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor." Mol Endocrinol **10**: 979-86.

Baldwin J. M. (1993). "The probable arrangement of the helices in G protein-coupled receptors." Embo J **12**: 1693-703.

Baldwin J. M. (1994). "Structure and function of receptors coupled to G proteins." Curr Opin Cell Biol **6**: 180-90.

Baldwin J. M., Schertler G. F. and Unger V. M. (1997). "An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors." J Mol Biol **272**: 144-64.

Ballesteros J., Kitanovic S., Guarnieri F., Davies P., Fromme B. J., Konvicka K., Chi L., Millar R. P., Davidson J. S., Weinstein H. and Sealfon S. C. (1998). "Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor." J Biol Chem **273**: 10445-53.

Ballesteros J. A. and Weinstein H. (1995). "Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors." Methods in Neurosciences, Receptor Molecular Biology **25**: 366-428.

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

Barak L. S., Menard L., Ferguson S. S., Colapietro A. M. and Caron M. G. (1995). "The conserved seven-transmembrane sequence NP(X)2,3Y of the G-protein-coupled receptor superfamily regulates multiple properties of the beta 2-adrenergic receptor." Biochemistry **34**: 15407-14.

Bennett T. A., Maestas D. C. and Prossnitz E. R. (2000). "Arrestin binding to the G protein-coupled N-formyl peptide receptor is regulated by the conserved "DRY" sequence." J Biol Chem **275**: 24590-4.

Bhattacharyya R., Saha R. P., Samanta U. and Chakrabarti P. (2003). "Geometry of interaction of the histidine ring with other planar and basic residues." J Proteome Res **2**: 255-63.

Black J. W. and Shankley N. P. (1995). "Drug receptors. Inverse agonists exposed." Nature **374**: 214-5.

Bockaert J. and Pin J. P. (1999). "Molecular tinkering of G protein-coupled receptors: an evolutionary success." Embo J **18**: 1723-9.

Bohm S. K., Khitin L. M., Smeekens S. P., Grady E. F., Payan D. G. and Bunnett N. W. (1997). "Identification of potential tyrosine-containing endocytic motifs in the carboxyl-tail and seventh transmembrane domain of the neurokinin 1 receptor." J Biol Chem **272**: 2363-72.

Bromme D., Bonneau P. R., Purisima E., Lachance P., Hajnik S., Thomas D. Y. and Storer A. C. (1996). "Contribution to activity of histidine-aromatic, amide-aromatic, and aromatic-aromatic interactions in the extended catalytic site of cysteine proteinases." Biochemistry **35**: 3970-9.

Chauvin S., Hibert M., Berault A. and Counis R. (2001). "Critical implication of transmembrane Phe310, possibly in conjunction with Trp279, in the rat gonadotropin-releasing hormone receptor activation." Biochem Pharmacol **62**: 329-34.

Chauvin S., Berault A., Lerrant Y., Hibert M. and Counis R. (2000). "Functional importance of transmembrane helix 6 Trp(279) and exoloop 3 Val(299) of rat gonadotropin-releasing hormone receptor." Mol Pharmacol **57**: 625-33.

Cho W., Taylor L. P., Mansour A. and Akil H. (1995). "Hydrophobic residues of the D2 dopamine receptor are important for binding and signal transduction." J Neurochem **65**: 2105-15.

Christopoulos A. and Kenakin T. (2002). "G protein-coupled receptor allosterism and complexing." Pharmacol Rev **54**: 323-74.

Coy D. H., Coy E. J. and Schally A. V. (1973). "Effect of simple amino acid replacements on the biological activity of luteinizing hormone-releasing hormone." J Med Chem **16**: 1140-3.

Coy D. H., Vilchez-Martinez J. A., Coy E. J., Nishi N., Arimura A. and Schally A. V. (1975). "Polyfluoroalkylamine derivatives of luteinizing hormone-releasing hormone." Biochemistry **14**: 1848-51.

Coy D. H., Coy E. J., Hirotsu Y., Vilchez-Martinez J. A., Schally A. V., van Nispen J. W. and Tesser G. I. (1974). "Investigation of the role of tryptophan in the luteinizing hormone- releasing hormone." Biochemistry **13**: 3550-3.

Davidson J. S., McArdle C. A., Davies P., Elario R., Flanagan C. A. and Millar R. P. (1996a). "Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide." J Biol Chem **271**: 15510-4.

Davidson J. S., Assefa D., Pawson A., Davies P., Hapgood J., Becker I., Flanagan C., Roeske R. and Millar R. (1997). "Irreversible activation of the gonadotropin-releasing hormone receptor by photoaffinity cross-linking: localization of attachment site to Cys residue in N-terminal segment." Biochemistry **36**: 12881-9.

De Lean A., Stadel J. M. and Lefkowitz R. J. (1980). "A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor." J Biol Chem **255**: 7108-17.

Dixon R. A., Sigal I. S., Candelore M. R., Register R. B., Scattergood W., Rands E. and Strader C. D. (1987). "Structural features required for ligand binding to the beta-adrenergic receptor." Embo J **6**: 3269-75.

Donnelly D., Johnson M. S., Blundell T. L. and Saunders J. (1989). "An analysis of the periodicity of conserved residues in sequence alignments of G-protein coupled receptors. Implications for the three- dimensional structure." FEBS Lett **251**: 109-16.

Erb L., Garrad R., Wang Y., Quinn T., Turner J. T. and Weisman G. A. (1995). "Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity." J Biol Chem **270**: 4185-8.

