The Role of Extracellular Loop Three of the Human Gonadotropin Releasing Hormone Receptor in Ligand Selectivity

by

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This thesis is dedicated to my wife, Gilda.

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Contents

Abstract

The hypothalamic neuropeptide, gonadotropin releasing hormone (GnRH) preferentially interacts with GnRH type I receptors on the gonadotropes in the anterior pituitary. Activation of the GnRH receptor is required for the biosynthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which regulate reproductive function. Multiple forms of GnRH are present in most vertebrate species and are thought to have physiological functions in addition to regulating pituitary hormone release. The mammalian type I GnRH receptor is proposed to discriminate between endogenous forms of GnRH (King and Millar, 1995). In this thesis the mechanism of the GnRH selectivity by a mammalian type I GnRH receptor is examined at molecular level and previous hypotheses are re-evaluated.

In GnRH, Arg^{8} has been proposed to be involved in an electrostatic interaction with an acidic residue in the mammalian type I GnRH receptor (Hazum, 1987). In the mouse GnRH receptor, $Glu^{7.32(301)}$ in extracellular loop three (ECL3) determines high affinity for Arg^{8}-containing GnRH. However, this interaction appears to be more complex than a direct electrostatic interaction (Flanagan et al., 1994). Moreover, $Glu^{7.32(301)}$ is substituted by $Asp^{7.32(302)}$ in non-rodent GnRH receptors and is proposed to determine selectivity for Arg^{8}-containing GnRH (Sealfon et al., 1997). The lack of conservation indicates a need to determine whether $Asp^{7.32(302)}$ has the same function in the human GnRH receptor as $Glu^{7.32(301)}$ has in the mouse receptor. Furthermore, the incorporation of a direct interaction of $Glu^{7.32(301)}$/$Asp^{7.32(302)}$ with Arg^{8} in models of receptor-ligand complexes (Chauvin et al., 2000; Flanagan et al., 2000; Hoffmann et al., 2000), in spite of evidence that a direct interaction may not always occur, shows that better definition of the mechanism by which the $Asp^{7.32(302)}$ determines binding specificity for GnRH is needed. Additionally, GnRH is found as a mixture of conformers in solution of which a small population bind the GnRH receptor with high affinity (Karten and Rivier, 1986). The biologically active conformation of GnRH is believed to be stabilised with conformational constraints (Monahan et al., 1973), which bind mammalian GnRH receptors with high affinity (Sealfon et al., 1997). Thus, the role of ligand conformation in binding the human GnRH receptor was also investigated.

In this thesis it is demonstrated that mutating $Asp^{7.32(302)}$ to Asn in the human GnRH receptor decreases affinity for GnRH, but not for analogs with substitutions for Arg^{8}. It is shown that a series of peptides with different structural constraints that stabilize a high affinity conformation of GnRH retain high affinity for the $Asp^{7.32(302)}$Asn mutant GnRH receptor. It is shown that an electrostatic interaction is not involved in the binding of these constrained analogs. It is also shown

that a conformationally-constrained analog with Gln^{8} has higher affinity than Arg^{8}-containing GnRH for the human GnRH receptor, indicating that ligand conformation plays a separate role to Arg^{8} in GnRH binding. These results are interpreted in terms of a sequential binding mechanism in which the Arg^{8} side chain of native GnRH interacts transiently with $Asp^{7.32(302)}$, before interacting with a final ligand binding pocket which also binds constrained analogs.

Moreover, non-mammalian GnRH receptors do not preferentially bind Arg^{8}-containing GnRH (Sealfon et al., 1997), but contrary to expectation, the acidic residue at position 7.32 is conserved in these receptors. Amino acid sequence alignment of ECL3 of mammalian and non-mammalian GnRH receptors revealed two distinct amino acid motifs. ECL3 of mammalian GnRH receptors contains the $Ser^{7.31}$-($Glu^{7.32}$/$Asp^{7.32}$)-$Pro^{7.33}$ motif and non-mammalian GnRH receptors have Pro-(Asp/Glu)-Tyr. Mutating $Pro^{7.33(303)}$ to Ala, or changing the position of Pro relative to $Asp^{7.32(302)}$ decreased affinity for GnRH, but not for GnRH analogues without Arg^{8} or with a conformational constraint. This suggests that $Pro^{7.33(303)}$ may constrain the conformation of ECL3, influencing the orientation of the Asp side chain.

The conformation of ECL3 was studied indirectly with site-directed mutagenesis and by spectroscopic studies of peptides corresponding to ECL3. Increasing the length of ECL3 with Ala-insertional mutagenesis decreased affinity for GnRH, suggesting that the entire ECL3 is important for GnRH binding. It is shown that peptides corresponding to the sequence of ECL3 are functional by being able to specifically inhibit GnRH stimulated signalling. Spectroscopic analysis of a model peptide, corresponding to the sequence of ECL3, revealed a superposition of predominantly random coil with a central ß-hairpin domain (Petry et al., 2001). This thesis proposes that a specific structure in ECL3 stabilises a conformation which aligns the carboxyl side chain at locus 7.32 in the mammalian type I GnRH receptor to interact with Arg^{8} of GnRH.

It is proposed that residues in ECL3 of the mammalian GnRH receptor which are implicated in ligand binding also have additional roles such as structural organisation of the receptor either in the inactive or active conformation. In this thesis evidence is provided that both $Asp^{7.32(302)}$ and $Pro^{7.33(303)}$ also play a role in receptor activation in addition to ligand binding. However, primarily this thesis proposes that in the human type I GnRH receptor Pro in locus 7.33 confers unique local conformational constraints on the conformation of ECL3 which influences the orientation of $Asp^{7.32(302)}$ thereby regulating GnRH selectivity.

List of abbreviations

AA, arachidonic acid;

BIA, Biomolecular Interaction Analysis

CAM receptor, constituitively active mutant receptor;

CD, circular dichroism;

DAG, diacylglycerol;

ECL, extracellular loop;

Emax, maximal agonist-stimulated IP production;

ER, endoplasmatic reticulum;

FSH, follicle stimulating hormone;

GAP, GnRH associated peptide;

GnRH, Gonadotropin releasing hormone;

GPCR, G protein-coupled receptor;

GTP, guanosine triphosphate;

ICL, intracellular loop;

IP, inositol phosphate;

IP_3, inositol 1,4,5-triphosphate;

Ipr-Lys, N^{ε}-isopropyllysine;

LH, luteinizing hormone;

LKT, leukotrienes;

L-VG channels, L-type voltage gated channels;

MAP kinase, mitogen activated protein kinase;

NK, neurokinin;

PC, phosphatidylcholine;

PIP_2, phosphatidylinositol 1,4 bisphosphate;

PKC, protein kinase C;

PLC, phospholipase C

PLD, phospholipase D;

PTH, parathyroid hormone;

Q, coupling efficiency;

RI, retro-inverso;

SE, standard error;

SPR, surface plasmon resonance;

TM, transmembrane;

TRH, thyroid releasing hormone.

Chapter 1

Introduction to GnRH Receptor-Ligand Interactions as a Model G Protein-Coupled Receptor

1.1 Summary

G protein-coupled receptors (GPCRs) represent an important class of drug discovery targets that exist on the surface membrane of all cells and are associated with a wide range of therapeutic agents which include inflammation, cancer, cardiovascular, metabolic, gastrointestinal, central nervous and reproductive system disorders/diseases. There are estimated over 1000 GPCRs in the human genome of which only a small proportion is well-characterised with known ligands and of which approximately half are current targets of commercial drugs. Detailed knowledge of how GPCRs interact with their ligands could facilitate structure-based drug design as an important drug discovery tool.

Inferences of the structure of GPCRs have been based on a low resolution structure of rhodopsin as well as from sequence alignments and experimantal results related to structure-function of these receptors. The crystallisation of rhodopsin is a landmark achievement which enabled a three-dimensional X-ray structure to be determined that provides a direct view of the ligand binding site (Palczewski et al., 2000). In this chapter a diversity of experimental results suggest that GPCRs have a similar overall fold and have a core function of signalling activation through a conformational change of the transmembrane (TM) domains which alters the conformation of the intracellular domains eliciting a response via G proteins and various other signalling pathways. Through this common signalling transduction mechanism different GPCRs can bind to specific ligands to convey extracellular signals to the intracellular mileu.

The protein structure of the GnRH receptor is characteristic of the rhodopsin family of GPCRs (Sealfon et al., 1997). Similar to other GPCRs, the α-helical TM helices of the GnRH receptor is believed to be arranged around a hydrophillic core as proposed by the crystal structure of rhodopsin (Palczewski et al., 2000). The ligand binding site of the GnRH receptor comprises elements of extracellular domains and superficial residues in the putative TM domains (Flanagan et al., 1997; Sealfon et al., 1997). The interaction of a ligand, like GnRH, with the binding domains of the receptor is proposed to induce conformational changes in the TM helices, which are associated with signal propagation. However, there are multiple forms of GnRH present in most vertebrate species that are thought to have different physiological functions. The GnRH receptor is able to discriminate between endogenous forms of GnRH through an unknown mechanism, which may regulate reproductive function.

1.2 Introduction to G protein-coupled receptors (GPCRs)

The GnRH receptors that have been cloned from several mammalian and non-mammalian species are highly conserved and belong to the superfamily of G protein-coupled receptors (GPCR) based on the structure of rhodopsin (Sealfon et al., 1997). This family of receptors is characterized by an extracellular N-terminal domain, seven alpha helical transmembrane domains, which are interconnected by alternating extra- and intra-cellular loops, and an intracellular C-terminal domain (Schertler et al., 1993). Consequently, a molecular model of the GnRH receptor is generated based on mutagenesis studies and a computer-based simulation of rhodopsin (Sealfon et al., 1997).

GPCRs comprise the largest superfamily of transmembrane proteins with over 1000 members, which have been cloned and characterised (Gether, 2000), and are grouped by their ability to couple to heterotrimeric GTP-binding proteins (G proteins). These serpentine seven transmembrane spanning receptors are important in conveying extracellular signals to the intracellular milieu of target cells, causing specific responses. Sensations of exogenous stimuli such as light, odors and taste are mediated by this class of receptors. The chemical diversity among the endogenous ligands is remarkable, which include biogenic amines, peptides, glycoprotein hormones, lipids, nucleotides, ions and proteases. GPCRs are involved in such major physiological systems, such as the central nervous-, neuroendocrine-, cardiovascular-, immune-, gastrointestinal- and reproductive systems, making them an important target for medical therapies.

This large functionally diverse class of receptors can be grouped into three major families according to their sequence alignment: the large rhodopsin family (family A), the secretin-calcitonin-PTH family (family B), and the metabotropic glutamate receptors (family C). In addition, minor subfamilies include the yeast pheromone receptors (families D and E) and cAMP receptors (family F) (Gether, 2000). Section 1.2 of this chapter, will focus on the molecular mechanisms underlying ligand binding in GPCRs and receptor activation of family A GPCRs. Section 1.3 will review aspects of GnRH and the mammalian type I GnRH receptor pertaining to ligand binding, selectivity and activation.

1.2.1 Structural Characteristics of GPCRs

The GPCRs share a high degree of sequence homology and there have been significant advances in collating information on GPCR structure-function in databases (www.cmbi.kun.nl, www.tgrap.uit.no and mgddk1.niddk.nih.gov). GPCRs characteristically have seven hydrophobic α-helical domains, which traverse the cell membrane, and are linked by alternating extracellular and intracellular loops. An

extracellular N-terminal segment and in most cases a C-terminal segment is also present. There are three extracellular and three to four intracellular loops, with the fourth cytoplasmic loop formed when certain Cys residues are palmitoylated at the C-terminal tail (eg. $Cys^{7.69(322)}$ and $Cys^{7.70(323)}$ in rhodopsin) (Fig. 1.1). The recent crystal structure of rhodopsin revealed that the fourth cytoplasmic loop has a helical structure (short eighth helix) extending from the C-terminal side of TM seven into the cytoplasm and appears to be terminated by $Gly^{7.71(324)}$ before continiung as the C-terminal tail (Palczewski et al., 2000).

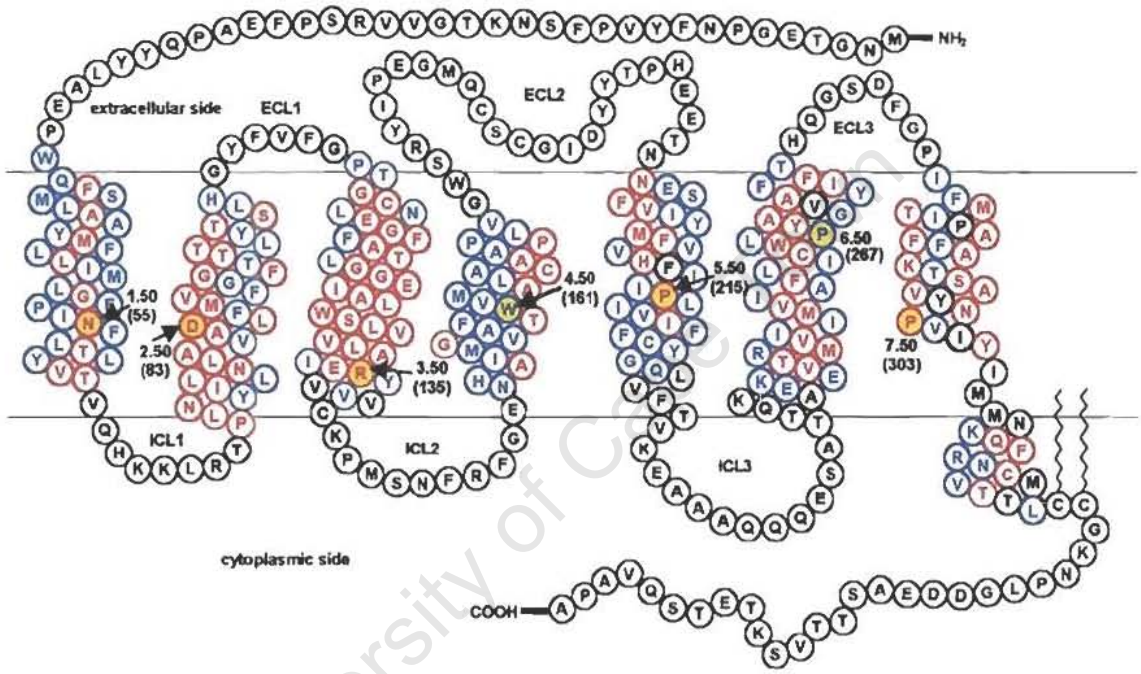

Figure 1.1 Primary structure of bovine rhodopsin, which is representative of a family A GPCR (adapted from Ballesteros et al., 2001a). The N-terminus is on the extracellular side and the C-terminus is on the intracellular side of the membrane. TM domains are connected by intra- and extra- cellular loops. Residues in yellow are the most conserved in the family A GPCRs. Residues in **red** are proposed to face the interior of the TM helix bundle and residues in **blue** are proposed to face the hydrophobic lipid environment (Ballesteros et al., 2001a).

1.2.1.1 Transmembrane (TM) Domains

The TM helices connect the extra- and intracellular loops, which have roles in ligand binding and signalling respectively (Schertler et al., 1993).

When TM domains were analysed for hydrophobicity, each of the seven TM helices ranged in size from 18 to 27 amino acids, with TM helices three, five, and six proposed to be the longest, extending from the lipid bilayer into the cytosol (Baldwin, 1993). The membrane boundaries vary

between the seven helices, of which TM helices three, five and six protrude more than others from the lipid bilayer toward the extracellular surface (Ji et al., 1998; Kyte and Doolittle, 1982). The TM helix ends of rhodopsin inferred from electron paramagnetic resonance spectroscopy studies of spin-labeled Cys substituted residues (Altenbach, 1996; Altenbach, 1999a; Altenbach, 1999b) are consistent with the cytoplasmic ends of the seven TM helices in the high resolution structure of rhodopsin (Palczewski et al., 2000). However, spin-labelling studies have predicted a continuation of α-helical structure at the cytoplasmic ends of TM helices five, six and seven into the cytoplasm (Altenbach, 1996; Altenbach, 1999a). A possible explanation for this discrepancy is that the crystal structure does not accommodate a highly dynamic structure (even in the inactive state) of this region. Another possibility is that the natural membrane environment in which rhodopsin is embedded, is not present in the crystal structure. The lipid membrane bilayer consists of two complex regions of variable dielectric behaviour which sandwich a third low dielectric core region corresponding to the hydrophobic lipid chains. The phospholipid headgroups form a mixed hydrophobic/hydrophilic phase of which the cytoplasmic side of the membrane contains an abundance of negatively charged phospholipid headgroups which may interact through ionic interactions with Arg/Lys residues of the TM domains (Ballesteros et al., 2001a). At the cytoplasmic boundary of membrane proteins basic residues are three times more abundant than acidic residues resulting in a significant positive charge at the cytoplasmic side of these receptors. It is proposed that the positions of these Arg/Lys residues can be used to predict the cytoplasmic ends of TM helices (Ballesteros and Weinstein, 1995; Visiers, 2001), especially since a recent revision thereof agrees extremely well with spin-labelling studies in rhodopsin (Jensen et al., 2001) (Fig. 1.1). It is possible that in the crystal structure (Palczewski et al., 2000) the absence of negatively charged headgroups of the membrane bilayer may result in unstable or unfolded helical ends at the cytoplasmic boundaries in the structure. On the extracellular side of the TM helices, residues facing the interior of the helical bundle are expected to participate in ligand recognition and are therefore divergent through evolution causing sequence conservation methods to be inaccurate in predicting the extracellular end of the TM domain. It is proposed that the helical ends of rhodopsin (Palczewski et al., 2000) at the extracellular side are minimal ends (Ballesteros et al., 2001a). The mixed hydrophilic/hydrophobic phospholipid headgroup regions is proposed to provide an environment that may support key interactions of GPCRs with ligands from the extracellular side and on the intracellular side, with G proteins and other interacting proteins (Ballesteros et al., 2001a).

The two-dimensional electron crystallography of bovine rhodopsin receptor confirmed the hydrophobicity prediction of the arrangement of the seven alpha-helical transmembrane domains. The projection maps are characterised by an arc-shaped feature, which has been interpreted as the presence of tilted helices (Schertler et al., 1993; Unger and Schertler, 1995). Using tilted two dimensional crystals allowed the generation of the first three-dimensional maps, which provided further insight into the packaging of the seven TM helix bundle and calculation of the tilting angles of helices. These calculations are in good agreement with the high resolution crystal structure of rhodopsin (Palczewski et al., 2000) (Fig. 1.2). In rhodopsin TM helices one to three are tilted by 27-30°, TM helix five is tilted 23° and TM helices four and seven are almost perpendicular to the membrane. TM helix six is perpendicular on the cytoplasmic side, but is bent towards TM helix five on the extracellular side (Unger et al., 1997). TM helices one, two, three, five, six and seven of rhodopsin are distributed in a counter-clockwise arrangement, buttressed by the interaction of TM four with TM two and three forming a three-dimensional helix bundle, as seen from the extracellular side. TM helices two, three, five and six are less hydrophobic and form a hydrophilic pocket at the core of the helix bundle (Unger et al., 1997). Baldwin identified hydrophobic and hydrophilic faces of each TM helix (amphiphilic) and proposed that the hydrophobic side chains of amino acids face outwards to the lipid bilayer, while polar side chains face inwards and/or towards other TM helices. The spatial organisation and amphiphilic character of TM helices forms a hydrophilic pocket at the centre of the helix bundle. This cavity is surrounded by TM helices three, four, five, six and seven, and is closed to the intracellular side by the tilted TM three. The more hydrophobic TM helices one, four and seven are exposed to the lipid bilayer (Baldwin, 1993). The arrangement of TM helices may provide specific mechanisms for ligand binding and receptor activation. For example, TM helices one, two and seven are generally opposed, and if TM helix seven associates more closely with TM helix two than TM helix one, a long crevice is formed between TM helix one and helix seven. Such a crevice may offer binding sites for certain ligands.

The crystal structure of rhodopsin revealed that there are a number of potential interhelical hydrogen bonds and hydrophobic interactions mediated by highly conserved residues of the different TM helices (Palczewski et al., 2000). For example, $Asn^{1.50(55)}$ interacts with $Asp^{2.50(83)}$ which are both highly conserved (see appendix). Another well-described motif is the (Glu/Asp)-Arg-Tyr motif (Fig. 1.1) at the bottom of TM helix three, which is often been implicated in receptor activation (see section 1.2.3). In rhodopsin $Glu^{3.49(134)}$ interacts with $Arg^{3.50(135)}$ in the inactive receptor (Palczewski et al., 2000) which has been proposed before in other GPCRs (Ballesteros et al., 1998). $Arg^{3.50(135)}$ is proposed to interact with residues in other TM helices (six and seven)

during receptor activation (Ballesteros et al., 1998; Gether, 2000). Another characteristic amino acid motif is the Asn-Pro-X-X-Tyr motif in TM helix seven (Fig. 1.1). In rhodopsin $Asn^{7.49(302)}$ and $Tyr^{7.53(306)}$ project to the inside of the helix bundle (Palczewski et al., 2000). From the crystal structure it appears that the side chain of $Asn^{7.49(302)}$ may interact with $Asp^{2.50(83)}$ via a water molecule (Palczewski et al., 2000). Another interaction between TM helices two and seven is that the side chains of $Asn^{2.60(73)}$ and $Tyr^{7.53(306)}$ are close enough for a hydrogen bond to occur (Palczewski et al., 2000). The conserved Asn-Pro-X-X-Tyr motif has been implicated in receptor activation and G protein coupling in other GPCRs (Gether, 2000). In general, the high resolution (2.8Å) three-dimensional crystal structure of rhodopsin has agreed extremely well with models based on a projection map of rhodopsin and experimental data (Palczewski et al., 2000) (Fig. 1.2).

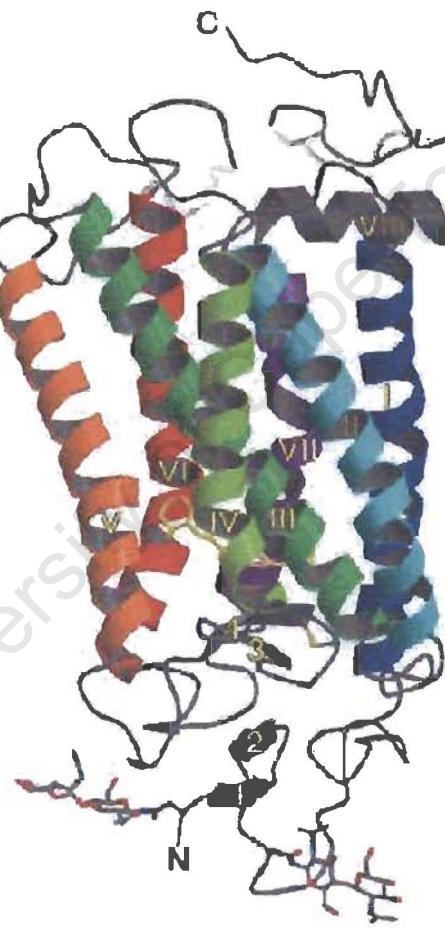

Figure 1.2 Three-dimensional representation of the Rhodopsin helical bundle drawn parallel to the membrane (reproduced from Palczewski et al., 2000). The TM helices are drawn as ribbons and extra- and intra-cellular domains as lines. The TM helices are in different colours and are respectively numbered in roman numerals. The N- and C- termini are indicated.

1.2.1.2 Structural Mimicry in GPCRs

The TM helices have the highest amino acid homology between the receptors of the same and different subgroups, which may contribute to a general receptor structure within a group of receptors. A given receptor can be identified as belonging to a specific GPCR family by the

presence of certain amino acid motifs conserved within the TM helices (Ji et al., 1998). However, is the percentage amino acid homology (~20%, Probst et al., 1992) between the different GPCRs enough to assume a similar structure? This also raises the question whether the recent crystal structure of rhodopsin is feasible as a structural template for other family A GPCRs. In a number of cases the sequence difference between rhodopsin and other GPCRs is remarkable such as the presence or absence of a Pro residue in a TM helix. A structural similarity seems unlikely if one considers that soluble proteins with a sequence homology of >25% share a clear structural similarity and that below this level of identity, structural divergence increases dramatically (Wilson et al., 2000; Yang and Honig, 2000). However, recently it has also been shown that different soluble proteins with different sequence patterns can assume the same fold (Yang and Honig, 2000). In GPCRs the structural similarity is not known as only one high-resolution structure has been determined from this superfamily. Nevertheless, recent analysis of molecular models and a host of experimental data on a variety of GPCRs combined with the high resolution structure of rhodopsin has revealed that the overall structures of the TM domains of rhodopsin and some GPCRs may be similar (Ballesteros et al., 2001a). It has been proposed that several of the unusual structural features of rhodopsin may also be present in other GPCRs despite that amino acids usually involved in these features are absent (eg. Pro). It has been proposed that different amino acids or alternate micro-domains may assume similar tertiary structures, through a form of structural mimicry (Ballesteros et al., 2001a). An example of structural mimicry has already been described in TM helix ends. Most GPCRs contain an abundance of basic residues at the cytoplasmic end of the TM domains. These positively charged residues are proposed to interact with negatively charged phospholipid headgroups as described. The function of this interaction may involve forming stable cytoplasmic boundaries for TM helices. The precise locus of the basic residues is not important to perform this function but its presence at the same region of the domain, which is proposed to result in the individual TM sizes across different GPCRs to be conserved (Ballesteros et al., 2001a).

The TM domains are proposed to assume defined structures, such as a β sheet in porins (Schirmer et al., 1995) and α-helices in rhodopsin (Unger and Schertler, 1995). Pro residues are frequently found in TM α-helices (Probst et al., 1992) and can kink the helix backbone by 26°, which may have an impact on protein structure (Barlow and Thornton, 1988). Pro residues are thought to influence packaging and organization of transmembrane helices (Vanhoof et al., 1995) and have been demonstrated to play key roles in GPCR expression, ligand binding and receptor activation (Hong et al., 1997; Sansom and Weinstein, 2000; Wess et al., 1993). Conserved Pro residues within TM helices are believed to be important in forming a hydrophilic binding pocket and being

involved in receptor activation by maintaining the correct position of helices and overall structure of GPCRs (Gether et al., 1997b; Konvicka et al., 1998). Generally, a Pro residue can distort an α-helix by preventing hydrogen bonding to the carbonyl oxygens of residues which are located 3-4 positions further in a turn of the helix. By bending the helices Pro may amplify conformational changes associated with receptor activation (Sansom and Weinstein, 2000).

As expected from experimental data and two-dimensional electron crystallography of rhodopsin, TM helices one, four, six and seven contain Pro-kinks in the recent rhodopsin crystal structure (Palczewski et al., 2000). However, Pro in TM helices does not always produce kinks as shown by TM helix five, which is almost straight. Moreover, the crystal structure of rhodopsin revealed that TM helices may display irregular helicy near Pro-kinks (eg. 3_{10}-helix in TM seven, see section 1.2.3.3 and Figs. 1.1 and 1.2) under the influence of other amino acids interacting with this region. Another interesting feature of rhodopsin is that TM helix two shows a typical Pro-kink structure in the absence of a Pro in this domain structure (Palczewski et al., 2000). The location of this kinked structure is defined by a Gly-Gly-X-X-Thr-Thr motif. The side chains of the two Thr residues in this motif can hydrogen bond to the carbonyls at the i-4 positions, stablising a bent conformation. Molecular simulation models of this structure revealed that a TM helix with this motif has the ability to achieve a conformation that can be superimposed on a TM helix with a regular Pro-kink (Ballesteros et al., 2001a). In the review of Ballesteros et al (2001a) many variations of structural mimicry are described. They proposed that structural mimicry is a mechanism by which a common ancestor could diverge sufficiently to develop the selectivity necessary to interact with diverse signals, while still maintaining the core function of GPCRs, which is signalling activation through a conformational change that is relayed to the cytoplasmic domain which interacts with G proteins.

1.2.1.3 Extra- and Intra-Cellular Loops of GPCRs

One of the major problems in modeling the loops is the low conservation pattern of these domains amongst GPCRs, compared to TM helices, which share 20-25% homology (Probst et al., 1992). Using indirect approaches, particularly the functional effect of site-directed mutagenesis and the construction of computational three-dimensional molecular models of the ligand receptor complexes sheds light on the mode of ligand binding and activation in GPCRs. These techniques provided insight to the function of two highly conserved Cys residues at the top end of TM domain three and in extracellular loop two (ECL2), respectively. These residues are proposed to form a disulphide bond important for structural integrity of many GPCRs (Davidson et al., 1997; Dixon et al., 1987; Karnik and Khorana, 1990; Karnik et al., 1988).

In the rhodopsin the extracellular domains associate to form a compact structure (Palczewski et al., 2000). The NH_2-terminus of the NH_2-terminal domain is located just below extracellular loop three (ECL3). The side chains of Asn^2 of the NH_2-terminal domain and $Asp^{7.29(282)}$ of ECL3 are in close opposition. Pro^{12} of the NH_2-terminal domain may also be in contact with ECL3. Pro^{23} of the NH_2-terminal domain is located close to $Tyr^{2.69(102)}$ of extracellular loop one (ECL1). These residues are proposed to maintain proper orientation between the NH_2-terminal domain and extracellular loop structure (Palczewski et al., 2000). ECL-1 and -3 run along the periphery of extracellular side of the helix bundle as opposed to ECL2 of which part folds deeply into the core of the helix bundle forming part of the agonist binding site. ECL2 contains two stretches of β-strand of which one (β_4 region) forms a lid over retinal, protecting it from the external environment (Fig. 1.2) (Palczewski et al., 2000). Residues in ECL2 have been demonstrated to determine pharmacological specificity of the α_1-adrenergic receptor for adrenergic ligands (Zhao et al., 1996) and differentiation between agonist and antagonist properties of a ligand in the GnRH receptor (Sun et al., 2001). Remarkably in the α_1-adrenergic receptor these residues seem to be aligned with the retinal contact residues in the β_4 region of ECL2, suggesting that there may be some structural similarity in this loop in other class A GPCRs (Ballesteros et al., 2001a).

The intracellular loops (ICLs) also exhibit structural organisation, for example, intracellular loops one (ICL1) and three (ICL3) are in close contact with the COOH-terminal tail and intracellular loop two (ICL2) has a distinct L-shaped structure (Palczewski et al., 2000). The role of extracellular loops (ECLs) in ligand-binding (Colson et al., 1998b; Perlman et al., 1997) and receptor activation (Zhao et al., 1998), mechanisms has been described for in GPCRs, and will be discussed in section 1.2.2.2.

1.2.2 Ligand Binding in GPCRs

GPCRs are generally activated by the binding of an agonist to a binding domain of the receptor, which is usually seated in the ECLs and TM binding pocket. Ligand binding and receptor activation are two distinct processes as shown by the existence of antagonists that competitively inhibit agonist binding. Interactions of ligands and receptors are proposed to involve hydrogen bonds, ionic interaction and hydrophobic contacts (Ji et al., 1998). The mechanics of receptor activation relies on agonist binding generating a conformational change in the receptor, which is transduced through the TM core to the cytoplasmic signal molecules (Gether and Kobilka, 1998).

Several distinct mechanisms have been described for receptor-ligand binding and resultant signal generation. These include high affinity binding; 1) exclusively to the TM core (retinal, biogenic amines, nucleosides, eicosanoids and lipid moieties), 2) to both the TM core and ECLs (peptides <40 amino acids), 3) to ECLs and N-terminal segments (polypeptides <90 amino acids), and 4) exclusively to the N-terminal segment (glycoproteins >30kDa) (Gether, 2000; Ji et al., 1998).

In GPCRs there is a large diversity of receptor-ligand interactions, and for this reason a general review will be presented on some GPCR ligand binding mechanisms with special focus on the family A class of GPCRs to which the GnRH receptors belong.

1.2.2.1 Ligand Binding Directly to the TM Domain

The classical small-molecule transmitters such as the biogenic amines (epinephrine, norepinephrine, dopamine and histamine) enter and bind the TM core of family A receptors (Gether and Kobilka, 1998; Strader et al., 1994). The charged amine of catecholamines /adrenergic ligands is thought to pair with the carboxyl group of $Asp^{3.32(113)}$ in TM helix three, which is found deep in the binding crevice formed by the TM helices (Strader et al., 1991). This aspartic acid is conserved among the biogenic amine receptors and is proposed to interact with the positively charged head group of other ligands such as dopamine (Mansour et al., 1992), serotonin (Wang et al., 1993), histamine (Gantz et al., 1992) and acetylcholine (Spalding et al., 1994). In the β_2-adrenergic receptor, $Asp^{3.32(113)}$ is important in ligand binding but not for signal generation. Additionally, the catechol ring of the ligand docks to a binding pocket consisting of TM helices five and six. It is proposed that the OH-groups of the catechol ring hydrogen bond to $Ser^{5.43(204)}$ and $Ser^{5.46(207)}$, which lie one α-helical turn apart on the same side of the TM five α-helix of the β_2-adrenergic receptor. This interaction would constrain the TM five α-helix influencing the TM structure (Strader et al., 1994). In TM helix six, $Phe^{6.52(290)}$ stabilises the catechol ring (Tota and Strader, 1990) and $Asn^{6.55(293)}$ forms a hydrogen bond with the β-OH group of epinephrine (Wieland et al., 1996). These proposed interactions with the catechol ring of the ligand may constrain the TM structure and packing resulting in receptor activation and generate a signal (Ji et al., 1998).

Similarly, the eicosanoids which are derived from arachidonic acid and include leukotrienes and prostanoids (prostaglandins, prostacyclins and thromboxanes) bind eiconsanoid receptors (family A) directly to the TM core (Kobayashi et al., 1997; Kobayashi et al., 2000). Other families of GPCRs also have ligands that bind directly to the TM core. For example, extracellular adenosine, adenine

nucleotides and uridine nucleotides are ligands that bind the TM core of P2Y receptors (Jiang et al., 1997; Kim et al., 1995; Rivkees et al., 1995).

Ligand binding and receptor activation are difficult to distinguish, and often receptor activation is considered a separate event from ligand binding. Rhodopsin (family A) has a unique receptor-ligand binding mechanism, in which its ligand, the retinal chromophore is covalently bound through the formation of a Schiff's base between the aldehyde moiety of retinal and the ε-amine of $Lys^{7.43(296)}$ of the receptor within a binding crevice formed by TM helices (Sakmar, 1998; Strader et al., 1994). The $Lys^{7.43(296)}$ side chain is directed along the longitudinal axis of rhodopsin and is supported by the two hydrophobic side chains of $Met^{1.44(44)}$ and $Leu^{1.52(47)}$ in TM helix one. This region is further stabilised by the phenyl rings of $Phe^{7.40(293)}$ and $Phe^{7.41(294)}$ interacting with other helices (Palczewski et al., 2000). Additional agonist-receptor interactions are the pairing of the protonated Schiff's base with conserved $Glu^{3.28(113)}$ which is proposed to bring TM helices three and seven in apposition (Zhukovsky et al., 1992), between retinal and aromatic residues at the top of TM helix six (Nakayama and Khorana, 1991), and between the C9 group of retinal and $Gly^{3.36(121)}$ (Han et al., 1997). From the high resolution crystal structure of rhodopsin it is evident that the retinylidene group runs almost parallel to TM helix three which provides side chains for the binding pocket consisting of residues, $Glu^{3.28(113)}$, $Gly^{3.29(114)}$, $Ala^{3.32(117)}$, $Thr^{3.33(118)}$, $Gly^{3.35(120)}$ and $Gly^{3.36(121)}$ (Palczewski et al., 2000). Absorption of a photon causes a change in the geometry of the retinal chromophore buried deep within the TM helix bundle (Rath et al., 1998). The light induced isomerisation of the inverse agonist, 11-cis-retinal, to the agonist, all-trans retinal, is thought to induce rigid body movements of the TM helices forcing a rapid transition of rhodopsin to the active metarhodopsin II (MII) state (Gether and Kobilka, 1998). This causes tertiary structural changes in the solvent exposed cytoplasmic interhelical loops opening high affinity sites for the binding and activation of various signalling proteins (Abdulaev and Ridge, 1998). After the photolysed chromophore transiently activates opsin, all-trans retinal is hydrolysed and dissociates from the opsin. This is followed by rhodopsin being regenerated by newly synthesised 11-cis-retinal delivered from adjacent retinal epithelial cells (Farrens and Khorana, 1995).

1.2.2.2 The Ligand Binding Domain Involving ECLs and TM Domain

Substance P, neurokinin A, neurokinin B are mammalian tachykinins which interact with extracellular domains as well with residues in the transmembrane regions of the neurokinin (NK)-1, NK-2 and NK-3 receptors (family A) respectively (Krause et al., 1997; Maggi et al., 1993). These receptors are homologous but have a distinct pharmacology between each other (Gether et al.,

1993; Maggi et al., 1993; Yokota et al., 1992). Chimeric NK-1/NK-3 receptors revealed that there are multiple epitopes scattered throughout the receptor structures, which contribute to different binding sites, thus enabling subtype selectivity of the tachykinin peptides (Gether et al., 1993; Yokota et al., 1992). Substance P is proposed to be mainly involved in binding the extracellular domains of the receptor as demonstrated with site-directed mutagenesis of amino acids in the N-terminus, and tops of TM helices three and seven (Fong et al., 1992b; Fong et al., 1992a; Huang et al., 1994). The importance of the extracellular loop regions in substance P binding is further supported by affinity cross-linking of a photolabile and radioactively labelled substance P to a Met residue in ECL3 (Boyd et al., 1996; Kage et al., 1996). On the other hand, mutational studies of neurokinin A binding to the NK-2 receptor provided evidence of interactions with the TM regions as well with residues in the extracellular domains. This suggests that neurokinin A may partially enter the TM binding crevice (Bhogal et al., 1994; Huang et al., 1995). Thus, the different ligand binding sites seem to contribute to different binding modes in a homologous group of receptors among homologous peptides.

