Structural Determinants of GnRH Ligand-Receptor Interactions

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Contents

Declaration

I declare that this thesis has been composed by myself, that the work is my own, and that when a substantial contribution has been made by another individual, his or her contribution has been clearly indicated. This work has not been submitted for any other degree or professional qualification, and all publications of material from this thesis prior to submission have been recorded in Appendix IV.

20/9/02

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Abbreviations

Amino Acids:

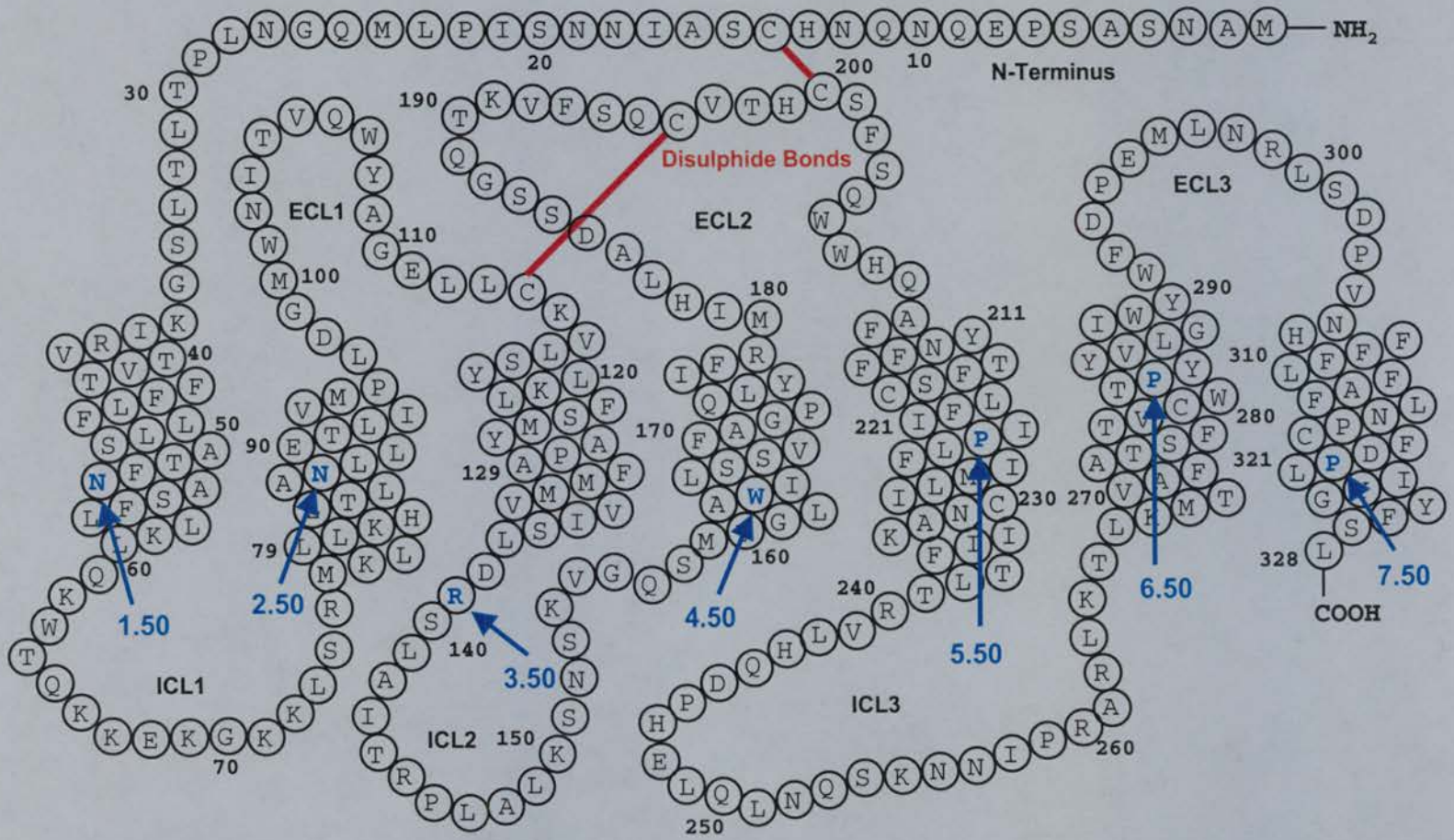
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartate
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamate
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

Other Abbreviations:

βAR	β-adrenergic receptor
$[Ca^{2+}]_i$	Intracellular calcium concentration
5-HT	5-Hydroxytryptamine (serotonin)
6,7 γ-lactam	γ-lactam ring between positions six and seven
ADP	Adenosine diphosphate
AP-1	Activating protein 1
ARF	ADP-ribosylation factor
BH	Bolton Hunter conjugated
BOSS	Bride of sevenless
bp	Base pairs
BSA	Bovine serum albumin
C5a	Complement component 5a
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CCK	Cholecystokinin
cDNA	Complementary DNA
cfGnRH	Catfish GnRH
cGnRH I	Chicken GnRH I ($[Gln^8]$-GnRH)
CIP	Calf intestinal alkaline phosphatase
CMV promoter	Human cytomegalovirus immediate-early gene
CRE	cAMP response element
C-terminus	Carboxy-terminus
D-aa^6	D-amino acid in position six
DAG	Diacylglycerol
ddNTP	2',3'-dideoxynucleoside triphosphate
DMEM	Dulbecco's modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside triphosphate
DTT	Dithiothreitol

EC_{50}	Effective concentration of an agonist that results in 50% of the maximal response
ECL	Extracellular loop
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
Egr-1	Early growth response protein 1
ET	Endothelin
fMLP	Formyl Met-Leu-Phe
FSH	Follicle-stimulating hormone
GAP	GnRH-associated peptide
GDP	Guanosine diphosphate
Gly-NH_2	Glycinamide
GnRH	Gonadotropin-releasing hormone
GnRH II	[His^5,Trp^7,Tyr^8]-GnRH (chicken GnRH II)
GnRH-R	GnRH receptor
GPCR	G-protein coupled receptor
G-protein	Guanyl nucleotide binding protein
GTP	Guanosine triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
IC_{50}	Inhibitory concentration of an unlabelled ligand that results in 50% of maximal labelled ligand binding
ICL	Intracellular loop
IP	Inositol phosphate
IP_3	Inositol 1,4,5-triphosphate
iPr	Isopropyl
IVF	*In vitro* fertilisation
kb	Kilobases
LH	Luteinising hormone
MAPK	Mitogen-activated protein kinase
mGnRH	Mammalian GnRH

NK	Neurokinin
NMR	Nuclear magnetic resonance
N-terminus	Amino-terminus
OD	Optical density
$P2Y_1$	Purinergic $2Y_1$
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pGlu	Pyroglutamate
pH	Potential of hydrogen ions ($-\log_{10}[H^+]$ in solution)
P_i	Inorganic phosphate
PIP_2	Phosphotidylinositol 4,5-bisphosphate
PKC	Protein kinase C
R, R*, R′, R″, R°	Receptor conformational states
RMS	Root mean squared
S.E.M.	Standard error of the mean
SDS	Sodium dodecyl sulphate
SF-1	Steriodogenic factor 1
sGnRH	Salmon GnRH ([Trp^7,Leu^8]-GnRH)
TAE	Tris-acetate/EDTA
TE	Tris-HCl/EDTA
TMD	Transmembrane domain
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin (thyroid-stimulating hormone)
VIP	Vasoactive intestinal peptide
WT	Wild type



Schematic representation of the human GnRH receptor. Residues indicated in blue are reference points for the amino acid numbering scheme (Ballesteros and Weinstein, 1995). Other residues are numbered relative to these positions, e.g. $Asp^{2.61(98)}$.

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Abstract

The aim of this thesis was to investigate how the spatial arrangements of amino acids in both the ligand and receptor influence gonadotropin-releasing hormone (GnRH) ligand-receptor interactions.

Mammalian GnRH (mGnRH) is believed to interact with mammalian GnRH receptors in a β-II′ turn conformation involving residues five to eight. This conformation can be constrained by substitution of a D-amino acid at position six or by a lactam ring involving residues six and seven, thereby increasing receptor binding affinity. It has been proposed that this is not the case for non-mammalian GnRH receptors. However, this thesis shows that this conformational constraint increases the binding affinity of mammalian, chicken and salmon GnRH for the chicken and catfish receptors, as well as for the mouse receptor. Therefore, the conclusion is that the β-II′ turn conformation enhances ligand binding for non-mammalian as well as mammalian GnRH receptors. An exception, however, is GnRH II where most substitutions of a D-amino acid in position six have limited effect on binding affinity. It is suggested that this ligand is pre-configured through intramolecular interactions, which accounts for its high binding affinity and total conservation of primary structure over 500 million years of evolution.

A number of residues in the receptor extracellular loops (ECLs) have been identified as interacting directly with ligands. Therefore, these regions play a crucial role in ligand binding. Chimeric receptors, consisting of the human receptor containing the three catfish receptor ECLs in all single, double and triple combinations were produced. Certain combinations of ECLs were found to have different effects on the binding of mGnRH, [D-Trp^6]-GnRH, GnRH II, [D-Lys^6]-GnRH II and Antagonist 135-18. ECL3 appears to influence the binding of mGnRH, but not [D-Trp^6]-GnRH, which correlates with previous studies. The difference in the binding affinity of these two ligands could be attributed to the ECLs, as their affinities at the triple-ECL substituted chimeric receptor were not significantly different to those at the wild type catfish receptor. Substitution of the ECLs did not simulate binding at the wild type catfish receptor for GnRH II, [D-Lys^6]-GnRH II or Antagonist 135-18. This implies that these ligands form different interactions with GnRH receptors compared with those formed by mGnRH and [D-Trp^6]-GnRH.

Potential interactions between ECLs were investigated with additional mutant receptors. ECL interactions may influence ligand binding by altering the spatial

arrangement or accessibility of ligand contact sites. Interactions between ECLs 2 and 3 appear to be particularly important: they are partly responsible for differences in the binding of GnRH II at the catfish compared with at the human receptor; a single residue in ECL2, namely $Glu^{5.35}$, is likely to be responsible for the different affinity of GnRH II at the rat receptor compared with at the mouse and human receptors as a result of repulsion between ECLs 2 and 3; and human and chicken GnRH receptors appear to have important differences in ECLs 2 and 3 such that agonist binding at human-chicken chimeric receptors is severely affected.

An appreciation of how ligand-receptor contact sites are configured is crucial to understanding how GnRH binds and activates its cognate receptor. This thesis provides insights into both ligand and receptor conformation thereby advancing our understanding of the ligand-receptor interaction.

1 Introduction

Gonadotropin-releasing hormone (GnRH) and its cognate receptor are molecules of considerable importance in the control of reproduction, and are therefore of particular scientific and clinical interest. This thesis aims to investigate how the spatial arrangements of amino acids in both the ligand and receptor influence GnRH ligand-receptor interactions.

Chapter 2 is a critical review of the current literature regarding: the role of GnRH in the control of human reproduction (including clinical uses of GnRH agonists and antagonists); the concepts and definitions of ligand-receptor pharmacology; G-protein coupled receptors in general (with particular emphasis on structure); similarities and differences between GnRHs and GnRH receptors from different species; the bioactive conformation of GnRH and substitutions that enhance its activity; the use of chimeric receptors in the study of ligand-receptor interactions; and the residues involved in interactions between GnRHs and GnRH receptors.

Chapter 3 describes the general materials and methods used in this thesis, with reagents, suppliers and oligonucleotide primers listed in Appendices I, II and III.

The experimental chapters (Chapters 4 to 6) describe studies investigating structural features of both GnRH ligands and receptors that determine their presentation for interaction with each other. Materials and methods specific to particular studies are described in each of these chapters.

Chapter 7 is a concluding discussion. There are two major findings. Firstly, conformational constraint of the β-II′ turn for residues five to eight in mammalian, chicken and salmon GnRHs, but not GnRH II, enhances binding at mammalian and non-mammalian GnRH receptors. This provides evidence that these central residues are pre-configured in a β-II′ turn in GnRH II. Secondly, the interactions of extracellular loops (ECLs) differentially affect GnRH, GnRH II, superagonist and antagonist binding to GnRH receptors. In particular, ECL3 conformation is important for the binding of mammalian GnRH, and the interaction of ECLs 2 and 3 appear to influence the binding of GnRH II, [D-Lys^6]-GnRH II and Antagonist 135-18. Indeed, a single residue in ECL2 is believed to be responsible for the different affinity of GnRH II at the rat GnRH receptor compared with at the human and mouse GnRH receptors.

2 Literature Review

2.1 GnRH and the Control of Human Reproduction

2.1.1 The Hypothalamic-Pituitary Axis

Mammalian gonadotropin-releasing hormone (mGnRH) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) that is synthesised as a prohormone in conjunction with GnRH-associated peptide (GAP). Following cleavage by processing enzymes (Wetsel et al., 1995) mGnRH is released into the hypophyseal-portal capillaries from nerve terminals located in the lateral palisade zone of the hypothalamic medial eminence (Johnson and Everitt, 1995; King et al., 1985). It passes to the anterior pituitary where it acts upon a cognate G-protein coupled receptor (GPCR) in the membrane of gonadotrope cells (Sealfon et al., 1997).

Activation of the GnRH receptor results in coupling to G_q. Initially, increases in phospholipase C activity lead to phosphotidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, forming inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Stojilkovic et al., 1994). Intracellular calcium concentration ($[Ca^{2+}]_i$) increases and conventional isoforms of protein kinase C (PKC) are activated. Subsequently, phospholipases A_2 and D are also activated, mediating diverse signalling pathways via arachidonic acid and different isoforms of PKC (Naor et al., 1998).

The complex calcium oscillations resulting from GnRH-induced calcium mobilisation cause the exocytosis of the gonadotropins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), from the gonadotrope cells into the peripheral circulation (Stojilkovic and Catt, 1992). Each elevation of $[Ca^{2+}]_i$ results in a burst of exocytosis and it is estimated that 540 vesicles are released due to a single 10 s application of GnRH (Tse et al., 1993). Calcium from IP_3-sensitive stores appears to be responsible, with little if any contribution from extracellular calcium.

GnRH secretion is pulsatile and results in pulsatile secretion of the gonadotropins (Leyendecker et al., 1990). The relationship between GnRH pulses and LH pulses appears to be direct, however, non-GnRH-associated pulses of FSH are evident (Padmanabhan et al., 1997). These may be caused by the interactions of locally produced activin, inhibin and follistatin; or by an unidentified FSH-releasing factor (Padmanabhan and McNeilly, 2001). Continuous exposure of gonadotrope

cells to GnRH results in desensitisation and consequent decreases in gonadotropin secretion (Conn and Crowley, 1994). The first GnRH pulse results in release of stored LH and FSH within minutes. There is also movement of secretory granules towards the plasma membrane, priming the cell for a much greater release of LH following exposure to a second GnRH pulse (Johnson and Everitt, 1995).

2.1.2 GnRH Regulation of Gonadotropin Synthesis

LH and FSH are heterodimers composed of a common α-subunit and a hormone-specific β-subunit. The α-subunit gene consists of 4 exons and 3 introns and encodes a mature peptide of 92 amino acids. The LH β-subunit gene consists of 3 exons and 2 introns and encodes a mature peptide of 121 amino acids. The FSH β-subunit gene consists of another 3 exons and 2 introns and encodes a mature peptide of 110 amino acids (Gharib et al., 1990).

Gene expression can be broadly divided into basal and GnRH-regulated (Brown and McNeilly, 1999). Activation of the α-subunit promoter by GnRH involves synergistic binding of a LIM homeodomain transcription factor and a factor stimulated via the mitogen-activated protein kinase (MAPK) pathway (Roberson et al., 1995). There is also evidence of α-subunit transcription stimulated by both cAMP and extracellular calcium (Saunders et al., 1998).

GnRH stimulates LH β-subunit transcription mainly via the PKC pathway, but also via MAPK and cAMP. The role of extracellular calcium is debatable (Saunders et al., 1998; Weck et al., 1998). Basal gene expression is controlled by such transcription factors as steriodogenic factor 1 (SF-1). GnRH-regulated transcription factors, such as early growth response protein 1 (Egr-1), synergise with these factors (Halvorson et al., 1999). GnRH stimulates FSH β-subunit transcription via the PKC pathway and activating protein 1 (AP-1) sites (Strahl et al., 1998).

2.1.3 Control of Gonadal Function

In the testis, LH binds to receptors on the Leydig cells. This results in steriodogenesis, particularly testosterone synthesis. LH also partially stimulates spermatogenesis, probably by elevating intratesticular testosterone levels (Amory and Bremner, 2001). FSH binds to receptors on the Sertoli cell and spermatogonial membranes. It is critical for stimulating seminiferous tubule growth during

development and initiating spermatogenesis during puberty. It is also required by adult men to produce the normal quantity of sperm (Amory and Bremner, 2001). Both LH and FSH receptors are GPCRs that signal via activation of adenylate cyclase.

In the ovary, lack of exposure to LH and FSH results in follicular atresia, and FSH concentration controls the number of early antral follicles that are recruited for development (Johnson and Everitt, 1995). LH binds to receptors on the theca interna cells, stimulating androgen synthesis via cAMP production. This action is inhibited by activin and enhanced by inhibin (Hillier, 1991). FSH binds to receptors on the granulosa cells and stimulates the aromatisation of androgens (from thecal cells) to oestrogens. Low concentrations of oestradiol have a negative feedback effect on gonadotropin secretion. This is exerted directly at the pituitary (Brown and McNeilly, 1999) and by altering GnRH secretion from the hypothalamus (Karsch and Evans, 1996). One antral follicle, the dominant follicle, now enters the pre-ovulatory phase when the combination of oestradiol and FSH stimulate the first appearance of LH receptors on the outer layer of granulosa cells (Shi and Segaloff, 1995). The rapid increase in oestradiol concentration resulting from aromatase activity switches negative feedback to positive feedback. A GnRH surge results that, combined with oestradiol action on the pituitary, triggers the LH surge (Kaynard et al., 1988) and subsequent ovulation of the pre-ovulatory follicle.

Following ovulation, the cycle enters the luteal phase. The remaining follicle becomes the corpus luteum, maintenance of which is dependent upon low levels of LH (Johnson and Everitt, 1995). The granulosa cells become granulosa-lutein cells and a proportion of thecal cells become thecal-lutein cells with an apparent increase in LH receptors. The granulosa-lutein cells produce progesterone, and oestrogens in primates, the latter from aromatisation of androgens produced by the thecal-lutein cells. The high plasma concentration of progesterone enhances the negative feedback effect of oestradiol and blocks the positive feedback effect (Johnson and Everitt, 1995; Leyendecker et al., 1990)

Testicular and ovarian function is controlled by GnRH via the control of LH and FSH. However, it is important to recognise that, in both the male and female, downstream hormones also exert influence over GnRH via multiple feedback mechanisms acting at both pituitary and hypothalamic levels (Amory and Bremner, 2001; Brown and McNeilly, 1999; Karsch and Evans, 1996). Therefore, GnRH has a pivotal role in a complex and highly integrated reproductive endocrine system.

2.1.4 Clinical Uses of GnRH Agonists and Antagonists

GnRH can be delivered in a pulsatile manner by infusion pumps, mimicking normal physiological secretion. This is an important therapy for restoration of reproductive function where there is aberrant endogenous GnRH secretion. In one study, over 90% of women with amenorrhea ovulated following administration of pulsatile GnRH (Santoro, 1990). The results were excellent in terms of single folliculogenesis, single pregnancy and predictable outcome. The treatment was very safe, even with prolonged intravenous administration. GnRH therapy is also effective for treating hypogonadal men with conditions such as hypogonadotropic hypogonadism and Kallmann's syndrome (Conn and Crowley, 1994).

Intramuscular depot delivery of a long-lasting GnRH agonist causes constant receptor occupancy and results in desensitisation. Daily intranasal or subcutaneous administration can be used, with transvaginal routes being another possibility. This effective, safe and reversible method of blocking the reproductive axis is beneficial in the treatment of conditions such as precocious puberty, endometriosis, uterine fibroids, prostate cancer, premenopausal breast cancer and polycystic ovarian disease (Barbieri, 1992; Conn and Crowley, 1994; Filicori, 1994). There are side effects to most GnRH therapies, particularly if administration is long-term (Lemay, 1989). These are expected and result from gonadal sex hormone deprivation. The most serious of these is bone demineralisation. One study found a significant 3% bone loss during the first 12 weeks of GnRH agonist treatment (Friedman et al., 1993), and this has been used as an index of patients likely to become osteoporotic after menopause. Carefully controlled replacement of selected steroid hormones can be used to alleviate most side effects, including bone demineralisation, depending on the condition and the individual (Cedars et al., 1990; Friedman et al., 1993). Precocious puberty may be an exception in that long-term GnRH therapy appears not to elicit noticeable side effects (Conn and Crowley, 1994).

Apart from prostate cancer and premenopausal breast cancer, the use of GnRH analogues in reproductive oncology is still at the experimental stage. However, there does appear to be great potential for improving treatment of breast, ovarian and endometrial cancers (Emons and Schally, 1994).

GnRH agonist and antagonist treatment is very important in assisted fertilization where blockade of the LH surge enables control of ovulation by exogenous gonadotropins (de Ziegler et al., 1987). Use of GnRH analogues for contraception has enormous potential, particularly in the male (Fraser, 1993). This

method may have important health benefits, particularly with regard to lowering the risk of reproductive cancers (Miller, 1991; Spicer et al., 1991).

Theoretically, a GnRH antagonist is a better agent for inhibiting the reproductive cascade. Competitive blockade of the GnRH receptor is more direct and lacks the problems of initial gonadotropin stimulation prior to desensitisation that occurs with agonists (Millar et al., 2000). Unfortunately, larger doses of antagonists are required with the associated increase in cost, and initial antagonists were found to have significant histamine-releasing activity (Conn and Crowley, 1994; Fraser, 1993). Clinical peptide GnRH antagonists are beginning to be used extensively in the clinic (for example, they are used for most *in vitro* fertilisation (IVF) procedures) and some are in phase III trials for prostate cancer. Recent advances in the development of non-peptide orally active GnRH antagonists indicate considerable therapeutic potential (Millar et al., 2000). The first orally active GnRH antagonist that suppresses LH and testosterone levels in healthy human volunteers (TAK-013) is currently undergoing further clinical trials (Suzuki et al., 2002).

2.1.5 Summary

Control of both male and female reproductive systems comprises a complex interplay of hormones that regulate each other via feedback mechanisms. GnRH is the key to this system with aberrant synthesis, storage, release or action resulting in partial or complete loss of reproductive function (Johnson and Everitt, 1995).