Faurholm B. J., Millar R. P. and Katz A. A. (2001). "The genes coding for the type II gonadotropin-releasing hormone receptor and the ribonucleoprotein RBM8 in humans overlap in two genomic loci." Genomics **76**: 15-18.

Feng W. and Song Z. H. (2003). "Effects of D3.49A, R3.50A, and A6.34E mutations on ligand binding and activation of the cannabinoid-2 (CB2) receptor." Biochem Pharmacol **65**: 1077-85.

Fernandez L. M. and Puett D. (1996). "Lys583 in the third extracellular loop of the lutropin/choriogonadotropin receptor is critical for signaling." J Biol Chem **271**: 925-30.

Fernandez-Recio J., Vazquez A., Civera C., Sevilla P. and Sancho J. (1997). "The tryptophan/histidine interaction in alpha-helices." J Mol Biol **267**: 184-97.

Filipek S., Stenkamp R. E., Teller D. C. and Palczewski K. (2003). "G protein-coupled receptor rhodopsin: a prospectus." Annu Rev Physiol **65**: 851-79.

Flanagan C. A., Fromme B. J., Davidson J. S. and Millar R. P. (1998). "A high affinity gonadotropin-releasing hormone (GnRH) tracer, radioiodinated at position 6, facilitates analysis of mutant GnRH receptors." Endocrinology **139**: 4115-9.

Flanagan C. A., Becker I. I., Davidson J. S., Wakefield I. K., Zhou W., Sealfon S. C. and Millar R. P. (1994). "Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone." J Biol Chem **269**: 22636-41.

Flanagan C. A., Rodic V., Konvicka K., Yuen T., Chi L., Rivier J. E., Millar R. P., Weinstein H. and Sealfon S. C. (2000). "Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction." Biochemistry **39**: 8133-41.

Flanagan C. A., Zhou W., Chi L., Yuen T., Rodic V., Robertson D., Johnson M., Holland P., Millar R. P., Weinstein H., Mitchell R. and Sealfon S. C. (1999). "The functional microdomain in transmembrane helices 2 and 7 regulates expression, activation, and coupling pathways of the gonadotropin-releasing hormone receptor." J Biol Chem **274**: 28880-6.

Fong T. M., Yu H., Huang R. R. and Strader C. D. (1992a). "The extracellular domain of the neurokinin-1 receptor is required for high-affinity binding of peptides." Biochemistry **31**: 11806-11.

Fraser C. M., Wang C. D., Robinson D. A., Gocayne J. D. and Venter J. C. (1989). "Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function." Mol Pharmacol **36**: 840-7.

Fredriksson R., Lagerstrom M. C., Lundin L. G. and Schioth H. B. (2003). "The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints." Mol Pharmacol **63**: 1256-72.

Freidinger R. M., Veber D. F., Perlow D. S., Brooks J. R. and Saperstein R. (1980). "Bioactive conformation of luteinizing hormone-releasing hormone: evidence from a conformationally constrained analog." Science **210**: 656-8.

Fritze O., Filipek S., Kuksa V., Palczewski K., Hofmann K. P. and Ernst O. P. (2003). "Role of the conserved NPxxY(x)5,6F motif in the rhodopsin ground state and during activation." Proc Natl Acad Sci U S A **100**: 2290-5.

Fromme B. F., Katz A. A., Millar R. P. and Flanagan C. A. (2004). "Proline$^{7.33(303)}$ in the third extracellular loop of the human GnRH receptor regulates selective binding of mammalian GnRH." Molecular and cellular endocrinology **In Press**.

Fromme B. J., Katz A. A., Roeske R. W., Millar R. P. and Flanagan C. A. (2001). "Role of Aspartate7.32(302) of the Human Gonadotropin-Releasing Hormone Receptor in Stabilizing a High Affinity Ligand Conformation." Mol Pharmacol **60**: 1280-1287.

Fujino M., Lobayashi S., Obayashi M., Shinagawa S. and Fukuda T. (1972a). "Structure-activity relationships in the C-terminal part of luteinizing hormone releasing hormone(LH-RH)." Biochem Biophys Res Commun **49**: 863-9.

Fujino M., Kobayashi S., Obayashi M., Fukuda T. and Shinagawa S. (1972b). "Synthesis and biological activities of analogs of luteinizing hormone releasing hormone (LH-RH)." Biochem Biophys Res Commun **49**: 698-705.

Funk C. D., Furci L., Moran N. and Fitzgerald G. A. (1993). "Point mutation in the seventh hydrophobic domain of the human thromboxane A2 receptor allows discrimination between agonist and antagonist binding sites." Mol Pharmacol **44**: 934-9.

Gantz I., DelValle J., Wang L. D., Tashiro T., Munzert G., Guo Y. J., Konda Y. and Yamada T. (1992). "Molecular basis for the interaction of histamine with the histamine H2 receptor." J Biol Chem **267**: 20840-3.

Gautron J. P., Leblanc P., Bluet-Pajot M. T., Pattou E., L'Heritier A., Mounier F., Ponce G., Audinot V., Rasolonjanahary R. and Kordon C. (1992). "A second endogenous molecular form of mammalian hypothalamic luteinizing hormone-releasing hormone (LHRH), (hydroxyproline9)LHRH, releases luteinizing hormone and follicle-stimulating hormone in vitro and in vivo." Mol Cell Endocrinol **85**: 99-107.

Gether U. (2000). "Uncovering molecular mechanisms involved in activation of G protein- coupled receptors." Endocr Rev **21**: 90-113.

Gether U. and Kobilka B. K. (1998). "G protein-coupled receptors. II. Mechanism of agonist activation." J Biol Chem **273**: 17979-82.