The GnRH receptor, which belongs to family A, binds its cognate ligand, GnRH, partially in the extracellular loops and TM regions (Sealfon et al., 1997). In the human GnRH receptor, mutation and affinity-labelling studies of GnRH suggest that the N terminus of GnRH binds the TM core (Davidson et al., 1990). It has been shown that residues $Asp^{2.61(98)}$, $Trp^{2.64(101)}$, $Asn^{2.65(102)}$, $Lys^{3.32(121)}$ and $Asn^{5.61(212)}$ have roles in ligand binding (Davidson et al., 1996a; Flanagan et al., 2000; Hoffmann et al., 2000; Zhou et al., 1995). Some of these receptor residues have been proposed to form part of the ligand binding pocket, interacting with the amino- and carboxy-termini of GnRH in a computational model of the receptor-ligand complex (Sealfon et al., 1997). $Asp^{2.61(98)}$ is proposed to interact with His^{2} of GnRH, while $Asn^{2.65(102)}$ interacts with Gly^{10}-NH_2. $Trp^{2.64(101)}$, $Lys^{3.32(121)}$, and $Asn^{5.61(212)}$ are important for binding of agonists, but not antagonists (Hoffmann et al., 2000; Zhou et al., 1995). In the mouse GnRH receptor, $Glu^{7.32(301)}$ in ECL3 was shown to have a role in recognizing the Arg^{8} residue of GnRH (Flanagan et al., 1994). In the human GnRH receptor this residue is $Asp^{7.32(302)}$ and the role of this residue in a ligand binding mechanism is investigated in this thesis. It is proposed that the interaction between $Asp^{7.32(302)}$ in ECL3 and Arg^{8}-containing GnRH serves to induce GnRH to assume a conformation that can interact with the binding pocket with a high affinity (Fromme et al., 2001). In section 1.3 a more detailed description of GnRH and the GnRH receptor binding pocket is given.

Other GPCRs belonging to family A also bind their cognate ligands partially in the extracellular loops and TM regions (Angiotensin and TRH receptors). For example, the hydrophobic C-terminal region of angiotensin II is proposed to enter the TM core of the angiotensin receptor, favouring Phe^{8} of angiotensin II to interact with $Lys^{5.42(199)}$ in TM helix five, which is 7-14 Å from the extracellular surface (Ji et al., 1998; Noda et al., 1995b; Noda et al., 1995a). Conversely, Asp-Arg of angiotensin II forms an ionic interaction with $His^{5.26(183)}$ in ECL2 and $Asp^{7.32(281)}$ in ECL3, the latter amino acid being important in signal generation (Feng et al., 1995).

It is evident that ligand binding to both the extracellular and TM domains may not always occur simultaneously. For example, the TRH receptor contains several amino acids in the ECLs and in the TM regions which form part of a multi-step ligand binding mechanism (Perlman et al., 1997). The extracellular domains are the first contact sites of a ligand with its receptor. This interaction may influence receptor activation directly (Parma et al., 1995), form part of a ligand binding pocket with other residues important for activation (Hoffmann et al., 1999) or be part of a ligand binding mechanism necessary for ligand entry into the TM binding core (Colson et al., 1998b). It was shown that $Tyr^{4.71(181)}$ in ECL2 of the TRH receptor is important for high affinity binding with the pyro-Glu moiety of TRH. Pyro-Glu of TRH also interacts with $Asn^{3.37(110)}$ in TM helix three and $Asn^{7.22(289)}$ in ECL3 (Perlman et al., 1997). With a computer-generated model of the TRH receptor, it was predicted that the small tripeptide TRH couldn't simultaneously interact with the proposed residues in ECL domains and TM regions. Kinetic analysis suggest that TRH initially interacts with residues $Tyr^{4.71(181)}$ and $Asn^{7.22(289)}$ in the ECLs and then moves into the TM binding pocket. It was suggested that the ECLs form an entry channel into the TRH-receptor-TM bundle and that $Tyr^{4.71(181)}$ projects into this channel and mainly accounts for high affinity binding (Perlman et al., 1997). The initial interaction may serve to position TRH for access into the TM domain. In the TM bundle, the pyro-Glu of TRH interacts with $Asn^{3.37(110)}$ in TM helix three, $Tyr^{7.15(282)}$ in TM helix six and $Arg^{7.39(306)}$ in TM helix seven, respectively, generating a signal (Colson et al., 1998a; Perlman et al., 1997). This group constructed a model of the ECLs of the TRH receptor and used molecular dynamics simulations and quasi-harmonic analysis to study the static and dynamic roles of the extracellular domain (Colson et al., 1998b). The model incorporated experimental evidence of the formation of an initial recognition site between TRH and the surface of its receptor. A "quasi-harmonic analysis of the vibrations of the extracellular loops" suggests that the low frequency motions of the loops will aid the access of the ligand to access the TM binding pocket. It was proposed that the initial interaction of TRH with the surface binding site contributes to the disruption of the interactions keeping the ECLs static, introducing anti-correlated vibrational

motion of ECL-2 and -3. Inducing conformational changes in the loops may open a pathway for TRH into the TM binding pocket (Colson et al., 1998b). They suggested that this binding mechanism might be applicable to other GPCRs that bind small ligands.

Residues in ECL3 seem to play an important role in ligand binding, ligand recognition and receptor activation. As shown in the TRH receptor this loop may also contribute to interactions, which keep the ECLs static in the inactive receptor conformation. Disruption of these interactions is proposed to be involved in exposing the TM core for further ligand binding, necessary for receptor activation (section 1.2.3). In rhodopsin $Asp^{7.29(282)}$ in ECL3 is located in close opposition to Asn^{2} of the NH_2-terminus and may have a role in the structural organisation of the extra-cellular domains (Palczewski et al., 2000). It is possible that residues in ECL3 which are implicated in ligand binding (eg GnRH and TRH receptor) may also have additional roles such as structural organisation of the receptor either in the inactive or active conformation. In support, it has been shown that ECL3 of the β_2-adrenergic receptor can alter receptor-G protein affinity (Zhao et al., 1998). In this study they demonstrated that replacing ECL3 in the β_2-adrenergic receptor with the corresponding loop of the α_1-adrenergic receptor resulted in a higher affinity for agonists, but not antagonists, increased agonist potency and an agonist-independent higher basal activity (constitutive activity). This result supports the emerging hypothesis that ECLs of GPCRs may play a role in receptor activation.

1.2.2.3 Multistep Ligand Binding to the N-terminal Segment and ECLs

Glycoprotein hormones, LH, FSH are large heterodimers (20-30 kDa) consisting of a common α-subunit and a hormone specific β-subunit. These hormones initially bind the N-terminus of the cognate GPCR (family A) with high affinity. The N-terminal segment of the hormone undergoes conformational changes once it binds to the N-terminus of the receptor, allowing it to further bind the membrane associated domain, generating a signal (Ji et al., 1998). The N-terminal segment initially forms multiple high affinity contact sites with both subunits of the glycoprotein hormone (Grossmann et al., 1997). Once the ligand is bound to the N-terminus, the liganded N-terminus further interacts with secondary contact sites on the ECLs of the receptor (Remy et al., 1996; Ryu et al., 1998).

It is proposed that partial entry of the ligand into the TM core is possible as found in the parathyroid hormone (PTH) receptors (family B). The C-terminal region of PTH is important for receptor binding, whereas the N-terminal region of the hormone is important for receptor activation. The N-terminal segment of the receptor is primarily important for ligand specificity and

a signal is generated in the membrane associated part of the receptor, involving $Ile^{3.39(244)}$ in TM helix three and $Tyr^{4.31(318)}$ in ECL2 (Adams et al., 1998; Bergwitz et al., 1997). It has been proposed that PTH bind its receptor in two steps. Firstly, to form a transient PTH-N-terminal segment complex, and next for the complex to interact with the membrane associated domain to generate a signal (Bergwitz et al., 1997).

In conclusion it seems evident from section 1.2.2 that GPCRs have evolved specific mechanisms to selectively bind their cognate ligands, which in turn induces a conformational change in the receptor, allowing the execution of a common function of GPCRs, to generate an intracellular signal.

1.2.3 GPCR Activation

G protein-coupled receptor activation is generally assumed to result in a significant structural rearrangement of the receptor, involving rigid body movement of TM helices (Hulme et al., 1999). Agonists are thought to induce or stabilise conformational changes in the receptor that facilitate interaction with cognate G proteins necessary for signal propagation (Gether, 2000).

1.2.3.1 Models of Receptor Activation

The extended ternary complex is a widely accepted model for GPCR activation. This two-state model proposes that there is an equilibrium between the inactive R and active R* states of GPCRs (Samama et al., 1993). In the absence of a bound agonist, the equilibrium is shifted to the inactive R state, resulting in low basal levels of ligand-independent signalling. Agonists are thought to preferentially bind the R* conformation and stabilise it, increasing the proportion of receptors in the R* state. Inverse agonists (or negative antagonists) are able to inhibit the agonist-independent receptor activity (basal levels) by preferentially binding and stabilizing the inactive R state. Inverse agonists preferentially bind the R state and consequently reduce the number of receptors in the R* state (Gether and Kobilka, 1998). Neutral antagonists bind the receptor with the same affinity to both R* and R states and cause no change in the equilibrium (Black and Shankley, 1995). However, it is evident that a model representing only two conformational states cannot accommodate the wide variety of complex behaviour in GPCRs (Gether and Kobilka, 1998). Furthermore, this model could not accommodate the observation that both agonists and inverse agonists protect some GPCRs against thermal denaturation (Gether et al., 1997b) and proteolysis (Kobilka, 1990). This led to the formulation of another model, which predicts that the unliganded receptor exists in a unique conformational state (R^o) that can undergo transitions to other

conformational states, such as R and R* (Strader et al., 1995). Strader et al, proposed that R^o is stabilised by inverse agonists and R* is stabilised by agonists. They also proposed that agonist binding occurs sequentially, resulting in a series of conformational states that are intermediates between the R and R* state. Different structural groups of agonists may bind to different receptor conformational states in a sequential order resulting in stabilising the R* state of the receptor (Strader et al., 1995). Conversely, there are various degrees of inverse agonism, which may induce various sequential structural changes and can stabilise the inactive conformation of a GPCR in a similar way (Gether, 2000). For example, the extended ternary complex model was used to estimate the fraction of constitutively active mutant (CAM) α_{2A}-adrenergic receptors in the active state by comparing the relative inverse efficacies of a series of antagonists (Wade et al., 2001). In contrast, neutral antagonists bind both R and R* equally well, have no effect on basal receptor activity and block the effects of both agonists and inverse agonists (Chidiac et al., 1994).

The discovery of different binding sites for peptide agonists and non-peptide antagonists suggested that GPCRs might exist in multiple-conformational states (Gether et al., 1995b; Schambye et al., 1995; Wiens et al., 1998). A multistate model was proposed, in which the receptor may alternate spontaneously between multiple active and inactive conformations. In this model, an agonist would bind a receptor conformation that activates the receptor, and an inverse agonist would bind the inactive receptor conformation (Gether, 2000). This model could accommodate two agonists sharing the same receptor without sharing a common binding site and both agonists must therefore stabilise an active conformation. Alternatively, competitive antagonists are proposed not to require any overlapping binding site with agonists and may stabilize distinctly different receptor conformations from agonists (Gether, 2000; Gether et al., 1995b; Schambye et al., 1995). Moreover, it has been shown that functionally different agonists induce distinct conformations in the G protein-coupling domain of the β_2 adrenergic receptor (Ghanouni et al., 2001). In this study, ligand-induced conformational changes in the G protein-coupling domain of the β_2 adrenergic receptor were measured with fluorescence lifetime analysis of a reporter fluorophore covalently attached to the G protein-coupling domain. The conformations induced by a full agonist were different from those induced by partial agonists, providing new insight into the basis of agonism and partial agonism (Ghanouni et al., 2001).

1.2.3.2 GPCRs Constrained in the Inactive Conformation

It is becoming increasingly evident from mutagenesis, biophysical and modelling studies that GPCRs contain a network of radial constraints, centred on TM helix three, which stabilise the

inactive ground state of the receptor. Upon agonist binding the receptor is induced to form a new set of axial interactions which help to stabilise the activated state (Hulme et al., 1999).

It has been encountered that specific mutations are able to increase constitutive agonist-independent GPCR activity (Kjelsberg et al., 1992; Scheer et al., 1996). Natural mutations that cause constitutive activation are linked to genetic diseases, such as mutations in the LH receptor leading to precocious puberty (Shenker et al., 1993). The molecular mechanism of constitutive activation of GPCRs was defined in a naturally occurring mutation ($Ala^{6.34(293)}$) in ICL3 of the α_{1B}-adrenergic receptor (Kjelsberg et al., 1992). Substitution of $Ala^{6.34(293)}$ with any other residue (except glycine) resulted in agonist-independent receptor activation. The Lefkowitz group formulated a hypothesis, which suggested that constraining intermolecular interactions have been conserved during evolution to maintain the receptor preferentially in the inactive conformation in the absence of an agonist (Kjelsberg et al., 1992). It was proposed that during agonist binding (or specific mutations) these inactivating constraints could be released as part of the receptor activation. This theory was supported by showing that proteins of constitutively activated β_2-adrenergic receptor mutants are structurally unstable and have enhanced conformational flexibility (Gether et al., 1997b; Rasmussen et al., 1999). They suggested that the mutational changes have disrupted the stabilising intramolecular interactions in the tertiary receptor structure, allowing the equilibrium of the receptor R state to be spontaneously shifted to the R* state, resulting in constitutive activation.

There is extensive evidence in GPCRs of the existence of a stabilising network of intramolecular interactions constraining the receptor in the inactive conformation. With the use of engineered metal binding sites, presumptive helix-helix interactions have been identified in the neurokinin (NK-1) (Elling and Schwartz, 1996), kappa-opioid (Thirstrup et al., 1996), rhodopsin (Sheikh et al., 1996), β_2-adrenergic (Sheikh et al., 1999) and m1-muscarinic acetylcholine (Lu and Hulme, 2000) receptors. In these studies a series of histidine substitutions were used to generate a zinc(II) binding site between TM helices five and six (NK-1 and kappa-opioid receptor), TM helices three and six (rhodopsin and β_2-adrenergic receptor), and TM helices three, six and seven (muscarinic acetylcholine receptor) which prevented receptor activation. The disruption of a presumed salt bridge between TM helices three and seven results in the constitutive activation of the rhodopsin receptor without its agonist (Robinson et al., 1992), which indicates that rhodopsin may have a predisposition to the active conformation. These studies suggest that rhodopsin-like receptors may be constrained by a stabilising network of interhelical-molecular interactions which upon agonist

activation is switched to a new set of contacts which define the activated state that binds the cognate G-protein.

1.2.3.3 Protonation Needed for GPCR Activation

One of the key events in activation of family A GPCRs is the protonation of the $Asp^{3.49}$ in the conserved Asp/Glu-Arg-Tyr motif. It was demonstrated that the $Glu^{3.49(134)}$Gln mutant rhodopsin receptor had improved coupling efficiency suggesting that protonation of $Glu^{3.49(134)}$ is connected to the formation of the activated metarhodopsin state (Arnis et al., 1994). It was observed that charge neutralising mutations (Asn/Gln), which mimic the protonated state of Asp or Glu at locus 3.49

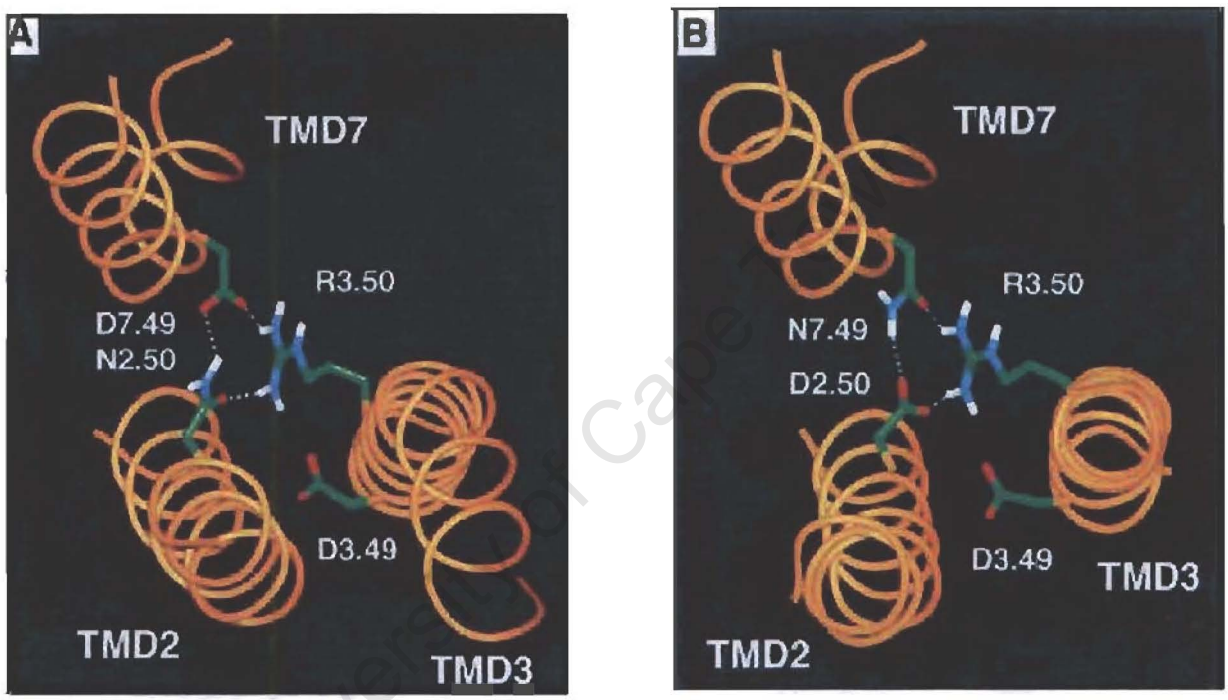

Figure 1.3 Three-dimensional model showing reciprocal interactions between TM helices two, three and seven of the GnRH and 5-HT_{2A} receptors (reproduced from Ballesteros et al., 1998). A) $Arg^{3.50}$ of the type I GnRH receptor can interact with $Asp^{3.49}$ as well as $Asp^{7.49}$ and $Asn^{2.50}$. B) $Arg^{3.50}$ of the 5-HT_{2A} receptor interacts $Asp^{3.49}$ and the carbonyls of $Asn^{7.49}$ and $Asp^{2.50}$. This set of interactions can be mimicked by a reciprocal configuration as in the type I GnRH receptor.

cause constitutive activation in the α_{1B}-adrenergic (Scheer et al., 1997) and β_2-adrenergic receptors (Rasmussen et al., 1999). In the GnRH receptor mutating $Asp^{3.49(138)}$ to Asn in the Asp-Arg-Ser (DRS) motif (Fig. 1.3 and 1.4) (equivalent to the Asp-Arg-Tyr) is associated with improved coupling (Ballesteros et al., 1998). One of the most conserved amino acids in the GPCR family is $Arg^{3.50}$ in this motif. This residue has been shown to play a role in coupling in the rhodopsin (Arnis et al., 1994), adrenergic (Rasmussen et al., 1999), muscarinic-acetylcholine (Jones et al., 1995), 5-HT_7 (Obosi et al., 1997), V2-vasopressin (Rosenthal et al., 1993), histamine (Alewijnse et al., 2000) and GnRH (Ballesteros et al., 1998) receptors. Scheer et al hypothethesized that $Arg^{3.50(143)}$

in the α_{1B}-adrenergic receptor is constrained in a hydrophilic pocket formed by the conserved polar residues in TM helices one, two and seven. In this model, the ionic counterpart of $Arg^{3.50(143)}$ is $Asp^{2.50(91)}$ in TM helix two (Scheer et al., 1996). Alternatively, Ballesteros et al proposed that in the GnRH receptor, the orientation of the highly conserved $Arg^{3.50(139)}$ side chain is constrained in the inactive conformation by an ionic interaction with $Asp^{3.49(138)}$ in the Asp-Arg-Ser (DRS) motif (Fig. 1.3A) (Ballesteros et al., 1998).

The high resolution crystal structure of rhodopsin in the inactive state show that $Glu^{3.49(134)}$ and $Arg^{3.50(135)}$ interact, supporting this proposal (Palczewski et al., 2000). In the 5-HT_{2A} and GnRH receptors it is proposed that agonist binding is associated with the protonation of $Asp^{3.49}$ resulting in the release of the $Arg^{3.50}$ side chain, which then under the guidance of $Ile^{3.54}$ interacts with $Asp^{2.50}$ (or $Asp^{7.49(319)}$ in the GnRH receptor), stabilising the R* conformation (Fig. 1.3). In order to interact with Asp in either position 2.50 or 7.49, the $Arg^{3.50}$ side chain has to move from is original interaction with $Asp^{3.49}$ by rotating TM helix three, thus inducing receptor activation (Ballesteros et al., 1998; Hulme et al., 1999). Recently, it has been shown that a specific intrahelical position and orientation of the Asp-Arg-Ser motif may be important in stabilising high affinity receptor conformations that are uncoupled from their cognate G proteins (Kitanovic et al., 2001). However, in the α_{1B}-adrenergic receptor there is evidence that $Arg^{3.50}$ is not directly involved in G protein activation. The charge-conserving mutation of $Arg^{3.50(143)}$ to Lys resulted in decreased signalling, but preserved the maximal agonist-induced response and conferred substantial constitutive activity to the α_{1B}-adrenergic receptor (Scheer et al., 2000). This suggests that $Arg^{3.50}$ may have a role in changing the overall GPCR conformation from the inactive to active state.

1.2.3.4 Conformational Changes in GPCRs during Activation

Over the last decade, the physical changes accompanying the transition of rhodopsin from the inactive to the active state have been analysed by different forms of spectroscopy including Fourier transform infrared resonance spectroscopy, (Garcia-Quintana et al., 1995), surface plasmon resonance spectroscopy (Salamon et al., 1994), tryptophan UV absorbance spectroscopy (Lin and Sakmar, 1996), and EPR spectroscopy (Farahbakhsh et al., 1993; Farrens et al., 1996; Resek et al., 1993). These approaches have provided evidence that conformational rearrangement occurs in the transition of rhodopsin to metarhodopsin II. Importantly, Sakmar and co-workers used tryptophan UV absorbance spectroscopy and mutagenesis studies to obtain evidence that photoactivation of rhodopsin involves movement of TM helix three relative to TM helix six (Lin and Sakmar, 1996). Consistent with this, EPR spectroscopy in combination with multiple cysteine substitutions provided further insight into the character of conformational changes in activated rhodopsin

(Farahbakhsh et al., 1993; Farrens et al., 1996; Resek et al., 1993). This method enabled the relative distance between TM helices three and six to be measured, by site-selective incorporation of pairs of sulfhydryl-reactive spin labels into a sequence of double cysteine mutants of rhodopsin (Farrens et al., 1996). They proposed that TM helix six undergoes a significant rotational movement and that the cytoplasmic end of this helix moves away from TM helix three during receptor activation.

Javitch et al provided evidence for conformational rearrangement of TM helix six in a constitutively active β_2-adrenergic receptor. Their data suggested that a cysteine in TM helix six is normally not accessible to modification by a charged hydrophilic sulfhydryl-specific reagent in the wildtype β_2-adrenergic receptor, but becomes accessible in a constitutively active mutant (Javitch et al., 1997). Gether et al used fluorescence spectroscopic techniques in the first direct structural analysis of conformational changes in a ligand-activated β_2-adrenergic receptor (Gether et al., 1997a; Gether et al., 1997b; Gether et al., 1995a). It was proposed that TM helices three and six are involved in a rearrangement during receptor activation. In support of Schwartz's multistate model (Section 1.3.2.1), Gether et al., provided evidence that not only agonists, but also inverse antagonists can induce various structural changes in GPCRs.

Thus, both biophysical and mutagenesis studies of rhodopsin and ligand-activated β_2-adrenergic receptor GPCRs provide evidence that the formation of the R* state involves movements of TM helices three and six. However, conformational changes may not be limited to ligand-activated movement of TM helices in relation to each other, but also involve movement within TM helices. Monte Carlo simulations have shown that the movement of TM helix six is associated with a change in the kink produced by the conserved Pro residue in this helix and that movement of TM helix three involves rigid body torsion. Rhodopsin, (Palczewski et al., 2000), shows a large kink produced by Pro in TM six in the inactive form of the receptor (Sansom and Weinstein, 2000). It has been proposed that this kink is significantly diminished in the activated β_2-adrenergic receptor (Ballesteros et al., 2001b). In rhodopsin TM helix six is proposed to rotate and its cytoplasmic side moves away from TM helix three, suggesting a change in the kink of TM helix six (Farrens et al., 1996). Furthermore, disulfide cross-linking of the helices prevented activation of transducin (G protein), which suggests the importance of this movement for activation of rhodopsin (Dunham and Farrens, 1999). It is proposed that the activation of a GPCR may induce a cascade of rearrangements in the conformation of TM helix six that use the flexibility of the Pro kink in the

transduction of the ligand-binding signal to the ICLs where G protein coupling takes place (Sansom and Weinstein, 2000).

In GPCRs, TM helix seven plays an important role in signal transduction. This TM helix contains a highly conserved (Asn/Asp-)-Pro-X-X-Tyr (N/DPXXY) motif (Fig. 1.1), which was proposed to introduce a perturbation in the α-helix and form part of a flexible hinge region (Konvicka et al., 1998). In rhodopsin this $Asn^{7.49(302)}$ and $Tyr^{7.53(306)}$ project towards the inside of the helical bundle, which is determined by an unusual structure observed in rhodopsin (Palczewski et al., 2000). Around $Pro^{7.50}$ there is a tightly wrapped 3_{10} helix at position 7.43-7.46 which is proposed to be stabilised by the covalent attachment of retinal to $Lys^{7.43(296)}$ (Ballesteros et al., 2001a). It was shown with molecular dynamics (MD) simulations that the Tyr in the (Asn/Asp-)-Pro-X-X-Tyr (N/DPXXY) motif (Fig. 1.1) sustains an intra-helical network of hydrogen bonds (Duong, 1999), and reaches out to a stabilising inter-helical network of hydrogen bonds in the receptor (Konvicka et al., 1998). However, this was not evident in rhodopsin (Palczewski et al., 2000), but may be present in receptors that do not have a tethered ligand. Ballesteros et al, suggested that when the receptor changes from the inactive to the active conformation, the structure of the (Asn/Asp-)-Pro-X-X-Tyr motif transforms between the Pro kink, as observed in rhodopsin (Palczewski et al., 2000) and a characteristic Pro-induced hinge conformation in the (Asn/Asp-)-Pro-X-X-Tyr motif (Konvicka et al., 1998). It has been proposed that in a specific motif within a helix, Pro may form a TM-helix-based hinge capable of two sorts of motion, twisting and kinking (Sansom and Weinstein, 2000). They proposed that molecular hinges or swivels in transmembrane α-helices may play an important role in receptor activation and signal transduction in GPCRs. Crystal structures of rhodopsin or other GPCRs in different states of activation would provide further insight to conformational changes in GPCRs during activation. It has also been proposed that several of the highly unusual structural features of rhodopsin are also present in other GPCRs, despite the absence of amino acids that are thought to be critical for the adoption of these features (Ballesteros et al., 2001a). This means that various combinations of amino acid motifs may support a similar receptor tertiary structure with a conserved function. Ballesteros et al, proposed that GPCRs have conserved a common core function of signalling and that through a conformational switch in the transmembrane domains, the cytoplasmic surface is orientated optimally for G protein interaction (Ballesteros et al., 2001a).

1.2.3.5 GPCR-G Protein Interaction

It is proposed that a specific network of amino acid interactions may keep the receptor constrained in the inactive state (Ballesteros et al., 1998; Gether, 2000; Hulme et al., 1999; Scheer et al., 2000). These constraints are released upon agonist binding causing conformational changes in the arrangement of the TM helices through rotation and/or protonation of intra- and inter-TM helix interactions. These physical changes in receptor conformation are proposed to modulate the three-dimensional arrangement of the intracellular domains, exposing ICL sequences which can bind one or more guanine nucleotide binding proteins (G proteins), previously inaccessible for binding (Samama et al., 1993). This ternary complex transmits this signal further via the G proteins (see next paragraph) by activating various effector enzyme cascades and/or ion channels. The activated effectors generate a large number of intracellular messenger molecules, which activate protein kinase signalling pathways, necessary for various cellular responses (Hall et al., 1999; Naor et al., 2000).

G proteins are heterotrimers consisting of α, β, and γ subunits (Taylor, 1990). The X-ray crystallography of a heterotrimeric G protein provided insight into its tertiary structure and revealed the mechanism of the nucleotide-dependent engagement of the α and β-γ subunits that regulates their interaction with the receptor and effector molecules (Lambright et al., 1996). In the basal state the α-subunit is bound to GDP which favours association of α- and β-γ-subunits. During receptor activation the specific sequences of ICLs are exposed which mainly associate with the α-subunit of the G protein. This stimulates the G protein to bind GTP instead of GDP. Binding of GTP causes the α-subunit to take on another conformation (Lambright et al., 1996), resulting in the dissociation of the G protein from the receptor and intra-molecularly, into a free GTP-bound α-subunit and β-γ complex (Conklin and Bourne, 1993). These products regulate the various effector molecules necessary for an intracellular response (Exton, 1994; Hall et al., 1999). The α-subunit has an intrinsic hydrolytic activity that converts GTP to GDP and returns the G protein to its inactive form (Lambright et al., 1996). There are different types of G protein subfamilies, which activate different signalling pathways. Gsα stimulate adenylate cyclase, Gtα activates a cyclic GMP-dependent phosphodiesterase, Gi and Go are involved in the regulation of ion channels and Gq regulates PLC dependent signalling pathways (Sternweis and Smrcka, 1992; Taylor, 1990).

Many studies have established pivotal roles of ICL-2 and -3 of GPCRs, and in some cases the proximal part of the C-terminal tail, in G protein-coupling. Chimeric substitutions have clearly defined ICL3 as the most important determinant in G protein-coupling selectivity (Wess, 1998).

Wess and co-workers have shown that a four amino acid epitope in ICL3 of the m2 muscarinic acetylcholine receptor can specifically recognise the C-terminal five amino acids of α-subunits of the Gi/o protein family (Liu et al., 1995). ICL2 is more important for the efficiency of G protein activation, than G protein specificity. In the m5 muscarinic receptor it was demonstrated that substituting residues clustering on one side of the presumed α-helix of ICL2 caused constitutive activation of the receptor. Residues clustering the opposite side of ICL2 affected G protein-coupling. This suggests that the one side of ICL2 plays a role in maintaining the receptor in the inactive state, while the opposite side is important in G protein activation. It was proposed that ICL2 could act as a switch that enables G protein-coupling (Burstein et al., 1998).

Although the majority of GPCRs can mediate their signal transduction through G proteins, there is accumulating evidence that GPCRs are capable of signalling through interactions with other proteins (reviewed by Lefkowitz, 1998). For example, some GPCRs can activate PLD through small G proteins (RhoA and Arf) and through transactivation of tyrosine kinase receptors. Additionally, there are seven transmembrane molecules that do not couple to G proteins (Ji et al., 1998). For the purpose of this thesis an overview pertaining to the signalling pathway of the GnRH receptor, which does couple to G proteins, will be described in section 1.3.2.6.

1.3 GnRH and its Receptor

The hypothalamic neuropeptide, GnRH interacts with GnRH receptors on the gonadotropes in the anterior pituitary. Activation of the GnRH receptor is required for the biosynthesis and release of the glycoprotein gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The gonadotropins target the ovaries and testis to regulate steroidogenesis and gametogenesis which is required for normal reproductive function (Fink, 1988).

Multiple forms of GnRH are present in most vertebrate species and are thought to have physiological functions in addition to regulating pituitary hormone release (King and Millar, 1995; Sherwood et al., 1993). The mammalian pituitary GnRH receptor is proposed to discriminate between endogenous forms of GnRH (King and Millar, 1995). Moreover, the cloning and characterisation of the non-mammalian GnRH receptors established that three different types of GnRH receptors exist, which display a unique ligand selectivity and pharmacology (Sun et al., 2001). Variations in the amino acid sequence between the mammalian and non-mammalian GnRH receptors have revealed possible mechanisms of receptor-ligand selectivity, which are investigated in this thesis (chapter three and four). This may provide the basis for differential gonadotropin

secretion. Recently, the type II GnRH receptor has been cloned from primate gonadotrope cells and has been shown to preferentially bind GnRH II (Millar et al., 2001; Neill et al., 2001). It has been proposed that GnRH, which preferentially binds the type I GnRH receptor, is more potent in stimulating LH release and that GnRH II preferentially stimulates FSH release. It is therefore likely that mammals have evolved a specialised reproductive system, which involves two GnRH receptors, which each binds a specific form of GnRH to regulate differential release of glycoprotein hormones.

1.3.1 GnRH

GnRH plays a pivotal role in vertebrate fertility as it initiates the reproductive cascade. It is generated in the hypothalamic neurosecretory cells from a precursor polypeptide by enzymatic processing. GnRH is released in a pulsatile manner into the portal vessels, which connect the hypothalamic region of the brain with the pituitary gland, integrating neural and endocrine systems. In the anterior pituitary gland it stimulates gonadotrope cells expressing GnRH receptors to release the gonadotropins, LH and FSH, which in turn regulate steroidogenesis and gametogenesis in male and female vertebrates. The gonadotrope cells represent only about 10% of pituitary cells and are divided into monohormonal cells (18% LH and 22% FSH cells) and 60% multihormonal (LH and FSH) cells (Naor et al., 1998), presenting a foundation for a complex regulatory system in selective gonadotropin secretion. In addition to stimulating gonadotropin release, GnRH stimulates gonadotrope cell growth, expression of GnRH receptors (Clayton, 1989), regulates GnRH receptor mRNA levels (Yasin et al., 1995), biosynthesis of LH (Clayton, 1989) and LH glycosylation (Ramey et al., 1987). GnRH secretion is regulated by gonadal steroids (estradiol, progesterone, and testosterone), glycoprotein hormones (FSH, LH, inhibin) and peptides (activen and follistatin). In turn these factors feedback and modulate the FSH and LH response to GnRH in gonadotropes.

1.3.1.1 GnRH Biosynthesis

The gene encoding GnRH is encoded by four exons necessary for the synthesis of the precursor hormone, which is enzymatically processed to the various decapeptide forms of GnRH (King and Millar, 1995). The first 23 amino acids of the 92 amino acid prepro-GnRH comprise a hydrophobic signal sequence (Adelman et al., 1986). This is followed by the decapeptide sequence of GnRH and a tripeptide sequence (Gly-Lys-Arg) necessary for enzymatic cleavage and amidation of Gly^{10} of GnRH. The amino-terminal pyro-glutamate is formed by spontaneous cyclisation of Glu^{1} in GnRH (Seeburg et al., 1987). The remaining 57-amino acids is known as the GnRH associated peptide (GAP), which is proposed to stimulate gonadotropin release and inhibit prolactin secretion

(Nikolics et al., 1985) through a mechanism that is independent of the GnRH receptor (Millar et al., 1986b).

1.3.1.2 The GnRH Pulse Generator

In mammals, GnRH is released from the median eminence of the hypothalamus into the portal system in a pulsatile manner, which influences the pattern of gonadotropin secretion (Conn and Crowley, 1991). The amplitude and frequency of these GnRH pulses vary with the reproductive states of the estrous and menstrual cycles (Dalkin et al., 1989). A high frequency of GnRH pulses preferentially stimulates LH release, mRNA synthesis for the LH β subunit, and synthesis of the common glycoprotein α subunit. Slower GnRH pulses favour the release of FSH and FSH β subunit synthesis (Dalkin et al., 1989). Thus, the pulse frequency of GnRH has a modulatory effect on individual gonadotropin secretion.

The pulsatile release of GnRH is proposed to be associated with the GnRH neurons operating in a synchronised manner. It is observed that the frequency of episodic electrical activity within the hypothalamus is associated with this pattern of GnRH secretion, which in turn results in an episodic pattern of gonadotropin secretion from the pituitary gland (Knobil, 1990). This morphologically disperse, but functionally interconnected network of GnRH neurons has been termed the GnRH pulse generator (Knobil, 1990). Immortalised GnRH secreting GT1 neurons have been shown to secrete GnRH spontaneously in perifused and static cell cultures (Mellon et al., 1990). It was demonstrated that such cultures release GnRH in an episodic manner, which can be modulated by several factors that influence GnRH release in hypothalamic tissue (Krsmanovic et al., 1992). Recently, experiments on long-term, multi-site recordings of GT1-7 cells revealed repeated episodes of increased firing rate. It was proposed that not every GnRH neuron participates in each secretory pulse and provides a possible mechanism for the variations in GnRH-pulse amplitude observed in vivo (Nunemaker et al., 2001). Moreover, it has been demonstrated that GnRH receptors are expressed in hypothalamic GnRH neurons, which respond to GnRH agonists and antagonists, and that local GnRH receptor activation regulates pulsatile GnRH release. Krsmanovic et al. proposed that this may form an ultrashort-loop autocrine feedback mechanism that contributes to the regulation of GnRH secretion from the hypothalamus (Krsmanovic et al., 1999). Nunemaker et al., proposed that the intrinsic rhythmic activity of GnRH neurons and the presence of autocrine GnRH action therein, are important elements in the nature of the GnRH pulse generator (Nunemaker et al., 2001). It was demonstrated that GnRH produced in the anterior pituitary resulted in low level activation of pituitary GnRH receptors, the induction of spontaneous

intracellular Ca^{2+} oscillations, and contributes to basal LH secretion in cultured pituitary cells. They suggested that the function of locally produced GnRH in the anterior pituitary gland, the major site of expression of GnRH receptors, provides a mechanism for the maintenance of optimal responsiveness of the gonadotropes to pulses of GnRH arising in the hypothalamus (Krsmanovic et al., 2000).

1.3.1.3 Functions of Naturally Occurring GnRHs

GnRH was first isolated as a hypothalamic peptide hormone and thought to exclusively regulate gonadotropin release from the pituitary (Matsuo et al., 1971; Schally et al., 1971). The discovery of multiple molecular forms of GnRH opened the pathway to elucidate the complex modulation of reproduction (King and Millar, 1979). The diversity of GnRH function was established by the discovery that multiple forms of GnRH are present in tissues of single species (Jones, 1987; King and Millar, 1982; Miyamoto et al., 1984). Various GnRH isoforms are expressed in extrahypothalamic tissue (midbrain, spinal cord, sympathetic ganglia, gonads, placenta and breast), and GnRH receptors are present in extrapituitary tissue which respond to GnRH by affecting cell activity in these tissues (Hsueh and Schaeffer, 1985; Kang et al., 2001; Sherwood et al., 1993). GnRH also stimulates the release of other pituitary hormones (growth hormone in fish) and is present in organisms that do not have pituitary tissue (tunicate) (Powell et al., 1996; Sherwood et al., 1993).