The use of GnRH analogues for clinical applications is now widespread, with sales exceeding $2 billion per annum (Millar et al., 2000). Despite this, there is room for improvement. Minor side effects and a lack of orally active compounds continue to present obstacles, however, there is great potential for future therapies. Improved understanding of how GnRH analogues interact with the GnRH receptor is the key to unlocking this potential. The binding of a ligand at its cognate receptor is dependent upon the spatial arrangement of key molecular features that form ligand-receptor interactions. The role of the rest of the molecule is to position these features (Millar et al., 2000). The nature of the molecule, be it peptide or non-peptide, is unimportant as long as the required molecular features are present and correctly positioned. Therefore, knowledge of the spatial arrangement of molecular features involved in GnRH ligand-receptor interactions is extremely important for the future design of pharmaceutical compounds, including non-peptide orally active compounds, that act at the GnRH receptor.

2.2 Ligand-Receptor Pharmacology

The success of multicellular organisms is dependent upon accurate signalling between their component cells. This accuracy must be spatial and temporal, i.e. a signal from one cell must initiate the required response in the target cell at the required time, for the required duration, and without initiating responses in non-target cells. Multicellular organisms such as humans have evolved complex nervous and endocrine systems so that accurate signalling can occur over short and long distances. The key to both these systems is the highly specific interaction between ligand and receptor.

'Ligands' are molecules that bind specifically to a 'receptor' and are classified as full agonists, partial agonists, inverse agonists or antagonists (Gether and Kobilka, 1998). 'Affinity' is a measure of the strength of interaction between the ligand and the receptor (Rang et al., 1999). All types of ligand can have high affinity for a receptor. 'Efficacy' is a measure of the cellular response initiated following binding of a ligand. Full agonists have high efficacy and generally require just a fraction of the receptor population to be occupied to initiate a maximal response. Partial agonists have intermediate efficacy, which by definition is submaximal even when the entire receptor population is occupied. Antagonists have zero efficacy and inverse agonists have negative efficacy, as discussed below (Chidiac et al., 1994).

Traditionally, agonist binding was believed to initiate a conformational change in the receptor, resulting in a switch from the resting state to the activated state (as suggested for enzyme-substrate interactions, such as between hexokinase and glucose (Bennett and Steitz, 1978)). This is known as the 'ligand induction' model and is likely to be the mechanism by which a photon activates rhodopsin (Gether and Kobilka, 1998) (See Section 2.3.3). However, for most GPCRs, the ternary complex model is more applicable. Originally, this involved the hormone (H), receptor (R) and G-protein (G) in the formation of two ligand-receptor complexes: low affinity (HR) and high affinity (HRG) (De Lean et al., 1980). More recently, it has been extended to incorporate the concepts of constitutive activation and inverse agonism (Chidiac et al., 1994; Samama et al., 1993). The extended ternary complex model includes an R* form of the receptor and assumes that only this form can bind the G-protein. This means that HR*G is the only possible active ternary complex, with the equilibrium between R (inactive state) and R* (active state) dependent upon both hormone and G-protein (Figure 2.1).

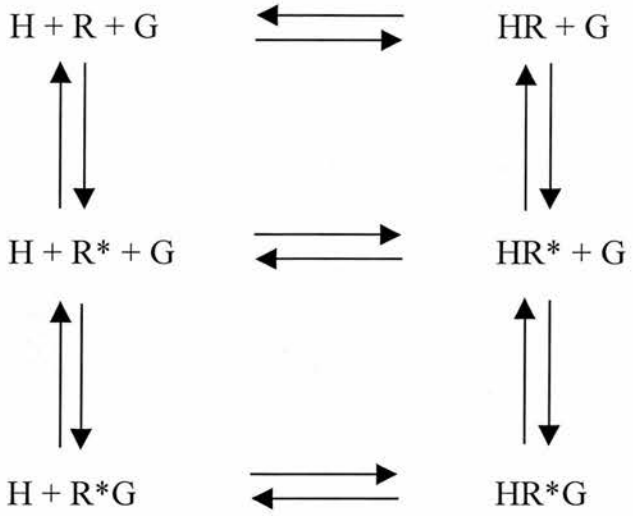

Figure 2.1. The extended ternary complex model (Samama et al., 1993).

H, hormone; R, inactive receptor conformation; R, active receptor conformation; G, G-protein; HR, inactive hormone-receptor complex requiring isomerisation to HR* before binding G-protein; R*G, hormone-independent receptor-G-protein complex (constitutively active); HR*G, active ternary complex.*

When the receptor is not bound to an agonist, the equilibrium is much closer to R than R*. Constitutively active receptors result from mutation (Lefkowitz et al., 1993). They are able to initiate a response without ligand binding, therefore, their equilibrium when not bound to a ligand is closer to R* than normal receptors (Samama et al., 1993). Agonists stabilise the R* conformation thereby shifting the equilibrium towards the active state. Inverse agonists stabilise the R conformation, thereby shifting the equilibrium towards the inactive state. Such ligands are able to reduce the response produced by constitutively active receptors, hence the concept of negative efficacy (Chidiac et al., 1994) (Figure 2.2). A true antagonist does not affect the equilibrium, binding to R and R* with equal affinity. In reality, there is probably no such entity; most antagonists are likely to be very weak partial agonists or inverse agonists, the effect of which is below the threshold of detection. It is highly unlikely that a molecule will bind to two different receptor conformations with exactly the same affinity. In practice, unless there is detectable constitutive activity, inverse agonists are indistinguishable from antagonists and are considered as such (Chidiac et al., 1994) (Figure 2.2).

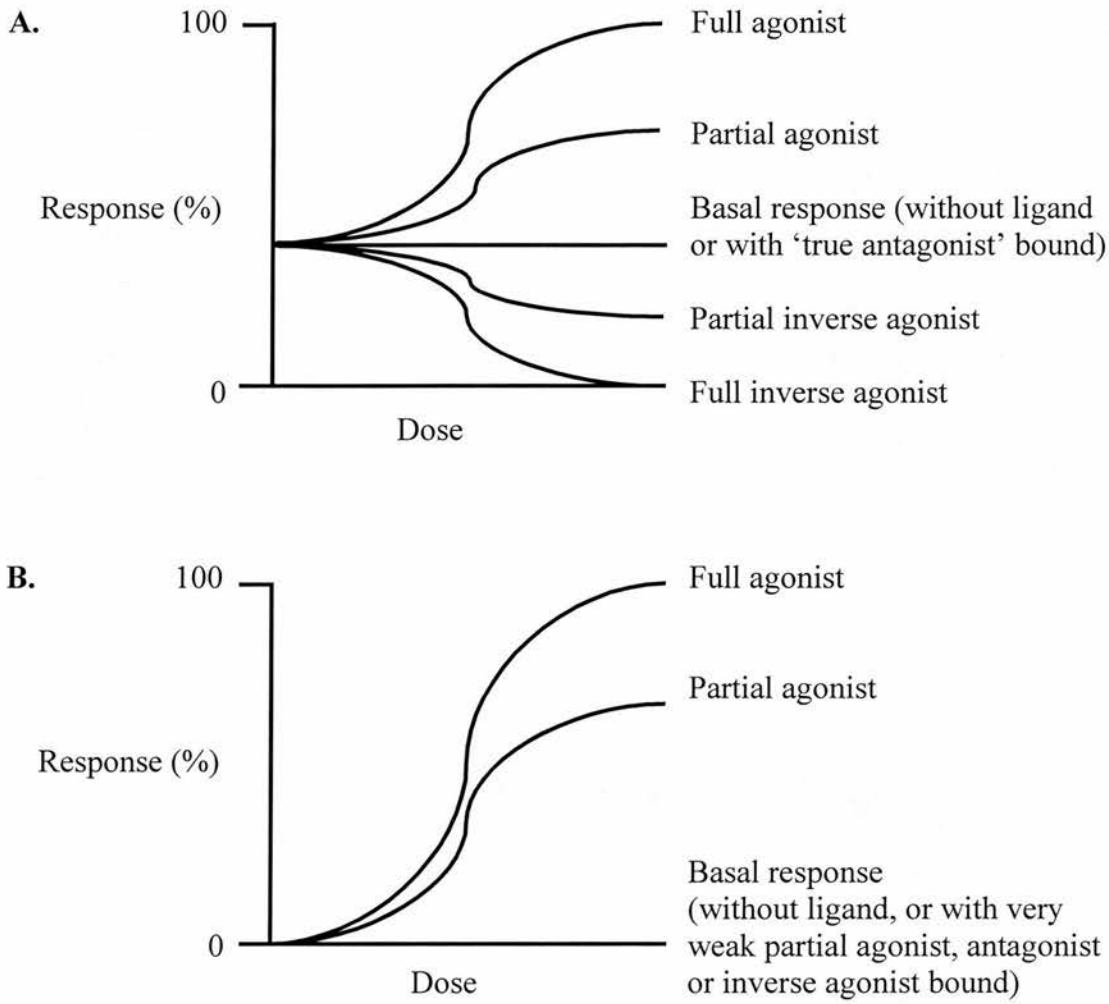

Figure 2.2. Theoretical dose-response curves for a constitutively active receptor **(A)** and a normal receptor **(B)**. Note that a 'true antagonist' does not increase or decrease the basal response initiated by a constitutively active receptor. Inverse agonists, partial inverse agonists, antagonists and very weak partial agonists cannot be distinguished at a normal receptor.

Ligand binding models can achieve an even greater level of complexity if intermediate transitional states between R and R* (R′, R″ etc.) are envisaged (Gether and Kobilka, 1998). These occur sequentially following each ligand-receptor interaction, several of which contribute to the binding energy required to stabilise R*. The formation of such intermediates is supported by the surprisingly slow kinetics of agonist-induced conformational changes observed with the β_2 adrenergic receptor (Gether et al., 1995). This model would also encompass sequential two-site

binding phenomena, evidence for which has been provided for the GnRH receptor (Fromme et al., 2001), as well as the TRH receptor (Colson et al., 1998; Perlman et al., 1997a) and muscarinic acetylcholine receptors (Jakubik et al., 2000). The model can be extended further by suggesting that the unbound receptor (R) has a different conformation to that stabilised by inverse agonists (R^o). This is to rationalise observations that inverse agonists as well as agonists protect the β_2 adrenergic receptor from thermal denaturation and proteolysis (Gether and Kobilka, 1998).

Competitive antagonists bind to the ligand recognition site without causing receptor activation. Therefore, they are able to interfere with the binding of agonists as they are 'competing' for the same site. Such antagonists can bind reversibly or irreversibly. Reversible competitive antagonism can be overcome by an excess of agonist, whereas irreversible competitive antagonism cannot. A partial agonist can act as an 'antagonist' if competing with a full agonist; although a submaximal response will be initiated, this will be lower than if the full agonist was present by itself. According to the model above, partial agonists may stabilise an intermediate state (such as R′ or R″) or stabilise a different conformation with lower affinity for the G-protein (Gether and Kobilka, 1998).

A non-competitive antagonist acts at a site other than the agonist recognition site. It does not compete with the agonist; therefore excess agonist cannot overcome its action. An example is when a molecule blocks the channel of a ligand-gated ion channel. Increased agonist concentration actually leads to increased blockade as more channels open (Rang et al., 1999). The above types of receptor antagonism should not be confused with physiological antagonism, where the effect of one ligand opposes that of another ligand acting on separate cells. The overall physiological result is antagonism, however, the ligands do not interact with the same receptors.

Occasionally the term 'receptor' is used to describe any target molecule, including enzymes and carrier molecules. In this thesis, a receptor is a specific molecule, the sole function of which is to bind a specific agonist and initiate a change in the target cell. Receptors can be divided into six superfamilies: G-protein coupled receptors (GPCRs), direct ligand-gated ion channels, tyrosine kinase-linked receptors, serine/threonine kinase-linked receptors, guanylate cyclase-linked receptors and intracellular steroid/thyroid receptors. The subject of this thesis, the GnRH receptor, is a member of the GPCR superfamily. The remaining superfamilies are beyond the scope of review in this thesis (see reviews by Evans, 1988; Goy, 1991; Schlessinger and Ullrich, 1992; Karlin, 1993; Miyazono et al., 2001).

2.3 G-Protein-Coupled Receptors (GPCRs)

A GPCR consists of a single polypeptide, which spans the plasma membrane with seven α-helices connected by three extracellular and three intracellular loops (Baldwin, 1994). The amino-terminus is extracellular and often contains glycosolation sites (Ulloa-Aguirre et al., 1999). The carboxyl-terminus is intracellular and, along with the intracellular loops, usually contains phosphorylation sites important for intracellular signalling, uncoupling, desensitisation and internalisation (Ulloa-Aguirre et al., 1999; Wess, 1998). Agonists stabilise the active receptor conformation by binding between the transmembrane domains and/or to sites in the extracellular loops/amino-terminus, depending on the particular ligand and receptor (Gershengorn and Osman, 2001; Gether, 2000; Ji et al., 1998).

2.3.1 G-Proteins

Guanyl nucleotide binding proteins (G-proteins) relay the signal initiated by agonist binding by associating with the intracellular C-terminal region and/or intracellular loops of the receptor (Spiegel et al., 1992; Ulloa-Aguirre et al., 1999; Wess, 1998). They consist of three subunits (α, β, and γ) of which there are several subtypes (Wess, 1998). In the inactive form, the α-subunit binds guanosine diphosphate (GDP) (Conklin and Bourne, 1993). In the active form, the GDP is replaced by guanosine triphosphate (GTP). An activated GPCR (R*) catalyses the exchange of GDP for GTP resulting in dissociation of the G-protein from the receptor (Figure 2.3).

The α-subunit is released and activates particular signalling pathways depending on its subtype (Table 2.1). The β and γ-subunits remain bound together and anchored to the plasma membrane. This complex is believed to have roles in signalling although these are largely undefined. There is evidence for βγ-subunits influencing adenylyl cyclase types I, II and IV, cardiac atrial potassium channels, phospholipase Cβ, phospholipase A_2 and the yeast mating factor pathway (Spiegel et al., 1992; Ulloa-Aguirre et al., 1999). βγ-subunits increase the affinity of GPCRs for α-subunits and are therefore likely to have a major role in regulating the selectivity of receptor/G-protein interactions (Wess, 1998).

There are at least 20 different α-subunits, 6 different β-subunits and 12 different γ-subunits, enabling a large number of combinations (Wess, 1998). G-

proteins are named after their α-subunits and can be classified into four major families based on sequence homology: G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (Ulloa-Aguirre et al., 1999; Wess, 1998). Subtypes can also be categorised according to their sensitivity to toxins (Table 2.1).

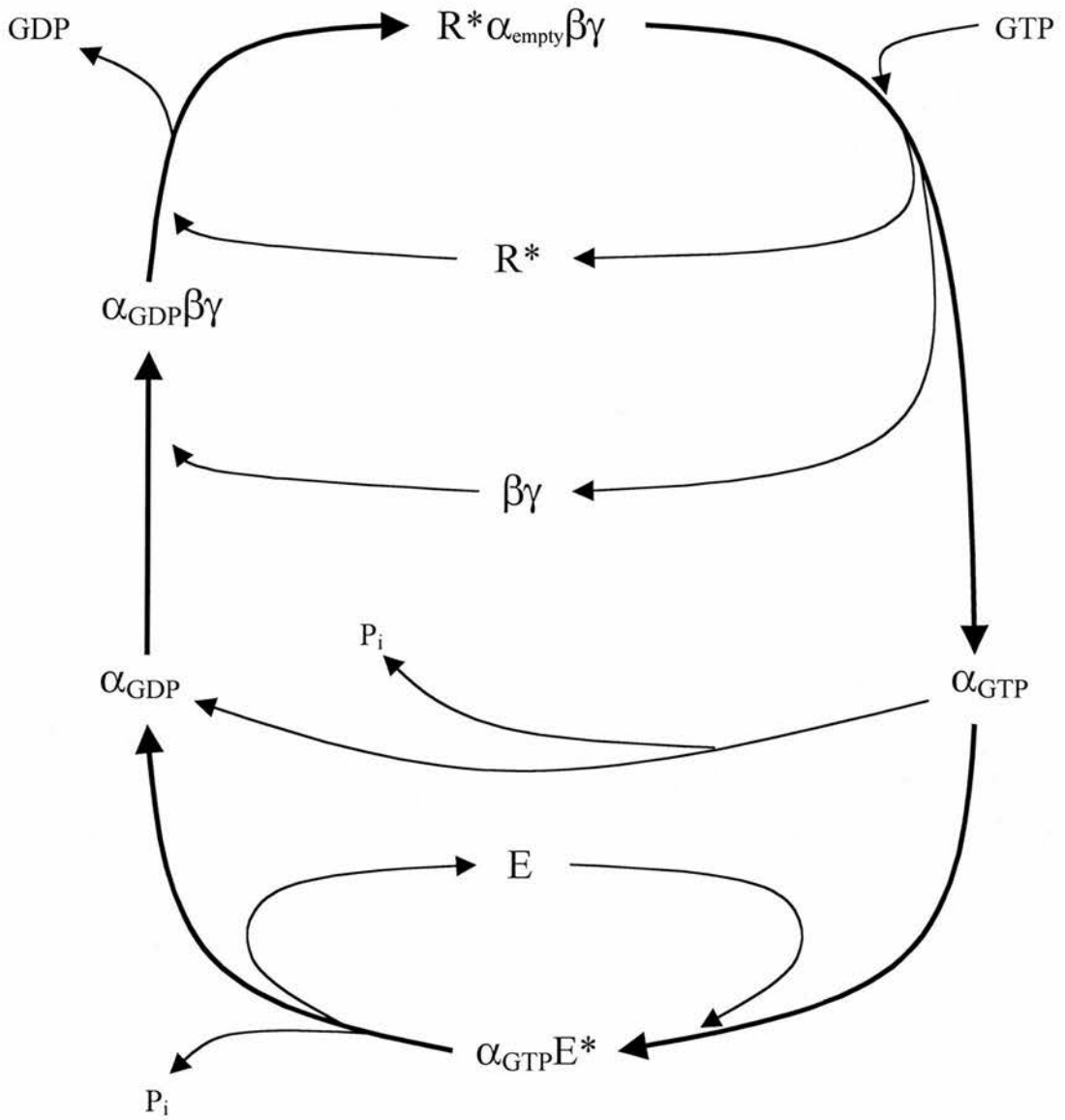

Figure 2.3. The G-protein cycle (Conklin and Bourne, 1993).
R*, activated receptor; α_{empty}, α-subunit with empty guanine nucleotide-binding site; E, effector; E*, activated effector; P_i, inorganic phosphate. α_{empty}, α_{GDP} and α_{GTP} have different conformations.

Table 2.1. Classification of G-Proteins

Family:	Gα-Subunit:	Effector:	Pertussis-Toxin Sensitive:	Cholera-Toxin Sensitive:
G_s	$G\alpha_{s1-4}$	↑ Adenylyl Cyclase, Ca^{2+} Channel		✓
	$G\alpha_{olf}$	↑ Adenylyl Cyclase		✓
$G_{i/o}$	$G\alpha_{i1-3}$	↓ Adenylyl Cyclase, ↑ K^+ Channel	✓	
	$G\alpha_{o1 \text{ and } 2}$	↓ Ca^{2+} Channel	✓	
	$G\alpha_{t1 \text{ and } 2}$	↑ cGMP-phosphodiesterase	✓	✓
	$G\alpha_{gust}$	↑ cGMP-phosphodiesterase	✓	
	$G\alpha_z$	↓ Adenylyl Cyclase, ↓ K^+ Channel		
$G_{q/11}$	$G\alpha_q$	↑ Phospholipase Cβ		
	$G\alpha_{11}$	↑ Phospholipase Cβ		
	$G\alpha_{14-16}$	↑ Phospholipase Cβ		
$G_{12/13}$	$G\alpha_{12 \text{ and } 13}$	Na^+, H^+ antiporter?		

Coupling to G-proteins has the capacity for considerable signal amplification and enables large changes to be triggered in the target cell in response to a very small external stimulus. Rhodopsin itself provides a good example of this sensitivity, as a single photon is sufficient to excite a rod cell (Khorana, 1992). Such amplification necessitates close control, which is provided by the intrinsic GTPase activity of the α-subunit (Conklin and Bourne, 1993). GTP is rapidly hydrolysed to GDP. Consequently, the α-subunit re-associates with the βγ complex, which in turn re-associates with the GPCR. This limits the number of signalling molecules each α-subunit can activate and enables the G-protein to return to its inactive state. Therefore, G-proteins are recycled for subsequent activation by the GPCR (Figure 2.3). Cholera toxin inhibits GTPase activity, thereby causing persistent effector activation (Spiegel et al., 1992).

More detailed consideration of G-proteins and intracellular signalling mechanisms is beyond the scope of this thesis (see reviews by Spiegel et al., 1992; Conklin and Bourne, 1993; Wess, 1998; Hur and Kim, 2002).

2.3.2 The Diversity of GPCRs

It is currently estimated that about one thousand genes (approximately 1-3% of a mammalian genome) code for GPCRs (Wess, 1998), each of which are structurally and functionally related but nonetheless distinct. Indeed, the ligands acting on these receptors are extremely diverse in size and structure, ranging from single photons, calcium ions, through small catecholamines, to large polypeptide hormones such as LH and FSH (see Section 2.1.2). With such ligand diversity, it is understandable that the GPCR superfamily is also structurally diverse. Two examples of classification schemes based on sequence homology, ligand structure and G-protein coupling are shown in tables 2.2 and 2.3.

Table 2.2. The five family classification scheme (Kolakowski, 1994):

Family:	Prototypical Receptor:
A	β_2 adrenergic receptor
B	Calcitonin receptor
C	Metabotropic glutamate receptors
D	Fungal pheromone receptors
E	*D. discoideum* cAMP receptors

Table 2.3. Classification scheme consisting of four major groups (Wess, 1998):

Class:	Receptor Type:
I	Rhodopsin-type receptor family (analogous to Family A)
II	Secretin/glucagon receptor family (analogous to Family B)
III	Metabotropic glutamate/calcium sensor receptor family, including basal vomeronasal pheromone (type 2) receptors (analogous to Family C)
IV	Subfamily of apical vomeronasal pheromone (type 1) receptors (not included in the five family classification scheme)

The major families are A, B and C (as these are analogous to Classes I, II and III, the classification scheme proposed by Kolakowski will be used henceforth). More than 90% of GPCRs belong to Family A, including the GnRH receptor (Gershengorn and Osman, 2001). It can be subdivided into six major subgroups, as shown in Table 2.4 (Gether, 2000; Kolakowski, 1994). The family can also be divided according to type of 'ligand', which include sensory stimuli, glycoprotein hormones, peptides, and biogenic amines (Wess, 1998). Family A receptors have a relatively short N-terminus and about 20 highly conserved residues in the transmembrane helices. Only the arginine residue in the Asp-Arg-Tyr (DRY) motif in helix 3 is completely conserved (Gether, 2000; Wess, 1998). Family B receptors have a longer N-terminus containing 6 conserved cysteine residues. An example is the VIP_1 receptor with an N-terminus of 144 amino acids (Laburthe et al., 1996). A few residues in the transmembrane helices are conserved, however, these differ from those conserved in Family A receptors. The DRY motif critical for the function of Family A receptors is absent (Laburthe et al., 1996). Family C receptors possess a much longer N-terminus than Family A or B receptors (500-600 amino acids including 19 conserved cysteine residues) and this region is largely responsible for ligand binding (Conn and Pin, 1997).