Gether U., Johansen T. E. and Schwartz T. W. (1993). "Chimeric NK1 (substance P)/NK3 (neurokinin B) receptors. Identification of domains determining the binding specificity of tachykinin agonists." J Biol Chem **268**: 7893-8.

Gomeza J., Joly C., Kuhn R., Knopfel T., Bockaert J. and Pin J. P. (1996). "The second intracellular loop of metabotropic glutamate receptor 1 cooperates with the other intracellular domains to control coupling to G-proteins." J Biol Chem **271**: 2199-205.

Guarnieri F. a. W., H. (1996). "Conformational memories and the exploration of biologically relevant peptide conformations: an illustration for the gonadotropin-releasing hormone." J. Am. Chem. Soc. **118**: 5580-5589.

Han M., Groesbeek M., Sakmar T. P. and Smith S. O. (1997). "The C9 methyl group of retinal interacts with glycine-121 in rhodopsin." Proc Natl Acad Sci U S A **94**: 13442-7.

Hannemann F., Bera A. K., Fischer B., Lisurek M., Teuchner K. and Bernhardt R. (2002). "Unfolding and conformational studies on bovine adrenodoxin

probed by engineered intrinsic tryptophan fluorescence." Biochemistry **41**: 11008-16.

Hausdorff W. P., Campbell P. T., Ostrowski J., Yu S. S., Caron M. G. and Lefkowitz R. J. (1991). "A small region of the beta-adrenergic receptor is selectively involved in its rapid regulation." Proc Natl Acad Sci U S A **88**: 2979-83.

Haviv F., Fitzpatrick T. D., Nichols C. J., Swenson R. E., Mort N. A., Bush E. N., Diaz G., Nguyen A. T., Holst M. R., Cybulski V. A. and et al. (1993). "The effect of NMeTyr5 substitution in luteinizing hormone-releasing hormone antagonists." J Med Chem **36**: 928-33.

Hazum E. (1981a). "Some characteristics of GnRH receptors in rat-pituitary membranes: differences between an agonist and and antagonist." Mol Cell Endocrinol **23**: 275-81.

He R., Browning D. D. and Ye R. D. (2001). "Differential roles of the NPXXY motif in formyl peptide receptor signaling." J Immunol **166**: 4099-105.

Hirotsu Y., Coy D. H., Coy E. J. and Schally A. V. (1974). "Stereoisomers of luteinizing hormone-releasing hormone." Biochem Biophys Res Commun **59**: 277-82.

Hjorth S. A., Schambye H. T., Greenlee W. J. and Schwartz T. W. (1994). "Identification of peptide binding residues in the extracellular domains of the AT1 receptor." J Biol Chem **269**: 30953-9.

Hoffmann S. H., ter Laak T., Kuhne R., Reilander H. and Beckers T. (2000). "Residues within transmembrane helices 2 and 5 of the human gonadotropin- releasing hormone receptor contribute to agonist and antagonist binding." Mol Endocrinol **14**: 1099-115.

Homburg R., Potashnik G., Lunenfeld B. and Insler V. (1976). "The hypothalamus as a regulator of reproductive function." Obstet Gynecol Surv **31**: 455-71.

Hovelmann S., Hoffmann S. H., Kuhne R., ter Laak T., Reilander H. and Beckers T. (2002). "Impact of aromatic residues within transmembrane helix 6 of the human gonadotropin-releasing hormone receptor upon agonist and antagonist binding." Biochemistry **41**: 1129-36.

Huang R. R., Yu H., Strader C. D. and Fong T. M. (1994). "Interaction of substance P with the second and seventh transmembrane domains of the neurokinin-1 receptor." Biochemistry **33**: 3007-13.

Huckle W. R. and Conn P. M. (1987). "Use of lithium ion in measurement of stimulated pituitary inositol phospholipid turnover." Methods Enzymol **141**: 149-55.

Hulme E. (1992). Receptor-ligand Interactions: a Practical Approach, Oxford University Press.

Hulme E. C., Lu Z. L., Ward S. D., Allman K. and Curtis C. A. (1999). "The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm." Eur J Pharmacol **375**: 247-60.

Hunyady L., Bor M., Balla T. and Catt K. J. (1995a). "Critical role of a conserved intramembrane tyrosine residue in angiotensin II receptor activation." J Biol Chem **270**: 9702-5.

Hunyady L., Bor M., Baukal A. J., Balla T. and Catt K. J. (1995). "A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type 1 angiotensin II receptor." J Biol Chem **270**: 16602-9.

Illing N., Troskie B. E., Nahorniak C. S., Hapgood J. P., Peter R. E. and Millar R. P. (1999). "Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (Carassius auratus)." Proc Natl Acad Sci U S A **96**: 2526-31.

Illing N., Jacobs G. F., Becker I. I., Flanagan C. A., Davidson J. S., Eales A., Zhou W., Sealfon S. C. and Millar R. P. (1993). "Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity among mammalian receptors." Biochem Biophys Res Commun **196**: 745-51.

Ji T. H., Grossmann M. and Ji I. (1998). "G protein-coupled receptors. I. Diversity of receptor-ligand interactions." J Biol Chem **273**: 17299-302.

Jiang Q., Lee B. X., Glashofer M., van Rhee A. M. and Jacobson K. A. (1997). "Mutagenesis reveals structure-activity parallels between human A2A adenosine receptors and biogenic amine G protein-coupled receptors." J Med Chem **40**: 2588-95.

Kaiser U. B., Zhao D., Cardona G. R. and Chin W. W. (1992). "Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor." Biochem Biophys Res Commun **189**: 1645-52.