Two forms of GnRH were isolated in neural tissue of a tunicate, which could activate the gonads directly. It was suggested that the direct regulation of the gonads was an early-evolved function in primitive animals and that the neuroendocrine system of regulating reproduction evolved later (Powell et al., 1996). An ancestral GnRH gene seems to have undergone early gene duplication, giving rise to an additional protein coding region, which evolved to give rise to another form of GnRH (King and Millar, 1995). It is proposed that ancestral GnRH diverged into two main branches, GnRH isoforms and GnRH II, which are encoded by separate genes and are not processed from a single precursor (King and Millar, 1995; White et al., 1998). Although the function of GnRH II remains uncertain, it is highly conserved and occurs ubiquitously in all the major vertebrate groups. On the other hand GnRH isoforms are found in particular classes and is known to have a regulatory role in reproduction (King and Millar, 1995). It has been established that the primate brain contains GnRH and GnRH II (White et al., 1998) and there is evidence for differential hormonal regulation in mRNA expression levels of the two forms (Kang et al., 2001). This supports that these two hormones may have different physiological functions. GnRH is

localised in the hypothalamic areas and functions mainly as hypophysiotropic factor. GnRH II predominates extra-hypothalamically and has been shown to inhibit the modulatory late-slow postsynaptic potential (M-current) in bullfrogs (Jones, 1987) and may have a role in reproductive behaviour/ neuromodulatory role (Maney et al., 1997; Rissman et al., 1997). To date, at least 15 GnRH forms have been isolated (Table 1.1) which have been co-opted in a diversity of regulatory functions, such as releasing gonadotropins (Schally et al., 1971) and growth hormone (Marchant et al., 1989) from pituitary cells, neurotransmitter in the central (Millan et al., 1986; Sherwood et al., 1993) and sympathetic (Jan et al., 1979; Jones, 1987; Troskie et al., 1997) nervous systems, as a paracrine regulator in the gonads (Hsueh and Erickson, 1979a; Hsueh and Erickson, 1979b) and placenta (Belisle et al., 1984) and as an autocrine regulator in tumour cells (Harris et al., 1991). In support, GnRH and GnRH receptors have been detected with RT-PCR to occur in non-reproductive tissue such as liver, heart, kidney, and skeletal muscle (Kakar and Jennes, 1995). However, it still remains to be proven that GnRH and GnRH receptors are functionally expressed in mentioned non-reproductive and tumour tissue.

Table 1.1. Amino acid sequences of naturally occurring GnRH forms. The conserved N- and C- termini are in blue. Position six is conserved as Gly in the higher vertebrates.

GnRH isoforms	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	Trp	Leu	Pro	Gly-NH_2
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	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	Trp	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

1.3.1.4 Primary Structure of Variant GnRH Forms

The amino acid sequence of GnRH has special conserved regions spanning over 500 million years of evolution. In particular the peptide length of 10 amino acids, and residues in the N-terminus (pGlu-His-Trp-Ser) and C-terminus (Pro-Gly NH_2) are conserved and have been shown to be essential in receptor binding and activation. The most variable amino acid is found at position eight, which seems to have an important function in the mammalian reproductive system (Millar et al., 1989). In contrast to mammalian GnRH, GnRH from most non-mammalian vertebrates contains uncharged residues at position eight (Table 1.1). Post-translational modifications may further result

in structural variation. For example, the hydroxylation of Pro^9 of mammalian GnRH has been demonstrated in rat and frog brains (Gautron et al., 1992). The 15 distinct GnRH forms sequenced from vertebrate and protochordate nervous tissue are often named after the species in which they were first identified (Table 1.1). Mammalian GnRH (GnRH) was first isolated from porcine brain (Matsuo et al., 1971). Another mammalian form of GnRH has been identified and sequenced in guinea pigs (gpGnRH) (Jimenez-Linan et al., 1997). In chicken brains two forms of GnRH were isolated; chicken I GnRH ([Gln^8]-GnRH) and chicken II GnRH (GnRH II) (King and Millar, 1982) (Miyamoto et al., 1984). Novel GnRH peptides have been isolated from brains of salmon (sGnRH) (Sherwood et al., 1983), catfish (cfGnRH) (Bogerd et al., 1992), dogfish (dfGnRH) (Lovejoy et al., 1992), herring (hrGnRH) (Carolsfeld et al., 2000), seabream (sbGnRH) (Powell et al., 1994) and medaka (mdGnRH) (Montaner et al., 2001; Okubo et al., 2000a). Two additional protochordate forms were isolated and characterised in tunicates (tGnRH-I and tGnRH-II) (Powell et al., 1996).

1.3.1.5 Activities of Variant GnRH Forms.

All the GnRH forms stimulate the release of gonadotropins, but the potencies differ within and between the vertebrate species. Native mammalian GnRH has the highest potency in mammalian pituitary cell cultures and cells transfected with the mammalian GnRH receptors. Non-mammalian GnRH exhibit diminished ability to stimulate the release of FSH and LH from mammalian pituitary cells (Millar and King, 1983) as well as a decreased capacity to stimulate inositol phosphate production in cells transfected with mammalian GnRH receptors (Sealfon et al., 1997). Since [Gln^8]-GnRH has relatively low activity in mammals, it was proposed that position eight may play an important role in high affinity binding to the mammalian GnRH receptors. A neutral amino acid in position eight is believed to reduce affinity of GnRH for mammalian type I GnRH receptors (Flanagan et al., 1997; Millar et al., 1989). However, endogenous GnRH II, which also does not contain Arg^8, is more active than [Gln^8]-GnRH in mammals. The combination of His^5, Trp^7 and Tyr^8 in GnRH II seems to partially compensate for the loss of Arg^8. Conversely, GnRH and non-mammalian GnRH have similar high activities in the non-mammalian vertebrates, suggesting that different receptor-ligand interactions exist between groups of species (Sealfon et al., 1997).

Although GnRH stimulates release of both LH and FSH there is evidence of a specific FSH-releasing factor (Millar et al., 2001; Padmanabhan and McNeilly, 2001). Recently, the type II GnRH receptor, which preferentially binds GnRH II, has been cloned from various primates (Millar et al., 2001; Neill et al., 2001). It has been shown that the human type I and marmoset type II GnRH receptors activate different MAP kinase pathways, a major component in the GPCR

signalling pathway (section 1.3.2.8). It has been demonstrated that GnRH II has preferential FSH releasing activity when compared to GnRH and [Gln^8]-GnRH (Millar et al., 1986a). Now, it has been demonstrated in sheep that administration of GnRH II results in a higher FSH to LH ratio, while GnRH stimulates a higher LH to FSH ratio. It is likely that gonadotrope cells express both type I and II GnRH receptors which co-ordinately regulate LH and FSH biosynthesis (Millar et al., 2001). It is proposed that the type I and II GnRH receptors have evolved different binding mechanisms for GnRH and that GnRH II is the elusive FSH-releasing factor (Millar et al., 2001). Establishing the binding mechanism of GnRH with its cognate type I GnRH receptor will provide a foundation for understanding ligand selectivity between these two receptors.

1.3.1.6 Structure-Function of Mammalian GnRH

The conservation of GnRH peptide length and the observation that any truncation of the GnRH results in decreased activity suggests that the entire peptide is required for gonadotropin activity-releasing activity (Karten and Rivier, 1986; Rivier et al., 1973). Comparing the amino acid sequence of 15 GnRH forms reveals that there is a high conservation in the N-terminus (pGlu-His-Trp-Ser) and C-terminus (Pro-Gly NH_2), indicating that these features are important for receptor binding and activation. The central region comprising residues five to eight is the most variable and they are not important in GnRH affinity but rather in receptor-ligand selectivity (Sealfon et al., 1997). Modifying the individual residues of GnRH and monitoring the effect on LH release and inositol phosphate production allowed the identification of residues necessary for receptor binding and activation (Sealfon et al., 1997).

During the original purification of GnRH, the amino terminal pGlu residue was found necessary for receptor activation (Amoss et al., 1971). It was shown that the -CO-NH-CHCO-group of acetylated Gly^1 analogs is the minimal structure required for GnRH activity (Okada et al., 1973). Substitution of pGlu is used most frequently in synthesising antagonists (Karten and Rivier, 1986).

Position two requires an aromatic amino acid as confirmed with the high activity of [Trp^2]-GnRH (Monahan et al., 1973) and lower activity of other substitutions (Monahan et al., 1973) (Sealfon et al., 1997). A naturally occurring GnRH, guinea pig GnRH ([Tyr^2,Val^7]-GnRH) has Tyr instead of His in position two and has similar high activity to GnRH (Jimenez-Linan et al., 1997). The imidazole ring of His has a number of features, which may benefit interaction with the receptor, such as its aromatic character, hydrogen-bonding capacity and acid-base properties (Coy, 1975). His^2 has been proposed to interact directly with $Lys^{3.32(121)}$ (Zhou et al., 1995), $Asp^{2.61(98)}$ (Flanagan

et al., 2000) of the GnRH receptor. Substitution of His^2 with bulky hydrophobic residues (eg. D-4-Cl-Phe) is another hallmark of antagonists, highlighting the importance of this residue in receptor activation (Rivier et al., 1995).

Position three of GnRH requires an aromatic residue for receptor activation, as supported by lamprey GnRH ([Trp^3, Lys^8]-GnRH) having Trp^3 substituted by Tyr^3 (Powell et al., 1996). Substituting Trp^3 with non-aromatic residues resulted in a complete loss of activity (Coy et al., 1974), while substitutions with Phe, Tyr or synthetic aromatic amino acids (2-naphthyl-Ala and pentamethyl-Phe) resulted in analogues with substantial potency (Coy et al., 1974; Sandow, 1978). The common feature between these amino acids is their ability to form π-π interactions with other aromatic compounds. It is proposed that Trp^3 interacts with an aromatic group on the receptor (Sealfon et al., 1997). D-Trp^3 analogues of GnRH bind the receptor with high affinity but lack activation capability. This suggests that the L-stereochemistry of Trp^3 is necessary for receptor activation (Karten and Rivier, 1986).

To date, position four is 100% conserved as Ser among the known GnRH forms (Table 1.1). The precise role of Ser^4 in GnRH activity is not known, but the size of the side chain seems to be important. Substitutions with amino acids with small side chains are tolerated, while substitutions with amino acids with larger side chains decrease activity (Sandow, 1978). It is proposed that Ser in position four introduces spatial constraints in GnRH (Sealfon et al., 1997). This property may be necessary for the folding of GnRH to fit in the receptor-binding pocket, which cannot accommodate larger side chains.

Tyr in position five is not highly conserved in vertebrate GnRH, and substitutions are tolerated. [His^5]-GnRH has high binding affinity for mammalian GnRH receptors (Millar et al., 1989) and [Phe^5]-GnRH has 44-64% activity (Coy et al., 1973), demonstrating that a hydroxyl group is not required. It was demonstrated that GnRH requires an aromatic side chain in position five for high activity in mammalian GnRH receptors and it may have an indirect role in receptor activation (Millar et al., 1989). Initial studies suggested that Tyr^5 forms a functional unit with the side chains of His^2 and Arg^8 when GnRH is bound to the receptor (Karten and Rivier, 1986; Shinitzky et al., 1976).

A Gly residue is highly conserved in position six in vertebrate GnRH isoforms, except in the primitive lamprey GnRH. The presence of an achiral amino at this position is vital in supporting the

biologically active conformation of GnRH, discussed in the next section (section 1.3.1.7) (Sealfon et al., 1997). The Gly residue introduces flexibility in the peptide backbone, which allows a beta II type turn to form involving the Tyr^5-Gly^6-Leu^7-Arg^8 residues (Monahan et al., 1973). Substitutions of Gly^6 with other L-amino acids decrease GnRH activity, as these residues are not energetically favoured in forming this bend (Coy et al., 1973; Monahan et al., 1973). The stereo chemistry of D-amino acids with large side chains is proposed to enhance the formation of a beta II type turn and increase the activity of GnRH in mammals (Freidinger et al., 1980; Millar et al., 1989; Monahan et al., 1973). Both Gly and D-amino acids have positive phi dihedral angles, and may favour a beta II type turn at the Gly^6 residue (Freidinger et al., 1980). D-amino acids in position six have their side chains facing away from the receptor during binding, which is favourable for iodination where the large iodine group cannot interfere with the binding of the GnRH analog (Flanagan et al., 1998).

Position seven is Leu in mammalian GnRH and often is substituted with large uncharged residues in other naturally occurring GnRH forms. Substituting position seven with amino acids which have small side chains (Ala, Gly) or charged residues (Lys, Arg) decreased activity (Fujino et al., 1972).

Position eight is the most variable residue in the known GnRH isoforms (Table 1.1), suggesting that virtually any residue is tolerated in this position (Millar et al., 1989). However, it has been demonstrated that the mammalian pituitary receptor requires a basic residue in position eight, which is proposed to play an important role in ligand-selectivity of mammalian type I GnRH receptors (Sealfon et al., 1997). In mammalian GnRH this residue is Arg and it has been postulated that the Arg^8 side chain may interact directly with a negatively charged Asp or Glu residue in the receptor (Hazum, 1987). It was shown that Arg^8 in GnRH and $Glu^{7.32(301)}$ of the mouse GnRH receptor play a role in receptor-ligand selectivity (Flanagan et al., 1994). The role of $Asp^{7.32(302)}$ of the human GnRH receptor in conferring selectivity for Arg^8-containing GnRH is investigated in this thesis (chapter three). The mammalian type I GnRH receptor seems to be more stringent in its requirements of ligand conformation. It has been reported that the conserved N- and C-termini of GnRH are closely apposed when GnRH binds to its receptor (Karten and Rivier, 1986; Sealfon et al., 1997). Thus, during receptor binding, GnRH is proposed to be in a folded conformation (Sealfon et al., 1997) in which Arg^8 is proposed to stabilize a functional unit (Shinitzky et al., 1976), which consists of a β-II-bend involving the central residues Tyr^5-Gly^6-Leu^7-Arg^8 (Monahan et al., 1973) (see next section 1.3.1.7). In this thesis it is postulated that the mammalian type I GnRH receptor has evolved a specific ligand binding mechanism, which determines selectivity for Arg^8-containing GnRH (chapter four).

In support, non-mammalian GnRH receptors do not preferentially select Arg^8-containing GnRH, instead they bind GnRH II with high affinity (Okubo et al., 2000b; Sun et al., 2001; Troskie et al., 2000; Wang et al., 2001). GnRH II ([His^5, Trp^7, Tyr^8]-GnRH) has Tyr in position eight and has 5-fold greater activity in birds than [Gln^8]-GnRH. Substituting position eight of [Gln^8]-GnRH with Tyr increases its activity by 3-fold. On the other hand substituting Tyr^8 in GnRH II with Arg or Glu results in a drastic decrease in activity (Habibi et al., 1992; Millar et al., 1989). This shows that position eight has a unique role in other GnRH forms in determining selectivity for their cognate receptors. This suggests that there may be a fundamental difference in the mechanism of hormone binding to the different GnRH receptors.

Position nine is conserved as Pro in all known forms of GnRH. Pro is expected to impose conformational limitations on GnRH (Coy, 1975). The few substitutions that have been tested decreased GnRH activity (Sandow, 1978). The discovery that Pro^9 may be hydroxylated in the fetal brain and decreases GnRH activity, emphasises the biological importance of this residue (Gautron et al., 1992).

The Gly^{10}-amide is not essential for activity (Coy, 1975) and small uncharged moieties are acceptable at the COOH-terminus (Fujino et al., 1972). Larger groups are not tolerated as they may sterically hinder ligand access to the binding pocket. It has been suggested that the total chain length may be important in GnRH binding to its receptor (Fujino et al., 1972). The carbonyl of Gly^{10}-amide is proposed to form a hydrogen bond with $Asn^{2.65(102)}$ of the GnRH receptor. In support, it was demonstrated that the GnRH analogs with Gly^{10}-amide had lower potency for the $Asn^{2.65(102)}$Ala mutant GnRH receptor than GnRH analogs with N-ethylamide in position 10. This suggests that $Asn^{2.65(102)}$ is important for determining potency for GnRH analogs with a C- terminal glycinamide, while GnRH analogs with C-terminal ethylamide are less dependent on $Asn^{2.65(102)}$ (Davidson et al., 1996a).

1.3.1.7 The Biologically Active Conformation of Mammalian GnRH

It was proposed that GnRH exists as a mixture of conformers as identified with conformational energy analysis (Momany, 1978; Momany, 1976). Using molecular modelling it was suggested that the low energy GnRH conformers have a beta II type turn at the Gly^6 residue (Guarnieri, 1996). In another study it was shown using 2D-NMR techniques and molecular mechanics calculations that GnRH as well as [Gln^8]-GnRH exist in a mixture of conformers. It was suggested that in GnRH the

N and C-termini are closely opposed, whereas [Gln^8]-GnRH has the two termini far apart (Maliekal, 1997). It has been demonstrated with tryptophan fluorescence that substitution of Arg^8 with Gln results in a loss of predominance of folded conformers (de L. Milton et al., 1983). These studies suggest that Arg^8-containing GnRH has a different conformation to that of other naturally occurring forms.

It has been proposed that Arg^8 stabilizes an active conformation of GnRH (Shinitzky et al., 1976), which consists of a β-II-bend involving the Tyr^5-Gly^6-Leu^7-Arg^8 residues (Monahan et al., 1973). In this conformation, Arg^8 is proposed to form stabilising intramolecular hydrogen bonds with side chains of other residues in GnRH and is the preferred conformation for binding with mammalian type I GnRH receptors (Karten and Rivier, 1986). The incorporation of a D-amino acid in position six (Momany, 1976) or a γ-lactam in positions six and seven (Freidinger et al., 1980) are proposed to stabilize the active GnRH conformation resulting in elevated potency (Freidinger et al., 1980; Monahan et al., 1973). It has been demonstrated that mammalian type I GnRH receptors have a high affinity for GnRH, which is decreased when Arg at position eight is replaced by the neutral Gln residue. GnRH analogs with a D-amino acid incorporated into position six have enhanced affinity for the mammalian type I GnRH receptor (Flanagan et al., 1994; Sealfon et al., 1997). This shows that the mammalian type I GnRH receptor has enhanced affinity for the conformational state of GnRH that is stabilized by incorporating the mentioned constraints.

Conversely, non-mammalian GnRH receptors bind the extended [Gln^8]-GnRH isoform and GnRH with high affinity (Okubo et al., 2000b; Sun et al., 2001; Troskie et al., 2000), do not have enhanced affinity for conformationally constrained GnRH analogs, and bind GnRH II with the highest affinity. All the known GnRH forms have the N- and C-termini highly conserved, which suggests that the different GnRH receptors may have overlapping binding sites. The mammalian type I GnRH receptor seems to be more stringent in its requirements of ligand conformation, which suggest that mammalian and non-mammalian receptors may have different ligand binding mechanisms. The biologically active conformation of GnRH is proposed to be induced or stabilised by the interaction of Arg^8-containing GnRH with an acidic residue in ECL3 of the mammalian GnRH receptor (Flanagan et al., 1994). However, the precise role of ligand conformation in the process of ligand selectivity is uncertain and is investigated in this thesis.

1.3.2 The GnRH Receptor

The amino acid sequence of the GnRH receptor was first deduced when it was cloned from cDNA of the αT3 mouse gonadotrope cell line (Tsutsumi et al., 1992) and confirmed by other research groups (Reinhart et al., 1992; Perrin et al., 1993). Since then, several mammalian GnRH receptors have been cloned in the past years and it was shown that they share over 80% amino acid homology with each other (Sealfon et al., 1997) (Fig. 1.5). Recently, many non-mammalian GnRH receptors have been cloned and they share 42-47% homology with the mammalian receptors (Illing et al., 1999; Okubo et al., 2000b; Sun et al., 2001; Troskie et al., 2000; Wang et al., 2001). It was discovered that a species may contain more than one type of GnRH receptor. To date, in non-mammalian vertebrates up to three variant GnRH receptor forms have been cloned in a single species (Wang et al., 2001). Recently, the type II GnRH receptor was cloned in two simian species, which has been shown to preferentially bind GnRH II (Neill et al., 2001). The genes encoding the type II GnRH receptor (Faurholm et al., 2001) and GnRH II (White et al., 1998) has been identified in humans, suggesting that primates have two GnRH receptors (type I and type II GnRH receptor, Fig. 1.5) which preferentially bind their cognate GnRH isoforms (GnRH and GnRH II). The conservation of amino acids between these GnRH receptors may reveal residues with overlapping functions in GnRH binding and receptor activation. Moreover, variations in the pattern of conservation of amino acids and/or amino acid-motifs may prove valuable in identifying the structure activity of the GnRH receptors (Fig. 1.5). These comparisons have proven useful in elucidating ligand receptor interactions, as well as classifying its structure in the rhodopsin like G protein-coupled receptor (GPCR) family (Sealfon et al., 1997).

1.3.2.1 Primary Structure of the GnRH Receptor

The first cDNA representing a GnRH receptor (mouse) was cloned from an α-T3 cell line (Tsutsumi et al., 1992). The protein structure of the GnRH receptor is characteristic of GPCRs consisting of a single polypeptide chain with a N-terminal domain followed by seven hydrophobic α-helical TM helices, which are connected by extracellular and intracellular loops (Sealfon et al., 1997) (Fig. 1.4).

Similar to other GPCRs, the α-helical TM helices of the GnRH receptor is believed to be arranged around a hydrophillic core as found in the crystal structure of rhodopsin (Palczewski et al., 2000). The ligand binding site of the GnRH receptor comprises elements of extracellular domains and superficial residues in the putative TM domains (Flanagan et al., 1997; Sealfon et al., 1997). The interaction of a ligand, like GnRH, with the binding domains of the receptor is proposed to induce

conformational changes in the TM helices, which are associated with signal propagation. This conformational change affects the configuration of the ICLs, which are involved in interacting with G proteins associated with signal transduction (Sealfon et al., 1997).

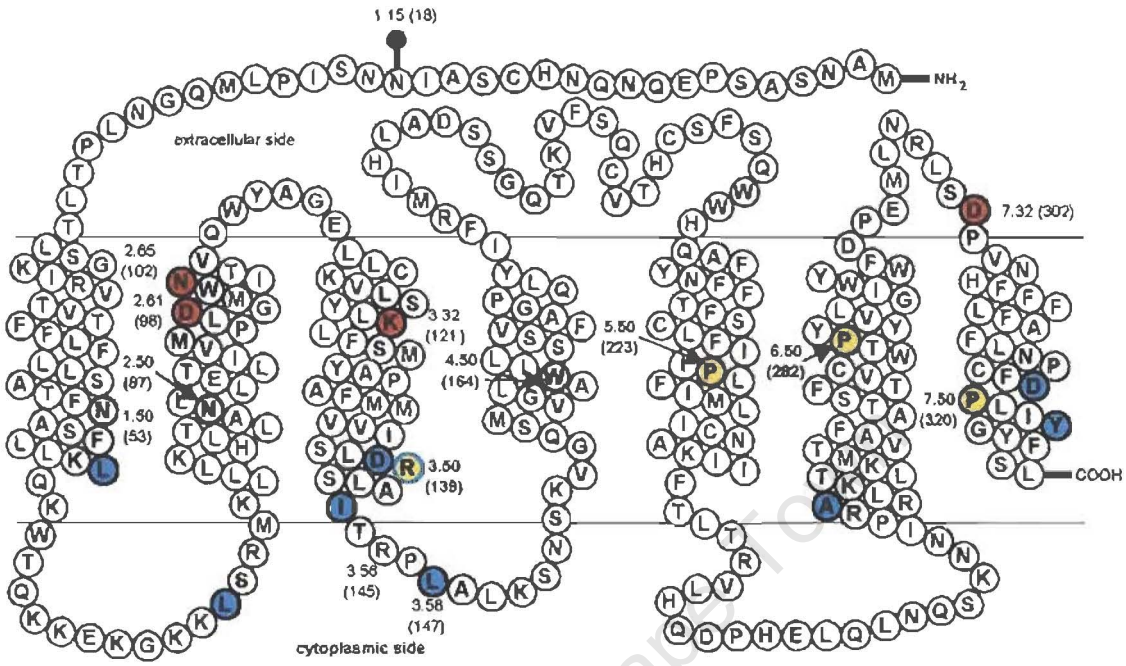

Figure 1.4: Primary structure of the human type I GnRH receptor. Yellow residues indicate the most conserved in the rhodopsin family A GPCRs. Red residues are important in ligand binding and blue residues are required for receptor activation and/ or G protein coupling.

The GnRH receptor is a relatively small GPCR, consisting of only 327 amino acid residues in rodents. The other mammalian species contain an insertion in ECL2 and have 328 residues (Sealfon et al., 1997) (Fig. 1.5). The mammalian GnRH receptor is unique in the rhodopsin class of receptors (family A) as it lacks the entire polar C-terminal intracellular tail, which is proposed to be involved in receptor desensitization by receptor kinases (Palczewski and Benovic, 1991). The ICL1 is unusually long and is proposed to be involved in various functions such as receptor internalisation (Thomas et al., 1995) and ligand induced receptor up-regulation (Loumaye and Catt, 1983). Another unique feature is that the highly conserved DRY sequence, found at the cytoplasmic side of TM3 in GPCRs, is DRS in the mammalian GnRH receptor (Sealfon et al., 1997).

Furthermore, $Asp^{2.50}$ and $Asn^{7.49}$ which are highly conserved in most GPCRs, are $Asn^{2.50(87)}$ and $Asp^{7.49(319)}$ in the GnRH receptor and are involved in receptor activation (Sealfon et al., 1997). $Asp^{7.49(319)}$ is part of the Asp-Pro-Leu-Ile-Tyr (DPLIY) motif (Fig. 1.4) conserved in GnRH receptors, which is conserved as the Asn-Pro-X-X-Tyr (NPXXY) motif (Fig. 1.1) in most GPCRs

(Probst et al., 1992). The functional significance of the unique features of the mammalian GnRH receptor is discussed in sections 1.4.2.5. and 1.4.2.6.

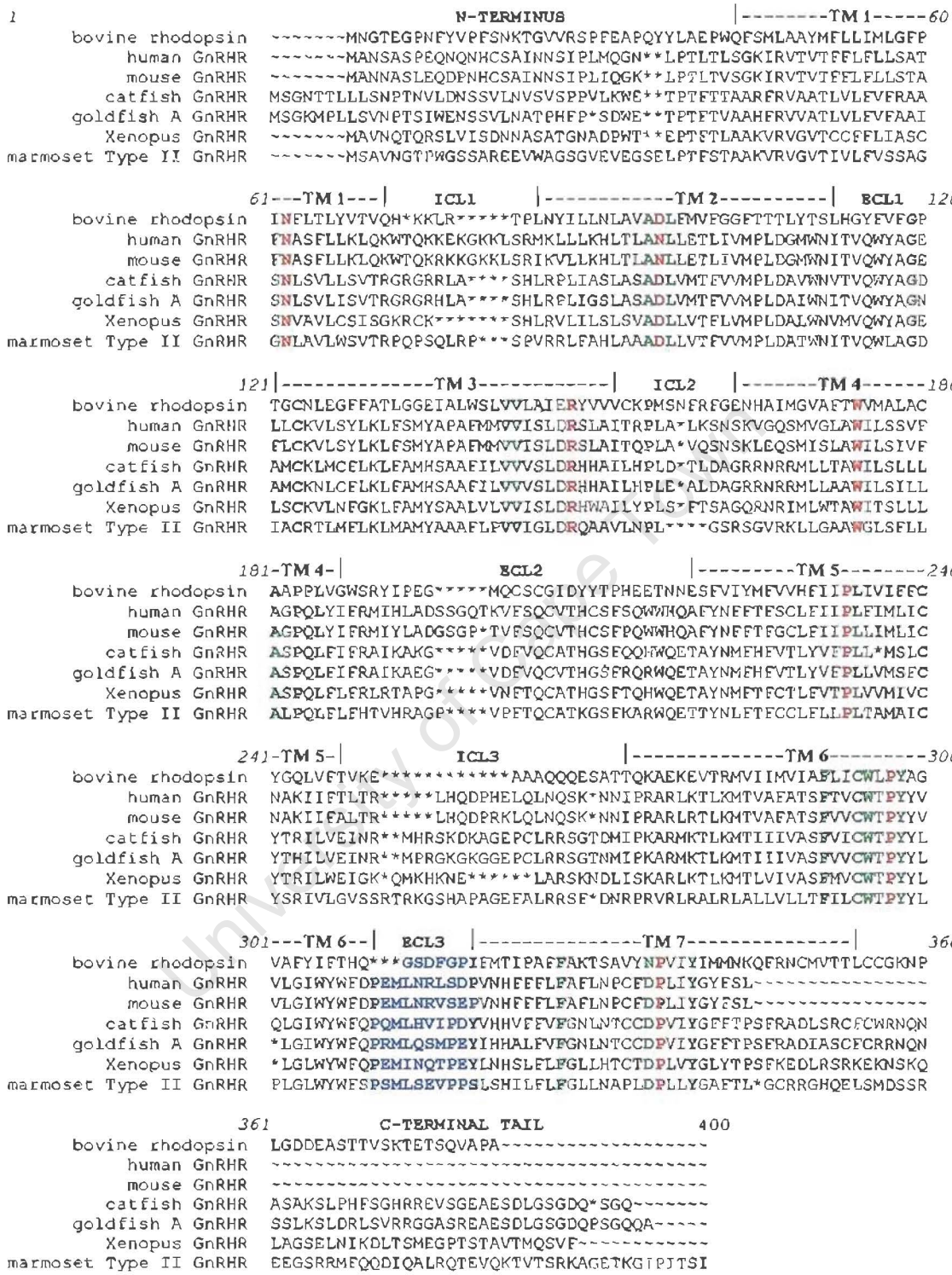

Figure 1.5 Amino acid alignment of GnRH receptors. TM helices as predicted by homology modeling with the rhodopsin crystal structure are indicated (Palczewski et al., 2000). Residues in **red** are the most conserved in the rhodopsin family of GPCRs. In **green** are the residues conserved between the GnRH receptor (GnRHR) and rhodopsin Numerical annotation refers to the catfish GnRH receptor. ECL3 is represented in **blue** and spaces for insertions are represented by *.

1.3.2.2 Post-Translational Modifications of the GnRH Receptor

Postranslational modifications characteristic of G protein receptors include N-linked glycosylation of the N-terminal domain (receptor expression) (Schvartz and Hazum, 1985), disulphide bridge formation between ECL2 and TM3 (receptor expression and proposed formation of ligand binding site) (Dohlman et al., 1990) and the palmitolation of cysteine residues in the carboxy-terminal domain (G protein interactions) (Lefkowitz et al., 1990; O'Dowd et al., 1989).

The rodent GnRH receptors contain three N-glycosylation consensus sequences (Asn-X-Ser/Thr). With mutagenesis it was shown that two of these sites in the N-terminus of the mouse GnRH receptor (Asn^4 and Asn^{18}) are glycosylated during transient expression in COS-1 cells. The third other potential glycosylation site is at $Asn^{2.65(102)}$ at the top of TM domain two (Davidson et al., 1995), which is not glycosylated. Most mammalian GnRH receptors have only one site (Asn^{18}-X-Ser/Thr) which is glycosylated in the N-terminus and is thought to contribute to receptor expression (Perrin et al., 1993; Sealfon et al., 1997) (Fig. 1.4). These polysaccharide moieties do not influence receptor binding affinity, but may contribute to receptor stability as mutations of Asn^4 and Asn^{18} decrease the amount of receptor expression (Davidson et al., 1995). However, from the crystal structure of rhodopsin it is evident that residues in the N-terminus that are glycosylated do not interact with other residues on the receptor (Palczewski et al., 2000). Nonetheless, the incorporation of the additional glycosylation site (Asn^4) of the mouse GnRH receptor into the N-terminus of the human GnRH receptor increased receptor expression (Davidson, 1996b).

1.3.2.3 The Tertiary Structure of the GnRH Receptor

The recent crystallisation of rhodopsin confirmed the fundamental basis of the three-dimensional structure of GPCRs (Palczewski et al., 2000). Previous structural information was derived from low-resolution electron microscopy of bacteriorhodopsin and rhodopsin (Donnelly et al., 1989; Schertler et al., 1993). This knowledge is essential to a complete understanding of the molecular functioning of GPCRs.

The GnRH receptor contains most of the amino acids that are highly conserved among the rhodopsin family of GPCRs (Baldwin, 1993). This high degree of homology in conserved residues and motifs suggest that this family of receptors, including the GnRH receptor, share a common structural framework and mechanism of activation. A three-dimensional model of the TM helix bundle of the GnRH receptor has been developed (Sealfon et al., 1997) using the combination of information gained from the analysis of sequence conservation patterns among GPCRs (Donnelly et

al., 1989), physio-chemical properties of conserved and partially conserved residues (Donnelly et al., 1989; Eisenberg et al., 1984), specific amino acid motifs such as Pro-kinks (Sansom and Weinstein, 2000; Williams and Deber, 1991) and the projection map of rhodopsin (Baldwin, 1993). The GnRH receptor model is consistent with the template of many GPCRs, which includes the counter-clockwise arrangement of TM helices as viewed from the extracellular side (Thirstrup et al., 1996), the proposed interactions between TM helices two and seven (5-HT2A receptor) (Sealfon et al., 1995b) and, TM helices one and seven mutually opposed (muscarinic acetylcholine receptors) (Liu et al., 1995b). The seven TM helices are arranged in a tight bundle enclosing a hydrophilic pocket and surrounded by the hydrophobic membrane environment (Sealfon et al., 1997). Consequently, several experimental studies have been designed to test hypotheses and refine the model. For instance, $Asp^{2.50}$ and $Asn^{7.49}$, which are highly conserved in most GPCRs, are $Asn^{2.50(87)}$ and $Asp^{7.49(319)}$ in the GnRH receptor and appear to have undergone a reciprocal mutation in mammalian GnRH receptors (Sealfon et al., 1997). Mutation of $Asn^{2.50(87)}$ to Asp eliminated receptor function, but a second mutation of $Asp^{7.49(319)}$ to Asn regenerated receptor expression (Zhou et al., 1994). This double mutation recreated the arrangement found in most GPCRs ($Asp^{2.50}$ and $Asn^{7.49}$). This restoration of receptor function by the reciprocal mutation suggests that the side chains of the two residues in different TM helices have complementary roles in maintaining GnRH receptor structure and therefore TM helices two and seven are proposed to be closely apposed (Zhou et al., 1994). However, in the crystal structure of rhodopsin in the inactive conformation the distance between $Asp^{2.50}$ and $Asn^{7.49}$ is too far to make a hydrogen bond. It appears that this interaction may be supported by a H_2O molecule near $Asp^{2.50}$ interacting with the side chain of $Asn^{7.49}$, thus mediating a contact between helices two, three and seven (Palczewski et al., 2000). It has been proposed that the $Asp^{2.50}$ - $Asn^{7.49}$ interaction in the GnRH receptor exist in the active conformation (Flanagan et al., 1999).

1.3.2.4 The GnRH Binding Site

In the course of systematic ligand modification and site-directed mutagenesis in the GnRH receptor, considerable advances have been made in identifying and characterizing putative ligand binding sites in the mammalian GnRH receptor. The ligand binding site for GnRH is located in the extracellular and TM domains of the GnRH receptor. Specific residues in the GnRH receptor have been identified as contact points for functional chemical groups in GnRH (Fig. 1.4). Studies of the GnRH receptor binding pocket have mostly relied on indirect approaches such as site directed mutagenesis, ligand modification and the construction of computational three-dimensional

molecular models of ligand receptor complexes. The combination of these approaches facilitates further understanding of the receptor and its ligand binding mode (Sealfon et al., 1997).

Lys$^{3.32(121)}$

The binding pocket of the GnRH receptor consists of residues mainly in TM helices two and three and is partially conserved with other GPCRs (Sealfon et al., 1997). From the crystal structure of rhodopsin, the retinylidene group (ß-ionone ring to C11) runs almost parallel to TM three providing contact sites for part of the binding pocket consisting of Glu$^{3.28(113)}$, Gly$^{3.29(114)}$, Ala$^{3.32(117)}$, Thr$^{3.31(118)}$, Gly$^{3.35(120)}$, Gly$^{3.36(121)}$ and Glu$^{3.37(122)}$ (Palczewski et al., 2000). Their structure of rhodopsin confirmed a proposed salt bridge linkage between the Schiff base and Glu$^{3.28(113)}$ and suggested that the interaction between the ß-ionone ring and TM three occurs at Glu$^{3.37(122)}$ (Palczewski et al., 2000). The biogenic amine receptors all contain an Asp at a homologous position in TM three, which acts as an counter-ion required for high affinity interaction with the cationic amine head group of the ligand (Strader et al., 1994). The equivalent position is Lys$^{3.32(121)}$, which is conserved, in all cloned GnRH receptors (Sealfon et al., 1997). Substitution of Lys$^{3.32(121)}$ with Arg preserved high affinity agonist binding, whereas substitution with Gln leads to a total loss in agonist binding, but does not affect antagonist binding, which indicates that Lys$^{3.32(121)}$ interacts with agonists but not antagonists (Zhou et al., 1995). GnRH receptor antagonists generally have modifications in the N-termini, where the pGlu-His-Trp sequence is substituted with D-amino acids, suggesting that this portion of GnRH may interact with Lys$^{3.32(121)}$. However, this interaction does not involve a salt bridge, as there are no negative charges in GnRH. It is proposed that either of the electron-dense aromatic rings of His^2 and Trp^3 or the imino group of His^2 in GnRH may form a hydrogen bond with the electropositive side chain of Lys at position 3.32(121) of the receptor (Zhou et al., 1995). In addition to this interaction, computational modelling has suggested that Lys$^{3.32(121)}$ may form an ionic interaction with Asp$^{2.61(98)}$ in TM helix two (Flanagan et al., 2000), as discussed below.

Asp$^{2.61(98)}$

It has been shown that the Asp$^{2.61(98)}$Asn mutant has a large decrease in GnRH-stimulated IP production (Flanagan et al., 1994) and had little effect on the potency of [Trp^2]-GnRH (Flanagan et al., 2000). It is proposed that Asp$^{2.61(98)}$ interacts with His^2 of native GnRH. Further studies have identified and characterized multiple interactions of the Asp side chain with the receptor and GnRH (Flanagan et al., 2000). Mutation of Asp$^{2.61(98)}$ to Glu decreased the affinity for a series of GnRH analogues containing the native His^2 residue, but less so for GnRH analogs with His^2 substitutions.

This shows that $Asp^{2.61(98)}$ confers specificity for His^2 of GnRH. Asp has a negative charge, which gives it potential ionic bonding properties, and its side chain has two oxygen atoms, which lend it hydrogen binding properties. It has been suggested that $Asp^{2.61(98)}$ interacts with His^2 through a hydrogen bond and may form another hydrogen bond with the backbone NH-group of Trp^3. It was proposed that the negative charge of $Asp^{2.61(98)}$ is required for an intramolecular interaction that regulates both ligand binding and receptor expression. Computational modeling shows that $Asp^{2.61(98)}$ may form an ionic interaction with $Lys^{3.32(121)}$ in TM helix three, which suggests that there is an interhelical interaction between TM helices two and three. It was proposed that $Asp^{2.61(98)}$ and $Lys^{3.32(121)}$ contributes to the structure of the agonist binding pocket and that $Asp^{2.61(98)}$ may have an additional role in agonist binding through an interaction with the unprotonated His^2 of GnRH (Flanagan et al., 2000).