Table 2.4. Classification of Family A receptors into six subgroups (Gether, 2000; Kolakowski, 1994)

Subgroup:	Members:
1	Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)
2	CCK, endothelin, tachykinin, neuropeptide Y, TRH, neurotensin, bombesin and growth hormone secretagogues receptors, as well as vertebrate opsins
3	Invertebrate opsins and bradykinin receptors
4	Adenosine, cannabinoid, melanocortin and olfactory receptors
5	Chemokine, fMLP, C5a, GnRH, eicosenoid, leukotriene, FSH, LH, TSH, galanin, nucleotide, opioid, oxytocin, vasopressin, somatostatin and protease-activated receptors
6	Melatonin receptors

There are other groups of proteins believed to consist of seven transmembrane helical domains not classified in the schemes above. These include an *Arabidopsis thaliana* receptor, Bride of sevenless (BOSS) and the Frizzled family (Josefsson, 1999). Much debate surrounds the classification of these groups, particularly due to a lack of experimentally demonstrated receptor function or G-protein coupling. The Frizzled family, for example, is increasingly believed to be part of the GPCR superfamily (Barnes et al., 1998), following demonstration of rat Frizzled-2 coupling to G-protein-linked inositol phosphate (IP) turnover (Slusarski et al., 1997).

As the number and diversity of GPCRs increases, databases are becoming increasingly important for integrating the huge body of data now available. A good example is the GPCRDB at http://www.gpcr.org, which also contains links to other GPCR databases (Horn et al., 1998).

2.3.3 The Structure of GPCRs

GPCRs exist in the hydrophobic environment of the lipid membrane and, except for rhodopsin, have low abundance. They are hard to isolate in their native form because of: their instability in environments lacking phospholipids; their tendency to aggregate and precipitate; and the heterogeneity of preparations. Therefore, crystals suitable for X-ray diffraction analysis are extremely difficult to produce (Findlay and Eliopoulos, 1990; Teller et al., 2001). A variety of methods have been used to gain insights into GPCR structure, largely involving rhodopsin and bacteriorhodopsin.

Rhodopsin is a visual photoreceptor present in rod cells. It consists of a 40 kD protein, opsin, covalently bound to 11-*cis*-retinal. Absorption of a single photon results in isomerisation of 11-*cis*-retinal to all-*trans*-retinal. This alters the conformation of the associated opsin causing activation of G-proteins (Khorana, 1992; Palczewski et al., 2000). Indeed, 11-*cis*-retinal is considered to act like an inverse agonist as it stabilises the inactive receptor conformation (Gether and Kobilka, 1998). Bacteriorhodopsin is a proton pump that utilises the energy of photons to provide an electrochemical potential (Pebay-Peyroula et al., 1997). It is an integral membrane protein found in *Halobacterium salinarium* (formerly *Halobacterium halobium*), however it is not a GPCR. The relevance of bacteriorhodopsin as a model for GPCRs is the topic of much debate, as discussed later.

The first map of bacteriorhodopsin was ascertained at 7 Å using electron microscopy and diffraction patterns (Henderson and Unwin, 1975). This showed the seven closely packed transmembrane α-helices. Three years later, proteolysis of bovine rhodopsin was found not to alter its circular dichroism spectrum, providing evidence that retinal bound covalently in the hydrophobic core of the opsin protein and the tertiary structure of this core was not influenced by the cleavage of extracellular regions (Albert and Litman, 1978). Furthermore, this work predicted that about 60% of the molecule was α-helical and that little if any of these helices were extracellular. This led to the suggestion that rhodopsin contained either seven or nine transmembrane α-helices and identified the possible structural similarity to bacteriorhodopsin.

The hydropathy profile of bovine rhodopsin implicated the existence of seven transmembrane domains (TMDs) (Nathans and Hogness, 1983). A similar hydropathy profile was observed with the mammalian β-adrenergic receptor (βAR), which also exhibited sequence and functional homology with rhodopsin (Dixon et al., 1986). As more GPCRs were cloned impressive sequence homology was discovered, particularly between the putative transmembrane domains. The first GPCR model was produced using the multiple sequence alignment of 23 receptors (Donnelly et al., 1989). The periodicity of conserved/non-conserved residues within the putative TMDs was analysed, assuming that conserved residues tend to occur on the internal facing side and the external side is more variable. These helices were termed 'amphipathic'. As multiple sequence alignments became important for identifying putative GPCRs and gaining structural insights, database pattern-scanning methods were used in an attempt to maximise the accuracy of such alignments (Attwood et al., 1991).

A map of bacteriorhodopsin was produced at 3.5 Å resolution, using electron cryo-microscopy (Henderson et al., 1990). This provided detailed structural information on the heptahelical membrane protein and the potential for new insights into GPCR structure. However, despite both having seven transmembrane helices, rhodopsin exhibited little sequence homology with bacteriorhodopsin, calling into question the validity of bacteriorhodopsin as a template for GPCRs (Pardo et al., 1992). It was even suggested that exon shuffling might have resulted in a change in helical sequential order, as greater homology was found if the order of helices was ignored (Pardo et al., 1992).

Rhodopsin remained the GPCR for which most structural data was available (Khorana, 1992) and the next breakthrough came when two-dimensional crystals of bovine rhodopsin were obtained, allowing a projection density map to be produced at

9 Å resolution (Schertler et al., 1993). Density peaks representing the seven transmembrane helices were observed, mostly oriented perpendicular to the plane of the membrane (Figure 2.4). The probable arrangement of GPCR TMDs was subsequently predicted based on this projection map (Baldwin, 1993). 204 GPCR sequences of 105 different types were aligned and analysed, enabling a variety of insights to be made. As each type of inter-helical loop was often short in length, it was deduced that helices proximal in the one-dimensional sequence were also proximal in the three-dimensional structure. This agreed with the model proposed by Donnelly and co-workers. Baldwin also exploited the concept of conserved/non-conserved residue periodicity as described previously (Donnelly et al., 1989). Additionally Baldwin identified positions where: variability was restricted; polar residues could be accommodated; closely related sequences were different; and ligands were believed to bind. The environment of the helices was found to be different, with TMDs 1, 4 and 5 most exposed to the lipid membrane and TMD 3 least exposed. The combination of all these data enabled the orientation of the helices to be predicted and their tentative assignment to the density peaks observed for rhodopsin (Baldwin, 1993). TMDs 1, 2 and 3 were believed to be sloping, thereby producing the elongated curved region observed in the projection map (Schertler et al., 1993). The least exposed helix, TMD 3, was assigned to the end of this region surrounded by other helices. TMDs 4, 5, 6 and 7, believed to be nearly perpendicular to the membrane, were assigned to the four distinct density peaks (Figure 2.4).

The predicted arrangement of TMDs in rhodopsin differed from that observed in bacteriorhodopsin (Baldwin, 1993). This was attributed to the helices sloping differently relative to the plane of the membrane, a conclusion supported by the different positions of conserved proline residues. An alternative explanation was that differences in helical packing or in crystallisation conditions had resulted in the map of rhodopsin (Schertler et al., 1993) being rotated approximately 15° relative to the map of bacteriorhodopsin (Henderson et al., 1990), thus showing very similar structures from a different angle (Hoflack et al., 1994). However, the lack of evidence for an evolutionary link between these proteins continued to undermine the use of bacteriorhodopsin as a template for GPCRs (Soppa, 1994).

Higher resolution projection maps of frog rhodopsin in two crystal forms were produced, at 7 and 6 Å (Schertler and Hargrave, 1995). These showed virtually identical structures to the 9 Å map, although helix 5 appeared to slope more than previously believed. TMDs 1, 2 and 3 were still not resolved, being represented by an elongated region of electron density as before. Evidence for a similar

arrangement of TMDs in adrenergic receptors compared with bacteriorhodopsin was presented (Mizobe et al., 1996), however, this study did not address the increasingly apparent differences in helical tilting and bending between bacteriorhodopsin and GPCRs. These differences became clearer following X-ray crystallography of bacteriorhodopsin at 2.5 Å resolution (Pebay-Peyroula et al., 1997) and electron cryo-microscopy of frog rhodopsin (Unger et al., 1997). The latter study produced a map with an effective resolution of 7.5 Å in the plane of the membrane and 16.5 Å normal to it, resolving all seven TMDs for the first time. Tilt angles for the helices were estimated, ignoring possible curvature or kinks (Table 2.5). TMD 4 was believed to be the shortest helix, being almost perpendicular to the plane of the membrane. As suggested previously, TMDs 1, 2, 3 and 5 were believed to be sloping significantly, particularly TMD 3. The helices were shown to be closely packed towards the intracellular side and more open towards the extracellular side, with TMDs 3, 4, 5, 6 and 7 forming a cavity for retinal binding.

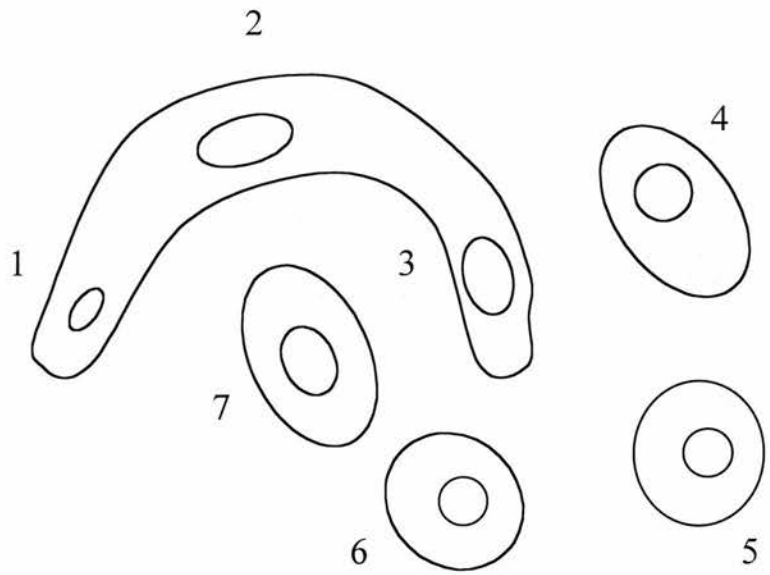

Figure 2.4. A simplified representation of the projection density map of rhodopsin at 9 Å resolution, showing the assignment of helices 1-7 to the density peaks (Baldwin, 1993; Schertler et al., 1993).

The major breakthrough came with the three-dimensional crystal structure of bovine rhodopsin at 2.8 Å resolution (Palczewski et al., 2000). This modelled 97.1% of the opsin molecule in the ground state, enabling the detailed structure of a GPCR to be visualised for the first time. Residues 236-240 in intracellular loop 2 (ICL2) and 331-333 in the C-terminus could not be mapped. Residues 334-338 had electron density, but of poor quality. These regions were believed to be particularly flexible and lack a single conformation. The remaining residues were successfully mapped, enabling their positions and interactions to be ascertained. A summary of key structural information is shown in Table 2.5. The structure, including overall helical tilt angles, was consistent with the low resolution map of frog rhodopsin (Unger et al., 1997). Following the superposition of rhodopsin on bacteriorhodopsin, TMDs 1, 2 and 3 superimposed reasonably well, however, TMDs 4 and 5 did not superimpose. Different twists and kinks, as well as slightly longer helices in rhodopsin, added to the disparity (Teller et al., 2001). Although studies of bacteriorhodopsin provided a useful framework for early GPCR models, it is clear that such a role in the future is inappropriate.

Table 2.5. Structural data for the transmembrane helices of rhodopsin.

Data produced from high resolution structure of bovine rhodopsin (Teller et al., 2001). Overall tilt angles estimated from low resolution structure of frog rhodopsin included in brackets for comparison (Unger et al., 1997).

TMD:	Residues:	Length: Å	Overall Tilt Angle[a]:	Bends within helix:	
				Angles:	Positions:
1	34-64	45	25° (28.4°)	12°	Pro^{53}
2	71-100	40	25° (27.2°)	30°	Gly^{89}-Gly^{90}
3	106-139	48	33° (29.6°)	12°, 11°	Gly^{120}-Gly^{121}, Ser^{127}
4	150-172	33	1° (3.8°)	30°	Pro^{170}-Pro^{171}
5	200-225	36	26° (22.7°)	25°, 15°	Phe^{203}, His^{211}
6	244-276	47	5° (7.4°)	36°	Pro^{267}
7	286-309	37	9° (13.4°)	24°, 21°	Pro^{291}, Pro^{303}

[a], 0° defined as perpendicular to the plane of the membrane.

Rhodopsin has appreciable sequence homology with other GPCRs, particularly in the TMDs (Josefsson, 1999; Probst et al., 1992). Therefore, models based on rhodopsin are likely to provide a good approximation of the membrane-spanning region of other GPCRs, particularly those with highest homology such as other members of Family A (Ballesteros et al., 2001). However, rhodopsin is fundamentally different from most GPCRs. The binding of a ligand does not activate the opsin protein as its 'ligand', 11-*cis*-retinal, is constantly bound (Gether and Kobilka, 1998; Palczewski et al., 2000). Most GPCRs are activated by diffusible ligands that enter a binding pocket between the TMDs and/or interact with sites in the extracellular domains (Baldwin, 1994; Gether, 2000; Strader et al., 1995). Little if any information regarding the spatial arrangements of extracellular loops (ECLs) or the N-terminus of other GPCRs can be derived from the crystal structure of rhodopsin, particularly as residues 177-190 in ECL2 form a twisted β-turn that 'plugs' the extracellular end of the retinal binding pocket. This structure would prevent a diffusible ligand entering the binding pocket unless it moved considerably in the transition from ground to high affinity state (Teller et al., 2001). The huge size range of extracellular domains in different GPCRs demonstrates the lack of structural as well as sequence homology in these regions (Gether, 2000).

2.3.4 Conserved Motifs

The vast majority of GPCRs appear to possess a disulphide bond connecting highly conserved cysteine residues in ECLs 1 and 2 (Gether, 2000; Probst et al., 1992) (Figure 2.5). This bond has been shown to be critical for stabilising correct receptor conformation in the GnRH receptor (Davidson et al., 1997), as it has with several other GPCRs (Table 2.6). It was predicted that all GPCRs would possess this motif (Strader et al., 1994). However, evidence from the VIP_1 receptor, a member of Family B, indicates otherwise. This receptor contains the highly conserved cysteine residues in ECLs 1 and 2, and mutation of the ECL2 cysteine abolishes ligand binding (Gaudin et al., 1995). However, mutation of either or both cysteines in ECL1 does not have this effect. Instead, it is postulated that the cysteine in ECL2 interacts with one of the cysteines in the N-terminus (Laburthe et al., 1996). This important structural difference may actually be due to a common function. In the GnRH receptor, a number of agonist contact sites have been identified in ECL1 and the adjoining TMDs (see Section 2.7.2). A critical residue for biogenic amine

binding is $Asp^{3.32}$ (Figure 2.5), again in one of the TMDs adjoining ECL1 (Strader et al., 1994). As with the GnRH receptor, these receptors require the disulphide bond linking ECLs 1 and 2 (Dohlman et al., 1990; Savarese et al., 1992). In contrast, the VIP_1 receptor ligand binding sites are believed to be primarily located in the N-terminus (Laburthe et al., 1996). Therefore, the postulated disulphide bond in this receptor would again link the key ligand binding region with ECL2.

Receptors show much greater similarity within families (see Section 2.3.2). The GnRH receptor belongs to Family A, whose members share a number of motifs (Figure 2.5). Most possess an intracellular C-terminal tail, usually containing at least one palmitoylated cysteine residue (O'Dowd et al., 1989) (Figure 2.5). Mammalian type I GnRH receptors are exceptional in not possessing such a tail (Sealfon et al., 1997).

Proline residues in TMDs 4, 5, 6 and 7 are highly conserved: the kinks they introduce are likely to be important for configuring the ligand binding pocket and structural changes involved in activation (Probst et al., 1992). The conformations of TMDs 4, 6 and 7 in rhodopsin resulting from proline kinks (Table 2.5) are likely to be similar in other Family A GPCRs (Ballesteros et al., 2001). TMD 5 in rhodopsin appears to possess an unusual local unwinding around $His^{211(5.46)}$ (number in brackets facilitates comparisons between GPCRs, as proposed by Ballesteros and Weinstein, 1995. See page xii and Section 3.1). This counteracts the kinking effect of $Pro^{215(5.50)}$ and is probably due to an unconserved interaction with $Glu^{122(3.37)}$ in TMD 3 (Beck et al., 1998). It has been suggested that the kinking of conserved $Pro^{5.50}$ in other GPCRs, in conjunction with local distortions around certain serine residues, results in a similar overall structure of TMD 5 to that observed in rhodopsin (Ballesteros et al., 2001). This has been termed 'structural mimicry'.

Table 2.6. Examples of receptors in which the disulphide bond connecting ECLs 1 and 2 has been shown to be critical for receptor configuration

Receptor:	Reference:
Rhodopsin	(Davidson et al., 1994; Karnik and Khorana, 1990)
β_2 adrenergic	(Dohlman et al., 1990)
M_1 muscarinic acetylcholine	(Savarese et al., 1992)
Angiotensin II	(Ohyama et al., 1995; Yamano et al., 1992)
GnRH	(Cook and Eidne, 1997; Davidson et al., 1997)
Thyrotropin-releasing hormone	(Cook et al., 1996; Perlman et al., 1995)

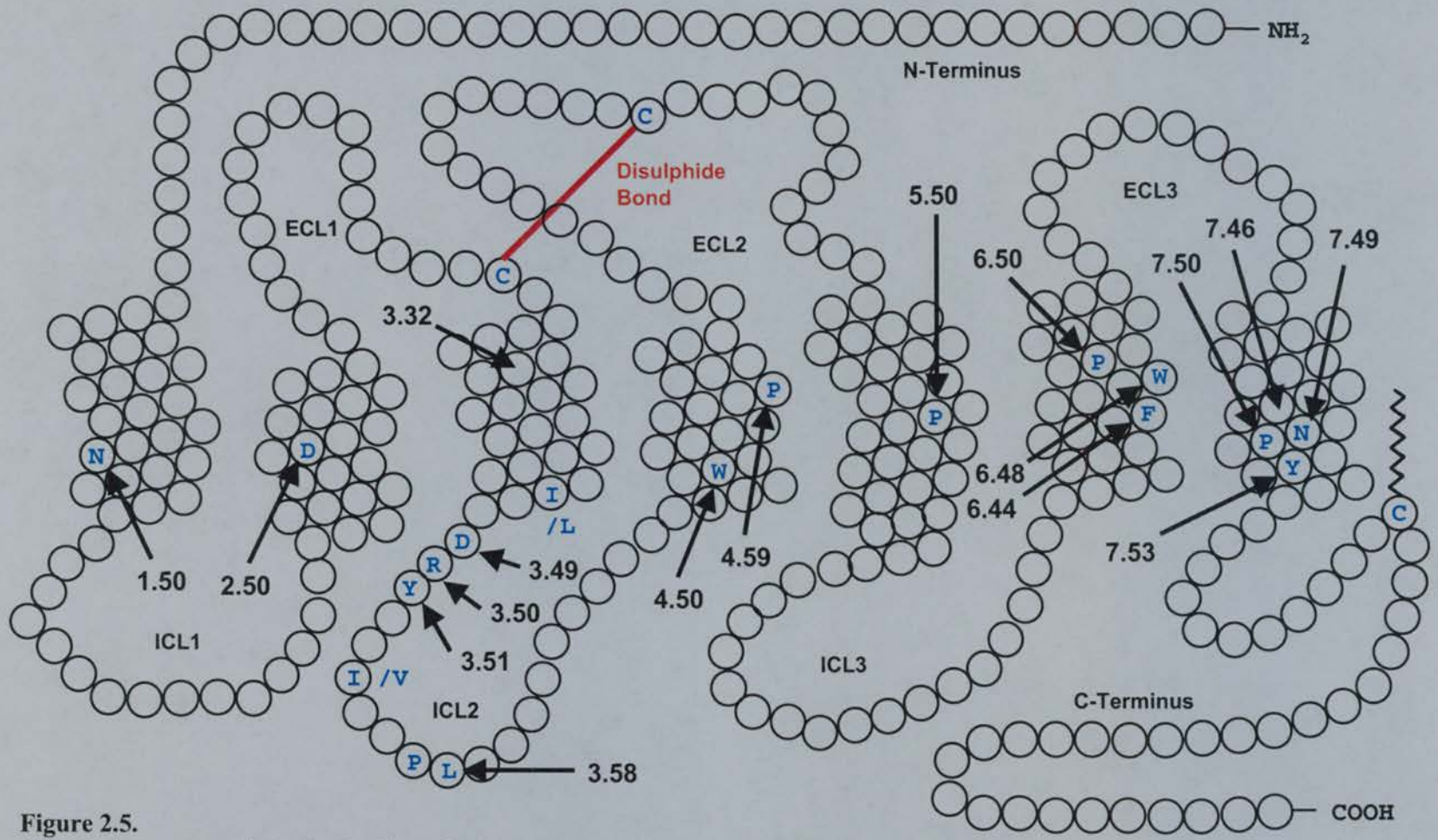

Figure 2.5.
Schematic representation of a Family A GPCR, showing the highly conserved residues

Conserved residues indicated in blue (see text for reference to specific residues)

Differences in the locality of the binding pocket may reflect differences in cognate ligands. The similar overall conformation of GPCRs enables them to signal via a common mechanism (Oliveira et al., 1994), meanwhile differences enable them to interact with a remarkable range of structurally diverse ligands (Gether, 2000). The concept that GPCRs are 'the same and not the same' (Sakmar, 2002) will be continually revisited throughout this thesis.