Kakar S. S., Musgrove L. C., Devor D. C., Sellers J. C. and Neill J. D. (1992). "Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor." Biochem Biophys Res Commun **189**: 289-95.

Karten M. J. and Rivier J. E. (1986). "Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective." Endocr Rev **7**: 44-66.

Kenakin T. (1997). "Agonist-specific receptor conformations." Trends Pharmacol Sci **18**: 416-7.

Keown W. A., Campbell C. R. and Kucherlapati R. S. (1990). "Methods for introducing DNA into mammalian cells." Methods Enzymol **185**: 527-37.

Kim J., Wess J., van Rhee A. M., Schoneberg T. and Jacobson K. A. (1995). "Site-directed mutagenesis identifies residues involved in ligand recognition in the human A2a adenosine receptor." J Biol Chem **270**: 13987-97.

King J. A. and Millar R. P. (1982). "Structure of chicken hypothalamic luteinizing hormone-releasing hormone. II. Isolation and characterization." J Biol Chem **257**: 10729-32.

King J. A. and Millar R. P. (1995). "Evolutionary aspects of gonadotropin-releasing hormone and its receptor." Cell Mol Neurobiol **15**: 5-23.

Kjelsberg M. A., Cotecchia S., Ostrowski J., Caron M. G. and Lefkowitz R. J. (1992). "Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation." J Biol Chem **267**: 1430-3.

Kobayashi T., Ushikubi F. and Narumiya S. (2000). "Amino acid residues conferring ligand binding properties of prostaglandin I and prostaglandin D receptors. Identification by site- directed mutagenesis." J Biol Chem **275**: 24294-303.

Kobayashi T., Kiriyama M., Hirata T., Hirata M., Ushikubi F. and Narumiya S. (1997). "Identification of domains conferring ligand binding specificity to the prostanoid receptor. Studies on chimeric prostacyclin/prostaglandin D receptors." J Biol Chem **272**: 15154-60.

Kolakowski L. F., Jr. (1994). "GCRDb: a G-protein-coupled receptor database." Receptors Channels **2**: 1-7.

Konvicka K., Guarnieri F., Ballesteros J. A. and Weinstein H. (1998). "A proposed structure for transmembrane segment 7 of G protein-coupled receptors incorporating an Asn-Pro/Asp-Pro motif." Biophys J **75**: 601-11.

Kubiak R. J., Yue X., Hondal R. J., Mihai C., Tsai M. D. and Bruzik K. S. (2001). "Involvement of the Arg-Asp-His catalytic triad in enzymatic cleavage of the phosphodiester bond." Biochemistry **40**: 5422-32.

Loewenthal R., Sancho J. and Fersht A. R. (1992). "Histidine-aromatic interactions in barnase. Elevation of histidine pKa and contribution to protein stability." J Mol Biol **224**: 759-70.

Lopata M. A., Cleveland D. W. and Sollner-Webb B. (1984). "High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment." Nucleic Acids Res **12**: 5707-17.

Lu Z. L., Saldanha J. W. and Hulme E. C. (2002). "Seven-transmembrane receptors: crystals clarify." Trends Pharmacol Sci **23**: 140-6.

Luthman H. and Magnusson G. (1983). "High efficiency polyoma DNA transfection of chloroquine treated cells." Nucleic Acids Res **11**: 1295-308.

Maggi C. A., Patacchini R., Rovero P. and Giachetti A. (1993). "Tachykinin receptors and tachykinin receptor antagonists." J Auton Pharmacol **13**: 23-93.

Maliekal J., Jackson, G.E., Flanagan, C.A., and Millar, R.P. (1997). "Solution conformations of gonadotropin releasing hormone (GnRH) and [Gln8]-GnRH." S. Afric. J. Chem. **50**: 217-219.

Mansour A., Meng F., Meador-Woodruff J. H., Taylor L. P., Civelli O. and Akil H. (1992). "Site-directed mutagenesis of the human dopamine D2 receptor." Eur J Pharmacol **227**: 205-14.

Matsuo H., Baba Y., Nair R. M., Arimura A. and Schally A. V. (1971). "Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence." Biochem Biophys Res Commun **43**: 1334-9.

Meurisse R., Brasseur R. and Thomas A. (2003). "Aromatic side-chain interactions in proteins. Near- and far-sequence His-X pairs." Biochim Biophys Acta **1649**: 85-96.

Millar R., Conklin D., Lofton-Day C., Hutchinson E., Troskie B., Illing N., Sealfon S. C. and Hapgood J. (1999). "A novel human GnRH receptor homolog gene: abundant and wide tissue distribution of the antisense transcript." J Endocrinol **162**: 117-26.

Millar R., Lowe S., Conklin D., Pawson A., Maudsley S., Troskie B., Ott T., Millar M., Lincoln G., Sellar R., Faurholm B., Scobie G., Kuestner R., Terasawa E. and Katz A. (2001). "A novel mammalian receptor for the evolutionarily conserved type II GnRH." Proc Natl Acad Sci U S A **98**: 9636-41.

Millar R. P. (2002). "GnRH II and type II GnRH receptors." Trends Endocrinol Metab **14**: 35-43.

Millar R. P., Milton R. C., Follett B. K. and King J. A. (1986a). "Receptor binding and gonadotropin-releasing activity of a novel chicken gonadotropin-releasing hormone ([His5, Trp7, Tyr8]GnRH) and a D-Arg6 analog." Endocrinology **119**: 224-31.

Millar R. P., King J. A., Davidson J. S. and Milton R. C. (1987). "Gonadotrophin-releasing hormone--diversity of functions and clinical applications." S Afr Med J **72**: 748-55.