$Asn^{2.65(102)}$

The $Asn^{2.65(102)}$ residue is located at the extracellular boundary of TM helix two and is proposed to interact with the glycine amide of GnRH (Davidson et al., 1996a). Mutation of $Asn^{2.65(102)}$ to Gln results in an increased affinity for GnRH. Mutation of $Asn^{2.65(102)}$ to Ala results in a 225-fold loss of potency for GnRH and a similar (95- to 750-fold) decrease in potency for GnRH analogs containing the polar Gly-NH_2 carboxy terminal residue present in native GnRH. However, the $Asn^{2.65(102)}$Ala mutant receptor only had a small decrease in potency (2.4- to 11-fold.) for GnRH analogues containing the apolar N-ethylamide substitution at the carboxy-terminus. Thus $Asn^{2.65(102)}$ determines specificity for ligands with C- terminal glycinamide. These results are consistent with a hydrogen bond between the side chain of $Asn^{2.65(102)}$ and the carboxy-terminal Gly-NH_2 moiety of GnRH analogues (Davidson et al., 1996a).

$Glu^{7.32(301)}$

In GnRH, Arg^8 may be involved in an electrostatic interaction with an acidic residue in the mammalian type I GnRH receptor (Hazum, 1987). The systematic mutation of extracellular acidic residues to their isosteric amides revealed that substituting $Glu^{7.32(301)}$ in the mouse GnRH receptor resulted in 60-fold decrease in affinity for GnRH and a loss in selectivity between GnRH and [Gln^8]-GnRH. It was proposed that an interaction involving Arg^8 and the $Glu^{7.32(301)}$ residue of the receptor plays a role in receptor-ligand selectivity (Flanagan et al., 1994). Mutation of the $Glu^{7.32(301)}$Gln mutant receptor had decreased affinity for GnRH, but not for analogs with substitutions for Arg^8. This suggests that $Glu^{7.32(301)}$ in ECL3 of the mouse type I GnRH receptor confers specificity for Arg^8-containing GnRH (Flanagan et al., 1994). The $Glu^{7.32(301)}$Gln mutant

receptor had more than 10-fold improved affinity for [Glu^{8}]-GnRH, which suggests that the positively charged Arg^{8} side chain of GnRH may interact via an electrostatic interaction with the negatively charged Glu$^{7.32(301)}$ residue (Sealfon et al., 1997). However, this decrease in affinity is small for the loss of an ionic interaction results in a decrease in affinity to the order of a 1000-fold (Wells et al., 1987). This suggests that the mechanism by which Glu$^{7.32(301)}$ determines selectivity for native GnRH is more complex than a simple electrostatic interaction.

It is established that various mammalian type I GnRH receptors selectively bind Arg^{8}-containing GnRH (Sealfon et al., 1997). However, Glu$^{7.32(301)}$ is not completely conserved in mammalian type I GnRH receptors. In the human type I GnRH receptor this residue is Asp$^{7.32(302)}$ (Chi et al., 1993; Cui et al., 2000; Illing et al., 1993; Kakar et al., 1992; Sealfon et al., 1997). Although this is a conservative substitution, it is surprising that such a functionally important residue is not absolutely conserved. In the monoamine receptors, the Asp$^{3.32}$ residue, which is important for ligand binding, is conserved as Asp not only in different species, but also in different receptor subtypes that recognize the same ligand and in different receptors that recognize distinct ligands ranging through acetylcholine, adrenalin, serotonin and histamine (Probst et al., 1992). This thesis investigates whether Asp$^{7.32(302)}$ of human type I GnRH receptor confers specificity for Arg-containing GnRH and defines the mechanism by which mammalian type I GnRH receptors selectively bind GnRH (chapter three).

Non-mammalian GnRH receptors do not preferentially bind Arg^{8}-containing GnRH (Sealfon et al., 1997), but surprisingly the acidic residue at position 7.32 is conserved in these receptors. Amino acid sequence alignment of ECL3 of mammalian and non-mammalian type I GnRH receptors revealed two distinct amino acid motifs involving Glu/Asp in position 7.32. ECL3 of mammalian type I GnRH receptors contains the Ser$^{7.31}$-(Glu$^{7.32}$/Asp$^{7.32}$)-Pro$^{7.33}$ [S(E/D)P] motif and non-mammalian type I GnRH receptors have Pro-(Asp/Glu)-Tyr [P(E/D)Y] (Fig. 1.4). The different position of Pro relative to Glu$^{7.32}$/Asp$^{7.32}$ in these motifs suggested that the conformation of ECL3 may differ between mammalian and non-mammalian receptors and that loop conformation may influence ligand selectivity. This thesis investigates the role of amino acid motifs in ECL3 involving Glu$^{7.32}$/Asp$^{7.32}$ in ligand selectivity and explores the potential influence of a specific conformation of ECL3 in playing a role in ligand binding and selectivity.

Recently, a second novel GnRH receptor (type II) has been cloned in primates and shown to preferentially bind GnRH II (Millar et al., 2001; Neill et al., 2001) (Fig. 1.5). The type II GnRH

receptor has also been cloned in non-mammalian species which contain up to three forms of GnRH receptors (Wang et al., 2001). The type II GnRH receptors and non-mammalian type I GnRH receptors preferentially bind GnRH II. The mammalian type I GnRH receptors preferentially bind GnRH. GnRH and GnRH II may have different physiological roles and therefore may need GnRH receptors that each can preferentially bind its cognate GnRH form to perform specific functions (Millar et al., 2001). ECL3 seems to play an important role in this selectivity in mammalian type I GnRH receptors and is investigated in this thesis.

1.3.2.5 GnRH Receptor Activation

The GnRH receptor conforms to the model of GPCR activation as discussed in section 1.2.3. This model may involve an initial binding step for agonists, which may overlap with antagonists, a further conversion binding step which is exclusive to agonists and leads to the formation of the ternary complex consisting of hormone, receptor and cognate G protein. The ternary complex also incorporates the spontaneous formation of an agonist-stabilised receptor-G protein complex, which has higher affinity for agonists. This complex dissociates when guanosine triphosphate (GTP) binds to the G protein, resulting in the receptor returning to the low affinity conformation. It is proposed that the unoccupied receptor fluctuates between the R and R* conformation and that agonist binding shifts the equilibrium to the activated receptor conformation (Gether and Kobilka, 1998).

The highly conserved $Arg^{3.50}$ residue at the cytosolic end of TM helix three is proposed to be involved in GPCR activation (Hulme et al., 1999; Oliveira et al., 1994; Scheer et al., 1996). In the GnRH receptor $Arg^{3.50(139)}$ is part of the conserved [(Ile/Leu)-X-X-Asp-Arg-X-X-X-(Ile/Val)] ([(I/L)XXDRXXX(I/V)]) motif (Fig. 1.5 and 1.6) at the intracellular end of TM helix three. Experimental and computational results have suggested that the $Arg^{3.50(139)}$ side chain is constrained in the inactive receptor through an ionic interaction with the preceding $Asp^{3.49(138)}$ residue (Ballesteros et al., 1998).

From the crystal structure of rhodopsin (inactive conformation) it was revealed that the carboxylate of $Glu^{3.49(134)}$ forms a salt-bridge with guanidium of $Arg^{3.50(135)}$ and is proposed to be one of the contraints keeping rhodopsin in the inactive conformation (Palczewski et al., 2000). In the GnRH receptor it was proposed that during agonist binding $Asp^{3.49(138)}$ is protonated, breaking the constraining interaction and releasing the $Arg^{3.50(139)}$ side chain, which is proposed to now interact with $Asp^{7.49(319)}$. It has been shown that a β-branched, bulky hydrophobic residue (Ile or Val) is required in locus 3.54 which is proposed to prevent movement of the $Arg^{3.50(139)}$ side chain to the

cytoplasm (Ballesteros et al., 1998) (Fig 1.6). Hypothetically, this steric clash promotes an interaction of $Arg^{3.50(139)}$ in TM helix three with $Asp^{7.49(319)}$ in TM helix seven ($Asp^{2.50}$ in other GPCRs) during receptor activation. $Asp^{7.49(319)}$ forms part of a pair of conserved residues ($Asn^{2.50(87)}$ and $Asp^{7.49(319)}$) which is involved in GnRH receptor activation (Zhou et al., 1994).

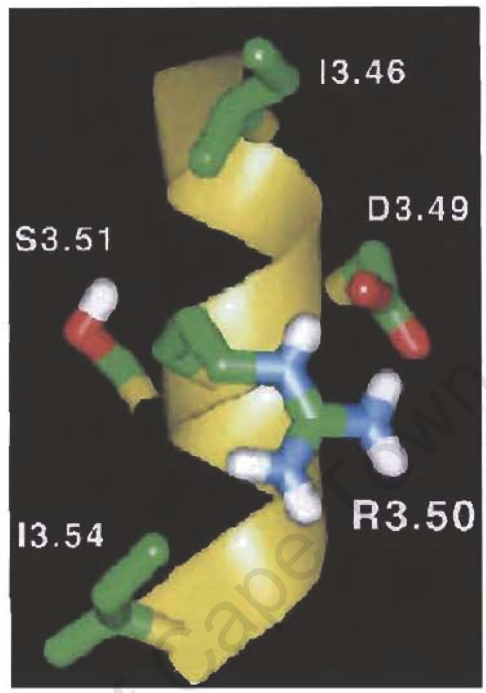

Figure 1.6 Spatial orientation of the conserved $Arg^{3.50}$ in the DRS motif of the human type I GnRH receptor (reproduced from Ballesteros et al., 1998). A three-dimensional computational simulation model of the human type I GnRH receptor shows the spatial proximity of the conserved residues in the Asp-Arg-Ser (DRS) motif.

In most GPCRs this pair of residues is $Asp^{2.50}$ and $Asn^{7.49}$, which are 98% and 95% conserved, respectively (Probst et al., 1992). As discussed in section 1.3.2.3, $Asp^{2.50}$ and $Asn^{7.49}$ in rhodopsin are proposed to interact via a H_2O molecule mediating a contact between helices two and seven (Palczewski et al., 2000). In the human type I GnRH receptor, a series of double mutations and molecular modeling have suggested that $Asn^{2.50(87)}$ and $Asp^{7.49(319)}$ interact and imply that $Asp^{7.49(319)}$ substitutes functionally for the conserved $Asp^{2.50}$ in other GPCRs (Ballesteros et al., 1998). $Asn^{2.50(87)}$ in the GnRH receptor is required for stable expression of the GnRH receptor, most likely through an interaction with the conserved $Asn^{1.50(53)}$ residue, which is also required for receptor expression (Flanagan et al., 1999). Moreover, $Asp^{7.49(319)}$ is involved in GnRH receptor activation. If a reciprocal mutation is introduced into the GnRH receptor, which recreates the arrangement found in most GPCRs ($Asp^{2.50}$ and $Asn^{7.49}$), binding is restored (Awara et al., 1996; Zhou et al., 1994). According to molecular modeling it has been proposed that the side chain of $Arg^{3.50(139)}$ is capable of extending from TM helix three toward TM helices two and seven, where it can

simultaneously interact with Asn$^{2.50(87)}$ and Asp$^{7.49(319)}$, stabilizing the active receptor conformation (Ballesteros et al., 1998). These physical changes in GPCR conformation regulate the three-dimensional arrangement of the intracellular domains, exposing ICL sequences which can bind to cognate G proteins, previously inaccessible for binding (Samama et al., 1993).

1.3.2.6 GnRH Receptor G Protein-Coupling

GnRH mediates its response through the activation of the GnRH receptor, which activates the phospholipase C signalling pathway via coupling to the $G_{q/11}$ class of G-proteins (Kaiser et al., 1997). Most GPCRs have a highly conserved [Asp-Arg-Tyr-X-X-(Ile/Val)-X-X-Pro-Leu] ([DRYXX(I/V)XXPL]) sequence in ICL2 (Fig. 1.1) that has been implicated in G protein coupling (Gether, 2000). In bovine rhodopsin this sequence is [Glu-Arg-Tyr-Val-Val-Val-X-X-Pro-Met] ([ERYVVVXXPM]) and Val$^{3.52(137)}$ Val$^{3.53(138)}$ Val$^{3.54(139)}$ are closely located to partly cover the cytoplasmic side of Glu$^{134(3.49)}$ and Arg$^{135(3.50)}$ which may be involved in receptor activation (Palczewski et al., 2000). In the GnRH receptors this motif is [Asp-Arg-X-X-X-(Ile/Val)-X-X-Pro-Leu] [DRXXX(I/V)XXPL] (Fig. 1.5 and 1.6). In the mammalian GnRH receptors Tyr$^{3.51}$ in this motif is replaced by Ser (Sealfon et al., 1997). Ser$^{3.51(140)}$ has been associated with receptor activation and internalization (Arora et al., 1995). The conserved hydrophobic Leu$^{3.58}$ residue, which is required for G protein-coupling and internalization of muscarinic receptors (Moro, 1993), also has a role in G protein-coupling and agonist-induced receptor internalization of the GnRH receptor (Arora et al., 1995). The GnRH receptor is also capable of coupling to adenylyl cyclase and studies have shown that ICL2 contains the Gs recognition motif, BBXXB (B is basic amino acids, Fig. 1.4) (Arora et al., 1998).

In the vertebrate GnRH receptors the C-terminal domain of ICL3 is conserved and residues in this region may be important for coupling to $G_{q/11}$. Within this region, Ala$^{6.29(261)}$ is proposed to play a role in allowing the assumption of a tertiary structure of ICL3 necessary for G protein-coupling and internalization (Myburgh et al., 1998). Neighboring Ala$^{6.29(261)}$ in ICL3 is Arg$^{6.30(262)}$, which is highly conserved and is also implicated in G protein coupling. Natural mutations of Arg$^{6.30(262)}$ to Gln have been shown to cause uncoupling of the receptor in families with hypogonadotropic hypogonadism (de Roux et al., 1999).

In TM helix seven, Tyr$^{7.53(322)}$ is part of the conserved Asp-Pro-Ile-Leu-Tyr (DPILY) motif (Fig. 1.5) in GnRH receptors (Asn-Pro-X-X-Tyr in other GPCRs) and if mutated to Ala results in full uncoupling. If Tyr$^{7.53(322)}$ is mutated to Phe the mutant receptor is supercoupled (Arora et al.,

1996). In other GPCRs this microdomain is proposed to be important in creating a flexible hinge involved in allowing the receptor to switch its three-dimensional arrangement between the inactive and active conformation (Konvicka, 1998; Sansom, 2000). Interestingly, most GPCRs are coupled to a small G protein, adenosine diphosphate ribosylation factor (ARF), which activates phospholipase D (PLD) (Mitchell et al., 1998). Mitchell proposed that GPCRs carrying the Asn-Pro-X-X-Tyr motif seem to form functional complexes with ARF and RhoA. Rhodopsin also carries the Asn-Pro-X-X-Tyr motif, however the importance of it remains unclear. In bovine rhodopsin the polar side chains of $Asn^{7.49(302)}$ and $Tyr^{7.53(306)}$ project to the inside of the molecule which may form an additional interhelical constraint with TM two (Palczewski et al., 2000). The GnRH receptor does not couple to ARF due to the substitution of $Asp^{7.49}$ (in other GPCRs) for $Asn^{7.49(319)}$. If $Asp^{7.49(319)}$ of the GnRH receptor is substituted with Asn as in the conventional GPCR arrangement, ARF coupling is induced (Mitchell et al., 1998).

1.3.2.7 GnRH Receptor Intracellular Signalling Pathways

The primary gateway of the intracellular signalling pathway of activated GnRH receptors is through an interaction with $G_{q/11}$ proteins that activate the Ca^{2+}-dependent phospholipase Cβ (PLC) (Fig. 1.7). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a small water soluble molecule, which diffuses from the membrane to the cytosol, where it binds to IP_3 receptors in the endoplasmic reticulum. Activated IP_3 receptors induce Ca^{2+} channels to open, releasing Ca^{2+} from these intracellular stores into the cytosol which elicits a rapid spike of LH (Kaiser et al., 1997; Stojilkovic et al., 1994) (Fig. 1.7). GnRH receptor activation also induces L-type voltage-operated Ca^{2+} channels to open and allow the influx of extracellular Ca^{2+}, restoring intracellular Ca^{2+} stores (Fig. 1.7). On the other hand, DAG remains in the plasma membrane where it activates Ca^{2+}-dependent protein kinase C (PKC) which phosphorylates proteins involved in a sustained LH release and gonadotropin biosynthesis (Kaiser et al., 1997). Although the GnRH receptor does not activate the G proteins, ARF and RhoA (Section 1.3.2.6) (Mitchell et al., 1998), agonist-stimulated gonadotropes display a sequential activation of PLC and PLD (Zheng et al., 1994). In the GnRH receptor, it is proposed that PLD hydrolyses phosphatidylcholine to DAG by unknown mechanism, resulting in a biphasic production of DAG in tandem with PLC (Zheng et al., 1994). GnRH has also been shown to stimulate adenylate cyclase resulting in cAMP production, which in turn activates protein kinase A (PKA) (See section 1.3.2.6) (Arora et al., 1998). Another potential signalling pathway of GnRHs through mitogen-activated protein kinase (MAPK) which is involved in the transcriptional regulation of genes encoding gonadotropins. MAPK also phosphorylates and activates phospholipase A_2

(PLA_2). PLA_2 hydrolyses phosphatidylcholine forming arachidonic acid which in turn stimulates leukotriene production in pituitary cells (Naor et al., 2000; Stojilkovic and Catt, 1995). It has been shown that GnRH directly activates the four MAPK cascades; extracellular signal-related kinase (Reiss et al., 1997), Jun N-terminal kinase (JNK) (Levi et al., 1998), p38MAPK (Roberson et al., 1999) and big MAPK (BMK) simultaneously, via Gq and PKC phosphorylation of receptor tyrosine kinases/RAF-1. It is proposed that the MAPK cascades are activated to various degrees which dictates the specificity of GnRH signalling (Naor et al., 2000; Naor et al., 1998). Ultimately, the signalling pathways of the GnRH receptor are all variously involved in gonadotropin gene expression, biosynthesis, and exocytosis. As discussed in section 1.3.1.2, the GnRH pulse frequency modulates the signalling pathways and together with the differential phasing and duration of the various signalling pathways provides fine regulation of gonadotropin secretion. Various forms of GnRH (GnRH and GnRH II), GnRH receptors (GnRH receptor and type II GnRH receptor), and feedback from gonadal steroid and peptide hormones, seem to be the primary regulators of gonadotropin secretion.

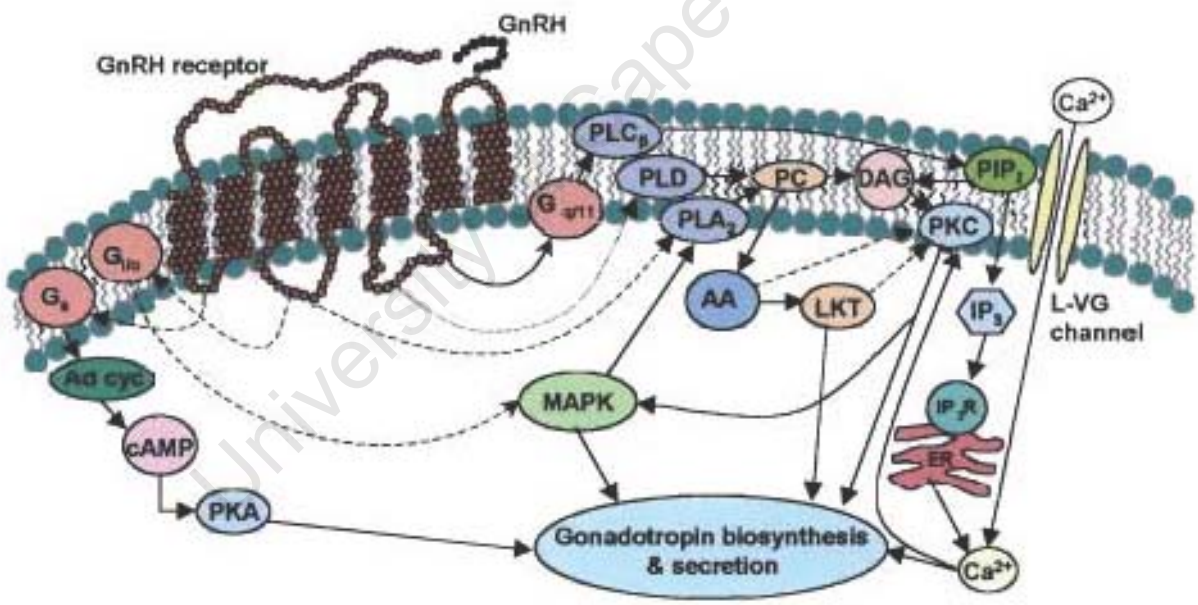

Figure 1.7 Signalling pathways of the GnRH receptor. The primary signalling pathway involves the activated GnRH receptor interacting with $G_{q/11}$. This G protein activates phospholipase C (PLC) which hydrolyses phosphatidylinositol 1,4 bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG activates protein kinase C (PKC) and IP_3 stimulates the release of Ca^{2+} from the (ER). Ca^{2+} also enters the cytosol through L-type voltage gated (L-VG) channels. The stimulated GnRH receptor also activates phospholipase D (PLD) which hydrolyses phosphatidylcholine (PC) to DAG which activates PKC and mitogen activated protein kinase (MAPK). PC generates arachidonic acid (AA) and leukotrienes (LKT) which also activate PKC. Dashed lines show proposed signalling pathways via G_s and $G_{i/o}$ proteins as discussed in section 1.3.2.8. The end products of the GnRH signalling pathways are gonadotropin biosynthesis and secretion.

1.3.2.8 GnRH Receptor Internalisation

Continuous high doses of GnRH lead to gonadotrope desensitisation which includes GnRH receptor down regulation, receptor uncoupling from cognate G proteins, down stream uncoupling, inhibition of gonadotropin synthesis, and alterations in glycosylation of gonadotropins.

In most GPCRs, receptor activation leads to protein kinases phosphorylating ICL domains which results in uncoupling of G proteins and internalisation of the receptor (Leeb-Lundberg et al., 1987; Sibley et al., 1987). In GPCRs the C-terminal tail is the prime target for phosphorylation by protein kinases which results in docking of arrestins, followed by uncoupling and receptor internalisation. The GnRH receptor lacks the C-terminal tail and consequently has a lack of rapid desensitisation in inositol phosphate production and a relatively slower rate of receptor internalisation (Davidson et al., 1994; Naor et al., 2000). Non-mammalian GnRH receptors have a C-terminal tail which mediates rapid agonist-promoted receptor internalization. It is proposed that the slower internalisation rate of the mammalian type I GnRH receptor may be associated with the physiological need of a prolonged LH surge, which is required for ovulation in mammals (Pawson et al., 1998).

1.4 Concluding Remarks

GPCRs comprise the largest superfamily of transmembrane proteins with over 1000 members, which have been cloned and characterised (Gether, 2000). The chemical diversity among the endogenous ligands is remarkable, which include biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions and enzymes. These seven transmembrane spanning receptors are important in conveying extracellular signals to the intracellular milieu of target cells, causing specific responses, which govern a wide variety of metabolic processes. To no surprise, 65% of current drugs target these receptors (Hamm, 2001). Using structural data obtained from X-ray crystallography of GPCRs may prove useful in providing a direct view of the drug-binding site. Although an X-ray diffraction analysis of rhodopsin has recently been published (Palczewski et al., 2000), most other G protein-coupled receptors (GPCR) are more difficult to purify. Consequently, understanding of the structure of these GPCRs is likely to depend on indirect methods, such as computational modeling and mutagenesis, for some time to come.

In the past decade vast strides have been made in the utilization and diversity of GnRH and its analogues in a wide therapeutic application in infertility, cryptorchidism, polycystic ovarian syndrome, leiomyomata, acute intermittent porphyria, and breast, ovarian and prostate cancer

(Casper, 1991; Millar et al., 1987). Researching GnRH and the GnRH receptor has been an intense and productive field providing further understanding in reproductive biology and developing improved medical therapies (Millar et al., 2000; Sealfon et al., 1997).

In the human GnRH receptor, residues $Asp^{2.61(98)}$, $Trp^{2.64(101)}$, $Asn^{2.65(102)}$, $Lys^{3.32(121)}$ and $Asn^{5.61(212)}$ (residue numbering is described in chapter two) have been shown to have roles in ligand binding (Davidson et al., 1996a; Flanagan et al., 2000; Hoffmann et al., 2000; Zhou et al., 1995). In a computational model of the receptor-ligand complex some of these receptor residues have been proposed to form part of the ligand binding pocket, interacting with the amino- and carboxy-termini of GnRH (Sealfon et al., 1997). In this model $Asp^{2.61(98)}$ is proposed to interact with His^2 of GnRH, while $Asn^{2.65(102)}$ interacts with Gly^{10}-NH_2. $Trp^{2.64(101)}$, $Lys^{3.32(121)}$, and $Asn^{5.61(212)}$ are important for binding of agonists, but not antagonists (Hoffmann et al., 2000; Zhou et al., 1995). In GnRH, Arg^8 is required for high affinity binding to mammalian type I GnRH receptors and has been proposed to be involved in an electrostatic interaction with an acidic residue in the GnRH receptor (Hazum, 1987). Mutation of the $Glu^{7.32(301)}$ residue of the mouse GnRH receptor to Gln decreased the receptor affinity for GnRH, but not for analogs with substitutions for Arg^8 (Flanagan et al., 1994). Subsequent models of GnRH receptor-ligand complexes have incorporated an interaction of the acidic residue of the receptor with Arg^8 of the ligand (Chauvin et al., 2000; Flanagan et al., 2000; Hoffmann et al., 2000). However, conformationally-constrained GnRH analogues bind the $Glu^{7.32(301)}$Gln mutant receptor with high affinity, suggesting that the mechanism by which $Glu^{7.32(301)}$ of the mouse GnRH receptor determines selectivity for native GnRH may be more complex than a simple electrostatic interaction. This study investigates whether $Asp^{7.32(302)}$ has the same function in the human GnRH receptor as $Glu^{7.32(301)}$ has in the mouse receptor. The mechanism by which the $Asp^{7.32(302)}$ determines selectivity for GnRH is examined by mutating $Asp^{7.32(302)}$Asn and ligand modification. This study further examines the role of various conformational constraints incorporated into GnRH in determining high affinity for the human type I GnRH receptor. The enhanced affinity of a series of peptides with different D-amino acid substitutions in position six and a peptide with a γ-lactam incorporated in positions six and seven (Freidinger et al., 1980), suggests that the GnRH receptor recognises a ligand conformation. These conformational constraints were also applied to antagonists, extending our earlier observations that various GnRH analogs can be stabilized in a high affinity conformation. Furthermore, the role of conformationally-constrained GnRH analogues in binding $Asp^{7.32(302)}$ of the human GnRH receptor is investigated. This thesis proposes that a ligand–receptor interaction induces a preferred ligand conformation which is necessary for effective GnRH binding.

Aligning the amino acid sequence of the ECL 3 of mammalian and non-mammalian type I GnRH receptors revealed some fundamental differences in amino acid sequence which may contribute to various conformations of ECL3 in the different GnRH receptor and play a role in a ligand binding and selectivity. The conformation of ECL3 in the mammalian GnRH receptor and its significance in GnRH binding and selectivity is explored in this thesis. The recent crystal structure of rhodopsin revealed that the extracellular loops are in a highly organized structure (Palczewski et al., 2000), which is proposed to be conserved in the family A type GPCRs (Ballesteros et al., 2001a), to which the GnRH receptor belongs. There is an emerging hypothesis that extracellular loops of GPCRs may modulate G protein interaction (Zhao et al., 1998) and this thesis also investigates the role of ECL3 of the GnRH receptor in receptor activation.

The primary question this thesis examines is the mechanism by which ECL3 of the human GnRH receptor determines high affinity binding for GnRH and how mammalian GnRH receptors confer selectivity for native GnRH.

Chapter 2

Materials and Methods

This chapter outlines the materials and methods used in this thesis.

2.1 Consensus Residue Numbering Scheme

In this thesis, a consensus numbering scheme (appendix I, p.146) is used to facilitate the comparison of equivalent amino acid residues in the different rhodopsin-like GPCRs (Ballesteros and Weinstein, 1995).

2.2 Site Directed Mutagenesis

The human GnRH receptor gene was cloned into a pcDNAI/AMP expression vector (Invitrogen, San Diego, USA) using Eco RI and Xho I. A polymerase chain reaction-based mutagenesis method was used to substitute various amino acids in ECL3 of the human GnRH receptor. Primers contained a silent restriction endonuclease site (Cla I: ATCGA, see appendix II, p.147) followed by the desired mutation(s) and flanked by at least 18 bases of the wild type receptor sequence. Sense primers were constructed to align with bases encoding the same silent restriction site (Cla I) as that of the antisense primers. Both sense and antisense primers had four to five randomly chosen bases (ATGC/ ATGCT, see appendix II) preceding the Cla I sites to form a large enough region for the enzyme to attach. Sense primers containing the silent site and desired point mutation(s) were used together with SP6 antisense primer, and antisense primers (containing the same silent site) were used with T7 sense primers. Polymerase chain reaction products were digested with appropriate restriction enzymes (Eco RI and ClaI, and ClaI and Xho I) to expose the appropiate ligation sites, agarose gel purified (Amersham, Arlington Heights, England) to remove the additional primer insertions (ATGC/ ATGCT), ligated (at Cla I site) using T4 DNA Ligase (Amersham, Arlington Heights, England), subcloned into the Eco RI and Xho I sites of the mammalian expression vector pcDNAI/AMP (Invitrogen, San Diego, USA), and transformed into competent XL-1 blue *Escherichia coli*. Plasmid DNA was extracted with the PC100 or PC500 kits (Machery-Nagel, Düren, Germany) from ampicilin-resistant clones. All mutant DNA constructs were manually sequenced (Epicentre Technologies, Madison, USA) and those with the intended mutation(s) were used in experiments.

2.3 Insertional Mutagenesis

To increase the length of ECL3 in the human GnRH receptor, Ala residues (one, two, and three) were inserted between $Pro^{6.37(294)}$ and $Glu^{6.38(295)}$. The cDNA of the human GnRH receptor was cloned into the phagemid pBluescript II SK (Stratagene, La Jolla, USA) by using EcoRI and XhoI restriction endonuclease sites. Oligonucleotide-directed mutagenesis (Kunkel et al., 1991) was

performed using single stranded Uracil-containing DNA with the human GnRH receptor gene (prepared by Mr. Peter Davies from above mentioned DNA construct) and primers (appendix II, p.147) containing the desired mutations and a silent restriction endonuclease site (Cla I: ATCGA). The primers were annealed to the single stranded DNA for five minutes at 70°C, followed by 30 minutes on ice (4°C). Primer extension was performed at 37°C for two hours. PCR products were cloned into pcDNAI/AMP (Invitrogen, San Diego, USA). The mutagenesis products were transformed into competent XL-Blue *Escherichia coli* (CJ236). The plasmid DNA was extracted using PC100 kit (Machery-Nagel, Düren, Germany) from ampicilin-resistant clones. The different mutant GnRH receptor constructs were subcloned into the Eco RI and Xho I sites of the mammalian expression vector pcDNAI/AMP, and transformed into competent XL-1 blue *Escherichia coli*. The plasmid DNA was extracted from ampicilin-resistant clones using PC100 or PC500 kits (Machery-Nagel, Düren, Germany). The mutations were confirmed by manual DNA sequencing (Epicentre Technologies, Madison, USA).

2.4 Substitution of ECL3 of the Human GnRH Receptor with ECL3 of the Chicken GnRH Receptor

ECL3 of the human GnRH receptor was substituted with the equivalent loop of the chicken GnRH receptor. It was necessary to introduce unique restriction enzyme sites into the human GnRH receptor gene to facilitate exchange of extracellular loop domains (Ott, 2000). Thus, a human GnRH receptor containing silent mutations that introduce Sna BI and Hpa I sites at the N- and C-terminus, respectively, of ECL3 was constructed (Ott, 2000). The resultant receptor showed binding properties and second messenger responses identical to those of the wild-type human GnRH receptor (Ott, 2000). ECL3 of the chicken GnRH receptor was amplified by PCR using proof-reading Deep Vent DNA polymerase (New England Biolabs, Hertfordshire, UK) and mutagenic primers flanked by Sna BI and Hpa I restriction endonuclease sites. The resulting ECL3 of the chicken GnRH receptor was agarose gel purified (Amersham, Arlington Heights, England) and ligated into the appropriately digested human GnRH receptor in pBluescript SK(-) (Stratagene, La Jolla, USA). This produced the chimeric receptor, which was subcloned into pcDNAI/Amp the expression vector and sequenced (Epicentre Technologies, Madison, USA).

2.5 GnRH Analogs

The GnRH analogs were either purchased, prepared by, or gifts from members of the various laboratories as indicated. GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), [Gln^8]-GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2) [His^5,D-Tyr^6]-GnRH, [D-Ala^6,N-Me-

Leu^7,Pro^9-NHEt]-GnRH, [D-Trp^6,Pro^9-NHEt]-GnRH, [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH, GnRH II ([His^5,Trp^7,Tyr^8]-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}-NH_2]-GnRH) were prepared on a Beckman System 990 peptide synthesizer by other members (R. Milton) of the Cape Town laboratory using conventional solid phase methodology and purified (>96%, at 210nM) by preparative C-18 reverse phase HPLC. Antagonist 129-62 ([Ac-D-Nal(2)^1,D-4-Cl-Phe^2,D-Trp^3,3-Pal^5,6,7 γ-lactam,Ipr-Lys^8,D-Ala^{10}-NH_2]-GnRH) and [Glu^8]-GnRH were prepared by Dr R Roeske (Indiana University School of Medicine, Indianapolis, Indiana, USA) using solid phase synthesis on an MBHA resin using Boc/Benzyl chemistry. The Boc-γ-lactam (Freidinger et al., 1980; Appendix III) was added as one amino acid unit. After removal from the resin by HF, the peptides were purified to homogeneity by reverse phase HPLC on a C-18 preparative column. Antagonist 26 ([Ac-D-4-Cl-Phe^{1,2},D-Trp^3,D-Lys^6,D-Ala^{10}-NH_2]-GnRH) was a gift from Dr D Coy (Tulane University School of Medicine, New Orleans, USA). [6,7 γ-lactam]-GnRH was a gift from Dr R Freidinger (Merck & Co., West Point, USA). Somatostatin (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys) was purchased from Sigma–Aldrich, SA.

2.6 Peptides Corresponding to the Sequence of ECL3 of the GnRH Receptor

Model peptides were designed and synthesised by Dr David Craik (University of Queensland, Brisbane, Australia), which correspond to residues 293(7.25)-302(7.33) of the mouse GnRH receptor. This peptide was used for spectroscopic and binding studies (chapter five). Gly and Cys residues were added at the carboxyl and amino termini, respectively (Cys-Gly-Pro-Glu-Met-Leu-Asn-Arg-Val-Ser-Glu-Pro-Gly-Cys) to allow the introduction of a disulfide bridge to reduce the conformational space and simulate a loop-like constrained form as is present in the native receptor. This peptide was synthesised by conventional solid phase peptide synthesis using t–Boc chemistry. The linear peptide was cleaved from the resin using HF (10% cresol) and purified using reversed phase HPLC on a C18 column. The disulfide bond in the cyclic peptide was produced by air oxidation (1:1 DMF–H_2O) of the linear peptide for 12 hrs, followed by HPLC purification. DL–dithiothreitol (DTT), 10–camphorsulphonic acid (CSA) and 2,2,2–trifluoroethanol (TFE) were purchased from Sigma–Aldrich, South Africa.

Retro-inverso (RI) peptides was synthesised by Dr Jean-Paul Briand (Immunochemistry Laboratory, Institut de Biologie Moleculaire et Cellulaire du CNRS, Strasbourg, France), using Fmoc technology and was purified by HPLC and checked with mass spectrometry. The sequence of the RI-ECL3 peptide corresponding to ECL3 of the human type I GnRH receptor (RI-ECL3) was AcAsp-Ser-Leu-Arg-Asn-Leu-Met-Glu-Cys-$CONH_2$ (all D amino acids) which included the

additional cysteine residue at the C-terminus for conjugation purposes. Control RI-peptides used in the various assays (chapter five) were ECL2 of the muscarinic receptor (AcVal-Arg-Thr-Val-Glu-Asp-Gly-Glu-Cys-$CONH_2$), ECL1 of the human GnRH receptor (AcLeu-Glu-Gly-Ala-Tyr-Trp-Gln-Val-Cys-$CONH_2$) and GnRH (AcGly-Pro-Arg-Leu-Gly-Tyr-Ser-Trp-His-Glu-Cys-$CONH_2$). RI peptides are composed of D-amino acids assembled in the reverse order (C to N terminus) in relation to the parent L-peptide, which leads to the orientation of the side chains in a RI-analog to be very similar to that in the parent peptide. These peptides are immunogenic and produce antibodies, which can cross-react with the parent peptide with high specificity (Van Regenmortel et al., 1998a).

2.7 Transfection and Cell Culture

COS-1 cells were maintained in Low Glucose DMEM media (10% heat inactivated fetal calf serum/FCS, Delta Bioproducts, Kempton Park, South Africa) at 37°C and 10% CO_2 and trypsin (0.05%). COS-1 cells were transiently transfected using the DEAE-Dextran method (Keown et al., 1990), as previously described (Millar et al., 1995). For 12-well plates (Corning, Cambridge, USA) 2.0µg DNA was used for transfection of 2 x 10^5 cells/well and 15µg DNA was used for 100 mm culture dishes (Corning, Cambridge, USA), containing 3.10^6 cells. After transfection, COS-1 cells were cultured for ±40 hours (Millar et al., 1995) in Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland) containing 10% FCS and antibiotics (2mg/ml streptomycin sulphate, 4000U/ml sodium Benzylpenicilin) in a 10% CO_2 incubator at 37°C.