At this stage, it is important to reiterate that the 2.8 Å resolution map of rhodopsin shows the inactive state (Palczewski et al., 2000) and that retinal has been described as functioning as an inverse agonist (Gether and Kobilka, 1998). Activation of rhodopsin results in significant TMD conformational changes, particularly of TMDs 3 and 4, but also of others (Meng and Bourne, 2001). Structural inferences from ligand binding studies of other GPCRs may imply a different conformation to rhodopsin, however, as agonists preferentially bind to the active receptor conformation, such discrepancies may also be due to the difference in functional state (Ballesteros et al., 2001). Until another GPCR is crystallised, and rhodopsin is crystallised in the active conformation, one can only speculate.

The rhodopsin crystal structure indicates that the conserved $Asn^{55(1.50)}$ and $Asp^{83(2.50)}$ residues in TMDs 1 and 2 respectively are able to hydrogen bond with the peptide carbonyl of $Ala^{299(7.46)}$ in TMD 7 (Palczewski et al., 2000). The conserved $Asn^{7.49}$ is positioned one helical turn from the residue in position 7.46 (Figure 2.5). Evidence for the proximity of $Asp^{2.50}$ and $Asn^{7.49}$ had been presented for the serotonin 5-HT_{2A} receptor (Sealfon et al., 1995). Mutation of $Asp^{2.50}$ to Asn eliminated coupling to IP turnover, however, this mutation in combination with $Asn^{7.49}$ to Asp partially restored function. Aspartate residues in both positions were tolerated, albeit with a lower maximal response. Interactions between $Asn^{1.50}$, $Asp^{2.50}$ and $Asn^{7.49}$ in the 5-HT_{2A} receptor, as well as in the thyrotropin-releasing hormone receptor, were proposed to influence receptor activation (Konvicka et al., 1998; Perlman et al., 1997b). Mutagenesis studies of many GPCRs have shown the importance of these three positions in G-protein coupling (Table 2.7). $Asp^{2.50}$ has been particularly well studied, with mutation resulting in decreased agonist affinity in the majority of cases (van Rhee and Jacobson, 1996).

The GnRH receptor is unusual, as the $Asp^{2.50}$/$Asn^{7.49}$ motif is not conserved. In mammalian type I GnRH receptors, the reciprocal arrangement is present ($Asn^{2.50}$/$Asp^{7.49}$). Mutation of $Asn^{2.50}$ to Asp in the mouse GnRH receptor abolished detectable ligand binding, hence the inability to establish the role of this particular residue in G-protein coupling (Flanagan et al., 1999; Zhou et al., 1994).

Table 2.7. Examples of receptors in which mutations of conserved residues in TMDs 1, 2, 3 and 7 have been shown to influence G-protein coupling

Residue:	Receptor:	Reference:
$Asn^{1.50}$	Thyrotropin-releasing hormone	(Perlman et al., 1997b)
$Asp/Asn^{2.50}$	β_2 adrenergic	(Chung et al., 1988)
	M_1 muscarinic acetylcholine	(Fraser et al., 1989)
	α_2 adrenergic	(Wang et al., 1991)
	D_2 dopaminergic	(Neve et al., 1991)
	Angiotensin II	(Bihoreau et al., 1993)
	Serotonin, 5-HT_{2A}	(Sealfon et al., 1995)
	ET-A and ET-B endothelin	(Rose et al., 1995)
	Thyrotropin-releasing hormone	(Perlman et al., 1997b)
	CB_1 and CB_2 cannabinoid	(Tao and Abood, 1998)
$Arg^{3.50}$	β_2 adrenergic	(Fraser et al., 1988)
	M_1 muscarinic acetylcholine	(Fraser et al., 1989)
	Rhodopsin	(Franke et al., 1990)
	α_2 adrenergic	(Wang et al., 1991)
	Angiotensin II	(Ohyama et al., 1992)
	V_2 vasopressin	(Rosenthal et al., 1993)
	GnRH	(Arora et al., 1997; Ballesteros et al., 1998)
$Asn/Asp^{7.49}$	β_2 adrenergic	(Barak et al., 1995)
	Angiotensin II	(Hunyady et al., 1995)
	Serotonin, 5-HT_{2A}	(Sealfon et al., 1995)
	GnRH	(Arora et al., 1996; Awara et al., 1996; Flanagan et al., 1999; Zhou et al., 1994)
	Platelet-activating factor	(Parent et al., 1996)
	Thyrotropin-releasing hormone	(Perlman et al., 1997b)
	Cholecystokinin B	(Gales et al., 2000)
	Thyrotropin	(Govaerts et al., 2001)

Ligand binding at the $Asp^{7.49}Asn$ mutant mouse receptor was similar to that at the wild type mouse receptor, as was binding at the reciprocal $Asn^{2.50}Asp/Asp^{7.49}Asn$ mutant receptor. However, both mutant receptors exhibited poor coupling efficiency (3-4% of wild type) (Flanagan et al., 1999; Zhou et al., 1994), and so $Asp^{7.49}$ is essential for receptor activation. Mammalian type II and non-mammalian GnRH receptors have aspartate residues in both positions (see Section 2.5), a situation tolerated in many GPCRs, but not in mammalian type I GnRH receptors (Flanagan et al., 1999; Zhou et al., 1994). Mutation of $Asp^{2.50}$ to Asn, singly or in combination with $Asp^{7.49}$ to Asn, abolished ligand binding at the catfish GnRH receptor (Blomenrohr et al., 1997). A subsequent study found that the double mutation of $Asp^{2.50}$ to Asn and $Met^{2.53}$ to Glu in the catfish GnRH receptor, thereby recreating the situation in mammalian type I GnRH receptors, restored binding and coupling to IP turnover (Blomenrohr et al., 2001). The overall structure and agonist binding mode appears to be similar in mammalian and non-mammalian GnRH receptors. However, localised differences in structure may contribute to differences in ligand selectivity (Blomenrohr et al., 2001). Mutation of $Asp^{7.49}$ in the mouse GnRH receptor resulted in the abolition of initial receptor down-regulation in certain cell lines. Coupling to IP turnover was attenuated, but not coupling to cAMP (Awara et al., 1996). Additionally this locus excludes coupling to phospholipase D, as mutation of $Asp^{7.49}$ to Asn resulted in sensitivity to inhibitors of ARF, a small G-protein that controls phospholipase D (Awara et al., 1996; Flanagan et al., 1999). The opposite mutation in the 5-HT_{2A} receptor resulted in decreased sensitivity to the inhibitor (Mitchell et al., 1998). In the β_2 adrenergic receptor, mutation of $Asn^{7.49}$ to Ala resulted in loss of high affinity agonist binding and abolition of receptor sequestration, down-regulation and phosphorylation, in addition to uncoupling (Barak et al., 1995).

The $Asn/Asp^{7.49}$ residue is part of the highly conserved $(N/D)PX_{2-3}Y$ motif in TMD 7 (Figure 2.5). As well as mutations of $Asn/Asp^{7.49}$ influencing G-protein coupling, studies have also shown impaired coupling following substitution of $Pro^{7.50}$ or $Tyr^{7.53/7.54}$, indicating the importance of the motif and not just $Asn/Asp^{7.49}$ (Hunyady et al., 1995; Arora et al., 1996; Barak et al., 1995). Mutation of $Pro^{7.50}$ or $Tyr^{7.53}$ in the β_2 adrenergic receptor had milder effects than mutation of $Asn^{7.49}$, but insertion of an alanine, thereby separating the proline and tyrosine residues by three rather than two residues, resulted in an inactive receptor (Barak et al., 1995). The GnRH receptor exhibited G-protein activation, IP turnover and internalisation similar to wild type despite mutation of $Tyr^{322(7.53)}$ to Phe. This was in contrast to the $Tyr^{322(7.53)}$ to Ala mutation abolishing coupling to IP turnover (Arora et al., 1996). The aromatic nature of this residue appears to be critical in the GnRH receptor.

Family A GPCRs characteristically contain $Arg^{3.50}$ (Figure 2.5) and there are many examples of receptors in which mutation of this residue abolishes G-protein coupling (Table 2.7), including the GnRH receptor (Arora et al., 1997; Ballesteros et al., 1998). $Asp^{3.49}$ and $Tyr^{3.51}$ are present in most Family A GPCRs (Probst et al., 1992). GnRH receptors are in the minority as they have Ser, Phe, Gln or His in place of $Tyr^{3.51}$ (see Section 2.5). Mutation of $Ser^{140(3.51)}$ to Tyr in the mouse GnRH receptor did not affect IP turnover, however, both agonist binding affinity and ligand-induced internalisation increased (Arora et al., 1995). Mutation of $Ser^{140(3.51)}$ to Ala did not affect agonist binding, expression, signalling or internalisation, whereas mutation of $Asp^{3.49}$ to Asn or Glu decreased expression, and increased signalling and internalisation, without affecting agonist binding (Arora et al., 1997). The position and orientation of the 'DRS' motif in the mouse GnRH receptor was shown to be critical for G-protein coupling by inserting successive alanine residues before or after the motif (Kitanovic et al., 2001). The 'DRY' motif is part of a larger '(I/L)XXDRYXX(V/I)XXPL' consensus sequence (Figure 2.5), which appears to have an important role in receptor function (Arora et al., 1995; Ballesteros et al., 1998). In the GnRH receptor, internalisation and IP turnover were both reduced by mutation of $Leu^{147(3.58)}$ to Ala or Asp (Arora et al., 1995).

Two other highly conserved residues are $Phe^{6.44}$ and $Trp^{6.48}$ in TMD6 (Probst et al., 1992) (Figure 2.5). $Trp^{6.48}$ is part of an aromatic locus believed to have a role in agonist binding at the GnRH receptor (Chauvin et al., 2000) (see Section 2.7.2).

2.3.5 Summary

The GnRH receptor belongs to a superfamily characterised by a common overall structure (consisting of seven TMDs) and signalling mechanism (involving G-proteins). This GPCR superfamily is large, structurally diverse, interacts with many types of ligand and potentially couples to multiple combinations of G-protein subunits, as well as non-G-protein mediators of signalling. Despite this diversity, many features are conserved and insights into the structure and function of an individual GPCR can be gained from analysis of other GPCRs. Residues conserved between the members of Family A are usually involved in activation or overall receptor configuration, illustrating that structurally diverse agonists initiate signalling through common mechanisms. Structural data derived from studies of rhodopsin are particularly important as a framework for modelling the GnRH receptor, although it is equally important to appreciate the limitations of such a framework.

2.4 Comparison of Different GnRHs

14 structural forms of GnRH have been identified in chordates and a further 2 forms in a protochordate (tunicate) (Table 2.8). The peptide length of 10 amino acids has been completely conserved for more than 500 million years of evolution (Millar, 2002). The N ($pGlu^1$-His^2-Trp^3-Ser^4) and C (Pro^9-Gly^{10}-NH_2) termini (shown in red in Table 2.8) are totally conserved, except for guinea pig and lamprey I GnRHs where a conservative substitution occurs in position 2 or 3. The N-terminus is believed to be involved in receptor binding and activation, while the C-terminus is believed to be involved only in receptor binding (Sealfon et al., 1997). The residue in position 8 is the most variable, but has an important role in the binding of mammalian GnRH (mGnRH) to its cognate receptors (see Section 2.7.2).

The remainder of the peptide is important for configuring the N and C-termini so that they are correctly oriented for interaction with the receptor. The residue in position 6 is particularly important and is glycine in all GnRHs from jawed vertebrates (shown in orange in Table 2.8) (see Sections 2.6.2 and 2.6.4).

Multiple forms of GnRH have been demonstrated in the same organism (King and Millar, 1992). Generally, fish have GnRH II, salmon GnRH (sGnRH) and a third form; birds and reptiles have GnRH II and chicken GnRH I (cGnRH I); and amphibians and mammals have GnRH II and mGnRH (King and Millar, 1992). Amphibians may possess a third form that is yet unidentified. The gene coding for GnRH II has been demonstrated in the human on chromosome 20, distinct from the gene coding for mGnRH on chromosome 8 (White et al., 1998). GnRH II has historically been called chicken GnRH II (cGnRH II) as it was first identified in the chicken (Miyamoto et al., 1984). However, since it has now been identified in the majority of vertebrates, it will be termed GnRH II in this thesis.

Algorithms have been used to produce phylogenetic trees of GnRH peptide evolution by comparing prepro-GnRH sequences (King and Millar, 1992; Okubo et al., 2000a; White et al., 1995). Jawed vertebrate GnRHs were originally grouped into two evolutionary branches: GnRH II was separated from other GnRHs as its primary structure has been completely conserved through 500 million years (King and Millar, 1992). More recently, three branches have been proposed, namely GnRH I (releasing forms), GnRH II (mesencephalic forms) and GnRH III (sGnRH) (telencephalic forms) (Okubo et al., 2000a; White et al., 1995). None of these schemes included the invertebrate tunicate GnRHs or the jawless vertebrate lamprey GnRHs.

Table 2.8. GnRHs identified to date (see text for reference to colours)

Species:	Sequence:										Reference:
	1	2	3	4	5	6	7	8	9	10	
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2	(Matsuo et al., 1971; Burgus et al., 1972)
Guinea Pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH_2	(Jimenez-Linan et al., 1997)
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2	(King and Millar, 1982; Miyamoto et al., 1983)
Rana	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly-NH_2	(Yoo et al., 2000)
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH_2	(Powell et al., 1994)
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly-NH_2	(Okubo et al., 2000; Montaner et al., 2001)
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly-NH_2	(Carolsfeld et al., 2000)
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH_2	(Ngamvongchon et al., 1992)
Whitefish	pGlu	His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	Gly-NH_2	(Adams et al., 2002)
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2	(Sherwood et al., 1983)
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH_2	(Lovejoy et al., 1992)
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2	(Miyamoto et al., 1984)
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH_2	(Sower et al., 1993)
Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH_2	(Sherwood et al., 1986)
Tunicate I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH_2	(Powell et al., 1996)
Tunicate II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH_2	(Powell et al., 1996)

2.5 Comparison of Different GnRH Receptors

Type I GnRH receptors have now been cloned from at least 21 different species of chordates (12 mammalian and 9 non-mammalian) (Table 2.9). At least 6 Type II (3 mammalian and 3 non-mammalian) and 3 Type III GnRH receptors have also been cloned (Table 2.9). Additionally, GnRH receptor orthologues have recently been identified in *Drosophila melanogaster* (Hauser et al., 1998) and *C. elegans* (Swanson et al., unpublished results).

GnRH receptors have been classified as type I, II or III based on sequence homology (Table 2.9). It should be noted that the three types of bullfrog GnRH receptor were originally classified according to tissue distribution, and not according to sequence homology with previously cloned GnRH receptors (Wang et al., 2001). Therefore, the bullfrog receptor classified as type II by Wang and co-workers is a type I receptor according to sequence homology (Table 2.9), and the bullfrog receptor classified as type III by Wang and co-workers is a type II receptor according to sequence homology. This leaves the bullfrog type I receptor that should be classified as a type III receptor according to sequence homology. This situation is extremely confusing and so for the sake of simplicity, the bullfrog receptors will be designated as type I, II and III according to sequence homology throughout this thesis. Similarly, Okubo and co-workers named the two medaka GnRH receptors that they cloned 'Medaka GnRH-R1' and 'Medaka GnRH-R2'. However, according to sequence homology, Medaka GnRH-R1 is a Type III GnRH receptor and Medaka GnRH-R2 is a Type I GnRH receptor (Table 2.9). They will also be designated according to sequence homology throughout this thesis.

Type I GnRH receptors cloned from eutherian mammals exhibit high overall amino acid sequence homology (85% or greater) with almost identical TMD amino acid sequences (Sealfon et al., 1997). The lack of an intracellular C-terminal tail and the particular Asp/Asn arrangement in TMDs 2 and 7 are exceptional for GPCRs. Key functional motifs (see Section 2.3.4) are conserved, with the exception of the marmoset type I receptor that has $Ser^{3.51}$ of the 'DRS' motif replaced by Phe. This substitution may have a role in desensitisation/internalisation (Byrne et al., 1999).

The Australian brushtail possum is a metatherian marsupial mammal, and is therefore distinct from the eutherian mammals from which GnRH receptors have been cloned (Table 2.9). Despite this, the possum GnRH receptor is very similar to other mammalian type I GnRH receptors in both structure and pharmacology. It exhibits 80% sequence homology and lacks an intracellular C-terminal tail (King et

al., 2000). However, like the marmoset, it has $Ser^{3.51}$ replaced by Phe (King et al., 2000). A second marsupial GnRH receptor was recently cloned from the wallaby (Cheung and Hearn, 2002) and is apparently very similar to the possum receptor.

Mammalian type II GnRH receptors have $Gln^{3.51}$, and non-mammalian GnRH receptors have $Gln^{3.51}$ or $His^{3.51}$ (see Table 2.9 for references and Figure 2.6 for examples). The impact of these substitutions has yet to be established.

A cysteine residue in ECL2 that is highly conserved amongst Family A GPCRs was discussed in Section 2.3.4. A second cysteine residue is conserved in ECL2 of all mammalian type I GnRH receptors, with the apparent exception of the pig. Furthermore, a cysteine residue is conserved in the N-terminus of all mammalian type I GnRH receptors (at position 14, except in the dog where it is at position 13) (Figure 2.6). Evidence has been presented for these cysteine residues forming a disulphide bond (Davidson et al., 1997), although the disruption of this bond appeared to have little affect on the binding of [D-Trp^{6},des-Gly^{10}]-GnRH (Cook and Eidne, 1997). The N-terminus/ECL2 disulphide bond is not present in mammalian type II and non-mammalian GnRH receptors as the cysteines are not conserved. Therefore, the configuration of the extracellular domains of these receptors is likely to differ significantly from that of the mammalian type I receptors.

An important locus for Family A GPCR configuration involves the residues in positions 2.50 and 7.49 in TMDs 2 and 7 respectively (see Section 2.3.4). Mammalian type I GnRH receptors have $Asn^{2.50}$ and $Asp^{7.49}$, whereas mammalian type II and non-mammalian GnRH receptors have $Asp^{2.50}$ and $Asp^{7.49}$ (Figure 2.6). Therefore, as discussed in Section 2.3.4, these differences between mammalian type I receptors and other GnRH receptors at this locus suggest that interactions in this microdomain differ, and that the TMD configuration of these receptors may also differ (Blomenrohr et al., 1997; Sun et al., 2001).

ECL3 in mammalian type I GnRH receptors is likely to have a different configuration to ECL3 in other GnRH receptors, due to the altered positioning of proline residues. Mammalian type I GnRH receptors have the motif 'S(D/E)P', whereas non-mammalian type I GnRH receptors have 'P(D/E)Y'. This may alter the presentation of $Asp/Glu^{7.32}$ (Flanagan et al., 1997; Petry et al., 2002), which has been shown to interact with Arg^{8} in mGnRH (Blomenrohr et al., 2002; Flanagan et al., 1994; Fromme et al., 2001) (see Section 2.7.2). Type II GnRH receptors have the motif 'PPS'; and type III GnRH receptors have the motif 'S(H/Q)S'. The lack of an acidic residue at position 7.32 is likely to contribute to the relatively low IP turnover following binding of mGnRH at such receptors (Wang et al., 2001).

Table 2.9. Classification of GnRH Receptor cDNA based on sequence homology (Bullfrog and Medaka original classifications in brackets)

	Species:	Reference:
Type I Mammalian	Mouse	(Reinhart et al., 1992; Tsutsumi et al., 1992)
	Rat	(Eidne et al., 1992; Kaiser et al., 1992)
	Sheep	(Brooks et al., 1993; Illing et al., 1993)
	Cow	(Kakar et al., 1993)
	Human	(Chi et al., 1993; Kakar et al., 1992)
	Pig	(Weesner and Matteri, 1994)
	Marmoset I	(Byrne et al., 1999)
	Dog	(Cui et al., 2000)
	Bonnet Monkey	(Santra et al., 2000)
	Horse	AF 018072
	Possum	(King et al., 2000)
	Wallaby	(Cheung and Hearn, 2002)
Type I Non-Mammalian	Catfish	(Tensen et al., 1997)
	Goldfish (Ia and Ib)	(Illing et al., 1999)
	Xenopus laevis I	(Troskie et al., 2000)
	Trout	(Madigou et al., 2000)
	Japanese eel	(Okubo et al., 2000b)
	Cichlid	(Robison et al., 2001)
	Medaka I (Okubo: 2)	(Okubo et al., 2001)
	Chicken	(Sun et al., 2001)
	Bullfrog I (Wang: II)	(Wang et al., 2001)
Type II Mammalian	Marmoset II	(Millar et al., 2001)
	Green monkey II	(Neill et al., 2001)
	Rhesus monkey II	(Neill et al., 2001)
Type II Non-Mammalian	Bullfrog II (Wang: III)	(Wang et al., 2001)
	Typhlonectes natans II	AF 174481
	Xenopus laevis II	Troskie *et al.* (unpublished results)
Type III	Striped bass III	(Alok et al., 2000)
	Bullfrog III (Wang: I)	(Wang et al., 2001)
	Medaka III (Okubo: 1)	(Okubo et al., 2001)
	Amberjack III	CAB 65407

Figure 2.6. Sequence alignment of GnRH receptors examined in this thesis (the marmoset type II GnRH receptor is included for comparison). Note the differences in structural motifs between mammalian type I GnRH receptors and other forms of GnRH receptor, both mammalian and non-mammalian. In particular, note the lack of a C-terminal domain in mammalian type I GnRH receptors.

Transmembrane domains (TMDs) indicated in red (precise TMD boundaries are unknown). Extracellular domains (ECLs) indicated in **black** and intracellular domains (ICLs) indicated in green. Residues involved in structural motifs that differ between mammalian type I GnRH receptors and other forms of GnRH receptors indicated in blue.