Millar R. P., Flanagan C. A., Milton R. C. and King J. A. (1989). "Chimeric analogues of vertebrate gonadotropin-releasing hormones comprising substitutions of the variant amino acids in positions 5, 7, and 8. Characterization of requirements for receptor binding and gonadotropin release in mammalian and avian pituitary gonadotropes." J Biol Chem **264**: 21007-13.

Millar R. P., Davidson J., Flanagan C. A. and Wakefield I. (1995). "Ligand binding and second messenger assays for cloned Gq/G11-coupled neuropeptide receptors: the GnRH receptor." Methods in Neurosciences, Receptor Molecular Biology **25**: 145-162.

Millar R. P., Lu Z., Pawson A. J., Flanagan C. A., Morgan K. and Maudsley S. R. (2004). "Gonadotropin-Releasing Hormone Receptors." Endocrine reviews **In press**.

Miller B. T., Collins T. J., Nagle G. T. and Kurosky A. (1992). "The occurrence of O-acylation during biotinylation of gonadotropin- releasing hormone and analogs. Evidence for a reactive serine." J Biol Chem **267**: 5060-9.

Miyamoto K., Hasegawa Y., Nomura M., Igarashi M., Kangawa K. and Matsuo H. (1984). "Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species." Proc Natl Acad Sci U S A **81**: 3874-8.

Momany F. A. (1976). "Conformational energy analysis of the molecule, luteinizing hormone- releasing hormone. I. Native decapeptide." J Am Chem Soc **98**: 2990-6.

Momany F. A. (1978). "Conformational analysis of the molecule luteinizing hormone-releasing hormone. 3. Analogue inhibitors and antagonists." J Med Chem **21**: 63-8.

Monahan M. W., Amoss M. S., Anderson H. A. and Vale W. (1973). "Synthetic analogs of the hypothalamic luteinizing hormone releasing factor with increased agonist or antagonist properties." Biochemistry **12**: 4616-20.

Montero-Moran G. M., Lara-Gonzalez S., Alvarez-Anorve L. I., Plumbridge J. A. and Calcagno M. L. (2001). "On the multiple functional roles of the active site histidine in catalysis and allosteric regulation of Escherichia coli glucosamine 6-phosphate deaminase." Biochemistry **40**: 10187-96.

Myburgh D. B., Millar R. P. and Hapgood J. P. (1998). "Alanine-261 in intracellular loop III of the human gonadotropin- releasing hormone receptor is crucial for G-protein coupling and receptor internalization." Biochem J **331**: 893-6.

Noda K., Saad Y. and Karnik S. S. (1995b). "Interaction of Phe8 of angiotensin II with Lys199 and His256 of AT1 receptor in agonist activation." J Biol Chem **270**: 28511-4.

Noda K., Saad Y., Kinoshita A., Boyle T. P., Graham R. M., Husain A. and Karnik S. S. (1995a). "Tetrazole and carboxylate groups of angiotensin receptor antagonists bind to the same subsite by different mechanisms." J Biol Chem **270**: 2284-9.

Okada T., Fujiyoshi Y., Silow M., Navarro J., Landau E. M. and Shichida Y. (2002). "Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography." Proc Natl Acad Sci U S A **99**: 5982-7.

Okada Y., Kitamura K., Baba Y., Arimura A. and Schally A. V. (1973). "Luteinizing hormone-releasing hormone analogs lacking N-terminal pGlu ring structure." Biochem Biophys Res Commun **53**: 1180-6.

Okubo K., Suetake H., Usami T. and Aida K. (2000b). "Molecular cloning and tissue-specific expression of a gonadotropin- releasing hormone receptor in the Japanese eel." Gen Comp Endocrinol **119**: 181-92.

Palczewski K., Kumasaka T., Hori T., Behnke C. A., Motoshima H., Fox B. A., Le Trong I., Teller D. C., Okada T., Stenkamp R. E., Yamamoto M. and Miyano M. (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor." Science **289**: 739-45.

Perez-Canadilllas J. M., Garcia-Mayoral M. F., Laurents D. V., Martinez del Pozo A., Gavilanes J. G., Rico M. and Bruix M. (2003). "Tautomeric state of alpha-sarcin histidines. Ndelta tautomers are a common feature in the active site of extracellular microbial ribonucleases." FEBS Lett **534**: 197-201.

Perlman S., Schambye H. T., Rivero R. A., Greenlee W. J., Hjorth S. A. and Schwartz T. W. (1995). "Non-peptide angiotensin agonist. Functional and molecular interaction with the AT1 receptor." J Biol Chem **270**: 1493-6.

Petry R., Craik D., Haaima G., Fromme B., Klump H., Kiefer W., Palm D. and Millar R. (2002). "Secondary structure of the third extracellular loop

responsible for ligand selectivity of a mammalian gonadotropin-releasing hormone receptor." J Med Chem **45**: 1026-1034.

Pettei M. J., Yudd A. P., Nakanishi K., Henselman R. and Stoeckenius W. (1977). "Identification of retinal isomers isolated from bacteriorhodopsin." Biochemistry **16**: 1955-9.

Powell J. F., Reska-Skinner S. M., Prakash M. O., Fischer W. H., Park M., Rivier J. E., Craig A. G., Mackie G. O. and Sherwood N. M. (1996). "Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications." Proc Natl Acad Sci U S A **93**: 10461-4.

Prioleau C., Visiers I., Ebersole B. J., Weinstein H. and Sealfon S. C. (2002). "Conserved helix 7 tyrosine acts as a multistate conformational switch in the 5HT2C receptor. Identification of a novel "locked-on" phenotype and double revertant mutations." J Biol Chem **277**: 36577-84.