2.8 Radioligand Binding Assay

Crude membrane binding assays were performed as this method makes it possible to optimize receptor concentration by varying the amount of transfected membranes used in the assay (Millar et al., 1995). The agonist peptide, [D-Ala^6,N-Me-Leu^7,Pro^9-NHEt]-GnRH was radioiodinated on Tyr^5 (Flanagan et al., 1998) with the Chloramine-T method as described. Previous studies showed that iodinated GnRH analogs had similar affinities to their equivalent peptides (Clayton et al, 1979, Perrin, 1983). Specific activity ranged between 900 and 1800 µCi/µg and 70% of the radioactivity could be bound by GnRH receptors (Flanagan et al., 1998). This label was used in initial experiments and was later replaced by a high affinity agonist peptide, [His^5,D-Tyr^6]-GnRH, which was radioiodinated at D-Tyr^6 (Flanagan et al., 1998). Specific activity for iodinated [His^5,D-Tyr^6]-GnRH ranged between 900 and 1800 µCi/µg and 69% of the radioactivity could be bound by GnRH receptors. Using this high affinity label allowed accurate determination of IC_{50}s for mutant receptors, which had low total binding. Additionally, low total binding with mutant receptors was

compensated for by increasing the membrane concentration per reaction (see separate chapters for details). Transfected COS-1 cells were harvested in binding buffer (1mM EDTA, 10mM HEPES, pH 7.4, 0.1% BSA) with a cell scraper (Corning, Cambridge, USA), manually homogenized in a Dounce homogenizer (Kontes, Vineland, USA) and centrifuged at 15000 x g for 30 minutes at 4°C. The resultant crude membrane pellet was resuspended in binding buffer to various membrane concentrations (see above). The membrane suspension (triplicate, 400µl/tube) was incubated overnight at 4°C with 50µl ^{125}I-[His^5,D-Tyr^6]-GnRH (50 000 cpm, 50 pM) and varying concentrations (50µl) of unlabelled GnRH analogs. We have previously found that equilibrium binding is achieved after 21 hours and stable for up to 30 hours (Flanagan et al., 1998). The incubation was terminated by the addition of 3ml cold polyethylenimine (0.01%, PEI), which also reduced non-specific binding, followed by immediate filtration through glass fibre filters (GF/C, Whatman, Maidstone, England) which were pre-soaked (15-30 minutes) in 1% PEI (Flanagan et al., 1998). The filters were washed twice with 3ml 0.01% PEI and the retained radioactivity was counted. Non-specific binding was determined in the presence of 1µM antagonist 27. Depending on the membrane concentration, non-specific binding accounted on average 20-40% of the total binding. As an additional control for non-specific binding, mock-transfected cells (expression vector only) were incubated under the same conditions as described above and at the specific membrane and label concentrations used for each receptor construct. The amount of ^{125}I-[His^5,D-Tyr^6]-GnRH (50 000 cpm, 50 pM) that bound cells transfected with the wildtype/mutant GnRH receptor in the presence of 1µM antagonist 27, was similar to the amount of label that bound mock transfected cells.

2.9 Inositol Phosphate (IP) Accumulation Assay

Transfected COS-1 cells (2 x 10^5 cells/well) in 12-well plates were cultered for ±24hours at 37°C in Dulbecco's modified Eagle's medium (as described in section 2.7) before overnight incubation with *myo*-[2-^{3}H] inositol (1µCi/well, Amersham, Arlington Heights, England) in 0.5ml Medium 199 (Gibco, Paisley, Scotland) with antibiotics. The labelled cells were washed twice with 1ml/well buffer I (140mM NaCl, 4mM KCl, 20mM Hepes, 8mM glucose, 0.1% BSA, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 7.4) and incubated with various concentrations of ligand (in duplicate) for one hour at 37°C in the presence of LiCl (10mM) as described (Millar et al., 1995). Basal IP accumulation was measured in the absense of ligand. Aspirating the medium and addition of 10mM formic acid (1ml/well) terminated the incubation and left on the cells for 30 minutes at 4°C to extract inositol phosphates. Inositol phosphates were separated from 1ml sample extracts on 10ml columns filled with 1ml 1X8-200 DOWEX-1 ion exchange resin, mesh 100-200 (Sigma–Aldrich, South Africa).

The columns were saturated with 3ml 3M ammonium formate with 0.1M formic acid, washed with 10ml dH_2O, loaded with 1ml sample extract, washed with 10ml dH_2O, followed by 5ml 5mM myo-inositol with 0.1M formic acid. Total inositol phosphates were eluted with 3ml 1M ammonium formate with 0.1M formic acid into 18ml of scintillation liquid (Zinsser Analytical, Frankfurt, Germany) and the radioactivity was counted.

2.10 Inhibition of GnRH Stimulated IP Accumulation with Peptides Corresponding to the Sequence of Extracellular Loops of the GnRH Receptor

A RI peptide (all D amino acids) corresponding to the sequence of ECL3 of the human GnRH receptor was initially intended for the generation of anti-GnRH receptor antibodies targeting ECL3 of mammalian GnRH receptors, but surprisingly could specifically inhibit GnRH stimulated IP accumulation (chapter five). This prompted us to test whether the peptides (all L amino acids) designed for spectroscopic studies with the sequence of ECL3 of the mouse GnRH receptor could also inhibit GnRH stimulated IP accumulation.

The ECL3 peptides were tested for their ability to inhibit GnRH stimulated IP accumulation in COS-1 cells transiently transfected with human GnRH receptor. Adding 0.1nM GnRH to the labelled cells could stimulate 40-50% of the maximal IP response and 1.0nM of [Gln^8]-GnRH induced 40-50% of the total IP production after one hour incubation. Using a concentration of GnRH which stimulates under the Emax value of an IP assay prevents inhibiting a signal that is saturated at maximum stimulation. Various concentrations of ECL3 peptides (0.1 to 10 μM) were incubated with GnRH (0.1nM) and [Gln^8]-GnRH (1.0nM) for two hours at 37°C before adding to the cells transfected with human GnRH receptor to allow sufficient association between the peptides. The preincubation step had no adverse effects on GnRH alone. This mixture was added (0.5ml/well) to the transfected cells and incubated for one hour. The reaction was terminated with formic acid and inositol phosphates were separated and counted as described. The ability of the ECL3 peptides to inhibit GnRH and [Gln^8]-GnRH stimulated IP production was compared to the potency of these peptides alone as described above. A 14 amino acid (Somatostatin: Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys) control peptide was included to measure a specific interaction of GnRH with ECL3 peptides of the GnRH receptor (see chapter five and Fig. 5.3 for details). The ability of different RI-peptides non-specifically interacting with GnRH and its receptor was performed with RI-ECL1 and RI-GnRH peptides.

2.11 Measuring an Interaction between a Retro-Inverso Peptide Corresponding to the Sequence of ECL3 and GnRH using a Surface Plasmon Resonance System

The ability of GnRH and [Gln^8]-GnRH to interact with a RI-peptide, corresponding to ECL3 of the human GnRH receptor sequence was analysed with a biomolecular interaction (BIA) system. This biosensor technology based on surface plasmon resonance (SPR) makes it possible to visualise the binding process as a function of time by following the refractive index that occurs when peptides/antibodies binds to another molecule immobilised on the surface of a sensor chip. This interaction is measured as resonance units (RU) (Van Regenmortel, 1998b). This system was employed to measure the binding kinetics of GnRH and [Gln^8]-GnRH to the RI-peptide, corresponding to ECL3 of the human GnRH receptor. A RI-peptide corresponding to the ECL2 of the muscarinic receptor (section 2.6) served as a control to measure a specific interaction of GnRH with the RI-ECL3 peptide of the GnRH receptor (chapter five).

All equipment, products and reagents for the BIACORE 1000 system were purchased from BIACORE^{TM} (Pharmacia Biotech, Uppsala, Sweden). Reagents used were P20 (used as a surfactant), an amine coupling kit containing N-N-hydroxysuccinimide (NHS), N-ethyl-N'-dimethylaminopropyl carbodiimide (EDC), 2-(2-pyridinyldithioethaneamine) (PDEA) and cysteine. All biosensor assays were performed in Hepes-buffered saline (HBS) running buffer (10mM Hepes, 150mM NaCl, 3.4mM EDTA, 0.005% P20). The carboxylated dextran matrix on the sensor chip was activated with 50µl of a mixture containing 0.2M EDC and 0.05M NHS. To introduce a reactive disulfide group onto the carboxyl groups of the dextran layer 40µl of 80mM PDEA in borate buffer (pH 8.5) was injected. The unoccupied sites of the matrix were saturated by injecting 35µl of ethanolamine hydrochloride (pH 8.5) into the system. The RI-ECL3 of the GnRH receptor or RI-ECL2 of the muscarinic receptor peptide (100µg RI-peptide /ml in 10mM acetate buffer, pH 5.0) was fixed for four minutes onto the matrix of the sensor chip by the standard thiol immobilisation protocol as briefly described (Biacore^{TM}, Uppsala, Sweden). The remaining reactive disulfide groups were saturated by injecting 30µl of 1M NaCl and 50mM L-cysteine in 0.1M formate buffer (pH 4.3). The matrix was washed of any excess reagents with 5µl 0.1N HCl (100µL/min). Above 300 RU were measured with the immobilised RI-peptides, indicating that the peptides were sufficiently immobilised. Increasing concentrations of GnRH or [Gln^8]-GnRH (250-4000 nM each) were injected over the sensor chip at a flow rate of 5µl/min and a total volume of 100µl for 15 minutes at room temperature (25°C). Rmax value (maximum SPR response) was 438.4 RU for GnRH with RI-ECL3, but no response measured with [Gln^8]-GnRH against RI-ECL3 (chapter five). No RU could be measured for the control RI-peptide (ECL2 of the muscarinic

receptor) exposed to either GnRH or [Gln^8]-GnRH. The kinetic tests were performed at a flow rate of 20µl/min. The sensorgrams were recorded and analysed by BiaEvaluation three software (Pharmacia Biotech, Uppsala, Sweden).

2.12 Data Reduction

IP assays were performed at least three times in duplicate and competition binding assays three times in triplicate. Four-parameter non-linear curve fitting (Prism, GraphPad Software Inc., San Diego, USA) was used to estimate the peptide concentrations required to stimulate half-maximal IP production (EC_{50}) and to half-maximally inhibit the binding of the radioligand (IC_{50}). The formulae for the sigmoidal dose response and one site binding competition values (unweighted) was defined as Y = Bottom + (Top - Bottom)/(1+10^(X - LogEC_{50})) (Prism, Version 3.0, GraphPad Software Inc., San Diego, USA). Coupling efficiency was defined as Q = 0.5 x [(Kd + EC_{50})/EC_{50}] x (E_{max}/B_{max}) (Ballesteros et al., 1998). Ki values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Kd and Bmax values were determined using non-linear curve-fitting (Prism, Version 3.0) of homologous competition binding assays (Klotz, 1982; Motulsky, 1999; Munson and Rodbard, 1980). The formulaes used were coldNM = 10^(x + 9); KdNM = 10^(logKD + 9) and Y = (Bmax*HotNM)/(HotNM + coldNM + KdNM) + NS (Prism, Version 3.0, GraphPad Software Inc., San Diego, USA). The high non-specific binding of the ^{125}I-[His^5,D-Tyr^6]-GnRH tracer at high concentrations makes saturation binding assays unreliable. Data in figures are from single experiments which are representative of at least three independent experiments. Data in tables are the mean ± SE of Ki values of at least three experiments and were provided as a measurement of range. P values were calculated using unpaired two tailed T-Tests performed on the negative log of Ki and EC_{50} values.

Chapter 3

The Role of Aspartate$^{7.32(302)}$ of the Human GnRH Receptor in Stabilizing a High Affinity Ligand Conformation

3.1 Summary

Mammalian gonadotropin-releasing hormone (GnRH) receptors preferentially bind GnRH (mammalian GnRH), which has Arg in position eight. The $Glu^{7.32(301)}$ residue, which determines selectivity of the mouse GnRH receptor for Arg^{8}-containing GnRH, is $Asp^{7.32(302)}$ in the human GnRH receptor. In chapter three, $Asp^{7.32(302)}$ of the human GnRH receptor is demonstrated to also confer selectivity for Arg^{8} of GnRH. The mechanism by which the $Asp^{7.32(302)}$ determines binding specificity for GnRH is examined by site-directed mutagenesis and ligand modification. It is revealed that although Arg^{8} and $Asp^{7.32(302)}$ are required for high affinity binding of GnRH, conformationally-constrained peptides, with D-amino acid substitutions in position six or with a 6,7 γ-lactam, bind the human GnRH receptor with high affinity, which is independent of the presence of $Asp^{7.32(302)}$ in the receptor or Arg^{8} in the ligand. The ability of the ligand constraints to increase affinity and to compensate for the absence of both Arg^{8} and $Asp^{7.32(302)}$ indicates that these residues both have roles in stabilizing a high affinity ligand conformation and that their roles are complementary. This suggests that the Arg^{8} and $Asp^{7.32(302)}$ side chains interact to induce a high affinity conformation of native GnRH. Thus, $Asp^{7.32(302)}$ of the human GnRH receptor determines selectivity for GnRH by its ability to induce a high affinity conformation of its native ligand. However, this initial interaction appears not to contribute to the final ligand-receptor complex. In this chapter it is proposed that Arg^{8} interacts transiently with $Asp^{7.32(302)}$ to induce a high affinity ligand conformation of GnRH, which then interacts with a binding pocket that is common for both constrained and unconstrained analogs of GnRH.

3.2 Introduction

In the human GnRH receptor, 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 have roles in ligand binding (Davidson et al., 1996a; Flanagan et al., 2000; Hoffmann et al., 2000; Zhou et al., 1995). Some of these receptor residues have been proposed to form part of the ligand binding pocket, interacting with the amino- and carboxy-termini of GnRH in a computational model of the receptor-ligand complex (Sealfon et al., 1997). In this model $Asp^{2.61(98)}$ is proposed to interact with His^{2} of GnRH, while $Asn^{2.65(102)}$ interacts with Gly^{10}-NH_2. $Trp^{2.64(101)}$, $Lys^{3.32(121)}$, and $Asn^{5.61(212)}$ are important for binding of agonists, but not antagonists (Hoffmann et al., 2000; Zhou et al., 1995). In the mouse GnRH receptor, $Glu^{7.32(301)}$ was shown to confer selectivity for the Arg^{8} residue of GnRH (Flanagan et al., 1994). Other mammalian GnRH receptors also have acidic side chains at the 7.32 locus in extracellular loop three (ECL3) and selectively bind Arg^{8}-containing GnRH (Sealfon et al., 1997). However, $Glu^{7.32(301)}$ is not completely conserved in mammalian GnRH receptors. In the human and other non-rodent type I

GnRH receptors this residue is $Asp^{7.32(302)}$ (Chi et al., 1993; Cui et al., 2000; Illing et al., 1993; Kakar et al., 1992; Sealfon et al., 1997). Although this is a conservative substitution, it is surprising that such a functionally important residue is not absolutely conserved.

Conversely, Arg^8 in GnRH is required for high affinity binding to mammalian type I GnRH receptors. Substitution of this residue decreases GnRH potency and affinity for the receptor (Millar et al., 1989). Two hypotheses have been proposed to explain the role of Arg^8 in determining high affinity binding. One is that the side chain of Arg^8 influences the structure of the ligand, stabilizing a high affinity conformation of the GnRH peptide (Shinitzky et al., 1976). The other proposal is that Arg^8 may be involved in an electrostatic interaction with an acidic residue in the GnRH receptor (Hazum, 1987). Mutation of the $Glu^{7.32(301)}$ residue of the mouse GnRH receptor to Gln decreased the receptor affinity for GnRH, but not for analogs with substitutions for Arg^8 (Flanagan et al., 1994). Subsequent models of GnRH receptor-ligand complexes have incorporated an interaction of the acidic residue of the receptor with Arg^8 of the ligand (Chauvin et al., 2000; Flanagan et al., 2000; Hoffmann et al., 2000). However, a GnRH analog with D-Trp substituted in position six showed only a small decrease in affinity for the $Glu^{7.32(301)}$Gln mouse receptor. This suggested that although $Glu^{7.32(301)}$ determines selectivity for native GnRH, the mechanism by which it does so may be more complex than a simple electrostatic interaction of Glu^{301} with Arg^8. It also indicates a need for caution in extrapolating experimental results to molecular models of GPCRs.

The lack of conservation indicates a need to determine whether $Asp^{7.32(302)}$ has the same function in the human GnRH receptor as $Glu^{7.32(301)}$ has in the mouse receptor. Furthermore, the incorporation of a direct interaction of $Glu^{7.32(301)}$/$Asp^{7.32(302)}$ with Arg^8 in models of receptor-ligand complexes, in spite of evidence that a direct interaction may not always occur, shows that better definition of the mechanism by which the $Asp^{7.32(302)}$ determines binding specificity for GnRHs needed. In this chapter it is demonstrated that mutating $Asp^{7.32(302)}$ in the human GnRH receptor decreases affinity for GnRH, but not for analogs with substitutions for Arg^8. In contrast, a series of peptides with different structural constraints that stabilize a high affinity conformation of GnRH retain high affinity for the mutant receptor. This indicates that an electrostatic interaction is not involved in the binding of these constrained analogs. It is also shown that a conformationally-constrained analog with Gln^8 has higher affinity than Arg^8-containing GnRH for the human GnRH receptor, indicating that ligand conformation plays a separate role to Arg^8 in GnRH binding. These results are interpreted in terms of a sequential binding mechanism in which the Arg^8 side chain of native GnRH

interacts transiently with Asp$^{7.32(302)}$, before interacting with a final ligand binding pocket which also binds constrained analogs.

3.3 Results

The role of Asp$^{7.32(302)}$ in the GnRH receptor in ligand binding was investigated with a polymerase chain reaction-based mutagenesis method used to replace Asp$^{7.32(302)}$ with Asn (see materials and methods, chapter two). Membrane binding assays were performed to estimate relative affinity of the mutant to wild type GnRH receptor. This method was used with a high affinity tracer to assess the binding characteristics of the Asp$^{7.32(302)}$Asn mutant GnRH receptor.

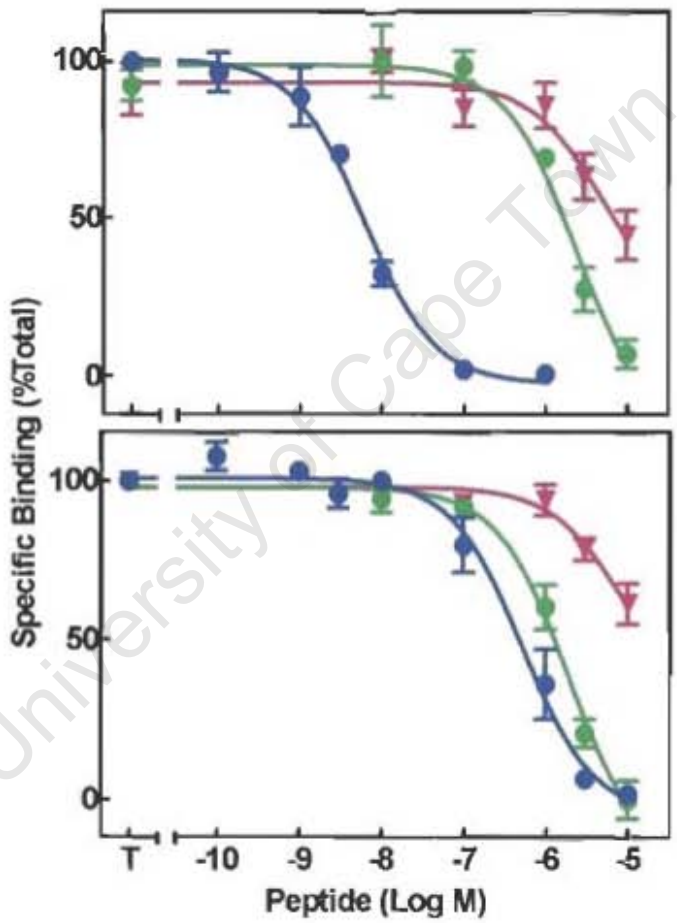

Figure 3.1 Competition binding of GnRH and GnRH analogs in human wild type and Asp$^{7.32(302)}$Asn mutant GnRH receptors. COS-1 cell membranes expressing the human wild type (top) and Asp$^{7.32(302)}$Asn mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), [Gln^8]-GnRH (●), and [Glu^8]-GnRH (▼). Data are mean ± SE of representative experiments performed in triplicate and expressed as percentage of specific binding in the absence of unlabeled ligand, the latter being indicated as total specific binding (T).

Membrane binding assays were performed because different membrane concentrations can be used to compensate for the lower total binding of the $Asp^{7.32(302)}Asn$ mutant GnRH receptor. A higher membrane concentration was used for the mutant GnRH receptor ($1/20^{th}$ dish per reaction tube) relative to membranes expressing the wild type receptor ($1/50^{th}$ dish per reaction tube).

3.3.1 The $Asp^{7.32(302)}Asn$ Mutant has Decreased Affinity for GnRH

The wild type GnRH receptor bound [His^5,D-Tyr^6]-GnRH, which was used as a radiolabelled ligand, with high affinity (Kd 0.35 ± 0.06 nM). The $Asp^{7.32(302)}Asn$ mutant receptor showed 2.8-fold lower affinity for ^{125}I-[His^5,D-Tyr^6]-GnRH (Kd 0.99 nM ± 0.01 nM). Receptor number was unaffected by the $Asp^{7.32(302)}$ mutation (wild type, 1.31 ± 0.23 x 10^5 sites/cell; $Asp^{7.32(302)}Asn$ mutant, 1.31 ± 0.06 x 10^5 sites/cell). The similar expression suggests that the lower total binding of the mutant receptor reported in initial experiments (Flanagan et al., 1998) results from the slightly decreased affinity for ^{125}I-[His^5,D-Tyr^6]-GnRH.

Table 3.1 Summary of ligand binding results with $Asp^{7.32(302)}Asn$ mutant GnRH receptor. Competition binding assays were performed on COS-1 cells transiently transfected with wild type or mutant GnRH receptors, using ^{125}I-[His^5, D-Tyr^6]-GnRH and various concentrations of unlabeled ligands as described under "Materials and Methods" in chapter two. Ki values are the mean ± SE. Statistical analyses were performed using pKi values as described under "Materials and Methods". The fold change was calculated as the ratio of the Ki values of the mutant and wild type receptors.

GnRH analog	Ki		Fold change
	Wild type	Mutant	
	nM		
GnRH[a]	6.79 ± 1.08	212 ± 40.6**	31.2
[Gln^8]-GnRH	923 ± 222	1240 ± 227	1.35
[Glu^8]-GnRH	>10 000	>10 000	-
[His^5,D-Tyr^6]-GnRH	0.48 ± 0.12[b]	1.36 ± 0.20*[b]	2.83
[D-Ala^6,N-Me-Leu^7,Pro^9-NHEt]-GnRH	0.97 ± 0.35	1.55 ± 0.09	1.60
[D-Trp^6,Pro^9-NHEt]-GnRH	0.15 ± 0.04	0.27 ± 0.06	1.80
[D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH	1.86 ± 0.19	1.67 ± 0.43	0.82
[6,7 γ-lactam]-GnRH	1.37 ± 0.07	4.66 ± 0.36**	3.40
GnRH II ([His^5,Trp^7,Tyr^8]-GnRH)	193.7 ± 28.5	213.3 ± 72.4	1.10
Antagonist 129-62	0.86 ± 0.21	1.52 ± 0.28	1.76
Antagonist 26	0.80 ± 0.13	1.13 ± 0.19	1.41

* significantly different from wild type, $p<0.05$

** significantly different from wild type, $p<0.001$

[a] GnRH sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

[b] Kd values are reported for the homologous ligand, [His^5,D-Tyr^6]-GnRH

In competitive ligand binding assays, the affinities of the wild type human GnRH receptor for uncharged, [Gln^8]-GnRH (Ki 923 ± 222 nM) and negatively charged, [Glu^8]-GnRH (>10 000 nM)

were lower than that for Arg^8-containing GnRH (Ki 6.79 ± 1.08 nM, Table 3.1, Fig. 3.1). This shows that the human GnRH receptor preferentially binds Arg^8-containing GnRH. Mutating $Asp^{7.32(302)}$ to Asn decreased affinity for native GnRH (31.2-fold), but the affinities for uncharged [Gln^8]-GnRH or negatively-charged [Glu^8]-GnRH were unchanged (Table 3.1, Fig. 3.1). This indicates that $Asp^{7.32(302)}$ determines the specificity of the wild type GnRH receptor for native Arg^8-containing GnRH. This is consistent with the proposal that the carboxyl side chain of $Asp^{7.32(302)}$ may be involved in an electrostatic interaction with the positively-charged Arg^8 side chain in GnRH. However, the mutant receptor retained higher affinity for native GnRH than for [Gln^8]-GnRH (6-fold) and did not show enhanced affinity for [Glu^8]-GnRH (Table 3.1). This result suggests that the mechanism by which the receptor selects for Arg^8-containing GnRH may be more complex than a simple electrostatic interaction of Arg^8 with $Asp^{7.32(302)}$.

3.3.2 GnRH Analogs with D-amino Acid Substitutions in Position Six of GnRH Enhance Affinity to the Human GnRH Receptor and Overcome the Absence of Arg^8 and/or $Asp^{7.32(302)}$

It was previously found that binding of the conformationally-constrained analog, [D-Trp^6,Pro^9-NHEt]-GnRH, was not affected by the $Glu^{7.32(301)}$Gln mutation in the mouse GnRH receptor (Flanagan et al., 1994). To test whether this is a general phenomenon in mammalian GnRH receptors, binding affinities of a series of GnRH analogs with different D-amino acid substitutions in position six were characterized in the wild type human receptor and in the $Asp^{7.32(302)}$Asn mutant (Fig. 3.2). As expected for the wild type receptor (Karten and Rivier, 1986; Sealfon et al., 1997), three GnRH analogs, [His^5,D-Tyr^6]-GnRH, [D-Ala^6,N-Me-Leu^7,Pro^9-NHEt]-GnRH and [D-Trp^6,Pro^9-NHEt]-GnRH, showed higher affinity (7.0 to 45-fold) than native GnRH (Table 3.1, Table 3.2, Fig. 3.2). The affinity of the wild type receptor for the uncharged, but conformationally constrained analog [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (Ki 1.9 ± 0.19 nM) was 486-fold higher than the affinity for [Gln^8]-GnRH (Ki 923 ± 222 nM) (Table 3.1). This shows that, in the wild type receptor, incorporation of a D-amino acid in position six enhances the affinity of [Gln^8]-GnRH more than the affinity of Arg^8-containing GnRH and thus compensates for the absence of Arg^8 (Table 3.2).

All four GnRH analogs with D-amino acid substitutions in position six had similar high affinity for the $Asp^{7.32(302)}$Asn mutant receptor compared with the wild type receptor (Table 3.1). In the mutant receptor the affinities of the three analogs with D-amino acids in position six and Arg in position eight were 137- to 784-fold higher than the affinity for (native) GnRH (Table 3.2). The affinity of the uncharged [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH was 744-fold higher than for [Gln^8]-GnRH (Fig.

3.2, Table 3.2). Thus, incorporation of a D-amino acid in position six of GnRH peptides with Arg^8, enhances binding affinity more in the mutant receptor than in the wild type receptor (Table 3.2).

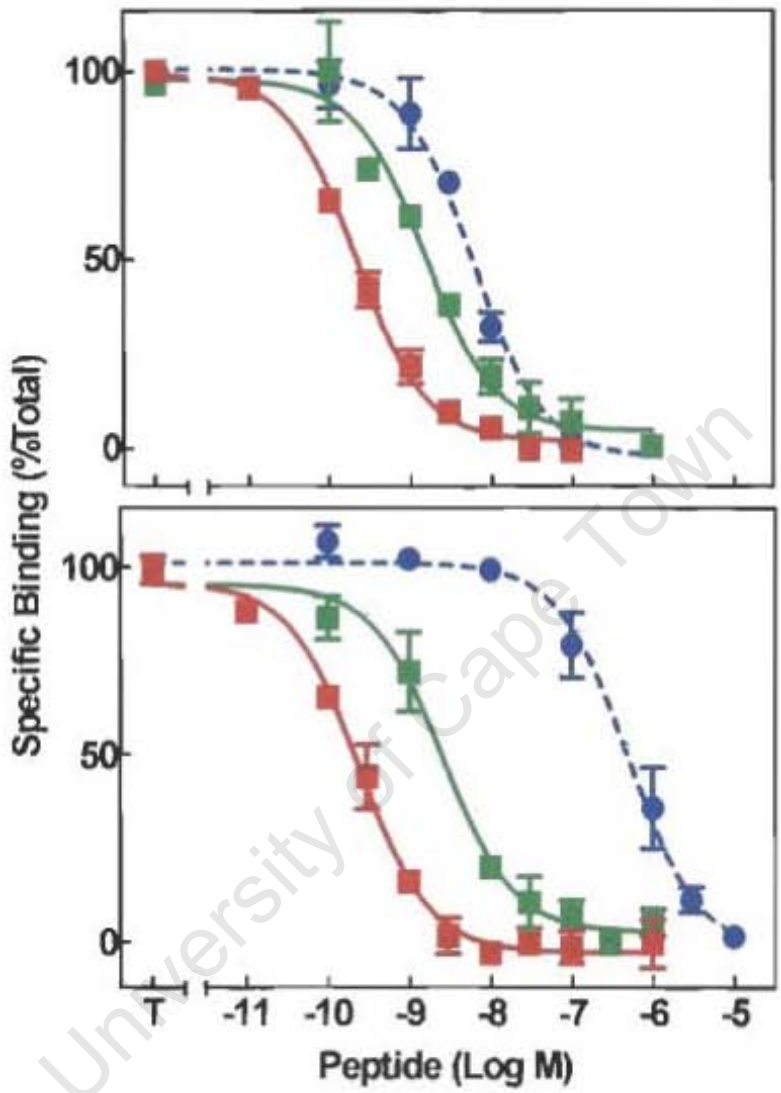

Figure 3.2 Competition binding of GnRH analogs with D-amino acid substitutions in position six. COS-1 cell membranes expressing the human wild type (top) and $Asp^{7.32(302)}Asn$ mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), [D-Trp^6, Pro^9-NHEt]-GnRH (■), and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (■). Data are mean ± SE of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand, the latter being indicated as total specific binding (T).

However, in the wild type receptor the affinity of [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (Ki 1.86 ± 0.19 nM, Table 3.1) remained significantly ($p<0.001$) lower (12.4-fold) than the affinity of [D-Trp^6,Pro^9-NHEt]-GnRH (Ki 0.15 ± 0.04 nM, Table 3.1). In the $Asp^{7.32(302)}Asn$ mutant receptor the affinity of

[D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (Ki 1.67 ± 0.43 nM) also remained lower (6.2-fold, $p<0.05$) than the affinity of [D-Trp^6,Pro^9-NHEt]-GnRH (Ki 0.27 ± 0.06 nM).

The affinity of [D-Trp^6,Pro^9-NHEt]-GnRH was similar ($p>0.05$) for the wild type- (Ki 0.15 ± 0.04 nM) and Asp$^{7.32(302)}$Asn mutant (Ki 0.27 ± 0.06 nM) receptors. Moreover, the affinity of [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH for the wild type (Ki 1.86 ± 0.19 nM) and Asp$^{7.32(302)}$Asn mutant (Ki 1.67 ± 0.43 nM) receptor was also similar ($p>0.05$, Table 3.1 and Fig. 3.2). This suggests that Arg^8 in [D-Trp^6,Pro^9-NHEt]-GnRH may still play a role in high affinity binding, but that it does not involve Asp$^{7.32(302)}$.

Table 3.2 Enhancement of GnRH affinity by conformational constraints. The fold enhancement of peptide affinity by conformational constraints was calculated as the ratio of the Ki of the parent peptide (GnRH or [Gln^8]-GnRH) to the Ki of the constrained peptide in the human (wild type) receptor or Asp$^{7.32(302)}$Asn mutant GnRH receptor.

Peptide	Wild type		Mutant	
	Ki	Fold enhancement	Ki	Fold enhancement
	nM		*nM*	
GnRH	6.8[a]	-	212	-
[D-Ala^6,N-Me-Leu^7,Pro^9-NHEt]-GnRH	0.97*	7.0	1.55*	137
[His^5,D-Tyr^6]-GnRH	0.48*	14.2	1.36*	156
[D-Trp^6,Pro^9-NHEt]-GnRH	0.15*	45.3	0.27*	784
[6,7γ-lactam]-GnRH	1.37*	5.0	4.66*	45.5
[Gln^8]-GnRH	923	-	1240	-
[D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH	1.86**	485.5	1.67**	744

[a] Ki values are the same as presented in Table 3.1
* significantly different from GnRH, $p<0.01$
** significantly different from [Gln^8]-GnRH, $p<0.001$

3.3.3 Incorporation of a 6,7 γ-Lactam Enhances Binding to the Receptor

Since D-amino acid substitutions in position six are thought to stabilize a high affinity conformation of GnRH (Monahan et al., 1973), the high affinity of the mutant receptor for peptides with D-amino acids in position six suggests that conformationally-constrained peptides may be less sensitive to the Asp$^{7.32(302)}$ mutation. However, part of the enhanced affinity may be contributed by an interaction of the amino acid side chain (e.g. D-Trp) with a receptor residue. To test whether the high affinity is due predominantly to the conformational constraint of the D-amino acid, peptides with a conformational constraint, in which there is no side chain, were examined. Introduction of a γ-lactam moiety in place of residues six and seven is reported to impose a peptide conformation,

which is similar to that stabilized by D-amino acid modifications (Freidinger et al., 1980). Consistent with the previous report (Freidinger et al., 1980), [6,7 γ-lactam]-GnRH exhibited higher affinity than GnRH for the wild type GnRH receptor (5.0-fold, Table 3.2). This result is similar to the increase found with D-amino acid substitutions in position six of GnRH (Table 3.2). [6,7 γ-lactam]-GnRH also had high affinity for the $Asp^{7.32(302)}$Asn mutant GnRH receptor (Ki 4.66 ± 0.36 nM, Table 3.3), which was 45.5-fold higher than the affinity for native GnRH (Table 3.2). This shows that the γ-lactam constraint enhances the affinity of GnRH for both the wild type and mutant receptors (Fig. 3.3, Table 3.1) and that the affinities for both receptors are similar. Incorporation of 6,7 γ-lactam enhanced binding affinity more in the mutant receptor than in the wild type receptor, which is similar to that found with D-amino acid substitution in position six.

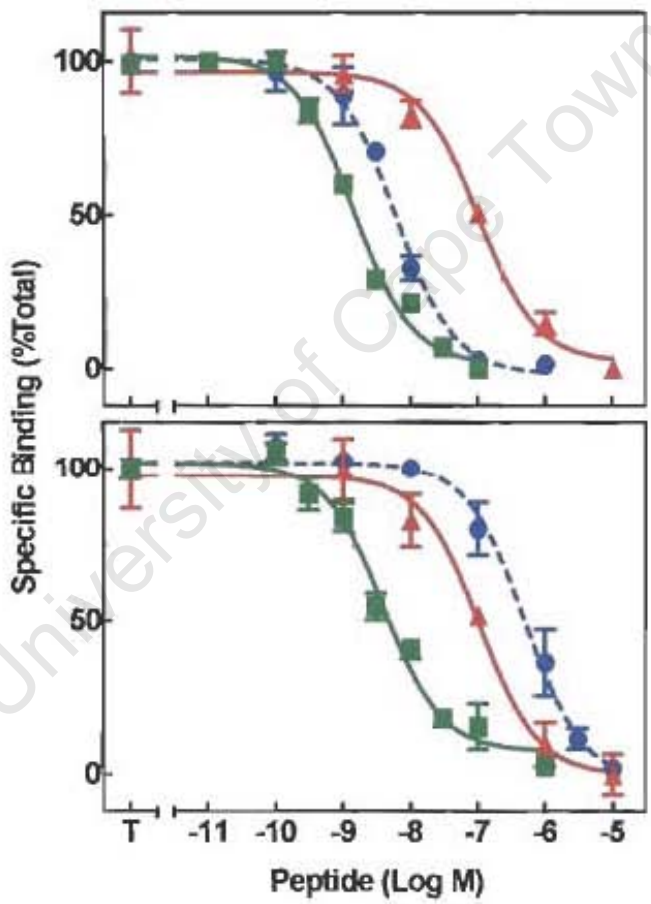

Figure 3.3 Competition binding of GnRH analogs with and without a 6,7 γ-lactam conformational constraint. COS-1 cell membranes expressing the human wild type (top) and $Asp^{7.32(302)}$Asn mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), [6,7 γ-lactam]-GnRH (■), and GnRH II (▲). Data are mean ± SE of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand, the latter being indicated as total specific binding (T).

These results show that when the conformation of GnRH is constrained (6,7 γ-lactam or D-amino acid in position six), $Asp^{7.32(302)}$ of the receptor is not required for high affinity binding.

Another class of conformationally-constrained GnRH analogs includes the GnRH antagonists. The novel antagonist, antagonist 129-62, which has a 6,7 γ-lactam, and antagonist 26, which has D-Lys^6, had similar high affinities for the wild type and mutant receptors (Table 3.1, Fig. 3.4).

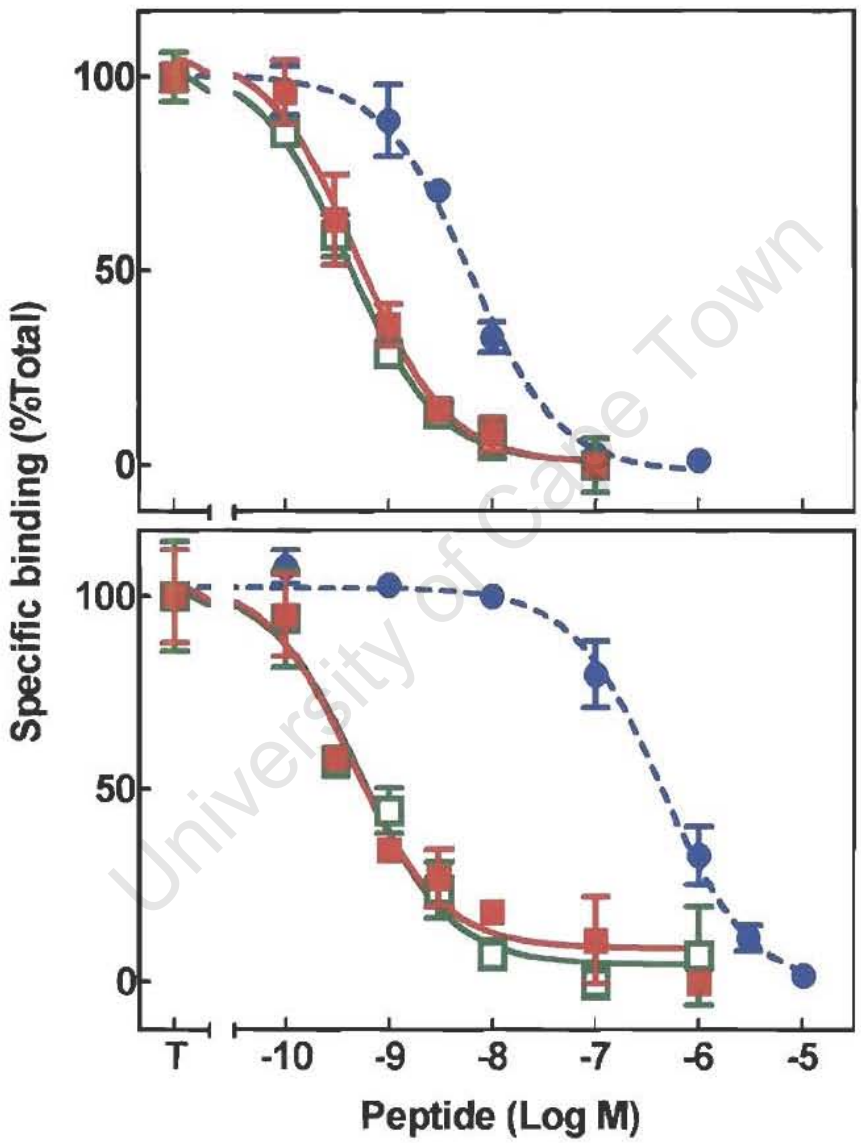

Figure 3.4 Competition binding of GnRH antagonists. COS-1 cell membranes expressing the human wild type (top) and $Asp^{7.32(302)}$Asn mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), antagonist 26 (■), and antagonist 129-62 (□). Data are mean ± SE of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand, the latter being indicated as total specific binding (T).