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                          N-Terminus
Mouse            MANNASLEQDPNH-------------CSAINN---- 19
Rat              MANNASLEQDQNH-------------CSAINN---- 19
Human            MANSASPEQNQNH-------------CSAINN---- 19
Catfish          M-SGNTTLLLSNPTN-------------VLDNSSVL 22
Chicken          M-CVPAALIEAEPPH-------------HPTTEGDT 22
Marmoset (II)    MSAVN-------GTPWGSSARE-----------EVW 18
Xenopus (II)     MDSQD-----------LCALNR---SCFHLKEQEKT 22
Bullfrog (II)    MNASDQPMGDGEAAPPGLCAFKGFNFSCVHANGFEKP 37

                       N-Terminus              TMD1
Mouse            ---SIPLIQGK---LPTLTVSGKIRVTVTFFLFLLST 50
Rat              ---SIPLTQGK---LPTLTLSGKIRVTVTFFLFLLST 50
Human            ---SIPLMQGN---LPTLTLSGKIRVTVTFFLFLLSA 50
Catfish          NVSVSPPV--LKWETPTFTTAARFRVAATLVLFVFRA 57
Chicken          NTSATHCL--EHWVEPRFTKAAKVRVAITAVFFLLAA 57
Marmoset (II)    AGSGVEVEGSE---LPTFSTAAKVRVGVTIVLFVSSA 52
Xenopus (II)     YGPNITVLNDKAFILPTFSTAAKIRVAITCVLFIFSA 59
Bullfrog (II)    HGPNITFLNEDHFVLPTFSTAAKIRVAITCVLFISSA 74

                 TMD1          ICL1          TMD2
Mouse            AFNASFLLKLQKWTQKRKKGKKLSRMKVLLKHLTLAN 87
Rat              AFNASFLVKLQRWTQKRKKGKKLSRMKVLLKHLTLAN 87
Human            TFNASFLLKLQKWTQKKEKGKKLSRMKLLLKHLTLAN 87
Catfish          ASNLSVLLSVTR---G-RGRRLASHLRPLIASLASAD 90
Chicken          CSNTAVLGSLLR-------KRRKCHVRPLILSLALAD 87
Marmoset (II)    GGNLAVLWSVTR---PQPSQLRPSPVRRLFAHLAAAD 86
Xenopus (II)     CFNIAALWTITY---KYK---KKSHIRILIINLVAAD 90
Bullfrog (II)    CFNMATLWTITY---KYR---KKSHIRILIINLVAAD 105
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Figure 2.6 (Continued)

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                    TMD2          ECL1              TMD3
Mouse           LLETLIVMPLDGMWNITVQWYAGEFLCKVLSYLKLFS 124
Rat             LLETLIVMPLDGMWNITVQWYAGEFLCKVLSYLKLFS 124
Human           LLETLIVMPLDGMWNITVQWYAGELLCKVLSYLKLFS 124
Catfish         LVMTFVVMPLDAVWNVTVQWYAGDAMCKLMCFLKLFA 127
Chicken         LLVTVAVMPLDAAWNVTVQWYGGDLSCKLLNFLKLFA 124
Marmoset (II)   LLVTFVVMPLDATWNITVQWLAGDIACRTLMFLKLMA 123
Xenopus (II)    LFITLVVMPLDAVWNVTLQWYAGDLACRVLMFLKLAA 127
Bullfrog (II)   LLITFVVMPLDAVWNVTIQWYAGDVACRILMFLKLVA 142

                      TMD3              ICL2          TMD4
Mouse           MYAPAFMMVVISLDRSLAITQPLAVQSNSKLEQSMIS 161
Rat             MYAPAFMMVVISLDRSLAVTQPLAVQSKSKLERSMTS 161
Human           MYAPAFMMVVISLDRSLAITRPLALKSNSKVGQSMVG 161
Catfish         MHSAAFILVVVSLDRHHAILHPLDTLDAGRRNRRMLL 164
Chicken         MYAAALVLVVISLDRHAAVLQPFA--RARRRNGLLLR 159
Marmoset (II)   MYAAAFLPVVIGLDRQAAVLNPLGSRSGVRK---LLG 157
Xenopus (II)    MYSSAFVTVVISLDRQAAILNPLGIGDAKKKNKIMLC 164
Bullfrog (II)   MYSSAFVTVVISLDRHAAILNPLGIGDAKKKNKAMLS 179

                      TMD4                ECL2
Mouse           LAWILSIVFAGPQLYIFRMIYLADGSGPT-VFSQCVT 197
Rat             LAWILSIVFAGPQLYIFRMIYLVDGSGPA-VFSQCVT 197
Human           LAWILSSVFAGPQLYIFRMIHLADSSGQTKVFSQCVT 198
Catfish         TAWILSLLLASPQLFIFRAIKAKG-VD----FVQCAT 196
Chicken         AAWLGSVLLASPQLFLFHVHTVPG-GN----FTQCVT 191
Marmoset (II)   AAWGLSFLLALPQLFLFHTVHRAGPVP----FTQCAT 190
Xenopus (II)    VAWFLSYLLAIPQLFVFHTVSRSEPIH----FVQCAT 197
Bullfrog (II)   VAWTLSLLLATPQLFVFHTVSRSQPVH----FVQCAT 212