Probst W. C., Snyder L. A., Schuster D. I., Brosius J. and Sealfon S. C. (1992). "Sequence alignment of the G-protein coupled receptor superfamily." DNA Cell Biol **11**: 1-20.

Rasmussen S. G., Jensen A. D., Liapakis G., Ghanouni P., Javitch J. A. and Gether U. (1999). "Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6." Mol Pharmacol **56**: 175-84.

Rath P., Delange F., Degrip W. J. and Rothschild K. J. (1998). "Hydrogen bonding changes of internal water molecules in rhodopsin during metarhodopsin I and metarhodopsin II formation." Biochem J **329**: 713-7.

Rhee M. H., Nevo I., Levy R. and Vogel Z. (2000). "Role of the highly conserved Asp-Arg-Tyr motif in signal transduction of the CB2 cannabinoid receptor." FEBS Lett **466**: 300-4.

Sakmar T. P. (1998). "Rhodopsin: a prototypical G protein-coupled receptor." Prog Nucleic Acid Res Mol Biol **59**: 1-34.

Samama P., Cotecchia S., Costa T. and Lefkowitz R. J. (1993). "A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model." J Biol Chem **268**: 4625-36.

Sandow J., Konig, W., Geiger, R., Uhman, R., and von Rechenberg, W. (1978). Structure-activity relationships in the LH-RH molecule. Control of ovulation. D. B. Crighton, Haynes, N.B., Foxcroft, G.R., and Lamming, G.E. London, Buttersworths: 47-70.

Sansom M. S. and Weinstein H. (2000). "Hinges, swivels and switches: the role of prolines in signalling via transmembrane alpha-helices.[In Process Citation]." Trends Pharmacol Sci **21**: 445-51.

Scheer A., Fanelli F., Costa T., De Benedetti P. G. and Cotecchia S. (1997). "The activation process of the alpha1B-adrenergic receptor: potential role of protonation and hydrophobicity of a highly conserved aspartate." Proc Natl Acad Sci U S A **94**: 808-13.

Scheer A., Costa T., Fanelli F., De Benedetti P. G., Mhaouty-Kodja S., Abuin L., Nenniger-Tosato M. and Cotecchia S. (2000). "Mutational analysis of the highly conserved arginine within the Glu/Asp- Arg-Tyr motif of the alpha(1b)-adrenergic receptor: effects on receptor isomerization and activation." Mol Pharmacol **57**: 219-31.

Scheiner S., Kar T. and Pattanayak J. (2002). "Comparison of various types of hydrogen bonds involving aromatic amino acids." J Am Chem Soc **124**: 13257-64.

Schertler G. F. and Hargrave P. A. (1995). "Projection structure of frog rhodopsin in two crystal forms." Proc Natl Acad Sci U S A **92**: 11578-82.

Schertler G. F., Villa C. and Henderson R. (1993). "Projection structure of rhodopsin." Nature **362**: 770-2.

Schwartz T. W. (1994). "Locating ligand-binding sites in 7TM receptors by protein engineering." Curr Opin Biotechnol **5**: 434-44.

Sealfon S. C., Weinstein H. and Millar R. P. (1997). "Molecular mechanisms of ligand interaction with the gonadotropin- releasing hormone receptor." Endocr Rev **18**: 180-205.

Sealfon S. C., Chi L., Ebersole B. J., Rodic V., Zhang D., Ballesteros J. A. and Weinstein H. (1995b). "Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT2A receptor." J Biol Chem **270**: 16683-8.

Sherwood N. M., Lovejoy D. A. and Coe I. R. (1993). "Origin of mammalian gonadotropin-releasing hormones." Endocr Rev **14**: 241-54.

Shinitzky M., Hazum E. and Fridkin M. (1976). "Structure-activity relationships of luliberin substituted at position 8." Biochim Biophys Acta **453**: 553-7.

Soderhall A., Polymeropoulos E., Paulini K., Gunther E. and Kuhne R. (2002). Molecular dynamics of human luteinizing hormone receptor:comparative modelling studies of ligand-receptor interactions. Designing drugs and crop protectans:Processes, Problems and Solutions, Bournemouth.

Stacey M., Lin HH., Gordon S.and McKnight AJ.(2000)"LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors".Trends Biochem Sci. **25**:284-9.

Stitham J., Stojanovic A., Merenick B. L., O'Hara K. A. and Hwa J. (2003). "The unique ligand-binding pocket for the human prostacyclin receptor. Site-directed mutagenesis and molecular modeling." J Biol Chem **278**: 4250-7.

Strader C. D., Fong T. M., Graziano M. P. and Tota M. R. (1995). "The family of G-protein-coupled receptors." Faseb J **9**: 745-54.

Strader C. D., Candelore M. R., Hill W. S., Sigal I. S. and Dixon R. A. (1989). "Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor." J Biol Chem **264**: 13572-8.

Strader C. D., Fong T. M., Tota M. R., Underwood D. and Dixon R. A. (1994). "Structure and function of G protein-coupled receptors." Annu Rev Biochem **63**: 101-32.

Struthers M., Yu H. and Oprian D. D. (2000). "G protein-coupled receptor activation: analysis of a highly constrained, "straitjacketed" rhodopsin." Biochemistry **39**: 7938-42.

Sun Y. M., Flanagan C. A., Illing N., Ott T. R., Sellar R., Fromme B. J., Hapgood J., Sharp P., Sealfon S. C. and Millar R. P. (2001). "A chicken gonadotropin-releasing hormone receptor that confers agonist activity to mammalian antagonists. Identification of D-Lys(6) in the ligand and extracellular loop two of the receptor as determinants." J Biol Chem **276**: 7754-61.