3.3.4 GnRH II Binds the Human GnRH Receptor Independently of $Asp^{7.32(302)}$

GnRH II ([His^5, Trp^7, Tyr^8]-GnRH), which occurs naturally in the human brain (White et al., 1998) does not contain Arg^8, but binds the GnRH receptor with higher affinity (Ki 193.7 ± 28.5 nM) than [Gln^8]-GnRH (Ki 923 ± 222 nM, Table 3.1, Fig. 3.3) or [Tyr^8]-GnRH (Millar et al., 1989). This suggests that the combination of substitutions in positions five, seven and eight allows the peptide to bind with relatively high affinity, independently of interactions that involve Arg^8, possibly by stabilizing a high affinity conformation (de L. Milton et al., 1983). GnRH II had similar affinity for the wild type (Ki 193.7 ± 28.5 nM) and mutant receptors (Ki 213.3 ± 72.4 nM, Table 3.1). Consequently, the $Asp^{7.32(302)}$Asn mutant receptor retained higher affinity for GnRH II (Ki 213.3 ± 72.4 nM) than for [Gln^8]-GnRH (Ki 1240 ± 227 nM).

In summary, these results show that mutating $Asp^{7.32(302)}$ of the human GnRH receptor to Asn decreases the affinity for GnRH in a manner that is specific to an interaction with Arg^8, and that a specific conformation of GnRHs important for high affinity binding of GnRH to its receptor.

3.3.5 Decreased IP Production by the $Asp^{7.32(302)}$Asn Mutant GnRH Receptor

The $Asp^{7.32(302)}$Asn mutant GnRH receptor coupled to the IP signalling pathway (Fig. 3.5). GnRH displayed a 44-fold decrease in potency at the mutant receptor (EC_{50} 12.6 ± 1.78 nM) relative to the wild type receptor (EC_{50} 0.29 ± 0.07 nM, Fig. 3.5). This decrease in potency is consistent with the decreased affinity of the mutant receptor for native GnRH.

Table 3.3 Summary of GnRH receptor agonist stimulated IP accumulation. EC_{50} and Emax values are mean ± SE. Statistical analyses were performed as described under "Materials and Methods". The fold change was calculated as the ratio of the EC_{50} values in the mutant and wild type receptors. Emax in the mutant is expressed as percent of Emax stimulated by the same ligand in wild type receptor in the same experiment.

GnRH analog	EC_{50}		Fold change	Emax
	Wild type	Mutant		
	nM			*% wt*
GnRH	0.29 ± 0.07	12.6** ± 1.80	43.5	68.7* ± 8.32
[Gln^8]-GnRH	5.19 ± 1.13	17.2* ± 2.84	3.3	69.3* ± 4.20
GnRH II	1.75 ± 0.55	6.66* ± 1.62	3.8	71.3* ± 7.70
[D-Ala^6,N-Me-Leu^7,Pro^9-NHEt]-GnRH	0.06 ± 0.002	0.31 ± 0.17	5.2	76.0 ± 2.89

* significantly different from wild type receptor, $p<0.05$.
** significantly different from wild type receptor, $p<0.001$.

However, the mutant receptor also exhibited decreased Emax for GnRH (Fig. 3.5, Table 3.3), suggesting that the mutation may induce partial uncoupling of the receptor from intracellular

signalling. In contrast, the relative Emax values of the different GnRH analogs were similar. Surprisingly, ligands that showed no decrease in affinity for the mutant receptor also exhibited decreased IP production in the mutant receptor (Table 3.3, Fig. 3.5). This shows that the effect of the mutation on cytosolic signalling is distinct from its effect on binding affinity for GnRH.

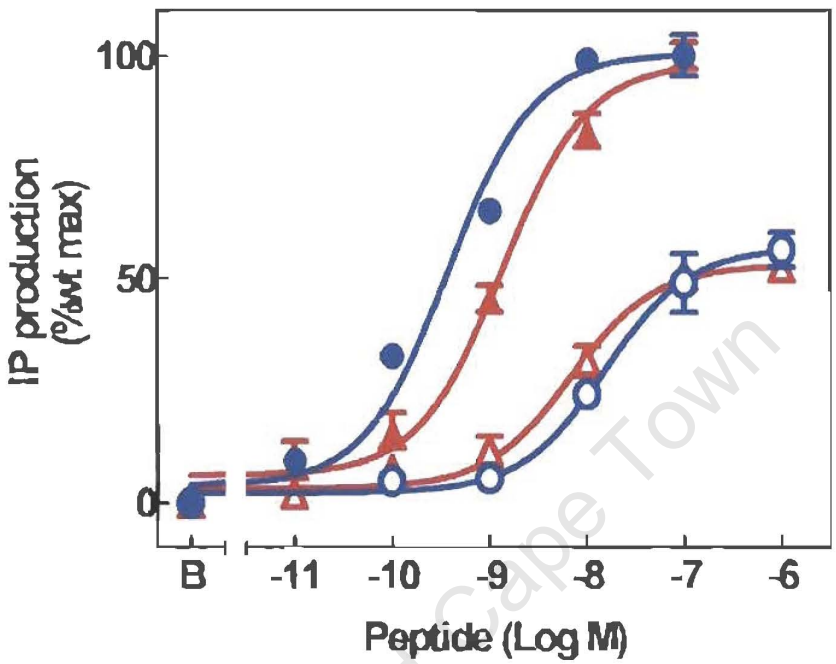

Figure 3.5 GnRH-stimulated IP production. COS-1 cells transiently transfected with the wild type human GnRH receptor (filled symbols) and with the $Asp^{7.32(302)}Asn$ mutant (open symbols) were incubated with various concentrations of GnRH (●, ○) and GnRH II (▲, △). Data are mean ± SE of a representative experiment performed in duplicate and expressed as percentage of Emax stimulated by each peptide in the wild type receptor. EC_{50} values in this experiment were 0.38 nM for GnRH in the wild type; 15.1 nM for GnRH in the mutant; 1.36 nM for GnRH II in the wild type and 6.76 nM for GnRH II in the mutant.

3.4 Discussion

The basic Arg residue in position eight of GnRH is required for high affinity binding to mammalian type I GnRH receptors (Millar et al., 1989). The proposal that Arg^8 may be involved in an electrostatic interaction with an acidic residue in the GnRH receptor (Hazum, 1987) was examined in the mouse GnRH receptor, where it was found that the $Glu^{7.32(301)}$ residue confers specificity for GnRH with Arg in position eight (Flanagan et al., 1994). In spite of the demonstrated functional importance of $Glu^{7.32(301)}$, this residue is not conserved in the human GnRH receptor, which has $Asp^{7.32(302)}$ instead (Chi et al., 1993). The carboxyl side chain of both residues suggests that $Asp^{7.32(302)}$ can potentially perform the same functions in the human GnRH receptor as $Glu^{7.32(301)}$

does in the mouse receptor and computational models of both rodent and human receptors have incorporated interactions of Glu or Asp with Arg^8. However, the study in the mouse receptor suggested that the mechanism of receptor selectivity for Arg^8-containing GnRH may be more complex than a simple electrostatic interaction in the receptor ligand complex. This demonstrates a need for detailed investigation of the mechanism by which $Asp^{7.32(302)}$ determines specificity for Arg^8. We show that an electrostatic interaction is not required for high affinity binding of certain GnRH analogs and we propose that $Asp^{7.32(302)}$ may stabilize a high affinity conformation of GnRH.

$Asp^{7.32(302)}$ of the Human GnRH Receptor Determines Selectivity for GnRH

The decreased affinity of the $Asp^{7.32(302)}$Asn mutant for native GnRH, but not for [Gln^8]-GnRH shows a loss of specificity for Arg^8 of GnRH. This shows that, like the $Glu^{7.32(301)}$ residue of the mouse GnRH receptor, the $Asp^{7.32(302)}$ side chain determines receptor preference for the Arg^8 side chain of GnRH. However, the 31-fold decrease in the affinity of the $Asp^{7.32(302)}$Asn mutant for GnRH is smaller than would be expected for a loss of an electrostatic interaction (Wells et al., 1987). The low affinity of the negatively-charged ligand, [Glu^8]-GnRH could potentially result from a repulsive interaction with the negatively-charged $Asp^{7.32(302)}$ of the wild type receptor, but the $Asp^{7.32(302)}$Asn human GnRH receptor mutant did not show increased affinity for [Glu^8]-GnRH. This suggests that the mutation does not remove an unfavourable interaction with [Glu^8]-GnRH and consequently, high affinity binding of native GnRH may not arise from an electrostatic interaction of the Arg^8 and $Asp^{7.32(302)}$ side chains. In support, the affinity of GnRH for the mutant receptor was higher than the affinity for [Gln^8]-GnRH (Table 3.1), also suggesting that the Arg^8 – $Asp^{7.32(302)}$ interaction may not arise from a direct electrostatic interaction.

Conformational Constraints Enhance GnRH Binding to the Wild Type GnRH Receptor

Arg^8 is proposed to stabilize an active conformation of GnRH (Shinitzky et al., 1976), which consists of a β-II-bend involving the Tyr^5-Gly^6-Leu^7-Arg^8 residues (Monahan et al., 1973). Incorporation of a D-amino acid in position six (Momany, 1976) or a γ-lactam in positions six and seven (Freidinger et al., 1980) is proposed to stabilize this conformation and results in an increased GnRH potency (Freidinger et al., 1980; Monahan et al., 1973). The current study has shown that GnRH analogs with D-amino acid substitutions in position six, or with a 6,7 γ-lactam, have higher affinities than native GnRH for the wild type human GnRH receptor. This study shows that the human GnRH receptor has enhanced affinity for the conformational state of GnRH that is stabilized by incorporating either constraint.

To test whether preferential binding of Arg^8-containing GnRH is retained in the presence of a conformational constraint in the wild type receptor, affinities of conformationally constrained peptides with Arg^8 or Gln^8 ([D-Trp^6,Pro^9-NHEt]-GnRH and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH) were compared. The modification enhanced the affinity of [Gln^8]-GnRH more than that of GnRH (Table 3.2). Thus, a conformational constraint not only enhances the affinity of [Gln^8]-GnRH, but also compensates for the absence of Arg^8. This shows that the wild type human GnRH receptor binds conformationally constrained GnRH analogs with high affinity and that this interaction is independent of Arg^8 in GnRH.

GnRH antagonists constitute another class of conformationally-constrained GnRH receptor ligands. Antagonist 26 has D-Lys in position six and bound the wild type receptor with high affinity. A novel antagonist (antagonist 129-62), which incorporates a γ-lactam constraint, had similar high affinity for the human GnRH receptor. Taken together these results indicate that the wild type human GnRH receptor recognizes a specific conformation of GnRH, which it binds with high affinity.

Constrained Ligands Retain High Affinity for the $Asp^{7.32(302)}$Asn Mutant

In contrast to native GnRH, GnRH analogs with conformational constraints retained high affinity for the $Asp^{7.32(302)}$Asn mutant receptor. Because of the lower affinity of the mutant receptor for GnRH, the enhancement of the affinity of the conformationally-constrained analogs, compared with GnRH, was greater in the mutant receptor than in the wild type receptor. The preservation of high affinity for constrained peptides in the mutant receptor shows that the $Asp^{7.32(302)}$ side chain is not required for high affinity binding of conformationally-constrained GnRH analogs.

Ligand Constraint Compensates for the Absence of Both Arg^8 and $Asp^{7.32(302)}$

The ability of conformational constraints to enhance GnRH binding to the wild type GnRH receptor and to retain high affinity for the $Asp^{7.32(302)}$Asn mutant, suggests that the conformational constraint can compensate for the absence of both Arg^8 in the ligand and $Asp^{7.32(302)}$ in the receptor.

This suggests that native GnRH interacts with the receptor differently than conformationally-constrained GnRH analogs. Thus, native GnRH and conformationally-constrained GnRH analogs may occupy different (although overlapping) binding pockets on the receptor. Two other GnRH receptor residues, $Asp^{2.61(98)}$ and $Asn^{2.65(102)}$, determine recognition of His^2 and Gly-NH_2 of GnRH respectively (Davidson et al., 1996a; Flanagan et al., 2000). Comprehensive analysis shows that the

interaction of these receptor residues with native GnRH is similar to their interaction with constrained analogs. Mutation of these residues decreases receptor recognition of native and constrained analogs of GnRH to the same extent, suggesting that both native GnRH and constrained analogs interact with these residues (Davidson et al., 1996a; Flanagan et al., 2000). Thus, the ability of the ligand constraint to compensate for the absence of both Arg^{8} and $Asp^{7.32(302)}$ suggests that the ligand residue and the receptor residue have complementary roles in stabilizing a high affinity ligand conformation.

The $Asp^{7.32(302)}$ - Arg^{8} Interaction Induces a High Affinity Conformation of GnRH

In this study it is demonstrated that substituting Arg^{8} of native GnRH or $Asp^{7.32(302)}$ of the receptor decreases GnRH affinity. The lack of an additive effect when both substitutions are combined suggests that these side chains interact with each other. However, conformational constraint of the ligand reverses the loss of affinity due to substitution of Arg^{8} and/or $Asp^{7.32(302)}$. This suggests that both residues have roles in stabilizing a high affinity conformation of unconstrained GnRH. Arg^{8} has been proposed to have two distinct roles in high affinity binding: an intramolecular interaction which stabilizes a high affinity peptide conformation (Shinitzky et al., 1976), and an intermolecular electrostatic interaction with an acidic group in the receptor (Flanagan et al., 1994; Hazum, 1987). The current results suggest that Arg^{8} both stabilizes peptide conformation and interacts with $Asp^{7.32(302)}$, and that $Asp^{7.32(302)}$ also affects peptide conformation. This, in turn, suggests that an interaction of Arg^{8} with $Asp^{7.32(302)}$ affects peptide conformation. The similar affinities of constrained peptides for the wild type and mutant receptors (with and without $Asp^{7.32(302)}$) suggest that once the ligand is in a high affinity conformation, the putative Arg^{8} - $Asp^{7.32(302)}$ interaction does not contribute to the binding energy of the final ligand-receptor complex. While this result suggests that an interaction between Arg^{8} and $Asp^{7.32(302)}$ may be required to induce a high affinity conformation in unconstrained, native GnRH, the absence of the Arg^{8} - $Asp^{7.32(302)}$ interaction with constrained analogs suggests that this interaction is transient. The affinity of a conformationally constrained GnRH peptide with Arg^{8} substituted ([D-Trp^{6},Gln^{8},Pro^{9}-NHEt]-GnRH) was lower than that of a conformationally constrained GnRH peptide with Arg^{8} ([D-Trp^{6},Pro^{9}-NHEt]-GnRH) for both the wild type and $Asp^{7.32(302)}$Asn mutant GnRH receptors. This suggests that Arg^{8} may contribute a minor role for high affinity binding of conformationally-constrained GnRH analogues, but that this interaction does not involve $Asp^{7.32(302)}$, which supports the proposal that the Arg^{8} - $Asp^{7.32(302)}$ interaction is transient.

However, the concept of the receptor affecting peptide conformation is speculative, as there is no direct data to support this assertation. Using conformational memories and computational exploration it was shown that GnRH consists of a population (±70%) sharing a distinctive ß-type turn in the backbone that involves residues five to eight (Guarnieri and Weinstein, 1996). This conformation is shown to correspond to the geometry of a constrained GnRH analog which has high affinity for the GnRH receptor. The remainder of the population consists of a mixture of conformers that do not contain the ß-type turn in the backbone and have low affinity for the receptor. GnRH analogs with Arg^{8} substituted do not readily assume this high affinity conformation (Guarnieri and Weinstein, 1996). It is possible that in solution Arg^{8}-containing GnRH fluctuates between low and high affinity conformations with the equilibrium shifted towards a high affinity conformation (±70%). Therefore, another proposal may include that $Asp^{7.32(302)}$ of the GnRH receptor selects rather than induces a high affinity conformation, which may further interact with the ligand pocket.

Thus, an initial interaction between Arg^{8} and $Asp^{7.32(302)}$ may be required to induce or select a high affinity conformation of native GnRH, which then interacts with a final binding pocket, which involves $Asn^{2.65(102)}$ and $Asp^{2.61(98)}$ (Davidson et al., 1996a; Flanagan et al., 2000). This final ligand-receptor complex does not include $Asp^{7.32(302)}$, but may include another receptor residue interacting with Arg^{8} of GnRH. It has been suggested that residues on the extracellular surface of the TRH receptor form a initial ligand recognition site (Perlman et al., 1997) and that TRH binds sequentially with the surface binding site, and then with the TM pocket (Colson et al., 1998b). Therefore, contrary to the initial hypothesis of a direct electrostatic interaction between Arg^{8} and $Asp^{7.32(302)}$ in the ligand-receptor complex, we show that the basis of receptor selectivity for mammalian GnRH appears to be the ability of $Asp^{7.32(302)}$ to induce or select a high affinity conformation in native GnRH.

Cytosolic signalling assays showed that GnRH had decreased potency at the $Asp^{7.32(302)}$Asn mutant receptor, consistent with its decreased affinity. However, the Emax for GnRH was lower in the mutant receptor, suggesting that the mutation destabilizes the activated receptor conformation (Samama et al., 1993). Several agonists, which showed no decrease in affinity for the mutant receptor, nevertheless showed decreased stimulation of IP production. This suggests that the $Asp^{7.32(302)}$ side chain has a role stabilizing the activated receptor conformation. The apparent decreased efficacy of ligands that had unchanged affinity, suggests that this function is distinct from its role in ligand selectivity, which will be discussed further in chapter four. A previous study

reported that mutagenesis of ECL3 of the $ß_2$-adrenergic receptor also affected receptor activation (Zhao et al., 1998).

In conclusion, the wild type human GnRH receptor has high affinity for agonist and antagonist ligands with certain conformational constraints. This suggests that the wild type human GnRH receptor recognizes and binds ligands in a specific conformation that can be stabilized by these ligand modifications. We show that the $Asp^{7.32(302)}$ side chain determines selectivity for Arg^8-containing GnRH. However, the ligand conformational constraints can compensate for the decrease in affinity that results from substitution of Arg^8 and $Asp^{7.32(302)}$. This suggests that $Asp^{7.32(302)}$ may determine selectivity for Arg^8-containing GnRH by its ability to induce a high affinity conformation in the ligand. This work also proposes that Arg^8-containing, native GnRH interacts transiently with $Asp^{7.32(302)}$, which induces or selects a high affinity conformation in GnRH, before it interacts with the ligand binding pocket, which excludes $Asp^{7.32(302)}$. This further definition of the mechanism of ligand recognition improves our conceptual models of GnRH receptor-ligand interaction.

Chapter 4

The Role of Proline$^{7.33(303)}$ of the GnRH Receptor in Ligand Selectivity and Receptor Activation

4.1 Summary

In mammalian GnRH receptors, ECL3 contains the amino acid sequence motif, $Ser^{301(7.31)}$-$Asp^{302(7.32)}$/$Glu^{301(7.32)}$-$Pro^{303(7.33)}$, of which the acidic residue in position 7.32 confers selectivity for Arg^8-containing GnRH. Non-mammalian vertebrate GnRH receptors do not preferentially bind Arg^8-containing GnRH, but also have an acidic residue in position 7.32. Contrastingly, ECL3 of non-mammalian GnRH receptors have an amino acid motif containing $Pro^{7.31(303)}$-$Asp^{7.32(302)}$/$Glu^{7.32(301)}$-$Tyr^{7.33(303)}$. The different positions of Pro, in relation to the acidic residue in position 7.32 in mammalian and non-mammalian GnRH receptors, suggested that the conformation of ECL3 affects ligand selectivity. In this study the role of the location of $Pro^{7.33(303)}$ in ECL3 of the human GnRH receptor is examined by receptor site-directed mutagenesis and ligand modification. Mutating $Pro^{7.33(303)}$ to Ala or introducing Pro into position 7.31 results in decreased affinity for GnRH but not for GnRH analogs with a conformational constraint or without Arg^8. These GnRH receptor mutants mimic a phenotype of mutants that have substitutions of $Asp^{302(7.32)}$/$Glu^{301(7.32)}$ in mammals. $Pro^{7.33(303)}$ may impose a constraint on the conformation of ECL3 influencing the neighboring $Asp^{7.32(302)}$ to present its side chain in an orientation that favors interaction with the Arg^8 side chain of GnRH. In the human GnRH receptor, it has been proposed that a transient $Asp^{7.32(302)}$ - Arg^8 interaction induces a high affinity GnRH conformation, which further interacts with a final ligand binding pocket. We propose that $Pro^{7.33(303)}$ forms part of the ligand binding mechanism in which mammalian GnRH receptors distinguish GnRH from other naturally occurring forms.

4.2 Introduction

The mammalian GnRH receptor has a high affinity for Arg^8 in GnRH, while non-mammalian GnRH receptors are less discriminatory and accepts basic or neutral amino acids in this position. In ECL3 of the mammalian type I GnRH receptor a conserved acidic residue in position 7.32 ($Glu^{7.32(301)}$/$Asp^{7.32(302)}$) interacts transiently with Arg^8-containing GnRH, determines selectivity for the mammalian form of GnRH (Fromme et al., 2001). Despite non-mammalian type I GnRH receptors not preferentially binding Arg^8-containing GnRH (Sealfon et al., 1997), the acidic residue at position 7.32 is conserved in these receptors. Amino acid sequence alignment of ECL3 of mammalian and non-mammalian type I GnRH receptors revealed two distinct amino acid motifs. ECL3 of mammalian type I GnRH receptors contains the $Ser^{7.31}$-($Glu^{7.32}$/$Asp^{7.32}$)-$Pro^{7.33}$ motif and non-mammalian type I GnRH receptors have Pro-(Asp/Glu)-Tyr (Fig. 4.2). The different position of Pro relative to $Glu^{7.32}$/$Asp^{7.32}$ in these motifs suggested that the conformation of ECL3 may differ between mammalian and non-mammalian receptors and that loop conformation may influence

ligand selectivity. GPCR loops are able to assume functional ordered structures independently of TM domains. This is proposed to depend on the interaction and /or properties of specific amino acid combinations within a particular loop (Yeagle et al., 2000). The crystal structure of Rhodopsin shows that the extracellular domain is a highly organised structure which is proposed to form a basis for the arrangement of the seven-helix TM motif (Palczewski et al., 2000). It is possible that interactions of amino acid residue within a loop or with residues of other TM domains could stabilize extracellular loops in a specific conformation, which favors specific ligand-receptor binding interactions. Thus, it is conceivable that ligand binding of some GPCRs depends on specific amino acid residues in extracellular loops, as well as a particular conformation of the loop, which in turn depends on combinations/ interactions of amino acids within a loop.

Multiple forms of GnRH are present in most vertebrate species and are thought to have physiological functions in addition to regulating pituitary hormone release (King and Millar, 1995). GnRH II which is predominantly found in extrahypothalamic areas of many vertebrate species (King and Millar, 1995; Latimer et al., 2000; White et al., 1998), is suggested to have a neuromodulator role (Jones, 1987; Troskie et al., 1997). The mammalian pituitary GnRH receptor is proposed to discriminate between GnRH related peptides (King and Millar, 1995). The recent cloning of a second form of GnRH, GnRH II, which is widely expressed in primates (Latimer et al., 2000; White et al., 1998) indicates a need for the reevaluation of how the mammalian GnRH receptor selects between naturally occurring forms of GnRH. In this study, the role of $Pro^{7.33\ (302)}$ of the human GnRH receptor in ligand selectivity is investigated. We show that if $Pro^{7.33(302)}$ is substituted or moved to the non-mammalian position 7.31, a specific decrease in affinity for mammalian GnRH is displayed. From our results we interpret that $Pro^{7.33(303)}$ influences the conformation of ECL3 in such a way that the side chain of $Glu^{7.32(301)}$/$Asp^{7.32(302)}$ is orientated favorably for interaction with Arg^{8} of GnRH.

4.3 Results

A PCR based mutagenesis method was used to introduce mutations $Pro^{7.33(303)}$Ala; $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Gly; $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Phe; $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Tyr; $Ser^{7.31(301)}$Pro/$Asp^{7.32(302)}$Glu/$Pro^{7.33(303)}$Tyr and the chicken ECL3 (His-Pro-Ala-Met-Ile-Gln-Arg-Met-Pro-Glu-Tyr-Ile) chimera in the human GnRH receptor (chapter two). Membrane binding assays were performed because different membrane concentrations can be used to compensate for the lower total binding of the mutant GnRH receptors. A membrane concentration of $1/20^{th}$ dish per reaction tube was used for the $Pro^{7.33(303)}$Ala; $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Gly;

$Ser^{7.31(301)}Pro/Pro^{7.33(303)}Phe$ mutant GnRH receptors and a $1/6^{th}$ dish per reaction tube was used for the $Ser^{7.31(301)}Pro/Pro^{7.33(303)}Tyr$; $Ser^{7.31(301)}Pro/Asp^{7.32(302)}Glu/Pro^{7.33(303)}Tyr$ and the chicken ECL3 chimera in the human GnRH (chapter two). The wild type receptor had $1/50^{th}$ dish per reaction tube. The mutant GnRH receptors were constructed to investigate the role of $Pro^{7.33(303)}$ of the human GnRH receptor in ligand binding. $Pro^{7.33(303)}$ was mutated to Ala to determine the effect of absence of this residue on GnRH binding. A double mutation, $Ser^{301(7.31)}Pro/Pro^{7.33(303)}Gly$ changes the position of Pro relative to $Asp^{7.32(302)}$, moving it from position 7.33 to position 7.31, equivalent to its position in non-mammalian receptors. The mutation to Gly maximizes flexibility in this position. The non-mammalian GnRH receptor motifs were introduced into the human GnRH receptor by mutating $Ser^{301(7.31)}Pro/Pro^{7.33(303)}Tyr$ and $Ser^{301(7.31)}Pro/Asp^{7.32(302)}Glu/Pro^{7.33(303)}Tyr$. Substituting ECL3 of the human GnRH receptor with that of the chicken GnRH receptor was performed to investigate the role of ECL3 on ligand selectivity in the human GnRH receptor.

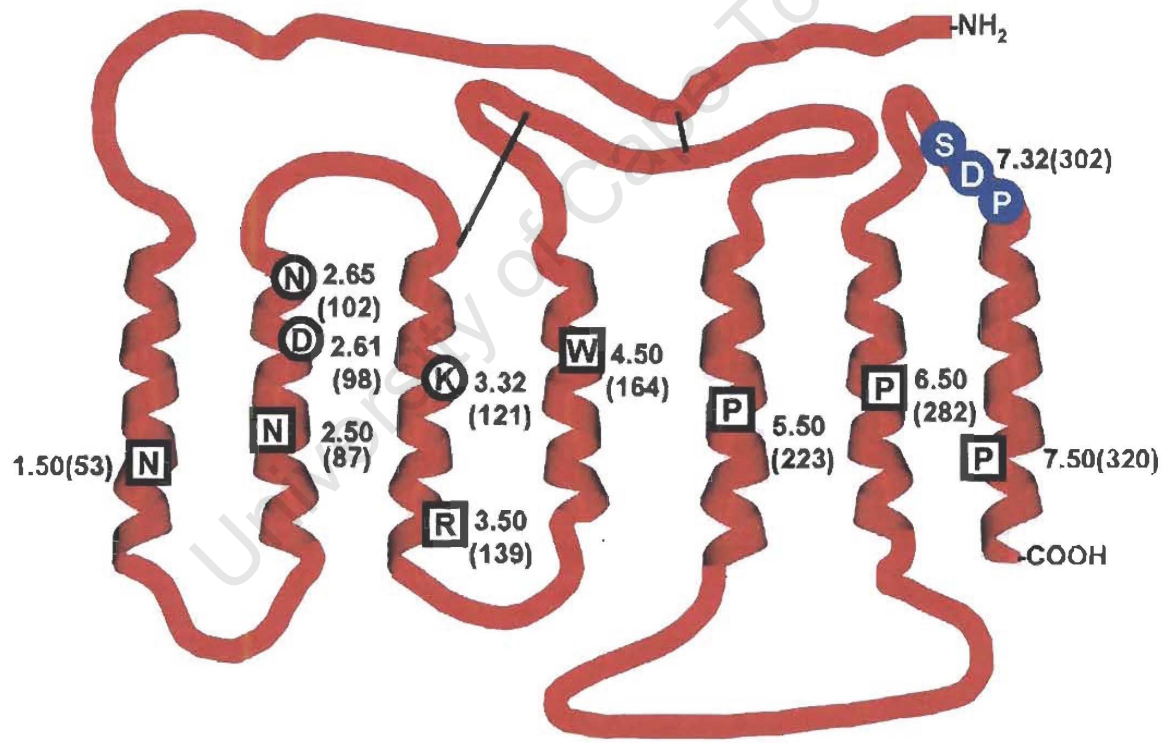

Figure 4.1 Schematic diagram of the human type I GnRH receptor. Residues that are important for ligand binding are indicated by circles. The most conserved residues in each transmembrane domain are indicated by squares, according to the consensus numbering scheme.

4.3.1 Mutating $Pro^{7.33(303)}$ Specifically Decreases Affinity for Arg^8-containing GnRH

The wild type human GnRH receptor exhibited high affinity for GnRH (Ki 5.62 ± 0.57 nM) and lower affinity for GnRH II (Ki 93.2 ± 2.60 nM) and [Gln^8]-GnRH (Ki 1009 ± 121 nM, Table 4.1, Fig. 4.3A), consistent with earlier reports that the human GnRH receptor preferentially binds Arg^8-containing

GnRH (Chi et al., 1993). The Pro$^{7.33(303)}$Ala mutant had decreased affinity for GnRH (Ki 44.0 ± 4.60 nM) and unchanged affinities for GnRH II (Ki 213 ± 49.2 nM) and [Gln^{8}]-GnRH (Ki 1873 ± 465 nM, Table 4.1, Fig. 4.3B). This suggests that Pro$^{7.33(303)}$ plays a role in discriminating GnRH from analogs that do not have Arg^{8}. This is similar to the phenotype of the Asp$^{7.32(302)}$Asn mutant, which also had decreased affinity for GnRH but not for analogs that lack Arg^{8} (Fromme et al., 2001). It was previously found that binding of conformationally-constrained analogs was not affected by the Glu$^{7.32(301)}$Gln mutation in the mouse GnRH receptor (Flanagan et al., 1994) or Asp$^{7.32(302)}$Asn mutation in the human GnRH receptor (Fromme et al., 2001). To test whether this is a general phenomenon in mammalian GnRH receptors, binding affinity of a GnRH analog with a D-amino acid substitution in position six was also characterized in the wild type human receptor and in the mutant receptors described in this chapter. The Pro$^{7.33(303)}$Ala mutant showed no loss of affinity for a conformationally-constrained analog, [His^{5},D-Tyr^{6}]-GnRH (Table 4.1, Fig. 4.4), suggesting that when the conformation of GnRH is constrained (D-amino acid in position six), Pro$^{7.33(303)}$ of the GnRH receptor is not required for high affinity binding.

```
                    301--TM6--| ECL3 |----------TM7----------| 350
          human GnRHR  LGIWYWFDPEMLNRLSDPVNHFFFLFAFLNPCFDPLIYGYFSL~~~~~~~
          mouse GnRHR  LGIWYWFDPEMLNRVSEPVNHFFFLFAFLNPCFDPLIYGYFSL~~~~~~~
        chicken GnRHR  LGLWYWFHPAMIQRMPEYINHSFFLFGLLHTCTDPIIYGLYTPSFREDVQ
        catfish GnRHR  LGIWYWFQPQMLHVIPDYVHHVFFVFGNLNTCCDPVIYGFFTPSFRADLS
      goldfish A GnRHR  LGIWYWFQPRMLQSMPEYIHHALFVFGNLNTCCDPVIYGFFTPSFRADIA
      goldfish B GnRHR  LGIWYWFQPEMLKVTPEYIHHLLFVFGNLNTCCDPVIYGLYTPSFRADLA
         Xenopus GnRHR  LGLWYWFQPEMINQTPEYLNHSLFLFGLLHTCTDPLVYGLYTPSFKEDLR
marmoset Type II GnRHR  LGLWYWFSPSMLSEVPPSLSHILFLFGLLNAPLDPLLYGAFTLGCRRGHQ
```

Figure 4.2 Alignment of the amino acid sequences of ECL3 of the mammalian and non-mammalian GnRH receptors. In red text is the highly conserved Pro residue at locus 7.50 according to the consensus numbering scheme (appendix I). In blue is ECL3 and in purple is the conserved acidic residue at locus 7.32.

4.3.2 Position of Pro is Important for GnRH Selectivity

The double mutant, Ser$^{301(7.31)}$Pro/Pro$^{7.33(303)}$Gly, had 23-fold decreased affinity for GnRH (Ki 129 ± 29.9 nM), but affinities for GnRH II (Ki 236 ± 86.2 nM) and [Gln^{8}]-GnRH (Ki 1683 ± 357 nM) were unchanged (Table 4.1, Fig. 4.3C). Mutating Ser$^{7.31(301)}$ to Ala had a similar phenotype to the human wild type receptor (Davidson, personal communication, unpublished) suggesting that Ser$^{7.31(301)}$ is not involved in GnRH binding. However, if the Ser$^{301(7.31)}$Pro/Pro$^{7.33(303)}$Gly mutations are introduced, a specific decrease in GnRH affinity is observed which is similar to that found with the Pro$^{7.33(303)}$Ala mutant receptor. Thus, substituting Pro$^{7.33(303)}$ or moving it to locus 7.31 in ECL3, mimics a decrease in GnRH affinity as observed in the Asp$^{7.32(302)}$Asn mutant receptor (Fromme et al., 2001), suggesting that the mammalian location of Pro in ECL3 is necessary for high affinity

binding of Arg^{8}-containing GnRH. However, the Ser$^{301(7.31)}$Pro/Pro$^{7.33(303)}$Gly mutant showed no loss of affinity for the conformationally-constrained agonist, [His^{5},D-Tyr^{6}]-GnRH (Table 4.1).

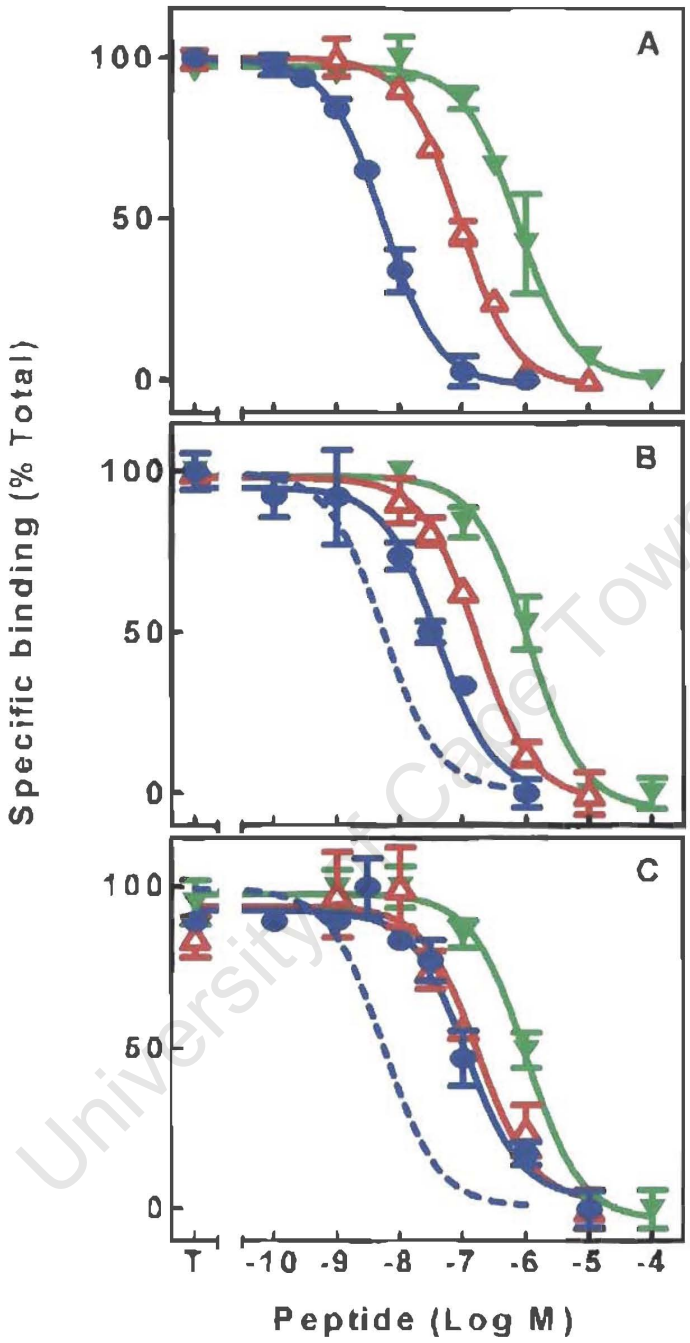

Figure 4.3 Competition binding of GnRH and GnRH analogs in human wild type and ECL3 mutant GnRH receptors. COS-1 cell membranes expressing the human wild type (A), Pro$^{7.33(303)}$Ala mutant (B) and, Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Gly mutant (C) GnRH receptors were incubated with ^{125}I-[His^{5},D-Tyr^{6}]-GnRH in the presence of various concentrations of GnRH (●), GnRH II (△) and [Gln^{8}]-GnRH (▼). The dotted line (---) in panels B and C represents a reference curve of GnRH binding with the human wild type GnRH receptor as in panel A (●). Data are mean ± SE of representative experiments performed in triplicate and expressed as percentage of specific binding in the absence of unlabeled ligand, the latter being indicated as total specific binding (T).

Table 4.1 Summary of ligand binding results of ECL3 mutants. Competition binding assays were performed on COS-1 cells transiently transfected with wild type or mutant GnRH receptors, using ^{125}I-[His^5, D-Tyr^6]-GnRH and various concentrations of unlabeled ligands as described under "Materials and Methods" (chapter two). Ki values are the mean ± SE. Statistical analyses were performed using pKi values as described under "Materials and Methods". The fold change was calculated as the ratio of the Ki values of the mutant and wild type receptors.