                    ECL2              TMD5
Mouse           HCSFPQWWHQAFYNFFTFGCLFIIPLLIMLICNAKII 234
Rat             HCSFPQWWHEAFYNFFTFSCLFIIPLLIMLICNAKII 234
Human           HCSFSQWWHQAFYNFFTFSCLFIIPLFIMLICNAKII 235
Catfish         HGSFQQHWQETAYNMFHFVTLYVFPLLVMSLCYTRIL 233
Chicken         HGSFRAHWEETVYNMFTFTTLYITPLSIMIVCYVRII 228
Marmoset (II)   KGSFKARWQETTYNLFTFCCLFLLPLTAMAICYSRIV 227
Xenopus (II)    VGSFQAHWQETIYNMFTFFCLFLLPLLIMVSCYTRIL 234
Bullfrog (II)   VGSFKAHWLETLYNMFTFCCLFLLPLLIMVFCYGRIL 249
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Figure 2.6 (Continued)

```
                    TMD5                ICL2                TMD6
Mouse               FALT-----RVLHQDPRKLQMNQSK-NNIPRARLRTL 265
Rat                 FALT-----RVLHQDPRKLQLNQSK-NNIPRARLRTL 265
Human               FTLT-----RVLHQDPHELQLNQSK-NNIPRARLKTL 266
Catfish             VEIN--RQMHRSKDKAGEPCLRRSGTDMIPKARMKTL 268
Chicken             WEIS--KQLKINKS------LVRSQNDHISKARMKTL 257
Marmoset (II)       LGVSSPRTRKGSHAPAGEFALRRS-FDNRPRVRLRAL 263
Xenopus (II)        MEIS--HKMKATCVSSKEIDLRRS-SNNIPRARMRTL 268
Bullfrog (II)       VEIS--RKMKKAEVSSREVNLRRS-YNNIPRARMRTF 283

                          TMD6                      ECL3
Mouse               KMTVAFATSFVVCWTPYYVLGIWYWFDPEMLN--RVS 300
Rat                 KMTVAFGTSFVICWTPYYVLGIWYWFDPEMLN--RVS 300
Human               KMTVAFATSFTVCWTPYYVLGIWYWFDPEMLN--RLS 301
Catfish             KMTIIIVASFVICWTPYYLLGIWYWFQPQML--HVIP 303
Chicken             KMTIVIVASFIICWTPYYLLGLWYWFHPAMI--QRMP 292
Marmoset (II)       RLALLVLLTFILCWTPYYLLGLWYWFSPSMLSE--VP 298
Xenopus (II)        KMSLVIVLTFIVCWTPYYLLGIWYWFSPEMLTEEKVP 305
Bullfrog (II)       KMSLVIVLTFIVCWTPYYLLGIWYWFSPEMLTSRKVP 320

                    ECL3            TMD7              C-Terminus
Mouse               EPVNHFFFLFAFLNPCFDPLIYGYFSL   327
Rat                 EPVNHFFFLFGFLNPCFDPLIYGYFSL   327
Human               DPVNHFFFLFAFLNPCFDPLIYGYFSL   328
Catfish             DYVHHVFFVFGNLNTCCDPVIYGFFTPSFRADLSRCF 340
Chicken             EYINHSFFLFGLLHTCTDPIIYGLYTPSFREDVQLCL 329
Marmoset (II)       PSLSHILFLFGLLNAPLDPLLYGAFTL---------G 326
Xenopus (II)        PSLSHILFLFGLLNTCLDPIIYGLFTIHFRREIRR-V 341
Bullfrog (II)       PSLSHILFLFGLFNTCLDPIIYGLFTIHFRREIRR-V 356

                                C-Terminus
Catfish             CWRNQNASAKS----------LPHFS---GHRREVSG 364
Chicken             -RGIEAAISQH----------VRHKPISVSEKTTKDG 355
Marmoset (II)       CRRGHQELSMDSSREEGSRRMFQQDIQALRQT----- 358
Xenopus (II)        CRCAAQGKDHDTASVGTGSFRITTTPAPIKRTVGVLG 378
Bullfrog (II)       CRCATQGKDADATSLGTGSFRISTAAVPLKRSAGASG 393

                          C-Terminus
Catfish             EA---ESDLGSGDQPSGQ                    379
Chicken             DV---NGQVTSGGSNGTT--------VNTVC       375
Marmoset (II)       -----EVQKTVTSRKAGE----TKDIPITSI       380
Xenopus (II)        GSGKFELEVTGHGLHSGKCDQCQGRIVESFM       409
Bullfrog (II)       GSCKFDLEVTGVGLHSGKCEHCKRQIVESFM       424
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2.6 GnRH Ligand Structure-Activity Relationships

2.6.1 The Bioactive Conformation of GPCR Ligands

Ligands such as GnRH are flexible molecules that will adopt a variety of conformations in solution (Maliekal et al., 1997; Momany, 1976a). Each 'conformer' will have a particular energy, with the most stable conformers having the lowest energy. An equilibrium will exist between the various conformers at any one time. The greater the percentage of molecules in the bioactive conformation, the greater the binding affinity at the cognate receptor (Freidinger et al., 1980). Intramolecular interactions can increase the stability of the bioactive conformation, and there is evidence for this phenomenon in GnRHs (Maliekal et al., 1997; Milton et al., 1983; Shinitzky and Fridkin, 1976; Shinitzky et al., 1976).

In addition to these intramolecular interactions, there is increasing evidence for the environment influencing ligand conformation (Laakkonen et al., 1996). This includes both the solvent and the receptor (Koerber et al., 2000; Mezei and Guarnieri, 1998). Changes upon binding at the receptor may be due to desolvation or specific interactions with receptor residues. There is evidence from the TRH and $P2Y_1$ receptors that multiple interactions occur sequentially (Colson et al., 1998; Moro et al., 1999; Perlman et al., 1997a). In the case of a flexible ligand, initial contacts may stabilise the bioactive conformation, lowering the energy requirement for subsequent interactions. Such 'induced-fit' may have evolved to increase ligand-receptor specificity and/or reduce the need for ligand pre-configuration. An interaction between Arg^8 in mGnRH and ECL3 of the cognate receptor is proposed to be such a ligand-conforming initial interaction (Fromme et al., 2001).

Various studies have provided insights into the bioactive conformations of ligands acting at GPCRs, and those involving GnRH will be discussed in Section 2.6.4. Due to the structural diversity of peptide GPCR ligands, it is unlikely that insights into the bioactive conformation of GnRH will be gained by comparison with ligands that act at other GPCRs. Tachykinin receptors bind peptide agonists of a similar size to GnRH, namely the undecapeptides substance P, eledoisin and physalaemin, and the decapeptides neurokinin A (substance K) and neurokinin B (Rang et al., 1999). However, the conformations of ^{125}I-Bolton Hunter-conjugated (^{125}I-BH) substance P and ^{125}I-BH eledoisin are unlikely to contain a β-II′ turn (Cascieri et al., 1986), as predicted for mGnRH (Momany, 1976a). A study of the C-terminal heptapeptide of neurokinin A, which is more active than the parent

compound, implied that a turn from Ser^5 to Gly^8 was present in the bioactive conformation, but was of the β-I type (Saviano et al., 1991).

2.6.2 Substitutions that Enhance the Activity of GnRH

A multitude of GnRH analogues have been generated to investigate the roles of different residues in GnRH function (Sealfon et al., 1997). As early as 1973, it was shown that replacement of Gly^6 with Ala resulted in an analogue with 4% of the activity of mGnRH both *in vivo* and *in vitro*. In contrast, replacement with D-Ala resulted in 350-450% of the activity of mGnRH (Monahan et al., 1973). These changes in potency have been attributed to effects on ligand conformation (see Section 2.6.4). Bulky hydrophobic D-amino acids were found to be the most biologically active, due to a combination of increased receptor affinity and increased biological half-life (Coy et al., 1976; Karten and Rivier, 1986; Nestor et al., 1984; Sealfon et al., 1997). However, the relationship between hydrophobicity and activity is not absolute: [D-Nal(2)^6]-GnRH was reportedly 200 times more potent than GnRH in suppressing estrus in rats, but analogues with greater hydrophobicity were less potent (Nestor et al., 1984). This was perhaps due to other factors such as steric hindrance, insolubility or partition to the lipid environment. Aromatic side chains appear to be favoured (Nestor et al., 1984), perhaps due to the formation of pi electron complexes. The effects of various D-amino acid substitutions in position 6 of mGnRH, cGnRH I, sGnRH and GnRH II are described in Chapter 4.

As well as position 6, activity-enhancing substitutions have been made at position 10. Replacing the C-terminus with Pro^9-ethylamide (Pro^9-NHEt) resulted in an analogue 5 times more potent than mGnRH at inducing ovulation (Fujino et al., 1972). Again, combining this modification with a D-amino acid at position 6 resulted in a cumulative increase in potency (Coy et al., 1974). In this study, [D-Ala^6]-GnRH was 8 times more potent than mGnRH, whereas [D-Ala^6,Pro^9-NHEt]-GnRH was 16 times more potent than mGnRH. Replacement of the C-terminus with aza-Gly^{10} is even more effective, as demonstrated by [D-Ser(tBu)^6,aza-Gly^{10}]-GnRH being at least 5 times more potent than [D-Ser(tBu)^6,Pro^9-NHEt]-GnRH at inducing ovulation (Dutta et al., 1978). Indeed, an extremely potent agonist is [D-Nal(2)^6,aza-Gly^{10}]-GnRH, which is about 230 times more potent than native mGnRH (Ho et al., 1984).

Other substitutions, such as 3-(1-naphthyl)alanine (Nal(1)) in place of Trp^3 (Yabe et al., 1976), gave small increases in potency. (N^{α}-Me)Leu in place of Leu^7 did not alter the biological activity of mGnRH on its own (Ling and Vale, 1975),

however, it did appear to increase the potency of some analogues containing a D-amino acid in position 6 (Karten and Rivier, 1986; Nestor et al., 1984).

Certain substitutions did not significantly improve the affinity of mGnRH for its cognate receptor, but were tolerated nonetheless. Notable examples were His^5 and Trp^7, singly or in combination (Millar et al., 1989). Tyr^8 substitution was not tolerated on its own, or in combination with Trp^7. However, the affinity of [His^5,Tyr^8]-GnRH for rat homogenised pituitary membranes was only 10-fold lower than that of mGnRH (Millar et al., 1989). The affinity of GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) for a similar membrane preparation was only 6.5-fold lower than that of mGnRH (Millar et al., 1986; Millar et al., 1989). It would appear that this combination of substitutions is largely able to overcome the requirement for Arg^8 in mGnRH, perhaps by introducing additional intramolecular interactions that stabilise the folded conformation (see Section 2.7.2). Evidence for pre-configuration of GnRH II is presented in Chapter 4.

2.6.3 GnRH Antagonists

GnRH antagonists have great therapeutic potential, as discussed previously in Section 2.1.4. Antagonistic properties are conferred by particular modifications to the N-terminus. Early studies demonstrated that deletion of $pGlu^1$ and/or His^2 resulted in very little, if any, agonist potency (Vale et al., 1972; Yanaihara et al., 1973). A large range of natural and unnatural amino acid substitutions in positions 1 to 3 have since been evaluated in an attempt to identify analogues with a total lack of agonist potency, but very high receptor affinity. Hydrophobic D-amino acids in all of these positions appear to be desirable, particularly in combination with a similar residue in position 6 (Karten and Rivier, 1986; Nestor et al., 1984). Such tetrasubstituted analogues can be enhanced slightly by substitutions at other positions, such as D-Ala^{10} (Erchegyi et al., 1981), and the most potent antagonists tend to differ from mGnRH in at least half the positions (Karten and Rivier, 1986).

In order to produce antagonists with stable high affinity conformations, many studies of mono- and dicyclic GnRH antagonists have been carried out, particularly by Rivier and co-workers (Dutta et al., 1978; Rivier et al., 1988; Rivier et al., 2000a; Rivier et al., 2000b; Rivier et al., 2000c). The most potent of these analogues involved bridges between the side chains in positions 4-10, 5-8, 4-10/5-8, 1-3, 1-3/4-10 or 1-5/4-10. The optimal conformation of the N-terminus was assessed using a bridge of varying lengths from position 1 to 5. Despite the apparent flexibility of the

N-terminus in linear GnRH analogues, only a small number of conformations were particularly potent, indicating the importance of N-terminal conformation to high affinity antagonist binding (Rivier et al., 2000a).

In this thesis, Antagonist 135-18 ([Ac-D-Nal(2)^{1},D-4-Cl-Phe^{2},D-Pal(3)^{3},Ile^{5},D-Lys(iPr)^{6},Lys(iPr)^{8},D-Ala^{10}-NH_2]-GnRH) is of particular interest because it acts as an antagonist at the human GnRH receptor, but as a full agonist at the chicken receptor (Sun et al., 2001) and a partial agonist at the *Xenopus* I receptor (Ott et al., 2002). This analogue was found to be a partial agonist at the human GnRH receptor containing the chicken GnRH receptor ECL2, implying that ECL2 is partly responsible for this phenomenon (Sun et al., 2001). ECL2 was also found to be the determining region in the *Xenopus* I receptor as Antagonist 135-18 was a full agonist at the human receptor containing the *Xenopus* I receptor ECL2, and a partial agonist when only portions of ECL2 were substituted (Ott et al., 2002). This study proposed that the D-Lys(iPr)^{6} side chain of Antagonist 135-18 formed a charge-supported hydrogen bond with His$^{5.32}$ in the *Xenopus* I receptor ECL2, and that this interaction was responsible for the agonist activity. However, the overall conformation of ECL2 was also clearly important. Chapter 5 describes the binding of Antagonist 135-18 to human GnRH receptors containing catfish GnRH receptor ECLs in all single, double and triple combinations.

2.6.4 Bioactive Conformations of GnRHs

Following the sequencing of mGnRH (Matsuo et al., 1971), various techniques have been used to investigate the bioactive conformation of the peptide. As discussed in Section 2.6.2, substitutions at position 6 have a dramatic effect on the binding affinity of mGnRH. This was first recognised by Monahan and co-workers, who proposed that D-Ala^{6} stabilised a β-II turn from residue 4 to 7 (Monahan et al., 1973).

Conformational energy analysis identified possible low energy conformers of the mGnRH peptide. The molecule was analysed in two sections (1-6 and 6-10) in order to reduce the number of variables. The resultant data was then combined to produce putative conformers of the whole sequence (Momany, 1976a). The most likely low energy structure deduced from this study, and therefore the likely bioactive conformer, was the 'CC conformer' that involved a modified β-II turn from residue 5 to 8. A series of tetrapeptide and decapeptide analogues were analysed using this method and the results correlated with experimental activity data. The

findings gave further support to the proposed 'CC conformer' being bioactive (Momany, 1976b). Substitution of Gly^6 with D-Ala was favoured in the proposed structure, with the added effect of reducing the freedom of opening at this position. The result would be an increase in the population of the bioactive conformer and therefore an increase in activity, as observed experimentally (Monahan et al., 1973).

[(N^{α}-Me)Leu^7]-GnRH was found to have approximately the same biological activity as native mGnRH (Ling and Vale, 1975). This substitution would not be allowed if the β-II turn was from residue 4 to 7 as proposed by Monahan and co-workers. However, it would be permitted in the 'CC conformer', with the modified turn from residue 5 to 8 (Momany, 1976b).

An alternative method of conformationally constraining the proposed turn structure involved the use of a γ-lactam bridge. The pro-*S* hydrogen atom of Gly^6 and the N^{α}-hydrogen of Leu^7 were replaced by methyl groups, which were connected by a single bond to form a five-membered lactam ring (Freidinger et al., 1980). This modification gave a 9-fold increase in potency relative to mGnRH, despite retaining an L-amino acid in position 6 and not introducing additional functional groups.

Another position of particular interest is residue 8. This is arginine in mGnRH, glutamine in cGnRH I, and leucine in sGnRH (Table 2.8). Analogues substituted with Glu^8 or Leu^8 have low activity in mammals (Millar et al., 1989), however, they are permitted in the 'CC conformer' (Momany, 1976b). Therefore, depending on the impact of Trp^7 in sGnRH, it is likely that both cGnRH I and sGnRH can adopt the modified β-II turn from residue 5 to 8. The low affinity of position 8-substituted analogues at mammalian type I receptors may be due to a low percentage of ligand in the 'CC conformer' and/or an inability to interact with $Asp/Glu^{7.32}$ in ECL3 of the receptor (see Section 2.7.2).

The bioactive conformation proposed by Momany had closely apposed N and C-termini (Momany, 1976a). Support for this was provided by studies using conformation-dependent mGnRH antisera. These bound to the N and C termini and tolerated certain amino acid substitutions in the central region of the ligand, but not in other positions (Millar et al., 1984).

The fluorescence intensity of Trp^3 in mGnRH has been used to provide insights into conformation. The resultant titration curve indicated that at physiological pH, only Arg^8 was ionised. The populations of His^2 and Tyr^5 were each found to be homogeneous, suggesting a relatively homogeneous population of conformers. Furthermore, the reduced basicity of His^2 and the increased acidity of Tyr^5 indicated that their side chains were in close proximity to the side chain of Arg^8 (Shinitzky and Fridkin, 1976). Indeed, the possibility of hydrogen bonds between

these residues was suggested. Further studies by the same group used various analogues substituted with other basic residues at position 8. These suggested a central role of the His^2-Tyr^5-Arg^8 structural unit (Shinitzky et al., 1976). The efficiency of collisional quenching was reduced by 36-39% with these analogues compared with native mGnRH, indicating a subtle spatial distortion of the proposed His^2-Tyr^5-Arg^8 unit relative to Trp^3. This may account for the low biological activity of [Lys^8]-GnRH (Chang et al., 1972). An analogue with a neutral residue at position 8, [ω-NO_2-Arg^8]-GnRH, was found to have a heterogeneous population of histidine residues and very low GnRH antibody cross-reactivity (Shinitzky et al., 1976). This was indicative of a heterogeneous population of ligand conformations, which was believed to be due to disruption of the His^2-Tyr^5-Arg^8 unit.

Subsequent fluorimetric titration of mGnRH and [Lys^8]-GnRH supported the findings of Shinitzky and co-workers (Milton et al., 1983). This study also showed that cGnRH I ([Gln^8]-GnRH) had a heterogeneous population of histidine residues similar to that observed with [ω-NO_2-Arg^8]-GnRH. Therefore, cGnRH I is also likely to exist as a series of conformers at physiological pH.

The technique of conformational memories was used to predict the relative conformational populations of mGnRH and [Lys^8]-GnRH in solution (Guarnieri and Weinstein, 1996). Approximately 70% of the mGnRH conformers were predicted to possess a β type turn for residues 5 to 8, with about 5% expected to have an extended backbone. These results again support the bioactive conformation proposed by Momany. More than 70% of the [Lys^8]-GnRH analogue was predicted to have an extended backbone, with a β type turn for residues 5 to 8 expected to occur with a frequency of just 3%. The low biological activity of [Lys^8]-GnRH (Chang et al., 1972; Milton et al., 1983) may be due to the low frequency of the bioactive conformer (Guarnieri and Weinstein, 1996), which may in turn be due to the disruption of intramolecular interactions as discussed above.

Two-dimensional proton NMR was used to predict the conformation of a cyclic decapeptide GnRH antagonist (Baniak et al., 1987). The analogue was found to exist as two conformers, both of which possessed a β-II′ turn for residues 6 to 7, and a β-II turn for residues 1 to 2. These were connected by extended antiparallel β-like strands. The backbone atoms from the Tyr^5–CO to the Pro^9–N of the bioactive conformer predicted by conformational memories could be superimposed on the structure of this high affinity analogue, with an RMS deviation in the range of just 0.6-0.8 Å (Guarnieri and Weinstein, 1996). The two structures diverge between the N-terminus and residue 4. As this analogue is an antagonist, divergence of the region believed necessary for receptor activation is unsurprising (see Section 2.6.3).

There are many more examples of cyclic antagonists that possess the β-II′ turn for residues 6 to 7, as demonstrated by NMR and molecular modelling (Koerber et al., 2000). Indeed, Rivier and co-workers were only able to identify one constrained high affinity GnRH antagonist that did not appear to have this structural motif (Rivier et al., 2000c). This particular analogue possessed a β-I′ turn for residues 6 to 7. Perhaps upon binding to the receptor, this conformation changed to form a β-II′ turn, or perhaps the unique configuration of side chains in this analogue enabled high affinity receptor interactions to occur despite the different backbone conformation (Koerber et al., 2000).

The study of conformational memories was extended by performing explicit water simulations to allow for the influence of the solvent on ligand conformation (Mezei and Guarnieri, 1998). As before, mGnRH was found to preferentially form a β-II turn structure and [Lys^8]-GnRH an extended structure. Additionally, it was predicted that the extended form of mGnRH is strongly solvated relative to the β-II turn and vice versa for [Lys^8]-GnRH. Assuming that the β-II turn structure must desolvate to bind the receptor, mGnRH pays a small desolvation penalty upon binding whereas [Lys^8]-GnRH pays a large penalty. These differences in desolvation penalty could contribute to the differences in binding affinity of the two ligands.

It is clear that Lys^8 is detrimental to the formation of the conformer believed to be bioactive at mammalian type I receptors (Guarnieri and Weinstein, 1996; Mezei and Guarnieri, 1998). Indeed, Lys^8 is not found in any of the naturally occurring GnRHs identified in jawed vertebrates (Table 2.8). Lamprey and tunicate GnRHs are likely to have different conformations compared with other GnRHs. This is reflected in the biological activity of these ligands at jawed vertebrate receptors. Lamprey I was shown to have a very low affinity for sheep and rat GnRH receptors, and very low gonadotropin-releasing activity at sheep and chicken GnRH receptors (Millar et al., 1989). Lamprey III was found to be 50-200 times weaker than mGnRH at increasing the serum LH and FSH levels in ovarectomized rats (Kovacs et al., 2002). Lamprey and tunicate GnRHs have an L-amino acid in position 6 that, unlike glycine, is likely to disrupt the formation of the β-II′ turn (Momany, 1976b; Monahan et al., 1973). It is interesting to note, however, that lamprey I, lamprey III and tunicate I have a negatively charged Glu^6, Asp^6 or Asp^5 along with the positively charged Lys^8 (shown in blue in Table 2.8). Perhaps a salt bridge between these residues provides some form of turn structure, which positions the N and C termini for cognate receptor binding (Powell et al., 1996).