Takeda S., Kadowaki S., Haga T., Takaesu H. and Mitaku S. (2002). "Identification of G protein-coupled receptor genes from the human genome sequence." FEBS Lett **520**: 97-101.

Takeuchi H., Okada A. and Miura T. (2003). "Roles of the histidine and tryptophan side chains in the M2 proton channel from influenza A virus." FEBS Lett **552**: 35-8.

Tan Y. V., Couvineau A., Van Rampelbergh J. and Laburthe M. (2003). "Photoaffinity labeling demonstrates physical contact between vasoactive intestinal peptide and the N-terminal ectodomain of the human VPAC1 receptor." J Biol Chem **278**: 36531-6.

Troskie B. E., Hapgood J. P., Millar R. P. and Illing N. (2000). "Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotropin-releasing hormone receptor

expressed in the pituitary and midbrain of Xenopus laevis." Endocrinology **141**: 1764-71.

Trumpp-Kallmeyer S., Hoflack J., Bruinvels A. and Hibert M. (1992). "Modeling of G-protein-coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors." J Med Chem **35**: 3448-62.

Tsutsumi M., Zhou W., Millar R. P., Mellon P. L., Roberts J. L., Flanagan C. A., Dong K., Gillo B. and Sealfon S. C. (1992). "Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor." Mol Endocrinol **6**: 1163-9.

Tuganova A., Yoder M. D. and Popov K. M. (2001). "An essential role of Glu-243 and His-239 in the phosphotransfer reaction catalyzed by pyruvate dehydrogenase kinase." J Biol Chem **276**: 17994-9.

Unger V. M. and Schertler G. F. (1995). "Low resolution structure of bovine rhodopsin determined by electron cryo-microscopy." Biophys J **68**: 1776-86.

Unger V. M., Hargrave P. A., Baldwin J. M. and Schertler G. F. (1997). "Arrangement of rhodopsin transmembrane alpha-helices." Nature **389**: 203-6.

Wang C., Yun O., Maiti K., Young Oh D., Kyu Kim K., Young Seong J. and Bang Kwon H. (2003). "Position of Pro and Ser near Glu7.32 in the Extracellular Loop 3 of Mammalian and Nonmammalian Gonadotropin-Releasing Hormone (GnRH) Receptors is a Critical Determinant for Differential Ligand Selectivity for Mammalian GnRH and Chicken GnRH-II." Mol Endocrinol.

Wang C. D., Buck M. A. and Fraser C. M. (1991). "Site-directed mutagenesis of alpha 2A-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists." Mol Pharmacol **40**: 168-79.

Wang C. D., Gallaher T. K. and Shih J. C. (1993). "Site-directed mutagenesis of the serotonin 5-hydroxytrypamine2 receptor: identification of amino acids necessary for ligand binding and receptor activation." Mol Pharmacol **43**: 931-40.

Wang L., Bogerd J., Choi H. S., Seong J. Y., Soh J. M., Chun S. Y., Blomenrohr M., Troskie B. E., Millar R. P., Yu W. H., McCann S. M. and Kwon H. B. (2001). "Three distinct types of GnRH receptor characterized in the bullfrog." Proc Natl Acad Sci U S A **98**: 361-6.

White R. B., Eisen J. A., Kasten T. L. and Fernald R. D. (1998). "Second gene for gonadotropin-releasing hormone in humans." Proc Natl Acad Sci U S A **95**: 305-9.

Wiens B. L., Nelson C. S. and Neve K. A. (1998). "Contribution of serine residues to constitutive and agonist-induced signaling via the D2S dopamine receptor: evidence for multiple, agonist- specific active conformations." Mol Pharmacol **54**: 435-44.

Wise A., Gearing K. and Rees S. (2002). "Target validation of G-protein coupled receptors." Drug Discov Today **7**: 235-46.

Xu W., Ozdener F., Li J. G., Chen C., de Riel J. K., Weinstein H. and Liu-Chen L. Y. (1999). "Functional role of the spatial proximity of Asp114(2.50) in TMH 2 and Asn332(7.49) in TMH 7 of the mu opioid receptor." FEBS Lett **447**: 318-24.

Yanaihara N. (1973). "[Chemical structure and synthesis of LH-RH]." Horumon To Rinsho **21**: 885-91.

Yanaihara N., Yanaihara C., Sakagami M., Tsuji K. and Hashimoto T. (1973). "Synthesis and biological evaluation of LH and FSH releasing hormone and its analogs." J Med Chem **16**: 373-7.

Yeagle P. L., Salloum A., Chopra A., Bhawsar N., Ali L., Kuzmanovski G., Alderfer J. L. and Albert A. D. (2000). "Structures of the intradiskal loops and amino terminus of the G-protein receptor, rhodopsin." J Pept Res **55**: 455-65.

Ying S. Y., Ling N., Bohlen P. and Guillemin R. (1981). "Gonadocrinins: peptides in ovarian follicular fluid stimulating the secretion of pituitary gonadotropins." Endocrinology **108**: 1206-15.

Yokota Y., Akazawa C., Ohkubo H. and Nakanishi S. (1992). "Delineation of structural domains involved in the subtype specificity of tachykinin receptors through chimeric formation of substance P/substance K receptors." Embo J **11**: 3585-91.

Zhou W., Flanagan C., Ballesteros J. A., Konvicka K., Davidson J. S., Weinstein H., Millar R. P. and Sealfon S. C. (1994). "A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor." Mol Pharmacol **45**: 165-70.