		GnRH analogs Ki-values (fold change)			
ECL3 Motif		nM			
	Binding sites (binding sites /cell x10^5)	GnRH[a]	GnRH II[b]	[Gln^8]-GnRH	[His^5,D-Tyr^6]-GnRH
(wt) Ser-Asp-Pro	1.11 ± 0.05	5.62 ± 0.57	93.2 ± 2.60	1009 ± 121	0.65 ± 0.03
Ser-Asp-**Ala**	1.17 ± 0.11 (1.05)	44.0 ± 4.60** (7.83)	213 ± 49.2 (2.29)	1873 ± 465 (1.86)	1.62 ± 0.22 (2.45)
Pro-Asp-**Gly**	1.35 ± 0.06 (1.21)	129 ± 29.9* (23.0)	236 ± 86.2 (2.54)	1683 ± 357 (1.67)	2.43 ± 0.75 (3.72)
Pro-Asp-**Phe**	1.33 ± 0.06 (1.19)	76.4 ± 2.87** (13.6)	136 ± 32.8 (1.48)	1590 ± 273 (1.58)	2.71 ± 0.71 (4.15)
Pro-Asp-**Tyr**	0.89 ± 1.57 (0.80)	193 ± 48.7* (34.3)	318 ± 94.5 (3.41)	2246 ± 505* (2.23)	12.2 ± 0.71** (18.7)
Pro-Glu-Tyr	1.45 ± 0.06 (1.30)	177 ± 18.9** (31.4)	242 ± 59.5 (2.60)	4059 ± 1255* (4.02)	9.45 ± 1.09** (14.5)
ECL3 chimera	-	3605 ± 1487** (642)	496 ± 157* (5.33)	2822 ± 309* (2.8)	281 ± 84.5** (206)
Ser-**Asn**-Pro	1.31 ± 0.06 (1.18)	212 ± 40.6** (37.8)	213 ± 72.4 (2.29)	1240 ± 227 (1.23)	1.36 ± 0.20 (2.08)

* significantly different from wild type, $p<0.05$

** significantly different from wild type, $p<0.001$

[a] GnRH sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

[b] GnRH II sequence: [His^5,Trp^7,Tyr^8]-GnRH

4.3.3 The Role of the Non-Mammalian Motif in ECL3 on GnRH Selectivity

The chicken GnRH receptor has high affinity for forms of GnRH with and without Arg in position eight and it does not preferentially bind Arg^8-containing GnRH (Sun et al., 2001). To investigate whether the incorporation of the entire non-mammalian GnRH receptor motif (Pro-Asp-Tyr and Pro-Glu-Tyr) into the human GnRH receptor would generate a high affinity for all forms of GnRH, GnRH receptor mutants with these motifs were constructed. The mutants, $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Tyr and $Ser^{7.31(301)}$Pro/$Asp^{7.32(302)}$Glu /$Pro^{7.33(303)}$Tyr showed decreased affinity both for GnRH (Ki 193 ± 48.7 and 177 ± 18.9 nM), and even more surprisingly, for the conformationally-constrained analog, [His^5,D-Tyr^6]-GnRH (Ki 12.2 ± 0.71 and 9.45 ± 1.09 nM), but minimal decreases in affinity for GnRH II (Ki 318 ± 94.5 and 242 ± 59.5 nM) and [Gln^8]-GnRH (Ki 2246 ± 505 and 4059 ± 1255 nM) (Table 4.1, Fig. 4.4).

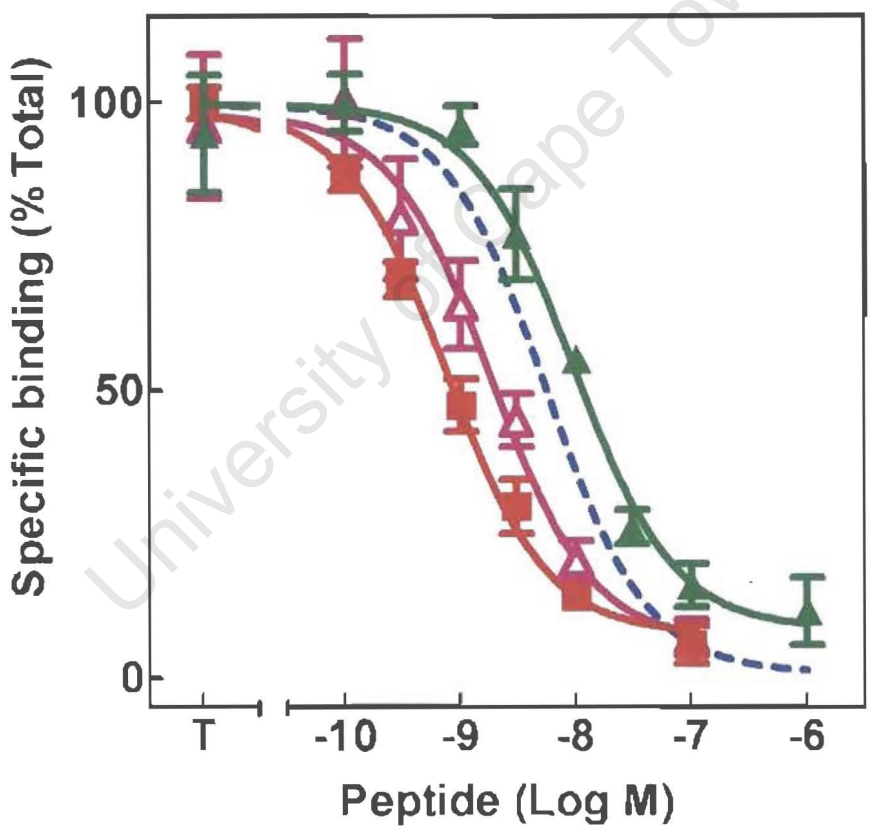

Figure 4.4 Competition binding of a conformationally-constrained GnRH analog in human wild type and ECL3 mutant GnRH receptors. COS-1 cell membranes expressing the human wild type (■), $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Phe (△), and $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Tyr mutant (▲) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of [His^5,D-Tyr^6]-GnRH. The dotted line (---) represents a reference curve of GnRH binding with the human wild type GnRH receptor. Data are mean ± SE of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand, the latter being indicated as total specific binding (T).

Thus, incorporation of the non-mammalian GnRH receptor motif did not enhance affinity for forms of GnRH which do not have Arg^8, but also decreased affinity for the constrained GnRH analog. This suggests that the Tyr residue of the chicken receptor in this motif plays a role in the observed decrease in affinity for the conformationally-constrained analog.

4.3.4 The $Pro^{7.33(301)}$-$Asp^{7.32(302)}$-$Phe^{7.31(303)}$ Motif in ECL3

The $Ser^{7.31(301)}Pro/Pro^{7.33(303)}Phe$ mutant receptor which has the non-mammalian motif but with Phe substituted for Tyr, was similar to the $Ser^{7.31(301)}Pro/Pro^{7.33(303)}Gly$ mutant, and different from the mutants containing the non-mammalian receptor motif. It had decreased affinity for GnRH (Ki 76.4 ± 2.87 nM), and minimally decreased affinity for the conformationally constrained analog (Ki 2.71 ± 0.71 nM) or the analogs that lack Arg^8 (Table 4.1, Fig. 4.4). Phe differs from Tyr in lacking a hydroxyl group. This result suggests that the hydroxyl group of the Tyr side chain is responsible for the decreased affinity of the $Ser^{7.31(301)}Pro/Pro^{7.33(303)}Tyr$ and $Ser^{7.31(301)}Pro/Asp^{7.32(302)}Glu/Pro^{7.33(303)}Tyr$ mutants for the conformationally-constrained GnRH analog. The larger side chain of Tyr may distort the conformation of ECL3.

4.3.5 Substituting ECL3 of the Human GnRH Receptor with ECL3 of the Chicken GnRH Receptor

To investigate whether the entire non-mammalian ECL3 GnRH receptor motif may generate high affinity for all forms of GnRH, the ECL3 sequence of the chicken GnRH receptor (His-Pro-Ala-Met-Ile-Gln-Arg-Met-Pro-Glu-Tyr-Ile) substituted ECL3 of the human GnRH receptor (Asp-Pro-Glu-Met-Leu-Asn-Arg-Leu-Ser-Asp-Pro-Val). This mutant receptor had decreased affinity for GnRH, [His^5,D-Tyr^6]-GnRH and GnRH II (Table 4.1). The poor total binding with labeled [His^5,D-Tyr^6]-GnRH led to using high membrane concentrations which resulted in high non-specific binding and wide standard deviations, resulting in receptor expression not being able to be calculated from homologous binding assays. This mutant receptor was also uncoupled to the PLC signalling pathway (Table 4.2). Therefore, this result was not included in subsequent calculations of Bmax and coupling efficiency.

4.3.6 The Role of ECL3 in Receptor Expression and PLC Activation

Mutant receptors showed significant increases in EC_{50} values compared to the wild type receptor (Table 4.2). The decrease in GnRH potency was higher than the loss of affinity for GnRH (Tables 4.1 and 4.2) and lower Emax values suggest that GnRH had a lower efficacy at the mutant receptors and/ or that mutations disrupted receptor expression.

To determine whether the low GnRH potency observed in the mutant receptors resulted from altered receptor expression, the Bmax values from homologous competition binding assays were determined (chapter two). Expression levels of all mutant receptors were comparable to that of the wild type receptor (Table 4.1) suggesting that GnRH has a decreased efficacy in the mutant receptors. The Emax values for GnRH stimulated IP accumulation of the $Pro^{7.33(303)}$Ala mutant was comparable with the wild type receptor, however, all other mutant receptors displayed significantly increased EC_{50} values and decreased Emax values (Table 4.2). To accurately measure how well a particular receptor is activated the coupling efficiency of the mutant receptors was calculated as described in chapter two. All mutant receptors had decreased coupling efficiency (< 59%) compared to the wild type receptor. The variations in the percentage coupling efficiency, relative to the wild type receptor, indicate that the mutant receptors were partially uncoupled. The $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Tyr and ECL3 chimeric mutant receptors were uncoupled.

4.3.7 Both $Asp^{7.32(302)}$ and $Pro^{7.33(303)}$ are Required for Efficient PLC Activation by GnRH and GnRH analogs

The $Asp^{7.32(302)}$Asn mutant receptor has decreased coupling efficiency relative to the wild type receptor (52.7%, Table 4.2). The $Pro^{7.33(303)}$Ala mutant receptor had a similar degree of decreased coupling efficiency (58.7%), suggesting that both $Asp^{7.32(302)}$ and $Pro^{7.33(303)}$ play a role in receptor activation. The Emax value of the $Pro^{7.33(303)}$Ala mutant receptor (96.7 ± 2.14 %) is higher than that of the $Asp^{7.32(302)}$Asn mutant receptor (73.0 ± 5.15 %, Table 4.2), which shows that the Emax value can be misleading if there other changes such as a decrease in EC_{50}. Both $Asp^{7.32(302)}$Asn and $Pro^{7.33(303)}$Ala mutant receptors showed a significant increase in EC_{50} values for GnRH (Table 4.2). However the higher EC_{50} value of the $Asp^{7.32(302)}$Asn mutant receptor compared to the $Pro^{7.33(303)}$Ala mutant receptor suggests that $Pro^{7.33(303)}$ may play a complementary role to $Asp^{7.32(302)}$ in receptor activation.

To test whether $Asp^{7.32(302)}$ and $Pro^{7.33(303)}$ or the entire ECL3 influences signalling via other GnRH analogs, the IP accumulation of the $Ser^{301(7.31)}$Pro/$Pro^{7.33(303)}$Gly, $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Tyr, ECL3 chimeric and $Asp^{7.32(302)}$Asn mutant receptors with GnRH II and [Gln^8]-GnRH was determined (Table 4.2). The $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Tyr and ECL3 chimeric mutant receptor showed no measurable stimulation with GnRH II or [Gln^8]-GnRH, suggesting that this mutant receptor was uncoupled for GnRH analogs lacking Arg^8. Both $Asp^{7.32(302)}$Asn and $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Gly mutant receptors had a significantly decreased coupling efficiency for

both GnRH II and [Gln^{8}]-GnRH, suggesting that these mutations result in partial uncoupling which is similar as found with GnRH (Table 4.2). These results suggest that both Asp$^{7.32(302)}$ and a Pro at locus 7.33 play a role in receptor activation that is commom to GnRH analogs with and without Arg^{8}.

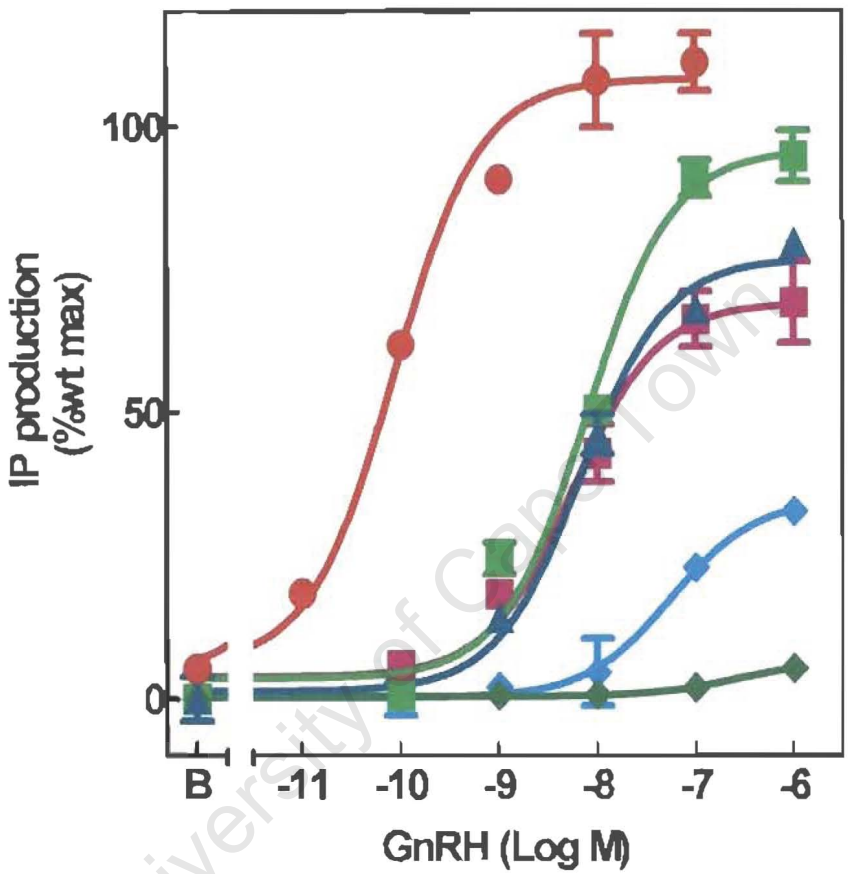

Figure 4.5 GnRH stimulated IP accumulation. COS-1 cells transiently transfected with the human wild type (●), Asp$^{7.32(302)}$Asn mutant (▲) (Fromme et al., 2001), Pro$^{7.33(303)}$Ala mutant (■), Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Phe (■), Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Tyr mutant (◆) and Ser$^{7.31(301)}$Pro/Asp$^{7.32(302)}$Glu/Pro$^{7.33(303)}$Tyr mutant (◆) GnRH receptors were incubated with various concentrations of GnRH. Data are mean ± SE of a representative experiment performed in duplicate and expressed as percentage GnRH stimulated E_{max} of the wild type receptor.

4.3.8 The Conformation of ECL3 may also Influence Receptor Activation

Mutant receptors with Pro in position 7.33 also had lower coupling efficiency relative to the wild type receptor (Table 4.2). This suggests that the position of Pro relative to Asp$^{7.32(302)}$ is important for receptor activation. However, this decrease in coupling efficiency is more than the loss of Asp$^{7.32(302)}$ or Pro$^{7.33(303)}$, suggesting that other residues in ECL3 or that the conformation of ECL3 may also play a role in receptor activation (Table 4.2). Moreover, mutant receptors with the non-mammalian receptor motif in ECL3 exhibited low or no measurable IP production,

suggesting that the mutations may uncouple the receptor from intracellular signalling (Table 4.2). The entire non-mammalian ECL3 and the hydroxyl group of Tyr at locus 7.33 seems not to be tolerated in the human GnRH receptor.

In summary, substitution of $Pro^{7.33}$ decreases affinity for GnRH but not for a constrained analog or GnRH analogs that do not contain Arg^8. Incorporation of the non-mammalian ECL3 or receptor motifs (Pro-Asp-Tyr or Pro-Glu-Tyr) or moving Pro to position 7.31 decreased affinity for GnRH and the constrained analog, but not for GnRH II and [Gln^8]-GnRH. These mutations also decreased agonist stimulated intracellular signalling for GnRH analogs with or without Arg^8.

Table 4.2 Summary of GnRH receptor agonist stimulated IP accumulation and coupling efficiency. EC_{50} and Emax values are mean ± SE. Statistical analyses were performed as described under "Materials and Methods". The fold change was calculated as the ratio of the EC_{50} values in the mutant and wild type receptors. Emax in the mutant is expressed as percent of Emax stimulated by the same ligand in wild type receptor in the same experiment. The coupling efficiency for GnRH was calculated from averaged values as described in "Materials and Methods" and expressed as a percentage of wild type.

ECL3 motif	EC_{50} nM (GnRH)	Emax (%wt)	Coupling efficiency (%wt)
(wt) Ser-Asp-Pro	0.29 ± 0.09	100	100
Ser-Asp-**Ala**	3.73 ± 1.83** (12.9)	96.7	58.9
Pro-Asp-**Gly**	9.88 ± 2.59** (33.6)	61.1 ± 5.60*	36.9
Pro-Asp-**Phe**	6.99 ± 1.08** (23.8)	77.1 ± 9.48	39.6
Pro-Asp-**Tyr**	NMS[a]	NMS	NMS
Pro-Glu-Tyr	68.7 ±23.1** (234)	25.3 ± 4.68**	3.36
ECL3 chimera	NMS	NMS	NMS
Ser-**Asn**-Pro	11.7 ± 2.06** (40.3)	73.0 ± 5.15*	52.7
ECL3 motif	EC_{50} nM (GnRH II)	Emax (%wt)	Coupling efficiency (%wt)
(wt) Ser-Asp-Pro	1.77 ± 0.42	100	100
Pro-Asp-**Gly**	7.13 ± 2.71* (4.04)	77.7 ± 1.25*	37.2
Pro-Asp-**Tyr**	NMS[a]	NMS	NMS
ECL3 chimera	NMS	NMS	NMS
Ser-**Asn**-Pro	6.66 ± 1.62* (3.77)	71.1 ± 8.24*	37.0
ECL3 motif	EC_{50} nM ([Gln^8]-GnRH)	Emax (%wt)	Coupling efficiency (%wt)
(wt) Ser-Asp-Pro	6.32 ± 1.27	100	100
Pro-Asp-**Gly**	21.8 ± 4.35* (3.44)	67.7 ± 8.65*	27.9
Pro-Asp-**Tyr**	NMS[a]	NMS	NMS
ECL3 chimera	NMS	NMS	NMS
Ser-**Asn**-Pro	17.2 ± 2.84*(2.72)	69.1 ± 2.30*	26.6

* significantly different from wild type receptor, $p<0.05$.
** significantly different from wild type receptor, $p<0.001$.
[a] construct in which no agonist stimulated IP accumulation could be determined (NMS).

4.4 Discussion

The mammalian type I GnRH receptor preferentially binds mammalian GnRH through an interaction between Arg^8-containing GnRH and an Asp/Glu residue in position 7.32 of ECL3 (Flanagan et al., 1994; Fromme et al., 2001). Non-mammalian type I GnRH receptors do not selectively bind Arg^8-containing GnRH (Sealfon et al., 1997), but surprisingly the acidic residue at position 7.32 is conserved. Amino acid sequence alignment of ECL3 of mammalian and non-mammalian GnRH receptors revealed two distinct amino acid motifs. Mammalian GnRH receptors contain the $Ser^{7.31}$-($Glu^{7.32}$/$Asp^{7.32}$)-$Pro^{7.33}$ motif and non-mammalian GnRH receptors have Pro-(Asp/Glu)-Tyr. Differences in the pharmacology of mammalian and non-mammalian GnRH receptors appear to be associated with the different positions of Pro in these ECL3 motifs. Pro residues are thought to influence packaging and organization of TM helices (Vanhoof et al., 1995) and have been demonstrated to play key roles in receptor expression, ligand binding and receptor activation (Hong et al., 1997; Wess et al., 1993; Sansom and Weinstein, 2000). Pro confers unique local constraints on peptide chain conformation, as the α-nitrogen atom of proline is part of the rigid pyrrolidine ring, and by means of a secondary amide bond is covalently bound to the preceding amino group (MacArthur and Thornton, 1991). This suggests that Pro may regulate the conformation of ECL3 of the GnRH receptor and this conformation may influence ligand binding. The role of $Pro^{7.33(303)}$ and its position in ECL3 has been investigated by site-directed mutagenesis.

Presence and Location of $Pro^{7.33(303)}$ is Required for GnRH Selectivity

The $Pro^{7.33(303)}$Ala mutant had decreased affinity for GnRH, but not for [Gln^8]-GnRH or GnRH II, showing that substitution of $Pro^{7.33(303)}$ specifically decreases affinity for Arg^8-containing GnRH. We have previously reported that $Asp^{7.32(302)}$ of the human GnRH receptor confers specificity for Arg^8 of GnRH (Fromme et al., 2001). The current results show that the specificity of the human GnRH receptor for Arg^8-containing GnRH depends on both $Asp^{7.32(302)}$ and $Pro^{7.33(303)}$.

The importance of $Pro^{7.33(303)}$ in ligand selectivity combined with the conservation of an acidic residue at locus 7.32 in both mammalian and non-mammalian vertebrate GnRH receptors, suggests that the position of the Pro residue may influence the Arg^8-$Asp^{7.32(302)}$ interaction. Mutant receptors in which Pro was moved from locus 7.33 to 7.31, as in the non-mammalian GnRH receptors, ($Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Gly and $Ser^{7.31(301)}$Pro/$Pro^{7.33(303)}$Phe mutants) also had decreased specificity for GnRH. This shows that Pro must be present and that it must be in the 7.33 locus to achieve high affinity binding of GnRH.

Conformationally-Constrained GnRH Overcomes the Absence of Pro$^{7.33(303)}$

The human GnRH receptor has enhanced affinity for a conformational state of GnRH, that is stabilized by the incorporation of a D-amino acid in position six. It is proposed that Asp$^{7.32(302)}$ of the GnRH receptor interacts transiently with Arg^{8} of GnRH to induce a high affinity conformation of the native ligand, which then interacts with a binding pocket that is common for both conformationally-constrained and -unconstrained analogs of GnRH (Fromme et al., 2001). Similar to the Asp$^{7.32(302)}$Asn mutant (Fromme et al., 2001), the Pro$^{7.33(303)}$Ala, Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Phe and Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Gly mutant receptors retained high affinity for the conformationally-constrained analog, [His^{5}, D-Tyr^{6}]-GnRH. This suggests that a conformational constraint of the ligand can overcome the absence of Pro$^{7.33(303)}$ as it can the absence of Asp$^{7.32(302)}$ (Fromme et al., 2001). The ability of [His^{5}, D-Tyr^{6}]-GnRH to compensate for the absence of Pro$^{7.33(303)}$, suggests that this residue does not play a role in binding conformationally-constrained GnRH.

The type I chicken GnRH receptor preferentially binds GnRH II, but binds Arg^{8}-containing GnRH, [Gln^{8}]-GnRH and conformationally-constrained GnRH analogs with similar affinity (Sun et al., 2001). This implies that Arg^{8} in GnRH, or a particular ligand conformation, does not perform the same role in binding non-mammalian GnRH receptors as it does with mammalian type I GnRH receptors. It is possible that the mammalian GnRH receptors have Pro conserved at locus 7.33, to present the Glu/Asp$^{7.32}$ side chain most favourable for interaction with Arg^{8} of GnRH. This supports the proposal that Pro may confer unique local conformational constraints on the conformation of ECL3 which influence the orientation of Asp$^{7.32(302)}$ thereby regulating ligand affinity. Therefore, the human GnRH receptor has evolved a ligand binding mechanism which requires both Asp$^{7.32(302)}$ and Pro$^{7.33(303)}$ for high affinity binding of GnRH, and this is the manner in which the mammalian GnRH receptor determines selectivity for GnRH.

Tyr at locus 7.33 Decreases Affinity for a Conformationally-Constrained GnRH Analog

The Pro$^{7.33(303)}$Ala, Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Gly and Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Phe mutant receptors had decreased selectivity for GnRH which resulted from decreased affinity for this native peptide. Although the chicken GnRH receptor does not selectively bind GnRH, it has high affinity for GnRH as well as GnRH II and [Gln^{8}]-GnRH. This suggested that entire non-mammalian GnRH receptor motif (Pro-(Asp/Glu)-Tyr) may be required for high affinity binding of these peptides. Mutant receptors containing the non-mammalian motifs (Pro-(Asp/Glu)-Tyr) in place of the

human GnRH receptor motif (Ser-(Asp/Glu)-Pro) showed no increase in affinity for GnRH II and [Gln^{8}]-GnRH, but had decreased affinity both for GnRH and conformationally-constrained GnRH. These results suggest that the hydroxyl group of the Tyr side chain in these motifs may account for the decreased affinity for the conformationally-constrained analog in the mutant receptors.

Both Pro$^{7.33(303)}$ and Asp$^{7.32(302)}$ Play a Role in PLC Activation

Similar to the Asp$^{7.32(302)}$Asn mutant, the Pro$^{7.33(303)}$Ala, Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Phe and Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Gly mutant receptors had decreased GnRH-stimulated IP accumulation, which was similar to or slightly higher than the decrease in affinity for GnRH. The similar receptor expression levels but lower coupling efficiency to the wild type receptor, suggests that Pro$^{7.33(303)}$ and Asp$^{7.32(302)}$ have a complementary role in receptor activation. Mutant receptors that had Tyr$^{7.33}$ included, by introducing the non-mammalian motif (Pro-(Asp/Glu)-Tyr) in place of the human GnRH receptor motif (Ser-(Asp/Glu)-Pro), showed a larger decrease in GnRH potency, than decrease in GnRH affinity. The decreased coupling efficiency of these mutant receptors suggests that they are less able to be activated. Mutating Pro$^{7.33(303)}$/Tyr in ECL3 appears to disrupt receptor activation. It is possible that the hydroxyl group of the Tyr side is interacting with other residues in the GnRH receptor tethering it in the inactive R-conformation. It has been reported that ECL3 of other GPCRs such as the β_2-adrenergic (Zhao et al., 1998) and M1 muscarinic acetylcholine (Huang et al., 1999) receptors can modulate G protein interactions. It is possible that ECL3, which connects TM helices six and seven, may interact with residues in other ECLs or TM domains (Palczewski et al., 2000) stabilising these TM domains in a specific position. TM helices six and seven in turn are important in the activation process of GPCRs, such as rhodopsin (Altenbach et al., 1996) and the GnRH receptor (Zhou et al., 1994), resulting in conformational changes in ICL3 which is important for G protein coupling (Samama et al., 1993).

There is evidence that ECLs of GPCRs play a direct role in receptor activation (Zhao et al., 1998). It is possible that residues in ECL3 which are implicated in ligand binding may also have additional roles such as structural organisation of the receptor either in the inactive or active conformation. Replacing ECL3 in the β_2-adrenergic receptor with the corresponding loop of the α_1-adrenergic receptor resulted in a higher affinity for agonists, but not antagonists, increased agonist potency and an agonist-independent higher basal activity (constitutive activity) (Zhao et al., 1998). Substituting the entire ECL3 in the human GnRH receptor with the ECL3 sequence of the chicken GnRH receptor results in low binding and no measurable signalling for GnRH analogs with or without Arg^{8}. If ECL-1 and -2 of the chicken receptor are separately substituted to ECL-

1 and -2 of the human GnRH receptor, the mutant receptors are still able to bind GnRH analogs with high affinity and they are still coupled to the PLC signalling pathway (Sun et al., 2001). If the mammalian Ser-(Asp/Glu)-Pro motif is substituted with the non-mammalian Pro-(Asp/Glu)-Tyr motif in the human GnRH receptor, the decrease in GnRH potency is greater than the decrease in GnRH affinity. We also show that GnRH receptors with substitutions at $Asp^{7.32(302)}$ or $Pro^{7.33(303)}$ in ECL3 are partially uncoupled. Moreover, we show that GnRH analogs that lack Arg^{8} also have decreased coupling efficiency with GnRH receptors with substitutions at $Asp^{7.32(302)}$ or $Pro^{7.33(303)}$ in ECL3. Together this suggests that ECL3 contain residues that have a common role in receptor activation. Rhodopsin has $Asp^{7.29(282)}$ in ECL3, which is in a similar position to $Asp^{7.32(302)}$ of the GnRH receptor. In the high resolution crystal structure of rhodopsin it is shown that $Asp^{7.29(282)}$ is located in close opposition to Asn^{2} of the NH_2-terminus and may have a role in the structural organisation of the extra-cellular domains (Palczewski et al., 2000). It is possible that in the GnRH receptor this interaction may also exist, however, in constraining the active conformation. This interaction is most likely indirect via stabilising one or more of the TM helices involved in receptor activation (eg. TM helix seven).

A Conformation of ECL3 in the Mammalian GnRH Receptor may Influence GnRH Binding

The secondary structure of the ECL3 of a mammalian GnRH receptor was investigated with CD and Micro-Raman techniques (Petry et al., 2002) (also see chapter five). In other GPCRs, segments of extra- and intra-cellular loops have been proposed to form functionally discrete conformations independently of the connecting TM domains (Abdulaev et al., 2000; Yeagle et al., 2000). It is proposed that ECL3 of the mammalian GnRH receptor exists predominantly as a random coil with a central ß-hairpin domain, consisting of residues Leu-Asn-Arg-$Ser^{7.31}$ (Petry et al., 2002) (Fig. 4.2). This conformation of ECL3 may, in addition to the local influence of $Pro^{7.33(303)}$, also be required for the selective interaction of the $Asp/Glu^{7.32}$ side chain in mammalian GnRH receptors with Arg^{8} of GnRH and play a role in receptor activation.

In summary, amino acid residues in ECL3 of the mammalian GnRH receptor confer high affinity for GnRH and have a role in agonist-stimulated receptor activation. An acidic residue at locus 7.32 in ECL3 has been demonstrated to confer specificity for Arg^{8}-containing GnRH. A Pro residue is required in the 7.33 locus of ECL3 to maintain GnRH selectivity. We propose that in the human GnRH receptor Pro in locus 7.33 confers unique local conformational constraints on the conformation of ECL3 which influences the orientation of $Asp^{7.32(302)}$ thereby regulating

selectivity for GnRH. Mutating residues in ECL3 which are important in GnRH binding also modulates G protein coupling. In conclusion, $Pro^{7.33(303)}$ of ECL3 of the human GnRH receptor is important in conferring high affinity for GnRH and play a separate role in receptor activation. We propose that ECL3 needs to be in a specific conformational structure which is stabilised by $Pro^{7.33(303)}$ and that Pro in position 7.33 forms part of a ligand binding mechanism in which mammalian GnRH receptors distinguish GnRH from other naturally occurring forms, such as GnRH II.

Chapter 5

The Conformation of the GnRH Receptor's ECL3 and its Role in GnRH Selectivity

5.1 Summary

It has been suggested that the conformation of ECL3 may also contribute to ligand selectivity (chapter four) and it has been proposed that ECL3 may exist in an α-helical conformation (Sankararamakrishnan, 2000). The mammalian ECL3 of the GnRH receptor is conserved as eight amino acid residues (Sealfon et al., 1997), which is sufficient to contain two full turns of a helix. With Ala-insertional mutagenesis we investigated whether the length of ECL3 has functional importance in GnRH binding. Introduction of a single Ala at the N-terminus side of ECL3 caused a significant decrease in GnRH affinity. Progressive insertion of a second and third Ala residue caused a further decrease in agonist binding, suggesting that the entire ECL3 is important in conferring high affinity for Arg^8-containing GnRH. This prompted the investigation of the secondary structure of a model peptide, representing ECL3 of mouse GnRH receptor using micro Raman and FT-Raman spectroscopy, ultraviolet circular dichroism (CD) spectroscopy, and two-dimensional (2D) NMR spectroscopy in a collaborative study (Petry et al., 2002). This spectroscopic analysis of the model peptide revealed a superposition of predominantly unordered structure and partial contributions from β-sheet structure (Petry et al., 2002). To determine whether the structure of ECL3 is important in interacting with GnRH, we used synthetic peptides corresponding to the sequence of ECL3, with and without conformational constraints, to measure the ECL3 peptides ability to inhibit GnRH stimulated signalling. It is shown that peptides corresponding to the sequence of ECL3 are functional by being able to specifically inhibit GnRH stimulated signalling. A specific structure in ECL3 is proposed to stabilise a conformation of ECL3 in which $Pro^{7.31}$ may also play a role (chapter four). This structure aligns the side chain of the acidic residue at locus 7.32 in the mammalian type I GnRH receptor to interact with Arg^8 of GnRH. Mammalian type I GnRH receptors have evolved a mechanism involving specific residues in ECL3 which determine high affinity for GnRH, which requires a ECL3 conformation that complements ligand selectivity for GnRH.

5.2 Introduction

Molecular models of the GnRH receptor have been based predominantly on inferences from biochemical approaches, mutagenesis, biophysical considerations and computational modelling (Sealfon et al., 1997). Although the TM domains of GPCRs, including the GnRH receptor, have been successfully modelled based on their high level of homology, little is known of the loop structures because of their large variation in length and composition. It has been proposed that segments of GPCR extra- and intra-cellular loops form functionally discrete conformations independently of the connecting TM domains (Abdulaev et al., 2000; Yeagle et al., 2000). The

ordered structures of ECLs may depend on the interaction and/or properties of specific amino acids and amino acid motifs within a particular loop (Yeagle et al., 2000). The crystal structure of rhodopsin revealed that the extracellular domains are highly organised (Palczewski et al., 2000). This ability of ECLs assuming a particular conformation may play a role in specific ligand-receptor binding mechanisms (chapter four and Yeagle et al., 2000). ECLs of GPCRs have been shown to contain residues implicated in ligand recognition (Gether, 2000) and the loops may have a physical role in a sequential ligand-receptor binding mechanism (Perlman et al., 1997; Colson et al., 1998b; Fromme et al., 2001).

The mammalian type I GnRH receptor is able to discriminate between GnRH and related peptides (King and Millar, 1995) through residues in ECL3 which play an essential role in determining high affinity for mammalian GnRH (Flanagan et al., 1994; Fromme et al., 2001). It was proposed that the secondary structure of ECL3 might also play a role in ligand recognition and receptor activation (chapter four). In the GnRH receptor, ECL3 connects the helical TM domains six and seven and possesses an Asp-Pro and (Asp/Glu)-Pro motif on its C- and N-termini, respectively. From molecular dynamics simulations the Asp-Pro sequence at the beginning of the loop was found to be a strong N–terminal helix cap. Asn-Pro and Asp-Pro motifs are proposed to induce hinges (helix breaker) in α-helices (Fig. 5.2) (Konvicka et al., 1998). Inferences from molecular dynamics simulations have proposed that ECL3 may exist in an α-helical conformation (Sankararamakrishnan, 2000). Additionally, the length of the mammalian ECL3 GnRH receptor is conserved as eight amino acid residues (Sealfon et al., 1997), sufficient to contain two full turns of a helix. However, the recent X–ray crystallography of bovine rhodopsin shows a lack of an α-helix in ECL3 (Palczewski et al., 2000). This prompted a collaborative study (Petry et al., 2002) in which, we used CD, NMR and Raman spectroscopy of synthetic peptides of ECL3 of the mouse GnRH receptor to gain more information about the conformation of this domain. Using Ala-insertional mutagenesis, the importance of the length of ECL3 of the GnRH receptor on GnRH binding was investigated. To determine whether the structure of ECL3 is important in interacting with GnRH, we used synthetic peptides correponding to the sequence of ECL3, with and without conformational constraints, to measure the ability of ECL3 peptides to inhibit GnRH stimulated signalling.

5.3 Results

5.3.1 Increasing the Length of ECL3 Decreases Affinity for GnRH

The functional importance of the length of ECL3 of the human GnRH receptor in GnRH binding

was investigated using insertional mutagenesis. Ala residues (one, two, and three) were inserted between residue $Pro^{6.38(294)}$ and $Glu^{6.37(295)}$ (Fig. 4.2) using oligonucleotide-directed mutagenesis (Kunkel et al., 1991) (chapter two). Membrane binding assays were performed to compensate for the lower total binding of the mutant GnRH receptors (chapter two). A membrane concentration of 1/5th dish per reaction tube was used for the mutant GnRH receptors, relative to the wild type receptor (1/20th dish per tube). In competion membrane binding assays with ^{125}I-[D-Ala^6,N-Me-Leu^7,Pro^9-NHEt]-GnRH, the Ki of the wild type GnRH receptor for GnRH was 1.06 ± 0.18 nM (Table 5.1).

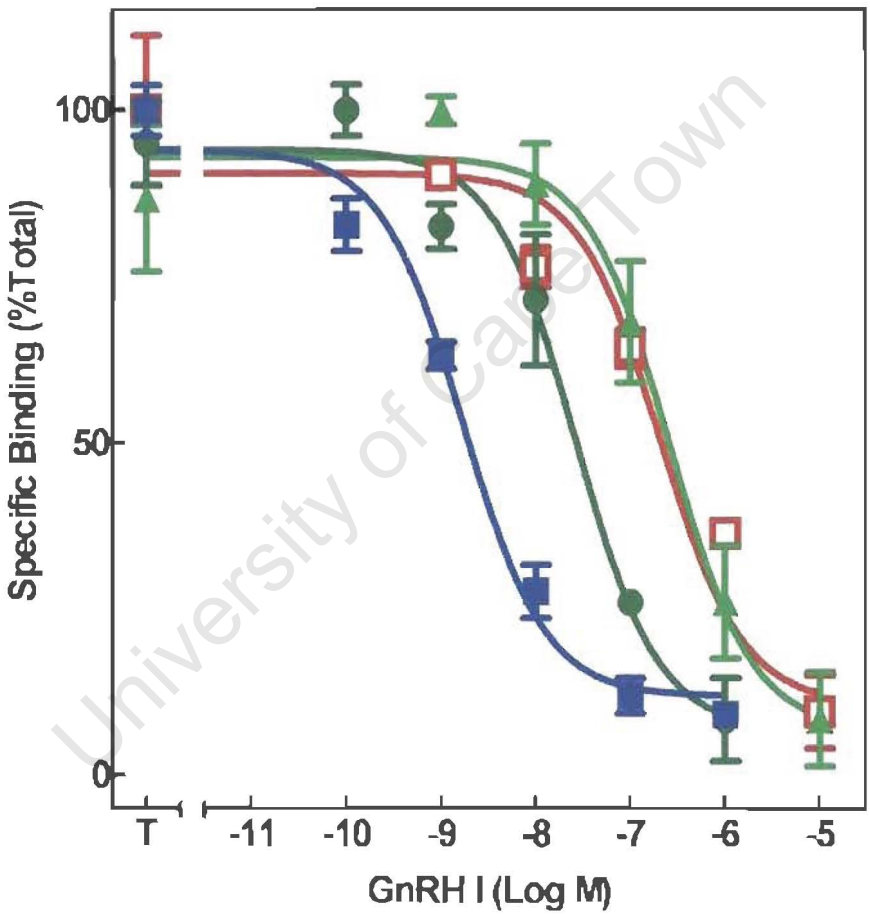

Figure 5.1 Inserting Ala residues into ECL3 of the human type I GnRH receptor decreases affinity for GnRH. COS-1 cell membranes expressing the human wild type I GnRH receptor (■) and mutant receptors which had one (●), two (▲), and three (□) Ala residues inserted after $Pro^{6.47(294)}$ in ECL3, were incubated with ^{125}I-GnRH-A in the presence of various concentrations of GnRH. Data are mean ± SE of a representative experiment performed in triplicate and expressed as percentage of specific binding in the absence of unlabeled ligand (T).