Tunicate II GnRH (tGnRH-II) is especially different from other GnRHs as it forms a dimer (Powell et al., 1996). Cys^6 residues (shown in green in Table 2.8)

were linked by a disulphide bond, which was broken under reducing conditions. It was suggested that each of the subunits binds to a receptor molecule, thereby enhancing receptor dimerisation (receptor dimerisation/oligomerisation is beyond the scope of review in this thesis: see reviews by Dean et al., 2001; Devi and Brady, 2000; Rios et al., 2001). The steric hindrance of side chains from each subunit could conceivably result in the decapeptides assuming turn structures, thus enabling the N and C termini to again interact with the receptor.

NMR studies have provided direct structural evidence for the conformations of mGnRH and cGnRH I (Maliekal et al., 1997). 192 structures of mGnRH were obtained, most of which could be grouped into three families. The backbone conformations were very similar in all these families (Figure 2.7a). They all exhibited a β type turn about Gly^6 and closely apposed N and C termini, thereby agreeing with the studies discussed above (Freidinger et al., 1980; Guarnieri and Weinstein, 1996; Mezei and Guarnieri, 1998; Millar et al., 1984; Momany, 1976a).

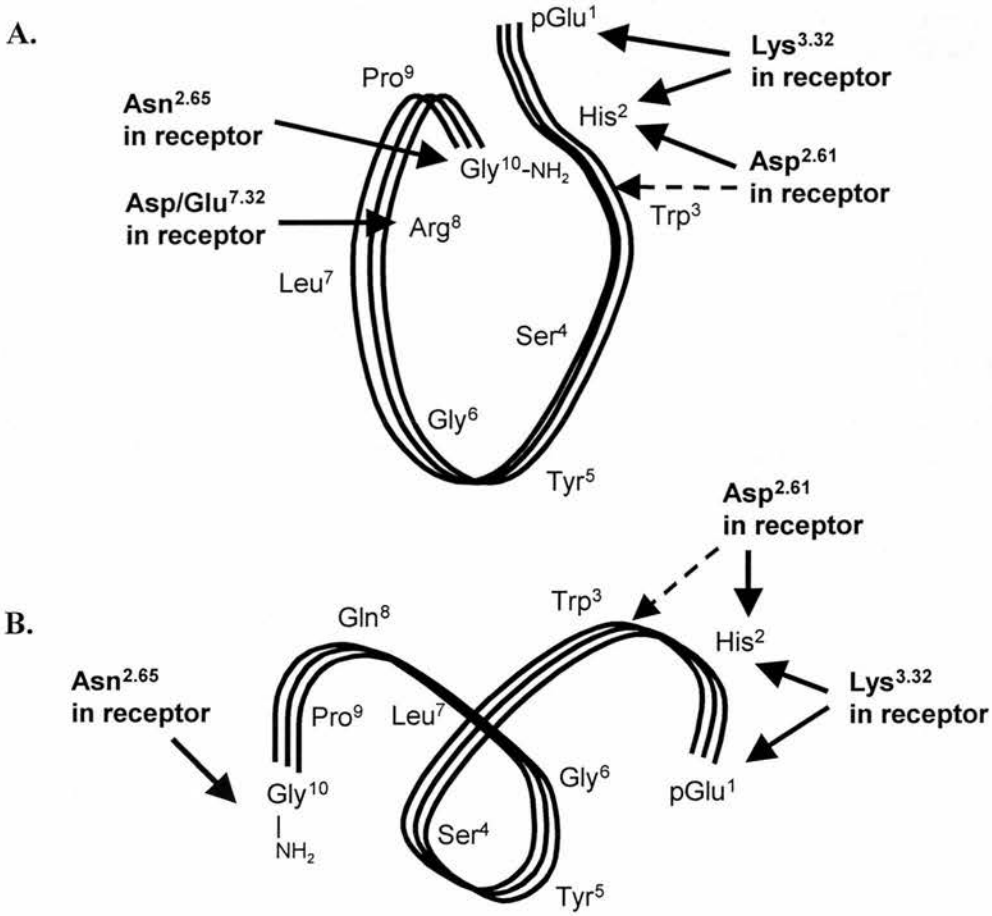


Figure 2.7. The mGnRH **(A)** and cGnRH I **(B)** backbones, as deduced from NMR (Maliekal et al., 1997), showing putative interactions with GnRH receptor residues

Several hydrogen bonds were present in all three mGnRH structures, although only two were in all of them: one between the carbonyl oxygen of Ser^4 and the side chain amino group hydrogen of Arg^8, and a second between the Gly^{10} NH hydrogen and the $pGlu^1$ carbonyl oxygen (Maliekal et al., 1997). Additionally, the Arg^8 side chain was involved in at least one other hydrogen bond in all of the structures: either with the side chain of His^2 or the side chain of Tyr^5. This supported the suggestions of a His^2-Tyr^5-Arg^8 structural unit based on fluorimetric titration data (Shinitzky and Fridkin, 1976; Shinitzky et al., 1976).

The 192 structures obtained for cGnRH I were largely grouped into four families (Maliekal et al., 1997). In all of these, a turn conformation was observed around Gly^6 as with mGnRH (Figure 2.7b). However, the conformations appeared to be more variable, and the N and C termini were not closely apposed. None of the conformers had a hydrogen bond between Ser^4 and Gln^8, or between Gly^{10} and $pGlu^1$. However, all conformers did possess several hydrogen bonds, with one between Ser^4 and $pGlu^1$ in all four families.

2.6.5 Summary

An enormous number of GnRH peptide agonists and antagonists have been characterised since mGnRH was first sequenced (Matsuo et al., 1971). Residues responsible for receptor binding and/or activation have been identified, along with a number of modifications that increase potency.

Conformational energy analysis, conformational constraint, fluorimetric titration, antibody recognition, conformational memories and NMR have all provided evidence of the bioactive conformation of mGnRH, in particular the presence of a β-II′ turn from residue 5 to 8 in mGnRH acting at mammalian type I GnRH receptors.

However, the current literature is unclear as to the conformation of this crucial central region in other forms of GnRH, or indeed the bioactive conformation of GnRHs interacting with non-mammalian receptors (Sealfon et al., 1997). The relatively low sequence homology and differing structural features (see Section 2.5) have led to suggestions that the bioactive conformational requirement of non-mammalian receptors may differ from that of mammalian type I receptors (Sun et al., 2001).

The study described in Chapter 4 investigates the effect of constraining the β-II′ turn on the binding affinity of mGnRH, cGnRH I, sGnRH and GnRH II at mammalian type I and non-mammalian GnRH receptors.

2.7 Receptor Structure-Activity Relationships

2.7.1 The Use of Chimeric Receptors

Generally, GPCRs that are very closely related in their primary amino acid sequence can still be distinguished pharmacologically. Such GPCRs may be subtypes of the same receptor (Frielle et al., 1988), or from different species (Beinborn et al., 1993). By using molecular techniques (see Chapter 3), regions of one receptor can be replaced with the corresponding regions of a related receptor (see Table 2.10 for examples and references). The resulting chimera can often be transfected and expressed successfully in cell lines. If a series of such chimeras are produced with different combinations of receptor regions, it is theoretically possible to establish the domains that account for the differences in pharmacology. This procedure can be useful for the rapid identification of functional domains, including those involved in ligand-receptor interactions (Frielle et al., 1988). Screening for contact sites by mutating individual amino acid residues may be more time consuming. Establishing the functional domain as a prelude to point mutations can considerably reduce the effort required in identifying important amino acids. For these reasons, chimeric receptors were utilised in this thesis to study the role of extracellular loops in the binding of agonists and antagonists at the GnRH receptor.

The use of chimeric receptors is not without problems. Firstly, such artificial combinations of receptor regions may not result in a functional protein correctly folded and expressed in the plasma membrane, particularly as TMDs in close proximity have probably evolved to maximise favourable interactions and minimise steric hindrance and electrostatic repulsion (Frielle et al., 1988). Secondly, replacement of large regions may result in conformational changes that complicate the interpretation of results (Huang et al., 1994). The use of chimeric receptors should be followed up with point mutation studies within the region of interest. Data derived from point-mutated receptors should also be interpreted with caution, as differences in local receptor conformation may cause incorrect presentation of a particular residue. Therefore, it is preferable to combine data from chimeric and point-mutated receptors (Huang et al., 1994).

Peptide hormones of the size of GnRH and neurokinins bind to both the TMDs and the extracellular domains of their cognate receptors (Fong et al., 1992; Strader et al., 1995). This is in contrast to the biogenic amines, which appear to bind entirely within the TMDs (Dixon et al., 1987; Strader et al., 1995).

Table 2.10. Examples of chimeric receptors used to identify regions involved in ligand-receptor interactions

Combination of Receptors:	Reference:
β_1 and β_2 adrenergic	(Dixon et al., 1989; Frielle et al., 1988; Marullo et al., 1990)
α_2 and β_2 adrenergic	(Kobilka et al., 1988; Mizobe et al., 1996)
M_2 and M_3 muscarinic acetylcholine	(Wess et al., 1990)
Neurokinin-1 and 2	(Gether et al., 1993b)
Neurokinin-1 and 3	(Fong et al., 1992; Gether et al., 1993a; Huang et al., 1994)
Human and canine cholecystokinin-B	(Beinborn et al., 1993)
α and β interleukin-8	(LaRosa et al., 1992)
Thyrotropin and luteinising hormone	(Nagayama et al., 1991)
C5a and formyl peptide	(Perez et al., 1993)

Substance P is selective for NK_1 (neurokinin-1) receptors, neurokinin A for NK_2 receptors, and neurokinin B for NK_3 receptors (Fong et al., 1992). Two studies of particular relevance to this thesis involved chimeric tachykinin receptors produced by substituting the extracellular domains of the NK_3 receptor into the NK_1 receptor. The second study (Huang et al., 1994) investigated point mutations in regions identified in the first study (Fong et al., 1992). None of the substitutions reversed the relative affinities of substance P and neurokinin B, or completely accounted for the specificity of an NK_1-selective non-peptide antagonist (L-703,606), leading the authors to conclude that some residues within the TMDs were also responsible for ligand selectivity. They did not consider possible interactions between extracellular domains, a concept investigated in this thesis with respect to the GnRH receptor (Chapters 5 and 6).

Several residues in the N-terminus, ECL1 and ECL2 of the tachykinin receptors were found to be required for high affinity agonist binding, but not binding of the non-peptide antagonist L-703,606 (Fong et al., 1992; Huang et al., 1994). Different residues, in ECLs 2 and 3, appeared to influence binding of this antagonist. A competitive antagonist interacts with a binding pocket or region that overlaps, but is not necessarily identical to, the agonist binding pocket (Flower, 1999). Therefore,

it is expected that some contact sites differ between agonists and antagonists. The tachykinin receptor studies also identified residues in ECLs 2 and 3 that appeared to affect the binding of neurokinin B, but not substance P (Fong et al., 1992; Huang et al., 1994). This illustrates that agonist binding can also involve different residues, although interactions that result in receptor activation are likely to be conserved. Results presented in this thesis highlight differences in the binding of ligands at the GnRH receptor, not only between agonists and antagonists, but also between different agonists (Chapters 5 and 6).

2.7.2 Residues Involved in Ligand-Receptor Interactions

Point-mutation studies of many GPCRs have established key residues in particular receptors that are important for ligand binding (van Rhee and Jacobson, 1996). Due to the diversity of ligands interacting with GPCRs, conservation of contact residues is generally limited to sub-groups of receptors that interact with similar ligands, such as the biogenic amines (Strader et al., 1995). However, it is now clear that certain loci are 'functionally conserved'. These positions are critical for ligand binding in a range of receptors and they differ in nature to complement their different cognate ligands (Ballesteros et al., 2001). The conserved overall structure of Family A GPCRs, particularly in the transmembrane region, indicates that residue positions important for binding in one receptor may well be exposed to the binding pocket/region in other receptors of the family. This provides a good starting point for the identification of ligand interaction sites in newly modelled receptors (Flower, 1999). Such residue positions may not interact with the physiological cognate ligand in other receptors, however, they do have the potential to influence the conformation of the binding pocket and may provide contact sites for novel ligands.

The biogenic amine receptors are the most widely studied sub-group of GPCRs (Strader et al., 1994). As mentioned in Section 2.7.1, chimeric receptor studies indicated that the ligand binding domain was located within the TMDs (Dixon et al., 1987). The basic nature of biogenic amines implicated the presence of an acidic counterion in the receptor. This was identified as $Asp^{113(3.32)}$ in TMD3 of the β_2-adrenergic receptor (Strader et al., 1987; Strader et al., 1988) and the chemical nature of the residue in this position was found to be critical (Strader et al., 1991). In the M_1 muscarinic acetylcholine (Fraser et al., 1989), H_1 histamine (Ohta et al., 1994), H_2 histamine (Gantz et al., 1992), D_2 dopamine (Mansour et al., 1992),

5-HT_{1A} (Ho et al., 1992) and 5-HT_2 receptors (Wang et al., 1993) the homologous residue was also identified as a ligand contact site. Studies of the α_2 adrenergic receptor were consistent with a similar role for the homologous residue in this receptor, however, alteration of normal receptor processing or insertion into the membrane were not discounted (Wang et al., 1991).

It is clear that $Asp^{3.32}$ is critical for binding biogenic amines, however, peptides such as GnRH have a very different structure (Strader et al., 1995). Despite this, the homologous $Lys^{3.32}$ in mammalian and non-mammalian GnRH receptors (Figure 2.8) has been identified as critical for binding GnRH agonists, although not antagonists (Blomenrohr et al., 2001; Zhou et al., 1995). Mutation of $Lys^{121(3.32)}$ to Arg had little effect on agonist binding, whereas, mutation to Gln abolished agonist, but not antagonist binding. These mutant receptors were able to stimulate IP turnover, although the EC_{50} obtained with $Lys^{121(3.32)}$Gln was 3 orders of magnitude higher than wild type (Zhou et al., 1995). No IP turnover was detected with $Lys^{121(3.32)}$Leu or $Lys^{121(3.32)}$Asp mutant receptors. Therefore, it would seem that the positively charged nature of $Lys^{121(3.32)}$ is important for high affinity agonist binding. As there is not a suitable counterion for $Lys^{121(3.32)}$ in mGnRH or GnRH II, the results are consistent with a charge-strengthened hydrogen bond interaction with these ligands. It was speculated that this involved the electron-dense aromatic rings of His^2 or Trp^3, or the polar imino group of His^2 (Zhou et al., 1995). Molecular modelling has subsequently indicated that $pGlu^1$ and/or His^2 are likely to form these interactions (Hoffmann et al., 2000) (Figure 2.7).

The TMD3/ICL2 boundary, (I/L)XXDR(Y/S)XX(I/V)XXPL, contains consensus sequences believed to be critical for receptor activation (Arora et al., 1995; Ballesteros et al., 1998). It is conceivable that agonist interaction with $Lys^{121(3.32)}$ is part of the mechanism of stabilising the active receptor conformation.

As discussed in Section 2.7.1, peptide hormones of the size of GnRH bind to both the TMDs and the extracellular domains of their cognate receptors. $Asp^{98(2.61)}$ at the TMD2/ECL1 boundary of the human GnRH receptor (Figure 2.8) is believed to interact with His^2 of GnRH/GnRH II, and possibly the backbone NH group of Trp^3 as well (Flanagan et al., 2000) (Figure 2.7). This was proposed because position 2-substituted analogues were found to be less sensitive to $Asp^{98(2.61)}$ mutations. The putative interaction of $Asp^{98(2.61)}$ with His^2 in the ligand was not believed to be ionic in nature. Nevertheless, it did appear that the charge of $Asp^{98(2.61)}$ had a function. It was suggested to either interact with one or more functional groups common to all the peptides tested and/or influence binding pocket configuration by forming intramolecular interactions (Flanagan et al., 2000).

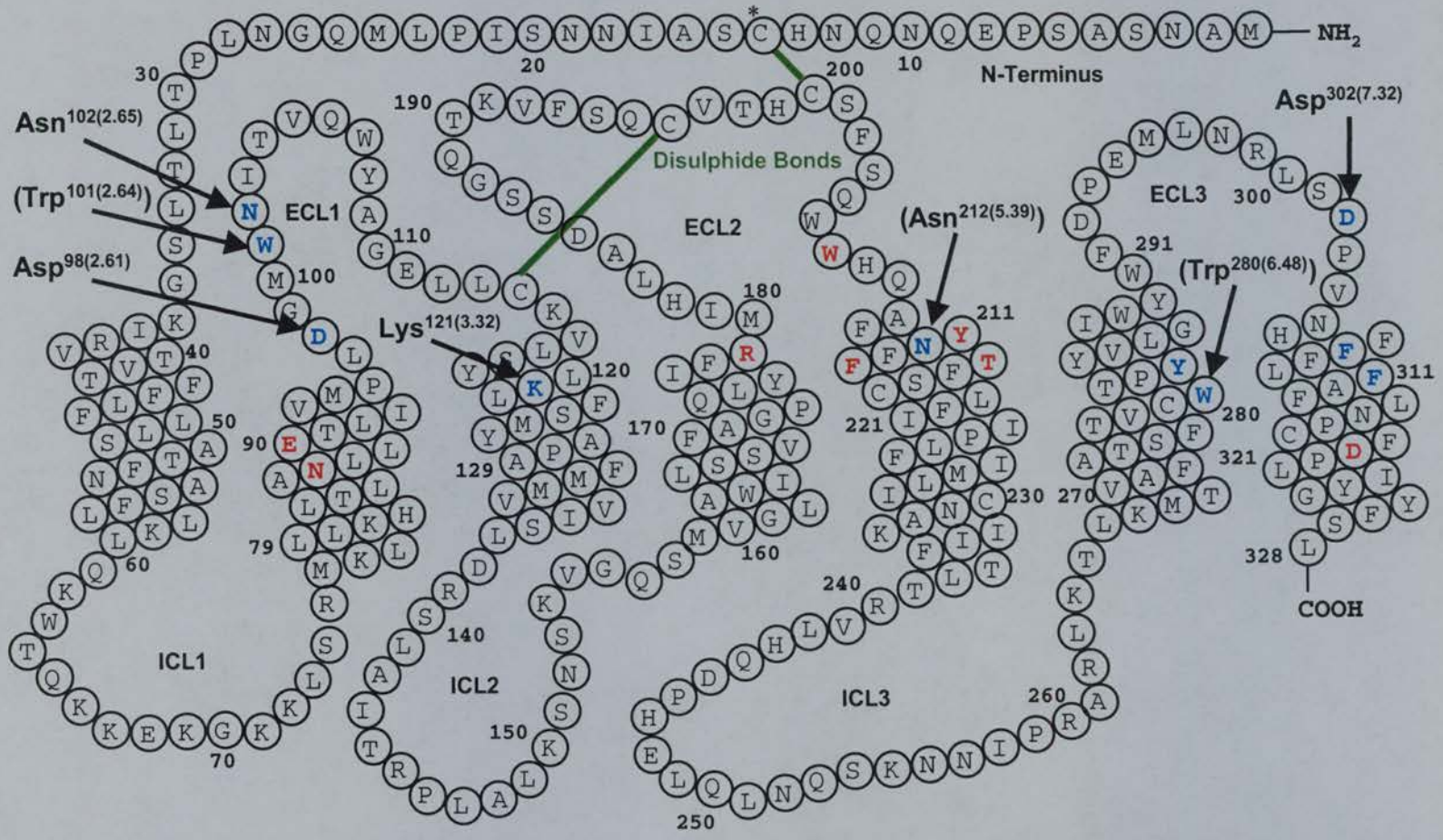


Figure 2.8.
Schematic representation of the human GnRH receptor, showing residues involved in binding and conformation

Putative mGnRH binding sites labelled with arrows
*, Interacts with [Azidobenzoyl-D-Lys^6]-GnRH and azido-labelled antagonist
Residues believed to be involved in the agonist binding pocket indicated in blue
Residues that appear to influence general receptor configuration indicated in red

$Asn^{102(2.65)}$ in ECL1 of the human receptor (Figure 2.8) is believed to interact with the C-terminal glycinamide of GnRH/GnRH II (Davidson et al., 1996) (Figure 2.7). Mutation of $Asn^{102(2.65)}$ to Ala resulted in potency losses of between 95 and 750-fold with a range of agonists possessing a C-terminal glycinamide, but had less of an impact on the binding of agonists and antagonists possessing a different C-terminus, such as [Pro^9-NEt]-GnRH and Cetrorelix (Davidson et al., 1996; Hoffmann et al., 2000). Given the high affinity of these analogues, their C-termini are likely to make alternative contacts (Millar, 2002).

In ECL3, $Asp/Glu^{7.32}$ in mammalian type I receptors (Figure 2.8) is believed to interact with Arg^8 in mGnRH (Flanagan et al., 1994; Fromme et al., 2001) (Figure 2.7). $Glu^{301(7.32)}$ in the mouse GnRH receptor was initially identified as a ligand binding site, following screening of conserved acidic acid residues in the extracellular domains (Flanagan et al., 1994). The specificity of $Glu^{301(7.32)}$ for Arg^8 was tested by comparing ligand binding and IP turnover at the wild type and $Glu^{301(7.32)}$Gln mutant mouse receptors, using a range of position 8-substituted GnRH analogues. A subsequent study identified a similar role for $Asp^{302(7.32)}$ in the human GnRH receptor, by testing position 8-substituted GnRH analogues at wild type and $Asp^{301(7.32)}$Asn mutant human receptors (Fromme et al., 2001). Both of these studies found that ligand conformational constraint by D-amino acid substitution for Gly^6 overcame the need for $Asp/Glu^{7.32}$ in the receptor and Arg^8 in mGnRH. It was proposed that the interaction of $Asp/Glu^{7.32}$ with mGnRH is transient, its role being to induce a high affinity ligand conformation in an initial binding step, which precedes formation of the final ligand-receptor complex (Fromme et al., 2001).

It was speculated that the chicken GnRH receptor would lack an acidic residue in position 7.32 due to its apparent inability to select between mGnRH and [Gln^8]-GnRH (Flanagan et al., 1994). However, its subsequent cloning revealed the presence of $Glu^{7.32}$ (Sun et al., 2001) and an alternative explanation regarding differences in receptor conformation was put forward. In mammalian type I GnRH receptors, $Asp/Glu^{7.32}$ is followed by proline (S(D/E)P), whereas in non-mammalian type I GnRH receptors it is preceded by proline (P(D/E)Y). This difference may alter the orientation of the Asp/Glu side-chain, thereby affecting the interaction with Arg^8 in mGnRH (Millar et al., 1997; Petry et al., 2002). NMR, Raman and FT Raman spectroscopy were used to analyse the structure of a cyclised peptide (CG PEMLNRVSEP GC) representing ECL3 of the mouse GnRH receptor (Petry et al., 2002). Glycine and cysteine residues were added to each end of the ECL3 peptide, and a disulphide bond connected the cysteines to simulate the distance between the extracellular ends of TMDs 6 and 7, as estimated from the crystal structure of

rhodopsin (Palczewski et al., 2000). The peptide was found to have a random coil conformation, except perhaps for a β-turn around the central residues, $L^{296}N^{297}R^{298}$ (Petry et al., 2002). This secondary structure was proposed to position the Glu^{301} residue for interaction with Arg^8 in mGnRH. The presence of a proline residue between the putative β-turn and Glu^{301}, as in non-mammalian type I receptors, may affect this presentation. However, the implication that in non-mammalian type I receptors the interaction does not occur is now questionable, as Arg^8 in mGnRH appears to interact with $Asp^{7.32}$ in the catfish receptor (Blomenrohr et al., 2002). In this study, mutation of $Asp^{304(7.32)}$ to Glu, thereby conserving the negative charge, had no affect on the binding of mGnRH, catfish GnRH (cfGnRH, [His^5,Asn^8]-GnRH) or GnRH II. In contrast, mutation to Asn or Ala reduced the binding affinity of mGnRH, but not that of cfGnRH or GnRH II. The study described in Chapter 5 investigates ligand-receptor interactions at the human and catfish GnRH receptors, including addressing this apparent contradiction.

GnRH II does not appear to interact with the $Asp/Glu^{7.32}$ residue, which is unsurprising considering the requirement for Arg^8 in the ligand (Blomenrohr et al., 2002; Flanagan et al., 1994; Fromme et al., 2001). However, GnRH II has a relatively high affinity for mammalian type I receptors, as discussed in Section 2.6.2. As conformationally constrained ligands also bind these receptors with high affinity without the $Asp/Glu^{7.32}$-Arg^8 interaction, perhaps native GnRH II is also somewhat constrained by intramolecular interactions. The study described in Chapter 4 provides evidence for this situation.

[Azidobenzoyl-D-Lys^6]-GnRH has been shown to photoaffinity cross-link with $Cys^{14(1.11)}$ in the N-terminal domain (Figure 2.8) causing irreversible activation of the receptor. This indicates that, when GnRH is positioned in the binding pocket in such a way that it stabilises the active receptor conformation, the residue in position six is in the proximity of $Cys^{14(1.11)}$ (Davidson et al., 1997). However, as an antagonist [Ac-(4-azidobenzoyl)-D-Lys^1,D-4-Cl-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]-GnRH, in which the reactive group is in position one, also cross-linked to $Cys^{14(1.11)}$ (Assefa et al., 1999), it appears that there is considerable movement of the azido-labelled D-Lys side chain, which enables it to target the highly reactive $Cys^{14(1.11)}$ regardless of the position in which the group is attached to GnRH. Thus, while photoaffinity labelling gives some idea of the binding position of GnRH, the flexibility of the labelled side chain is such that it does not give definitive information on contact sites.

$Trp^{6.48}$ in the GnRH receptor (labelled in brackets in Figure 2.8) may interact with Trp^3 in mGnRH in a model where it is part of an aromatic locus along with

$Tyr^{6.51}$, $Phe^{7.38}$ and $Phe^{7.41}$ (Chauvin et al., 2000). Mutation of $Trp^{279(6.48)}$ to Ser in the rat GnRH receptor reduced agonist binding affinity and receptor expression, and abolished IP turnover. Mutation to Arg abolished agonist binding and IP turnover, but expression was demonstrated using an enhanced green fluorescent protein (EGFP)-tagged $Trp^{279(6.48)}$Arg mutant receptor.