Zhou W., Rodic V., Kitanovic S., Flanagan C. A., Chi L., Weinstein H., Maayani S., Millar R. P. and Sealfon S. C. (1995). "A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites." J Biol Chem **270**: 18853-7.

Appendices

A.1
Primers designed for creating the His$^{7.36(305)}$ mutant receptors.

His$^{7.36(305)}$Ala (Bsm I-GAATGCN
Sense: GG GTG TCA GAG CCA GTG AAT GCA TTT CTC TTT CC
Antisense: GC AAA GAG AAA GAA TGC ATT CAC TGG CTC TGA CAC CC
Phe Leu Phe Phe Phe *Ala* Asn Val Pro Glu Ser Val

His$^{7.36(305)}$Asn (Xmn I – GAANN/NNTTC)
Sense: GG GTG TCA GAG CCA GTG AAT AAT TTC TTC TTT CTC TTT CC
Antisense: GC AAA GAG AAA GAA [illegible] CAC TGG CTC TGA CAC CC
Phe Leu Phe Phe Phe *Asn* Asn Val Pro Glu Ser Val

His$^{7.36(305)}$Gln (Xmn I – GAANN/NNTTC)
Sense: GG GTG TCA GAG CCA GTG AAT CAA TTC TTC TTT CTC TTT CC
Antisense: GC AAA GAG AAA GAA TTG ATT CAC TGG CTC TGA CAC CC
Phe Leu Phe Phe Phe *Gln* Asn Val Pro Glu Ser Val

His$^{7.36(305)}$Arg (mn I – GAANN/NNTTC)
Sense: GG GTG TCA GAG CCA GTG AAT CGA TTC TTC TTT CTC TTT CC
Antisense: GC AAA GAG AAA GAA TCG ATT CAC TGG CTC TGA CAC CC
Phe Leu Phe Phe Phe *Arg* Asn Val Pro Glu Ser Val

His$^{7.36(305)}$Phe (Apo I – R/AATY)
Sense: GG GTG TCA GAG CCA GTG AAT TTC TTT TTC TTT CTC TTT CC
Antisense: GC AAA GAG AAA GAA AAA GAA ATT CAC TGG CTC TGA CAC CC
Phe Leu Phe Phe Phe *Phe* Asn Val Pro Glu Ser Val

His$^{7.36(305)}$Trp (Xmn I – GAANN/NNTTC)
Sense: GG GTG TCA GAG CCA GTG AAT TGG TTC TTC TTT CTC TTT CC
Antisense: GC AAA GAG AAA GAA CCA ATT CAC TGG CTC TGA CAC CC
Phe Leu Phe Phe Phe *Trp* Asn Val Pro Glu Ser Val

The mutations introduced are indicated in boxes. The silent restriction sites for each mutation are shaded in red.

A.2

Dpn I mutagenesis used to generate His$^{7.36(305)}$ mutants.

Mouse GnRH receptor in pcDNA1/Amp with His$^{7.36(305)}$ site for mutation Mutagenic primers

Denature the plasmid and anneal the primers containing desired mutation.

Digest the methylated, nonmutated DNA template with DpnI restriction enzyme.

Transform mutated GnRH receptor in competent DH10B cells.

Automated sequencing to confirm the presence of the mutations was done using pcDNA1.1/Amp specific primers, T7:(5'TAATACGACTCACTATAGGG3') and Sp6:(5'GATTTAGGTGACACTATAG3').

A.3. Formulae for calculating IC_{50}, EC_{50}, coupling efficiency and Bmax values of the different $His^{7.36(305)}$ mutant receptors.

Sigmoidal dose response **(Nonlinear regression) curve were used to analyse data collected in IP assays.**

$$Y = \text{Bottom} + \frac{(\text{Top}-\text{Bottom})}{(1+10^{(\text{LogEC}_{50}-X)})}$$

Y is the response
X is the logarithm of agonist concentration

One Site Competition

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1+10^{(X - \text{LogEC}_{50})}}$$

Y is the response
X is the logarithm of agonist concentration

Homologous Competition Binding Curve.

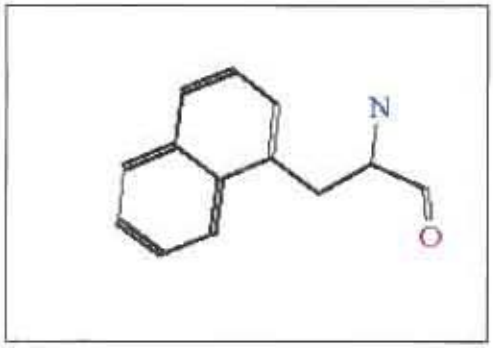
Data collected in ligand binding experiments were plotted on nonlinear regression curve using the following equations. These formulae were used to estimate the IC_{50} and maximum binding of the different mutants for estimating the number of receptors on the cell surface.

ColdNM=10^(x+9)
KdNM=10^(logKd+9)
Y=(Bmax*HotnM)/(HotnM + ColdNM + KdNM) + NS

Cold concentration in nM
Kd in nM
HotnM set to constant value (Calculated from total counts per reaction tube)
Y is total binding in cpm
X is log of unlabeled ligand
NS is non specific binding

Coupling efficiency Q. The mean values of Kd, EC_{50}, Emax and Bmax were used to calculate how well the mutant receptors activate effector PLC using the formula:
IC_{50} was for Kd values.
The calculated sites per cell were used for Bmax values.
$0.5 \times [(Kd + EC_{50})/EC_{50}] \times (Emax/Bmax)$ (Ballesteros *et al,* 1998).

A.4
Structures of unnatural amino acid side chains.



2-Naphthyl-Alanine