Mutant receptors with one, two or three Ala residues inserted after the N-terminal Asp-Pro-cap of ECL3 in the human GnRH receptor had significantly decreased affinity for GnRH ($p<0.001$,

Table 5.1). The insertion of one Ala residue into ECL3 resulted in a 19-fold decrease in affinity for GnRH (Ki 20.3 ± 7.43 nM). The additional insertion of another one (Ki 95.9 ± 43.6 nM) and two (Ki 77.4 ± 12.5 nM) Ala residues into ECL3, resulted in 90- and 77-fold decreases in GnRH affinity relative to the wild type receptor, respectively. The affinity for GnRH was significantly lower in the mutant receptor with three Ala insertions, compared to the mutant receptor that only had one Ala insertion ($p<0.05$, Table 5.1). This suggests that increasing the length of ECL3 in the GnRH receptor decreases GnRH affinity.

Table 5.1 Summary of ligand binding results of the human GnRH receptor with Ala-insertions in ECL3. Competition binding assays were performed on COS-1 cells transiently transfected with wild type or mutant GnRH receptors, using ^{125}I-[D-Ala^6-N-Me-Leu^7,Pro^9-NHEt]-GnRH and various concentrations of unlabeled ligands as described under "Materials and Methods" (chapter two). Ki values are the mean ± SE. Statistical analyses were performed using pIC_{50} values as described under "Materials and Methods". The fold change was calculated as the ratio of the Ki values of the mutant and wild type receptors.

Wild type	Pro^{294}/Ala^1/Glu^{295}	Pro^{294}/Ala^2/Glu^{295}	Pro^{294}/Ala^3/Glu^{295}
		nM	
1.06 ± 0.18	20.3 ± 7.43 (19.1)*	95.9 ± 43.6 (90.5)*	77.4 ± 12.5 (73.0)*[a]

* significantly different from wild type receptor, $p<0.001$.
[a] significantly different from mutant receptor with one Ala-insertion, $p<0.05$.

5.3.2 ECL3 Peptides are Able to Inhibit GnRH Stimulated IP-Accumulation and if Immobilised on a Sensor Chip are Shown to Specifically Interact with GnRH

ECL-3 peptides were tested for their ability to inhibit GnRH and [Gln^8]-GnRH stimulated IP accumulation in COS-1 cells transiently transfected with human GnRH receptor. The ability of an RI-peptide, corresponding to the mammalian ECL3 GnRH receptor sequence, to inhibit GnRH and [Gln^8]-GnRH stimulated IP accumulation was compared. RI peptides are composed of D-amino acids assembled in the reverse order (C to N terminus) in relation to the parent L-peptide, which leads the orientation of the side chains in a RI-analog to be very similar to that in the parent peptide. These peptides are immunogenic and produce antibodies, which can cross-react with the parent peptide with high specificity (Van Regenmortel et al., 1998a). This RI peptide of ECL3, AcAsp-Ser-Leu-Arg-Asn-Leu-Met-Glu-Cys-$CONH_2$ (all D amino acids), was initially intended for the generation of anti-GnRH receptor antibodies targeting ECL3, but surprisingly could specifically inhibit GnRH stimulated IP accumulation ($p<0.01$) in cells transfected with the human type I GnRH receptor. Adding 0.1 nM GnRH to the labelled cells could stimulate 44% of the maximal IP response and 1.0 nM of [Gln^8]-GnRH induced 40% of the total IP production after one hour incubation. Incubating (two hours) the RI-ECL3 peptide (10 M) with GnRH and using

this mixture to stimulate labelled COS-1 cells transfected with the human GnRH receptor decreased the agonist potency by 65.3 ± 12.6% ($p<0.01$, Fig. 5.2).

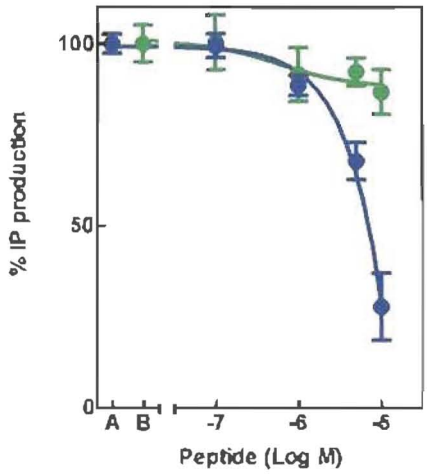

Figure 5.2 A RI-ECL3 peptide specifically inhibits GnRH stimulated IP-accumulation. COS-1 cells transiently transfected with the wild type human GnRH receptor were incubated alone with (A) 0.1 nM of GnRH (●) or (B) 1.0 nM or [Gln^8]-GnRH (●). These concentrations of GnRH and [Gln^8]-GnRH were also preincubated with increasing concentrations of RI-ECL3 peptide for two hours at 37°C and added to transfected cells as described ("Materials and Methods", chapter two). Values are expressed as a percentage IP accumulation relative to GnRH and [Gln^8]-GnRH that was incubated alone over the same period. Data are mean ± SE of at least three experiments each pe

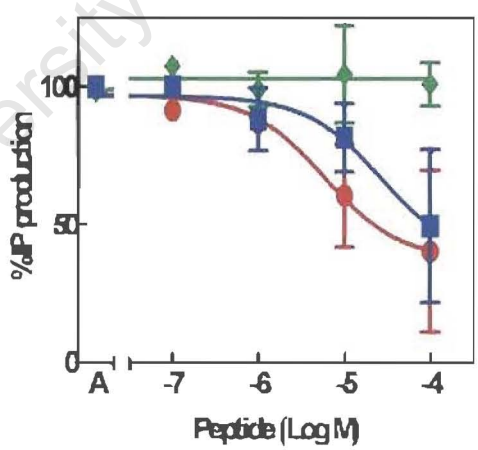

Figure 5.3 Inhibition of GnRH stimulated IP accumulation with L-peptides corresponding to the sequence of ECL3. COS-1 cells transiently transfected with the wild type human GnRH receptor were incubated alone with 0.1 nM of GnRH (A). This concentration of GnRH (0.1 nM) was preincubated (two hours, 37°C) with increasing concentrations of linear (■) and cyclic receptor peptides (●) and a control peptide, somatostatin (◆) before stimulating COS-1 cells (one hour, 37°C) transiently transfected with wild type human receptor with this mixture as described ("Materials and Methods", chapter two). Data are the mean ±SE of two experiments, each point performed in duplicate and expressed as a percentage IP accumulation relative to the 0.1 nM GnRH that was incubated in parallel over the same period of time and conditions.

The RI-ECL3 peptide (10 000 nM) could not inhibit [Gln^8]-GnRH stimulated IP accumulation, suggesting that it specifically affects GnRH potency (Fig. 5.2). Control peptides such as RI-GnRH and RI-ECL1 under the same conditions did not inhibit GnRH stimulated IP accumulation (chapter two). The preincubation of RI-peptides and GnRH allowed sufficient association between the peptides and this incubation step had no adverse effects on GnRH alone. The interaction of GnRH with the RI-ECL3 peptide was confirmed with Surface Plasmon Resonance (SPR) (chapter two). The fixed RI-ECL3 peptide bound GnRH (Kd=6390 nM) but not [Gln^8]-GnRH, supporting the proposal that this peptide specifically interacts with Arg^8-containing GnRH. In preliminary experiments it was found that the linear and cyclic ECL3 peptides may also inhibit GnRH stimulated IP accumulation (Fig. 5.3). This suggests that L-peptides of ECL3 may interact with GnRH and that structure of these peptides may play a role in GnRH binding.

5.3.3 The Secondary Structure of an ECL3 Peptide

The secondary structure of this model peptide, representing ECL3 of mouse GnRH receptor, has been studied by micro Raman and FT-Raman spectroscopy, by ultraviolet circular dichroism (CD) spectroscopy, and by two-dimensional (2D) NMR spectroscopy (Petry et al., 2002). These studies revealed that the cyclic ECL3 peptide contains a ß-hairpin loop amongst an ensemble of disordered structures (Petry et al., 2002). This data is not presented in this thesis as it was performed by Renate Petry as part of a collaborative study. In Fig. 5.4 the results are summarised of this part of the study.

5.4 Discussion.

Molecular cloning and pharmacological characterisation of GnRH receptors from a range of vertebrate species has shed light on the nature of the structure-activity relations of the receptor-ligand complex and together with mutagenesis of the receptor has contributed to the refinement of computational molecular models (Sealfon et al., 1997). The presence of a second form of GnRH, GnRH II, which is widely expressed in primates (Latimer et al., 2000; White et al., 1998) and the cloning of another GnRH receptor (type II) in primates (Millar et al., 2001; Neill et al., 2001) indicates a need for the reevaluation of how the mammalian GnRH receptor selects between naturally occurring forms of GnRH. There is evidence that the mammalian type I GnRH receptor has evolved a particular ligand binding mechanism that confers selectivity for GnRH (Fromme et al., 2001). Two residues in ECL3 have been identified as the micro-domain that confers selectivity for Arg^8 of GnRH in the mammalian type I GnRH receptor (chapter four). These residues are Asp$^{7.32(302)}$ and Pro$^{7.33(303)}$ in the human type I GnRH receptor. In the mammalian

GnRH receptor Pro in locus 7.33 is proposed to play a complementary role to $Glu^{7.32(301)}$/$Asp^{7.32(302)}$ in selectivity for Arg^{8}-containing GnRH. It is possible that this effect is exerted indirectly by influencing the alignment of the acidic residue's side chain to interact efficiently with Arg^{8} of GnRH. This raises the possibility that ECL3 may exist in a specific conformation which regulates the $Asp^{7.32(302)}$ - Arg^{8} interaction. This question was addressed in a collaborative study by synthesizing peptides which correspond to ECL3 of the GnRH receptor (Dr David Craik, University of Queensland, Brisbane, Australia), studying them with spectroscopic techniques (Petry et al., 2002), molecular modelling of ECL3 (Dr Phil Taylor, MRC Reproductive Sciences Unit, Edinburgh, Scotland), mutagenesis studies on ECL3, and by characterising interactions between GnRH and various peptides corresponding to the sequence of ECL3 (this chapter).

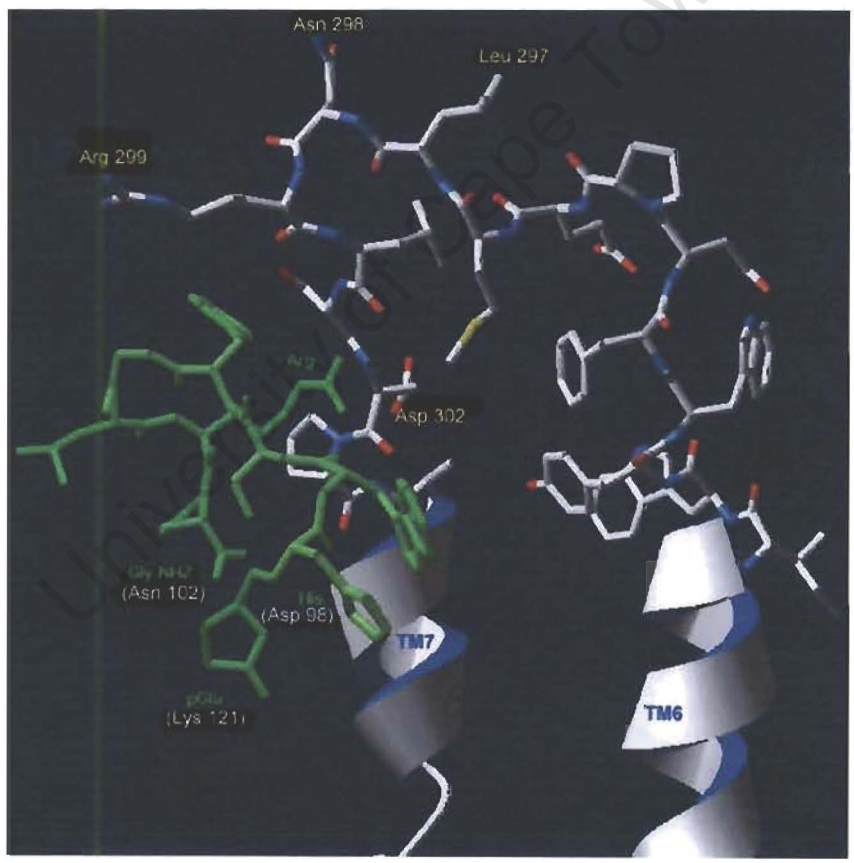

Figure 5.4 Interaction of Arg^{8}-containing GnRH with $Asp^{7.32(302)}$ of ECL3 of the human GnRH receptor (Petry et al., 2002). The interaction of GnRH with ECL3 of the human GnRH receptor was modelled by Dr Phil Taylor (MRC Reproductive Sciences Unit, Edinburgh, Scotland). The ß-hairpin turn of ECL3 was centered around residues Leu^{297}-Asn^{298}-Arg^{299} as determined with spectroscopic techniques (Petry et al., 2002) was included in this molecular model. The GnRH molecule is shown in its active ßII turn conformation and ready to dock with some of the residues ($Asp^{2.61(98)}$, $Asn^{2.65(102)}$, $Lys^{3.32(121)}$) in the agonist binding pocket as described (chapter three).

Synthetic Peptides Corresponding to the Amino Acid Sequence of ECL3 Interacts with GnRH

A synthetic RI peptide (RI-ECL3) corresponding to the amino acid sequence of ECL3 of the human GnRH receptor was initially intended for the generation of anti-GnRH receptor antibodies targeting ECL3. Surprisingly, this peptide specifically interacted with Arg^{8}-containing GnRH and distinguishes it from related peptides. This prompted us to test whether L-peptides corresponding to the sequence of ECL3 could also inhibit GnRH stimulated IP accumulation. Preliminary results indicated that the L-peptides used in this study may also interact with GnRH. Other studies have shown that peptides corresponding to the binding domain of a GPCR may reduce agonist potency (Yeagle et al., 2000). Thus, defining the structure of the binding domains of the GnRH receptor and other GPCRs may support design of novel peptides, which may directly interact with a ligand or be used as immunogens to elicit antibodies against specific structures and ligand binding domains of GPCRs.

The Conformation of ECL3 may Contribute to GnRH Selectivity

It has been proposed that ECL3 could exist as an α-helix (Sankararamakrishnan et al., 2000; Petry et al., 2002) and that this conformation could also contribute to the presentation of the side chain of the residues $Glu^{7.32(301)}$/$Asp^{7.32(302)}$ in a favourable position for a specific interaction with Arg^{8} of GnRH (Konvicka, communicated via Petry) (Petry et al., 2002). Moreover, the proposed length of ECL3 of mammalian and non-GnRH receptors is conserved, containing eight amino acid residues which is sufficient to contain two full turns of a helix (Konvicka et al., 1998). Despite this conservation there are numerous variations in the amino acid sequence of this loop, possibly imposing different conformations on ECL3 of mammalian and non-mammalian GnRH receptors, which may influence receptor ligand selectivity. Furthermore, Asn-Pro and Asp-Pro motifs are proposed to induce hinges (helix breaker) in alpha helices (Konvicka et al., 1998). In mammalian type I GnRH receptors ECL3 starts with an Asp-Pro and ends with an Asp/Glu-Pro motif (Fig. 4.2, chapter four). The second hinge found in mammalian receptors ($Ser^{7.31}$-($Glu^{7.32}$/$Asp^{7.32}$)-$Pro^{7.33}$) is replaced by a conserved (Pro-(Asp/Glu)-Tyr) sequence in non-mammalian receptors, and this may impose a different conformation on the loop, thereby influencing the orientation of the side chain of the conserved $Glu^{7.32}$/$Asp^{7.32}$. Introducing this (Pro-(Asp/Glu)-Tyr) motif of non-mammalian receptors in place of the ($Ser^{7.31}$-$Asp^{7.32}$-$Pro^{7.33}$) in ECL3 of the human GnRH receptor is proposed to distort the conformation (chapter four).

With Ala-insertional mutagenesis it is shown that the native length of the ECL3 plays a role in GnRH binding. It is possible that by inserting Ala-residues after Pro^{294} (before $Asp^{7.32(302)}$) distorts the conformation of ECL3 in such a way that the $Glu/Asp^{7.32}$ side chain is in a different orientation causing a decreased interaction with Arg^{8}-containing GnRH. However, these attempts to disrupt any theoretically predicted structure of the loop could not provide a direct answer as to whether the loop is helical or not. These experimental results, which were based on the suggestion of a helical ordered structure in ECL3, have thus prompted the investigation of the secondary structure of the loop by a set of spectroscopic techniques (Petry et al., 2002). The data obtained from these spectroscopic techniques have established that the synthetic ECL3 receptor domain has unordered conformation with some contributions from anti-parallel β-sheet and the presence of a β-hairpin turn centred on residues Leu-Asn-Arg (Fig. 5.4).

It is proposed that this conformation also exists in the complete native GnRH receptor (Petry et al., 2002). This structural data is consistent with two-dimensional NMR studies on ECL3 in rhodopsin, which exhibited a compact structure in solution with turns in the central region of the peptides (Yeagle et al., 2000). These results (Petry et al., 2002) also agree with X–ray structural information on bovine rhodopsin, where ECL3 has no α-helical structure and extracellular loops and TM domains are associated to form a compact structure with other transmembrane and extracellular domains (Palczewski et al., 2000). This conformation may also be influenced by micro-domains in ECL3 such as the conserved ($Ser^{7.31}$-($Glu^{7.32}$/$Asp^{7.32}$)-$Pro^{7.33}$) motif in mammalian GnRH receptors. In this motif Pro at locus 7.33 seems to exert a structural role in constraining a conformation of ECL3 that enhances an interaction between $Glu^{7.32(301)}$/$Asp^{7.32(302)}$ and Arg^{8} of GnRH. We propose that ECL3 of mammalian type I GnRH receptors has a specific functional conformation existing predominantly as a random coil with a central β-hairpin domain and that this conformation may be necessary to regulate the orientation of the acidic residue in ECL3 involved in selecting Arg^{8} –containing GnRH.

Chapter 6

Concluding Discussion

In this study two residues in ECL3 of the GnRH receptor were shown to play a regulatory role in a ligand binding mechanism, which determines specificity for Arg^{8}-containing GnRH. It was confirmed that $Asp^{7.32(302)}$, like $Glu^{7.32(301)}$ of the mouse GnRH receptor, confers selectivity of the human GnRH receptor for Arg^{8} of GnRH. Conformationally-constrained peptides bind the human GnRH receptor with high affinity, which is independent of the presence of $Asp^{7.32(302)}$ in the receptor or Arg^{8} in the ligand. The ability of conformationally-constrained ligands to compensate for the absence of both Arg^{8} and $Asp^{7.32(302)}$ indicates that these residues both have roles in stabilising a high affinity ligand conformation of native GnRH. In conformationally-constrained analogues the N- and C-termini of GnRH are closely apposed to allow interaction with residues $Asp^{2.61(98)}$, $Asn^{2.65(102)}$ and $Lys^{3.32(121)}$ on the GnRH receptor. It is proposed that GnRH may bind $Asp^{7.32(302)}$ in a transient step, necessary for the induction or selection of a high affinity GnRH conformation which binds with high affinity to a binding pocket located between the TM domains.

Non-mammalian vertebrate GnRH receptors do not preferentially bind Arg^{8}-containing GnRH, but also have an acidic residue in position 7.32. In mammalian type I GnRH receptors, ECL3 contains the amino acid sequence motif $Ser^{7.31(301)}$-$Asp^{7.32(302)}$/$Glu^{7.32(301)}$-$Pro^{7.33(303)}$, of which the acidic residue in position 7.32 confers selectivity for Arg^{8}-containing GnRH. Contrastingly, ECL3 of non-mammalian GnRH receptors have an amino acid motif containing Pro-(Asp/Glu)-Tyr. This study shows that the different positions of Pro, in relation to $Asp^{302(7.32)}$, affect ligand selectivity. It was shown that $Pro^{7.33(303)}$ is required for high affinity for GnRH but not for conformationally-constrained GnRH, mimicking the phenotype of mutants that have substitutions of $Asp^{7.32(302)}$/$Glu^{7.32(301)}$ in mammals. This suggests that $Pro^{7.33(303)}$ forms part of the ligand binding mechanism by which mammalian GnRH receptors distinguish GnRH from other naturally occurring forms. It is proposed that $Pro^{7.33(303)}$ may impose a constraint on the conformation of ECL3 influencing the neighboring $Asp^{7.32(302)}$ to present its side chain in an orientation that favors interaction with the Arg^{8} side chain of GnRH.

Although the amino acid sequence of ECL3 differs considerably between mammalian and non-mammalian GnRH receptors, the proposed length of ECL3 is conserved. Conversely, other extracellular loops of the GnRH receptor have different lengths conserved between the GnRH receptors. For example, ECL2 of a non-mammalian GnRH receptor was shown to confer agonist activity to an antagonist (antagonist 135-18) in the human GnRH receptor. When ECL2 of the human GnRH receptor was replaced with ECL2 of the non-mammalian GnRH receptor, it also

conferred agonist activity to antagonist 135-18. It was shown that altering the length of ECL2 has no influence on conferring agonist or antagonist binding (Ott, 2000). However, in chapter five it is shown that the length of the GnRH receptor's ECL3 plays a role in GnRH binding, suggesting that different ECLs have different requirements for interacting with ligands. It is possible that increasing the length of ECL3 distorts its conformation in such a way that the Glu/Asp side chain is in a different orientation causing a decreased interaction with Arg^{8}-containing GnRH. Although molecular modelling of the GnRH receptor TM domains has been accomplished based on rhodopsin structure, there is no knowledge of how the structure of the ECLs influences GnRH binding. The secondary structure of a model peptide, representing ECL3 of a mammalian GnRH receptor, has therefore been studied in a collaborative project by micro Raman and FT–Raman spectroscopy, by ultraviolet circular dichroism spectroscopy, and by two–dimensional NMR spectroscopy. It was proposed that ECL3 has a specific functional conformation existing predominantly as a random coil with a central ß-hairpin domain and that this conformation together with $Pro^{7.33(303)}$ may be necessary for the orientation of the acidic residue, at locus 7.32, in ECL3 involved in GnRH selectivity through an interaction with Arg^{8}. Moreover, peptides corresponding to the amino acid sequence of ECL3 were shown to specifically inhibit the GnRH stimulated signal, which supports the role of this loop in interacting with GnRH.

In GPCRs, ligands often form contact sites with residues in the ECLs which are necessary for full agonism. For example, in the angiotensin receptor, $Asp^{7.32(281)}$ in ECL3, is required to interact with Arg^{2} of angiotensin II to render it a full agonist (Feng et al., 1995). Moreover, residues in ECLs may also play a separate role in receptor activation. Replacing ECL3 in the β_2-adrenergic receptor with the corresponding loop of the α_1-adrenergic receptor resulted in a higher affinity for agonists, but not antagonists, increased agonist potency and led to constitutive activity (Zhao et al., 1998). It is possible that residues in ECL3 of the mammalian GnRH receptor, which are implicated in ligand binding, may have additional roles such as structural organisation of the receptor either in the inactive or active conformation. Substituting the entire ECL3 in the human GnRH receptor with a non-mammalian ECL3 sequence results in low binding and no measurable signalling (Sun et al., 2001). If the mammalian $Ser^{7.31(301)}$-$Asp^{7.32(302)}$/$Glu^{7.32(301)}$-$Pro^{7.33(303)}$ motif in ECL3 is substituted with the non-mammalian Pro-(Asp/Glu)-Tyr motif a decrease in GnRH affinity and coupling efficiency is observed. We also show that mutation of $Asp^{7.32(302)}$ and $Pro^{7.33(303)}$ results in decreased GnRH affinity and selectivity as well as causing partial uncoupling. Together this suggests that residues in ECL3 of the mammalian GnRH receptor have separate

roles in ligand binding and in receptor activation. In the high resolution crystal structure of rhodopsin it is shown that $Asp^{7.29(282)}$ in ECL3 is located in close opposition to Asn^{2} of the NH_2-terminus and may have a role in the structural organisation of the extra-cellular domains (Palczewski et al., 2000). It is possible that in the GnRH receptor this interaction may also exist, however, in constraining the active conformation. This interaction is most likely indirect via stabilising one or more of the TM helices involved in receptor activation (eg.TM helix seven). This thesis proposes that specific residues in ECL3 regulates a conformation which exposes an acidic residue in this loop to specifically interact with Arg^{8}-containing GnRH, and that this determines selectivity of GnRH for the mammalian GnRH receptor.

Chapter 7

Additional studies conducted during the course of this study and list of publications

7.1 A Retro-Inverso Peptide of GnRH Elicits Antibodies which Immunoneutralise Native GnRH and Inhibits Reproduction

This project was performed in parallel with this thesis. Since this work does not amalgamate with the theme of this thesis an abstract of the article (in preparation) is provided with a list of co-authors.

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Abstract

Gonadotropin releasing hormone (GnRH) regulates the reproductive hormone cascade by stimulating the release of gonadotropins from the anterior pituitary, which in turn regulates reproductive function. GnRH peptide analogs, which inhibit gonadotropin secretion, have found wide therapeutic application in infertility and sex steroid sensitive diseases, and show promise as a new generation contraceptives for men and women.

GnRH vaccines are an alternative approach to inhibit gonadotropin secretion. We show that a retro-inverso (RI) peptide can be used as a synthetic GnRH vaccine. Developing synthetic vaccines with RI peptides is attractive since it proposes stable and chemically defined products obtainable at low cost and biological risk. The RI peptides are composed of D-amino acids assembled in the reverse order (C to N terminus) in relation to the parent L-peptide. These peptides may be immunogenic and produce high titres of antibodies which bind the parent peptide with high affinity and specificity. RI-peptides are protease resistant and are therefore potentially orally active. We show that RI-GnRH peptides conjugated to ovalbumin and in the free form elicit high titres of anti-GnRH antibodies in rabbits and mice. The rabbit antibodies were affinity purified and demonstrated by ELISA to have high specificity for GnRH compared with other naturally occurring GnRH forms (chicken GnRH and GnRH II). The active antibody concentration and binding kinetics of antibody-peptide interactions were determined using biosensor technology (Biacore™). The anti-GnRH antibodies were able to significantly inhibit GnRH stimulated signal transduction in COS-1 cells which were transiently transfected with the human GnRH receptor. Furthermore, immunisation of mice with a RI-GnRH peptide significantly suppressed their fertility. The RI-GnRH immunogen was capable of raising antibodies in the

absence of Complete Freunds Adjuvant. This GnRH vaccine holds promise as a new generation of contraceptives in wild and domestic animals, and in treating sex-hormone dependent diseases in man.

7.2 List of Publications

1) Flanagan CA, **Fromme BJ**, Davidson JS and Millar RP (1998) A high affinity gonadotropin-releasing hormone (GnRH) tracer, radioiodinated at position 6, facilitates analysis of mutant GnRH receptors. *Endocrinology* **139:**4115-4119.

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

3) Sun YM, Flanagan CA, Illing N, Ott TR, Sellar R, **Fromme BJ**, Hapgood J, Sharp P, Sealfon SC and Millar RP (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. *Journal of Biological Chemistry* **276:**7754-7761.

4) **Fromme BJ**, Katz AA, Roeske RW, Millar RP and Flanagan CA (2001) Role of Aspartate7.32(302) of the Human Gonadotropin-Releasing Hormone Receptor in Stabilizing a High Affinity Ligand Conformation. *Molecular Pharmacology* **60:**1280-1287.

5) 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. *Journal of Medical Chemistry* **45:** 1026-1034.

6) **Fromme BJ**, Katz AA, Millar RP and Flanagan CA (2002) The role of Proline$^{7.33(303)}$ of the GnRH receptor in ligand selectivity. **(in preparation).**

7) **Fromme B**, Eftekhari P, van Regenmortel M, Hoebeke J, Katz A and Millar R (2002) A

retro-inverso peptide of GnRH elicits antibodies which immunoneutralise native GnRH and inhibits reproduction. **(in preparation)**.

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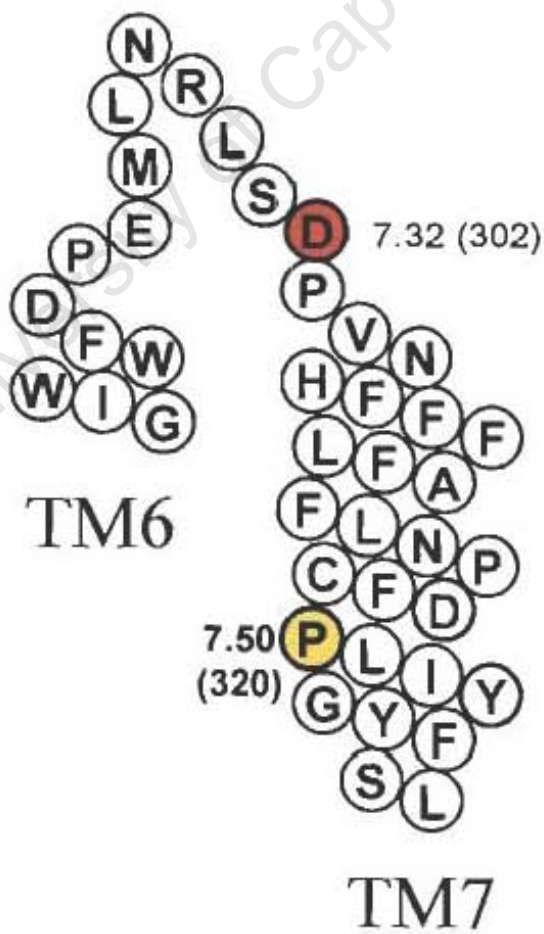
Appendix I

Consensus Residue Numbering Scheme

In this thesis, a consensus numbering scheme is used to facilitate the comparison of equivalent amino acid residues in the different rhodopsin-like GPCRs (Ballesteros and Weinstein, 1995). Amino acids were numbered relative to the most conserved residue in each transmembrane domain, which is assigned the number 50 (see illustration below and Fig. 1.1). A generic identifier consisting of the transmembrane helix number, followed by the number representing its position relative to the most conserved residue in the helix identifies individual amino acid residues. Its sequential number in the particular GPCR follows this. For example, the most conserved residue in helix seven of the GnRH receptor is Pro (in yellow on the illustration below), which is designated $Pro^{7.50}$. In the GnRH receptor $Pro^{7.50}$ is residue number 320 and is designated $Pro^{7.50(320)}$. Asp^{302} (in red on the illustration below) is 18 amino acids amino-terminal from $Pro^{7.50}$ and is therefore designated $Asp^{7.32(302)}$.



Appendix II

Nucleotide Sequence of Primers Used in this Thesis

In **bold and underlined** are the insertions/point mutations. In ***bold and italic*** are the silent restriction sites (Cla I). In the squares is the area of interest in ECL3.

a) Site-directed mutagenesis with "Kunkel" method (chapter five).

WT amino acid: Phe -Asp -Pro - Glu - Met -Leu - Asn - Arg - Leu -Ser - Asp

WT nucleotide: 5' TTT GAT CCT GAA ATG TTA AAC AGG TGG TCA GAC 3'

One Ala insertion: 5' TTT GAT CCT **GCA** GAA ATG TTA A***AT CGA T***GG TCA GAC 3'

Two Ala insertions: 5' TTT GAT CCT **GCA GCA** GAA ATG TTA A***AT CGA T***GG TCA GAC 3'

Three Ala insertions:5' TTT GAT CCT **GCA GCA GCA** GAA ATG TTA A***AT CGA T***GG TCA GAC 3'

b) Site-directed mutagenesis with PCR mutagenesis method (read anti-sense primers from 5' end).

An additional ATGC (sense primers) and ATGCT (anti-sense primers) nucleotide sequence was added (underlined) preceding the Cla I sites to allow sufficient space for the enzyme to attach.

Human wild type sequence as a reference:

Amino acid: Trp - Phe - Asp -Pro - Glu -Met -Leu - Asn - Arg - Leu - Ser - Asp - Pro - Val - Asn

Nucleotide:5'TGG TTT GAT CCT GAA ATG TTA AAC AGG TTG TCA GAC CCA GTA AAT3'

$Asp^{7.32(302)}Asn$ mutation (chapter three)

Sense: 5' A TGC A***AT CGA T***TG TCA **AAC** CCA GTA AAT CAC TTC TTC TTT C 3'

Amino acid: Asn - Arg -Leu - Ser - Asn - Pro - Val - Asn - His - Phe -Phe -Phe

Antisense 3' AT GCT ***AGC TA***A ATT GTA AAG TCC TAG TTT G 5'

Amino acid: Arg - Asn -Leu - Met -Glu- Pro - Asp - Phe

$Pro^{7.33(303)}Ala$ mutation (chapter four)

Sense: 5' A TGC A***AT CGA T***TG TCA GAC **GCA** GTA AAT CAC TTC TTC TTT C 3'

Amino acid: Asn - Arg - Leu - Ser - Asp - Ala - Val - Asn - His - Phe - Phe -Phe

Antisense 3' AT GCT ***AGC TA***A ATT GTA AAG TCC TAG TTT G 5'

Amino acid: Arg - Asn -Leu - Met -Glu- Pro - Asp - Phe

$Ser^{7.31(301)}Pro/Pro^{7.33(303)}Gly$ mutation (chapter four)

Sense: 5' A TGC A***AT CGA T***TG **CCA** GAC **GGA** GTA AAT CAC TTC TTC TTT C 3'

Amino acid: Asn - Arg - Leu - Pro - Asp - Gly - Val - Asn - His - Phe - Phe -Phe

Antisense 3' AT GCT ***AGC TA***A ATT GTA AAG TCC TAG TTT G 5'

Amino acid: Arg - Asn -Leu - Met -Glu- Pro - Asp - Phe

$Ser^{7.31(301)}Pro/Pro^{7.33(303)}Phe$ mutation (chapter four)

Sense: 5' A TGC A***AT CGA T***TG **CCA** GAC **TTT** GTA AAT CAC TTC TTC TTT C 3'

Amino acid: Asn - Arg - Leu - Pro - Asp - Phe - Val - Asn - His - Phe - Phe -Phe

Antisense 3' AT GCT ***AGC TA***A ATT GTA AAG TCC TAG TTT G 5'

Amino acid: Arg - Asn -Leu - Met -Glu- Pro - Asp - Phe

Ser$^{7.31(301)}$Pro/Pro$^{7.33(303)}$Tyr mutation (chapter four)

Sense: 5' A TGC A***AT CGA T***TG **CCA** GAC **TAC** GTA AAT CAC TTC TTC TTT C 3'

Amino acid: Asn - Arg - Leu - Pro - Asp - Tyr - Val - Asn - His - Phe - Phe -Phe

Antisense 3' AT GCT ***AGC TA***A ATT GTA AAG TCC TAG TTT G 5'

Amino acid: Arg - Asn -Leu - Met -Glu- Pro - Asp - Phe

Ser$^{7.31(301)}$Pro/Asp$^{7.32(302)}$Glu/Pro$^{7.33(303)}$Tyr mutation (chapter four)

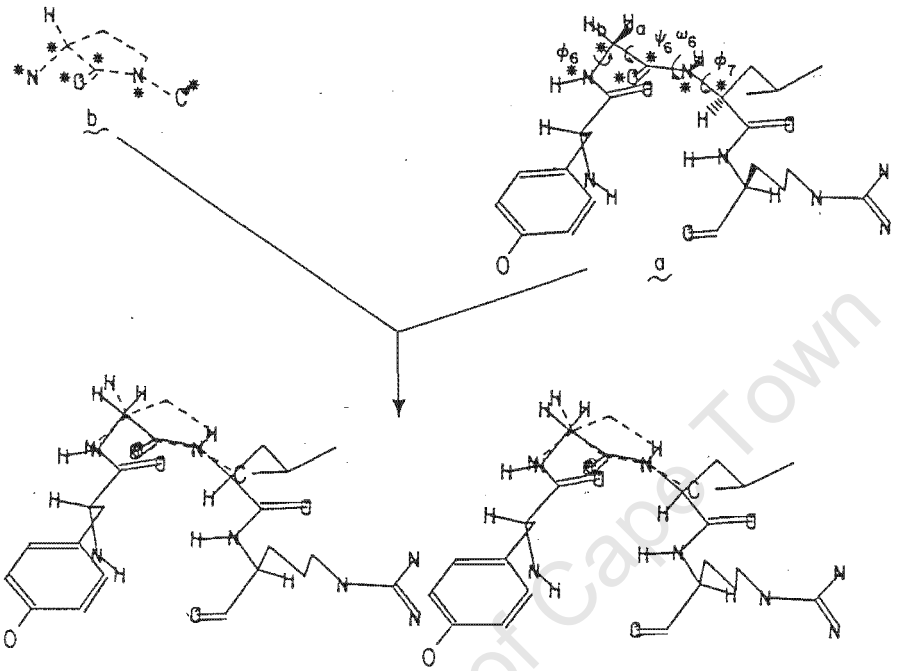
Sense: 5' A TGC A***AT CGA T***TG **CCA GAA TAC** GTA AAT CAC TTC TTC TTT C 3'

Amino acid: Asn - Arg - Leu - Pro - Glu - Tyr - Val - Asn - His - Phe - Phe -Phe

Antisense 3' AT GCT ***AGC TA***A ATT GTA AAG TCC TAG TTT G 5'

Amino acid: Arg - Asn -Leu - Met -Glu- Pro - Asp - Phe

Appendix III



Structure of a conformationally constrained GnRH analog, [6,7 γ-lactam]-GnRH. This figure was reproduced from Freidinger, 1980. In the figure (a) shows a computor superposition of the ß-turn of GnRH with (b) a γ-lactam conformational constraint.