Various other residues have been identified in the human receptor as affecting receptor expression and/or ligand binding. The effect of mutating $Asn^{2.50}$ and $Asp^{7.49}$ (indicated in red in Figure 2.8) was discussed in Section 2.3.4. Mutation of $Glu^{90(2.53)}$, $Arg^{179(4.65)}$, $Trp^{206(5.33)}$, $Tyr^{211(5.38)}$, $Phe^{214(5.41)}$ or $Thr^{215(5.42)}$ (indicated in red in Figure 2.8) to Ala abolished agonist binding, signal transduction and antagonist binding, probably due to incorrect receptor folding (Hoffmann et al., 2000). In the same study, $Trp^{101(2.64)}$ and $Asn^{212(5.39)}$ (labelled in brackets in Figure 2.8) were found to differentiate between agonists and antagonists, implying a role in the configuration of the agonist binding pocket. The model of [D-Trp^{6}]-GnRH binding to the human GnRH receptor proposed by Hoffmann and co-workers suggested an interaction of Trp^{101} with the Leu^{7} backbone oxygen via a hydrogen bond. It also suggested that Asn^{212} interacts with the His^{2} backbone oxygen, again via a hydrogen bond.

Residues in TMDs 5 and 6 of various Family A GPCRs have been shown to be important for ligand binding. Those in TMD5 include: $Thr^{5.39}$ and $Thr^{5.42}$ in M_1 and M_3 muscarinic acetylcholine receptors (Allman et al., 2000; Wess et al., 1991); $Ser^{5.42}$, $Ser^{5.43}$ and $Ser^{5.46}$ in β_2-adrenergic and D_1 dopamine receptors (Liapakis et al., 2000; Pollock et al., 1992; Strader et al., 1989); $Ser^{5.42}$ and $Ser^{5.46}$ in the α_{2A}-adrenergic receptor (Wang et al., 1991); $His^{5.39}$ in the NK_1 receptor (Fong et al., 1993); $Lys^{5.42}$ in the angiotensin II receptor (Yamano et al., 1992); $Asp^{5.42}$ and $Thr^{5.46}$ in the H_2 histamine receptor (Gantz et al., 1992); and $Asn^{5.46}$ in the H_1 histamine receptor (Ohta et al., 1994). Those in TMD6 include: $Trp^{6.48}$ and $Phe^{6.52}$ in the 5-HT_{2A} receptor (Roth et al., 1997); $Tyr^{6.51}$ in M_1 and M_3 muscarinic acetylcholine receptors (Ward et al., 1999; Wess et al., 1991); and $Phe^{6.51}$ in the α_{1B}-adrenergic receptor (Chen et al., 1999).

An "aromatic cluster" of residues has been shown to exist in the agonist binding pocket of the D_2 dopamine receptor by the substituted-cysteine accessibility method (Javitch et al., 1998). This cluster is centred around TMD6, but also extends to TMDs 5 and 7. It is believed to play a role in agonist binding and receptor activation in a number of GPCRs (Javitch et al., 1998; Roth et al., 1997).

The study by Hoffmann and co-workers discussed above identified the residue $Asn^{212(5.39)}$ in TMD5 of the human GnRH receptor as a potential ligand contact site, and four other residues that are likely to influence receptor folding. The study by Chauvin and co-workers, proposed that $Trp^{279(6.48)}$ in the rat GnRH receptor was also a potential ligand contact site. Combined with the body of evidence from other Family A GPCRs implicating TMDs 5 and 6 as important constituents of the ligand binding pocket, it is likely that the configuration of these TMDs is also important for ligand binding at the GnRH receptor. The studies described in Chapters 5 and 6 suggest interactions between ECLs 2 and 3 can influence the configuration of the ligand binding pocket in the GnRH receptor, perhaps by affecting the configuration of TMDs 5 and 6 to which they are anchored.

A non-peptide antagonist differentiated between the human and dog GnRH receptors, despite these receptors being 92% identical (Cui et al., 2000). The basis for the difference in affinity was found to be a single residue in TMD7, namely $Phe^{313(7.43)}$ in the human receptor and $Leu^{312(7.43)}$ in the dog receptor. One of the studies described in Chapter 6 provides evidence for a single residue in ECL2 causing GnRH II to differentiate between the human and rat GnRH receptors.

2.7.3 Summary

Both chimeric and point-mutated receptors can be utilised to establish receptor domains and/or residues involved in ligand-receptor interactions. Data from each of these types of construct should be interpreted with caution and ideally evaluated in combination. It is critical to consider the conformation of receptor domains and understand that ligand contact sites must be present **and** orientated correctly. A number of GnRH receptor residues have been identified as interacting with ligands and/or contributing to the conformation of the ligand binding pocket/domain. Recently, it has been proposed that residues from TMDs 5 and 6 have a similar role (Chauvin et al., 2000; Hoffmann et al., 2000). The studies described in Chapters 5 and 6 of this thesis increase our understanding of how the ECLs influence binding of mGnRH, [D-Trp^6]-GnRH, GnRH II, [D-Lys^6]-GnRH II and Antagonist 135-18 at the GnRH receptor, using a combination of chimeric and point-mutated receptors. The findings of these studies suggest that the spatial arrangement of TMDs 5 and 6 is important for the binding of GnRH II, [D-Lys^6]-GnRH II and Antagonist 135-18.

3 General Materials and Methods

3.1 Amino Acid Numbering Scheme

This scheme facilitates comparisons between GPCRs and has been described previously (Ballesteros and Weinstein, 1995). The most conserved residue in each TMD is assigned the TMD number followed by the index 50. Therefore, in the human GnRH receptor, the conserved residues Asn^{53}, Asn^{87}, Arg^{139}, Trp^{164}, Pro^{223}, Pro^{282} and Pro^{320} are designated $Asn^{1.50}$, $Asn^{2.50}$, $Arg^{3.50}$, $Trp^{4.50}$, $Pro^{5.50}$, $Pro^{6.50}$ and $Pro^{7.50}$ (See page xii). Other residues are then numbered relative to these positions, e.g. $Asp^{2.61(98)}$.

3.2 Molecular Cloning Techniques

The molecular cloning strategy is summarised in Figure 3.1.

3.2.1 The Polymerase Chain Reaction (PCR)

For each PCR reaction, two different oligonucleotide primers were designed. These annealed to opposite strands of the template DNA, at either end of the region to be amplified. The DNA was denatured by heating to 94°C and then cooled to allow the primers to anneal. The annealing temperature depended on the sequences of the primers, but was usually between 52 and 56°C. An approximate value was calculated as follows:

$$(A + T) \times 2 + (G + C) \times 4$$

where letters represent the number of bases of that type
(A=adenine, T=thymine, G=guanine, C=cytosine)

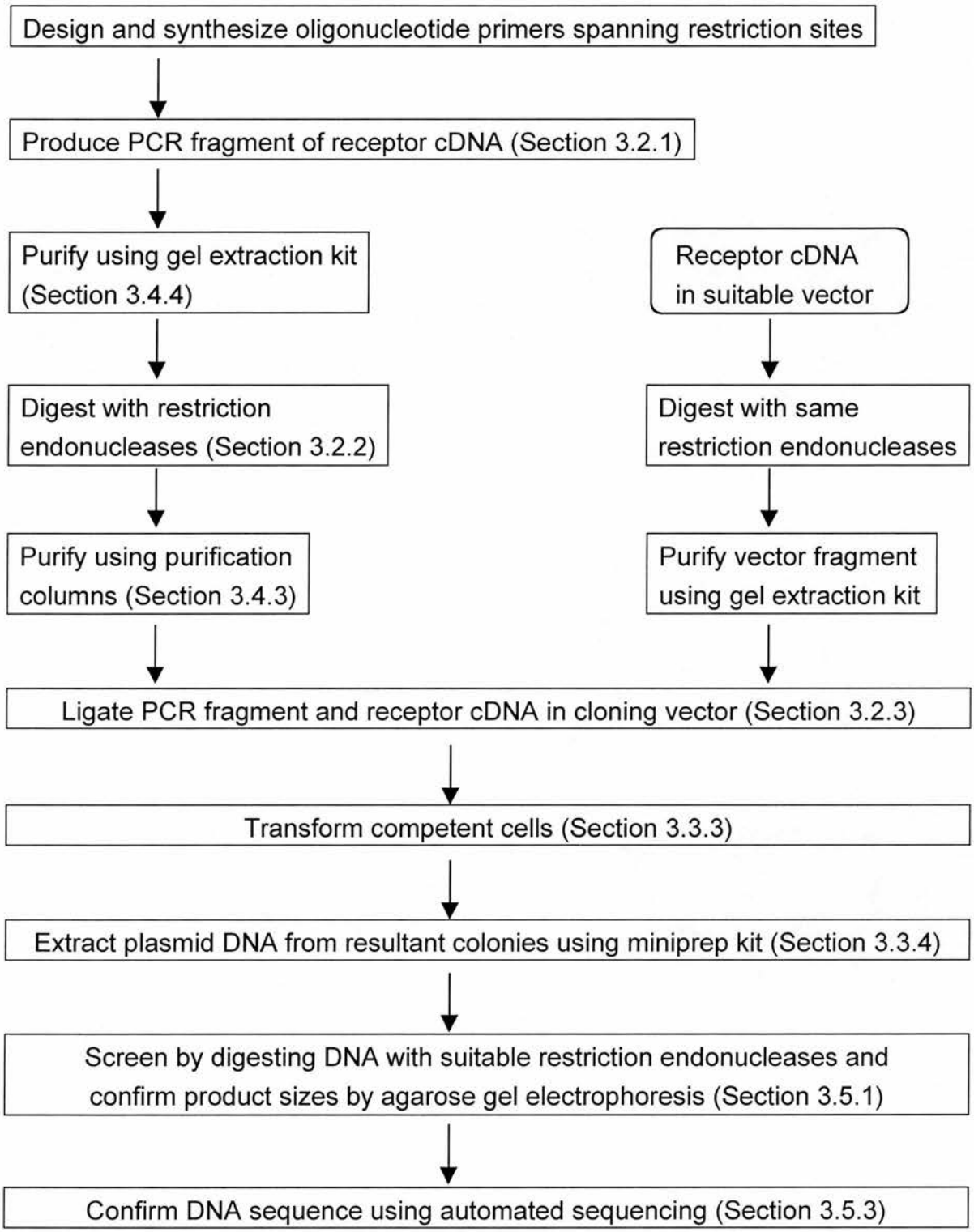


Figure 3.1. Flow Chart summarising the molecular cloning strategy

Increasing the annealing temperature reduced efficiency, but increased specificity (and vice versa). At 72°C, the primer sequences were extended by the thermostable *Taq* DNA polymerase, acting in the presence of excess deoxynucleoside triphosphates (dNTPs).

For example, the PCR used for producing the catfish receptor ECL cDNA (approximately 60 bp) consisted of an initial 5 min denaturing step at 94°C followed by 30 cycles of 1 min at 94°C, 30 s at the annealing temperature and 30 s at 72°C. There was then a final polymerisation step of 5 min at 72°C. The products from each round provided additional template for successive rounds. Therefore the segment of DNA between, and including, the primers was amplified exponentially. Longer PCR products require more time for extension at 72°C.

3.2.2 Digestion of DNA using Restriction Endonucleases

Restriction endonucleases used in molecular biology are able to cut double stranded DNA at a particular recognition site. This site generally has a specific sequence, but some sequences are degenerate. They are usually between four and six nucleotides in length, often with two-fold symmetry. The enzyme may digest both strands at the same position producing DNA fragments with blunt termini. Alternatively, opposite strands are digested on either side of the axis of symmetry, resulting in termini with one of the strands protruding.

Optimal conditions for digestion are specific for each endonuclease, with different enzymes requiring different salt concentrations. Most endonucleases act optimally at 37°C, but there are several exceptions. BSA is added to a final concentration of 0.1 mg/ml. This has been shown to improve the activity of many restriction endonucleases and there are no known examples of it impeding activity (see www.promega.com/pnotes/60/6079_28/). Digestion with two endonucleases at the same time is possible if both enzymes are able to act in similar conditions. The total concentration of glycerol (in which the endonucleases are stored) should not exceed 10%, otherwise non-specific digestion is likely.

3.2.3 Ligation of DNA

As described in Section 3.2.2, many endonucleases produce protruding single strands. As the recognition site is symmetrical, a second DNA fragment digested with the same endonuclease will have a complementary protruding strand. With the aid of the enzyme T4 DNA ligase, these fragments can be joined together. In this thesis, Ready-To-Go™ T4 DNA ligase was used, following the manufacturer's protocol (Amersham Pharmacia) (see Appendix I for composition of reagents and Appendix II for addresses of suppliers). 20 µl of DNA solution was added to the tubes, which were then incubated at 16°C for 45 min. The ratio of DNA fragments in this solution was calculated to account for relative concentration and size (smaller DNA fragments have more termini per µg of DNA). When subcloning receptor DNA into vector DNA, the ratio of receptor DNA termini to vector DNA termini was 3:1.

DNA fragments with blunt termini were also ligated with this enzyme, although efficiency was greatly reduced. There was also a lack of specificity and this was particularly important when both ends of a DNA fragment had blunt termini. To prevent self-ligation, the 5' phosphates were removed using the enzyme calf intestinal alkaline phosphatase (CIP) (New England Biolabs).

3.3 Bacterial Production of Plasmid DNA

3.3.1 Microbiological Sterile Technique

All work involving live bacteria was carried out in a microbiological laboratory under flame. Bench surfaces were swabbed with Presept before and after work. LB Broth, LB Agar (Anachem) and pipette tips were autoclaved and opened under flame. Pipette tips and inoculating loops were flamed before and after use. Glass spreaders were sterilised with burning ethanol. All plasticware used was sealed in sterile packaging. Control cultures, not inoculated with *E. coli,* were grown in parallel with

experimental cultures to check for contamination. Waste was decontaminated by Presept or autoclaving.

3.3.2 Preparation of LB Agar Plates

LB agar was heated to 60°C and 100 μg/ml of ampicillin or 50 μg/ml of kanamycin added (Sigma-Aldrich), depending on the antibiotic resistance gene present in the vector. The agar was allowed to set in 100 mm^2 dishes, dried in a Class I microbiological hood and incubated at 37°C before use.

3.3.3 Transformation of E. coli

Electroporation

DNA, electrocompetent Top 10 F′ cells, the electroporation cuvette and the holder were placed on ice. 40 μl of Top 10 F′ cells and 0.5 μl of DNA were pipetted into the bottom of the cuvette. The mixture was treated with a 15 s pulse (2.5 V, 25 μF) and added to 960 μl of SOC medium (Invitrogen) before shaking at 37°C for 1 h at 250 rpm. Using a glass spreader, 200 μl of culture was spread on an LB agar plate containing antibiotic.

Heat-Shock

DNA and One Shot™ Top 10 cells (Invitrogen) were placed on ice. 1 μl of DNA was added to 50 μl of cells and incubated on ice for 20 min. The cells were heat-shocked for 45 s in a 42°C water bath and returned to ice for 2 min. 250 μl of pre-warmed SOC medium was added before shaking at 37°C for 1 h at 250 rpm. Using a glass spreader, 200 μl of culture was spread on an LB Agar plate containing antibiotic.

3.3.4 Amplification and Purification

Miniprep

Small quantities of DNA were produced using the QIAprep Spin Miniprep Kit (Qiagen). A single bacterial colony was used to inoculate 4 ml of LB Broth containing 100 μg/ml of ampicillin or 50 μg/ml of kanamycin, depending on the antibiotic resistance gene present in the vector. This culture was shaken at 37°C for 8 h at 250 rpm and 1.6 ml was centrifuged for 5 min at 6000 rpm. The plasmid DNA was purified following the manufacturer's protocol, including all optional steps. DNA was eluted in 50 μl of distilled water giving a concentration of 300-400 ng/μl.

Maxiprep

Large quantities of DNA were produced using the Wizard® *Plus* Maxiprep DNA Purification System (Promega). The manufacturer's protocol was followed with some modifications to optimise yield.

A 10 ml starter culture was inoculated as for the miniprep protocol and shaken at 37°C for 8 h at 250 rpm. This was added to 250 ml of LB Broth for a further 16 h under the same conditions. The culture was centrifuged at 4°C for 15 min at 5000 rpm. The supernatant was discarded and the bacterial cell pellet resuspended. The cells were lysed for 15 min followed by neutralisation. 15 ml of the Wizard® resuspension, lysis and neutralisation solutions were used respectively. Following centrifugation at 4°C for 20 minutes at 6000 rpm, the supernatant was filtered through Whatman GF/A filter paper (Merck). 25 ml of isopropanol (-20°C) was added followed by incubation at 4°C for at least 1 h. Following centrifugation at 4°C for 15 min at 6000 rpm, the supernatant was discarded and the DNA pellet resuspended in 2 ml of TE buffer at 70°C. The suspension was mixed with Wizard® purification resin and drawn through a Wizard® maxiprep column using a vacuum manifold. The column was washed with 25 ml of Wizard® column wash solution and 5 ml of 80% ethanol. Residual solution was removed by spinning the column for 5 min at 2500 rpm using a swing-bucket rotor. The

DNA was eluted in 1.5 ml of TE buffer or distilled water at 70°C by spinning the column as before. The concentration was approximately 500-2000 ng/μl.

3.4 Additional DNA Purification Techniques

3.4.1 Ethanol Precipitation

0.1 volumes of sodium acetate (3 M, pH 5.2) and 2.5 volumes of ethanol were added to the DNA solution. This was vortexed and stood at -20°C for 45 min to precipitate the DNA, before centrifuging at 4°C for 15 min at 15000 rpm to pellet the DNA. The ethanol was aspirated off and 1 ml of 70% ethanol added to remove residual salt. The centrifugation was repeated, the ethanol removed, and the pellet air-dried at 37°C. The pellet was resuspended in a suitable volume of TE buffer or distilled water.

3.4.2 Extraction with Phenol/Chloroform

This procedure removes proteins from nucleic acid preparations. 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the preparation, vortexed and centrifuged at 15000 rpm for 1 min. The top (aqueous) layer was removed, added to 1 volume of chloroform, shaken, and centrifuged as before. The top (aqueous) layer was again removed and an ethanol precipitation carried out as described in Section 3.4.1.

3.4.3 Purification Columns

The QIAquick Spin PCR Purification Kit (Qiagen) was used to purify DNA from enzymatic reactions. The manufacturer's protocol was followed. DNA was adsorbed to

a silica-gel membrane that did not bind impurities. With the aid of centrifugation, the DNA was washed with an ethanol-based solution and eluted in distilled water.

3.4.4 Gel Purification

Agarose Gel Electrophoresis

A 1% agarose mini-gel (0.5 g of agarose and 0.5 μl of ethidium bromide in 50 ml of TAE buffer) was placed in a model HE 33 mini gel tank (Amersham) and submerged in TAE buffer. For particularly small DNA fragments a gel of up to 2% was used. DNA was mixed with loading dye (Promega) and loaded into the wells. A suitable DNA ladder (such as the Promega 1 kb or 100 bp ladder) was loaded in parallel and 120 V was applied to the gel for 30 min. The DNA fragments were visualised under ultra-violet light. Longer wavelengths were used as much as possible to minimise DNA damage.

Extraction and Purification

The DNA fragment of interest was excised from the agarose gel using a clean scalpel and the QIAquick Spin Gel Extraction Kit (Qiagen) used to purify it, following the manufacturer's protocol. The gel slice was dissolved and the DNA purified using similar columns to those described in Section 3.4.3.

3.5 Analysis of DNA

3.5.1 Agarose Gel Electrophoresis

DNA was cut at a single site by a suitable restriction enzyme (see Section 3.2.2). It was run on a mini-gel (see Section 3.4.4) with a marker of known concentration loaded in parallel (for example, 5 µl of the Promega 100bp DNA ladder contains approximately 150 ng of the 500 bp fragment and 50 ng of each of the other ten DNA fragments). Both size and concentration were estimated by comparison with suitable markers. Contamination with single-stranded nucleic acid could be identified as additional bands.

3.5.2 Spectrophotometric Analysis

The DNA solution was diluted to a suitable concentration, for example a maxiprep believed to be at a concentration of 500-2000 ng/µl was diluted 100-fold. The optical density (OD) of 1 ml of diluted DNA was analysed in a spectrophotometer at wavelengths of 260 nm and 280 nm. The ratio of optical density at these wavelengths (OD_{260}/OD_{280}) should be 1.8. Protein or phenol contamination results in a lower ratio. A DNA concentration of 50 ng/µl has an OD_{260} value of approximately 1.

3.5.3 DNA Sequencing

The Sanger Method

The Sanger method of DNA sequencing includes a low concentration of one of the four 2',3'-dideoxynucleoside triphosphates (ddNTPs) in the polymerase reaction.

This reaction is similar to PCR, however as one primer is used instead of two, the rate of DNA synthesis is linear rather than exponential. Due to the lack of a hydroxyl residue at the 3' position of deoxyribose, the ddNTP is not able to form a phosphodiester bond with a subsequent dNTP, therefore chain termination occurs. The concentration of ddNTP is sufficiently low that termination occurs infrequently. Therefore oligonucleotides of various sizes will be present and by using the four different ddNTPs in four parallel reactions, oligonucleotides can be produced that terminate at every A, C, G or T in the template strand. By including [^{32}P]dNTPs or [^{35}S]dNTPs in the reaction, the oligonucleotides can be visualised following separation by polyacrylamide gel electrophoresis. The order of the resultant band terminations reflects the DNA sequence.

Automated DNA Sequencing

An automated version of the Sanger method was used to sequence DNA used in this thesis. Fluorescent ddNTPs were used, with each type emitting light of a different wavelength. Therefore, one reaction containing all four fluorescently labelled termination ddNTPs was used instead of four separate reactions. The DNA was either run on a polyacrylamide gel (for analysis with the ABI Prism™ 373 Genetic Analyser) or through a capillary containing polymer (ABI Prism™ 310 Genetic Analyser). A laser beam was used to excite the fluorescent dyes. The light emitted was then recorded and analysed using ABI Prism™ base-calling software.

Polymerase Reaction Conditions

The sequencing reaction mixtures contained: 3 µl of ABI Ready Reaction Mix (v1.0); 5 µl of Half Term buffer; 5 µl of primer at 5 ng/µl (see Appendix III); and 200-1000 ng of DNA. The volume was made up to 20 µl with distilled water. The reaction began with a hot-start at 95°C. This was followed by 25 cycles, each consisting of 95°C for 15 s, 50°C for 25 s and 60°C for 4 min. The DNA was then ethanol precipitated (see Section 3.4.1) and resuspended in either 4 µl of sequencing loading buffer for analysis

on the ABI Prism™ 373 Genetic Analyser, or 8 µl of ABI Template Suppression Reagent for analysis on the ABI Prism™ 310 Genetic Analyser.

Analysis of Sequencing Data

Data was analysed using GeneJockey (Biosoft) sequence analysis software.

3.6 Engineered Human GnRH Receptor cDNA

The human GnRH receptor cDNA construct (Chi et al., 1993) had previously been engineered by T. Ott to include a number of silent mutations, thereby introducing restriction sites at the TMD/ECL boundaries (Ott et al., 2002). These sites are illustrated in Figure 3.2 with respect to the receptor amino acid sequence.

Point mutations and substitution of receptor ECL cDNA were carried out using this construct in one of two cloning vectors, pBluescript or pZErO-2 (Figure 3.3). These vectors contained less restriction sites compared with vectors such as pcDNA1, thereby avoiding the need to ligate more than two DNA fragments at any one stage. The ampicillin resistance gene in pBluescript contained a *Sca* I restriction site, however, as pZErO-2 was kanamycin-resistant instead of ampicillin-resistant, it lacked this site (Figure 3.3). This was advantageous when cloning into the *Sca* I restriction site at the ECL1/TMD3 boundary. Following engineering in the cloning vectors, the resultant cDNA was subcloned into pcDNA1/Amp for transfection into COS-7 cells, as this vector contains a eukaryotic promoter (Figure 3.3).

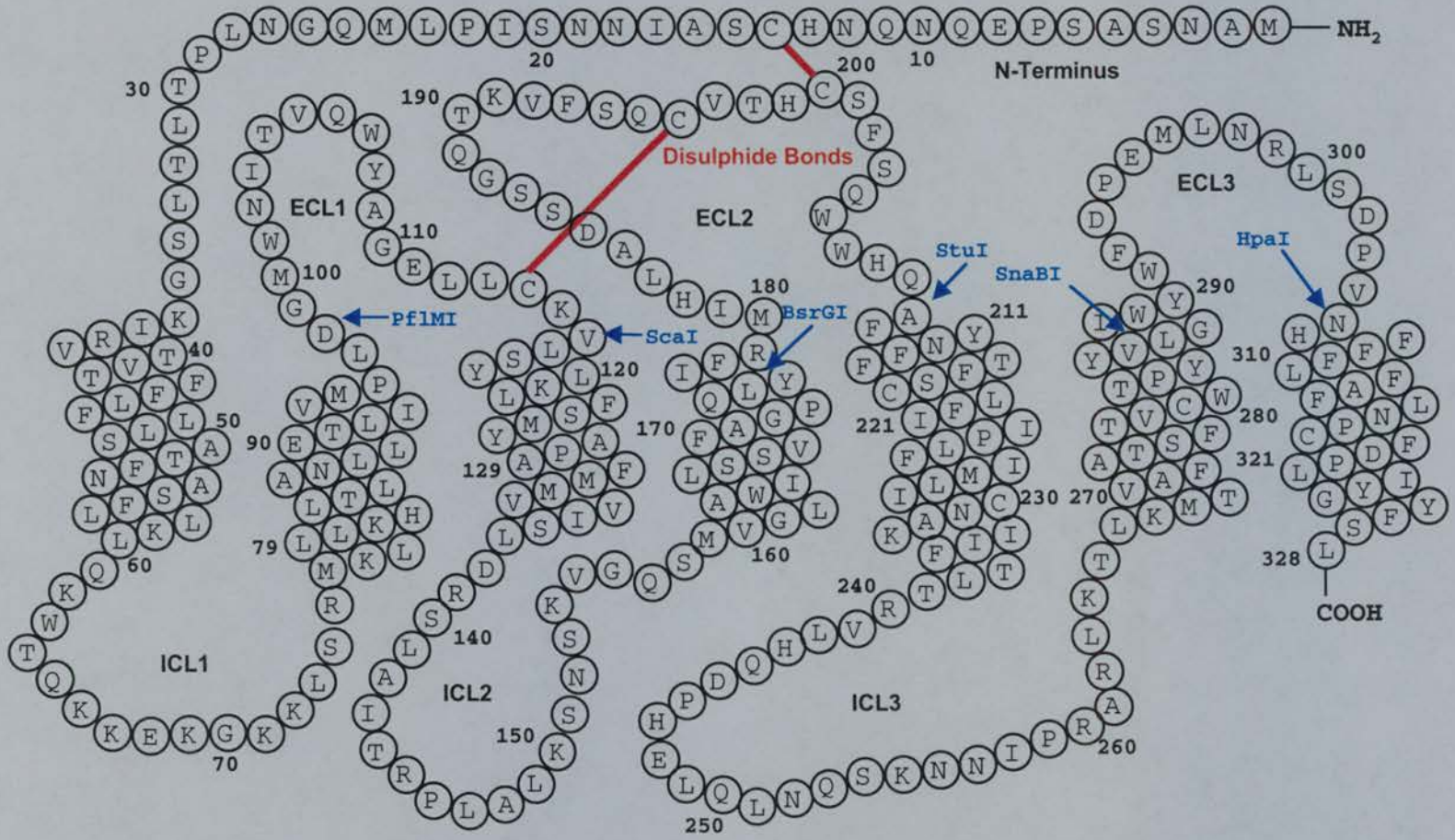


Figure 3.2. Schematic representation of the human GnRH receptor, with the engineered restriction sites indicated in blue

Figure 3.3. Schematic representations of the engineered human GnRH receptor in the three vectors used in this thesis: pBluescript SK(-), pZErO-2 and pcDNA1/Amp.

pBluescript was used for all cloning except substitution of ECL1.

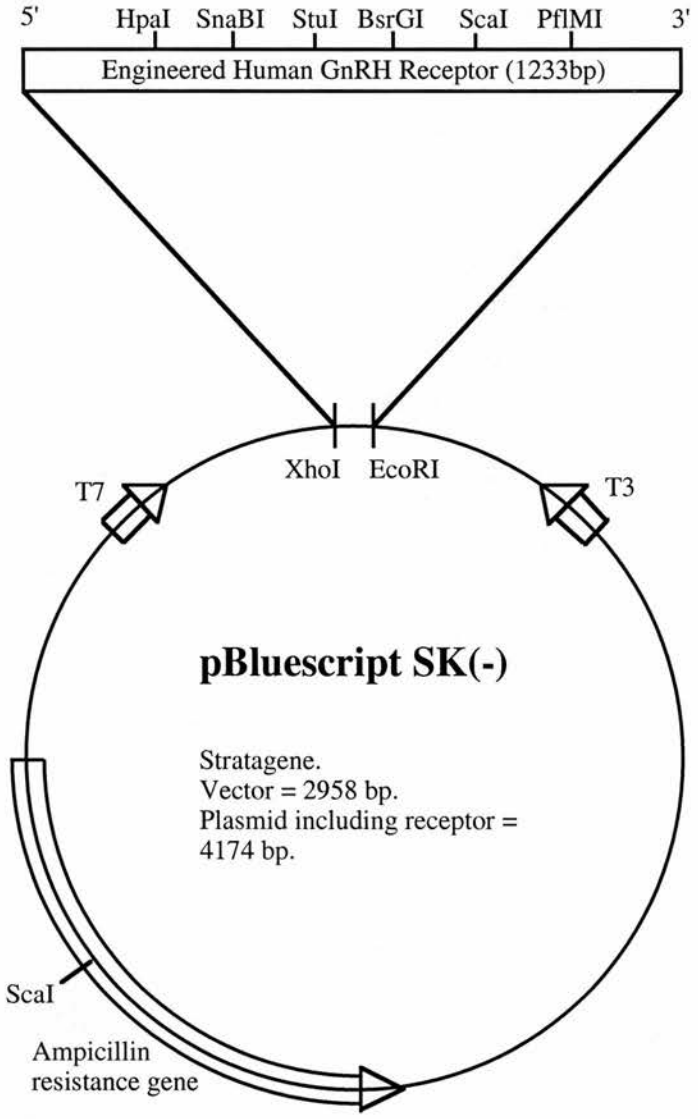
pZErO-2 was used for substitution of ECL1 as it lacks a Sca I restriction site in the vector sequence.

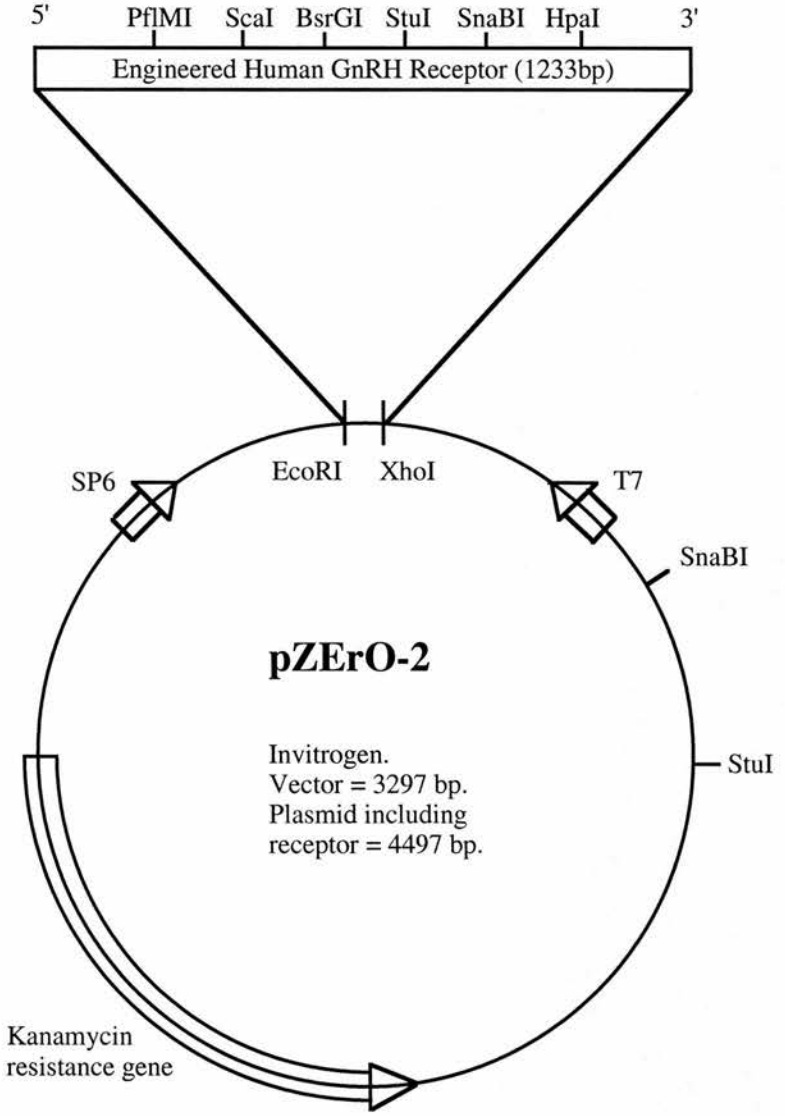
Constructs were subcloned into pcDNA1/Amp for transfection into COS-7 cells, as this vector contains the eukaryotic CMV promoter (human cytomegalovirus immediate-early gene).

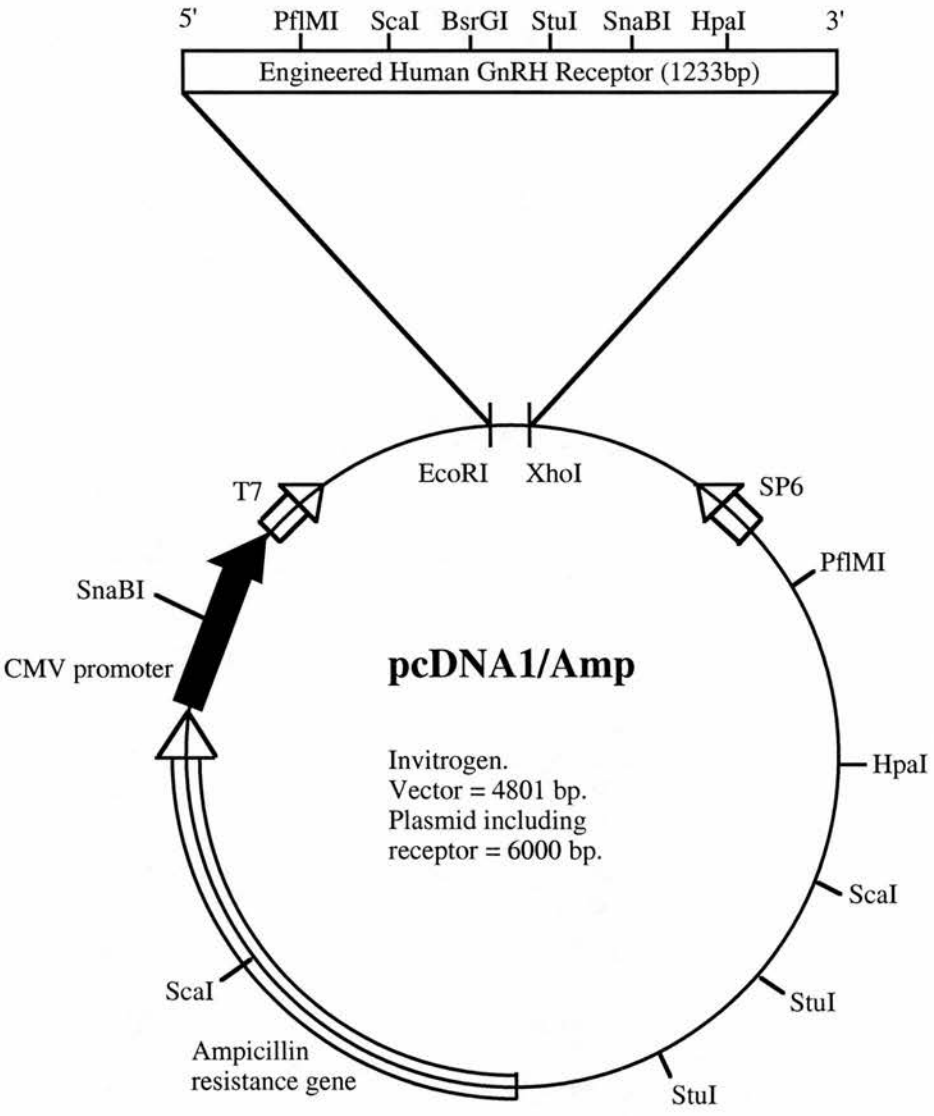
Restriction enzymes EcoR I, PflM I, Sca I, BsrG I, Stu I, SnaB I, Hpa I and Xho I were used to produce chimeric and point-mutated receptors. The restriction sites of these enzymes are shown in all three constructs.

Sequences of oligonucleotide primers, including T7, T3 and SP6, are shown in Appendix III.

(Figure 3.3 continues overleaf)




5'
PflMI
ScaI
BsrGI
StuI
SnaBI
HpaI
3'
Engineered Human GnRH Receptor (1233bp)
SP6
EcoRI
XhoI
T7
SnaBI
pZErO-2
Invitrogen.
Vector = 3297 bp.
Plasmid including
receptor = 4497 bp.
StuI
Kanamycin
resistance gene


5'
PflMI
ScaI
BsrGI
StuI
SnaBI
HpaI
3'
Engineered Human GnRH Receptor (1233bp)
T7
EcoRI
XhoI
SP6
PflMI
SnaBI
CMV promoter
pcDNA1/Amp
Invitrogen.
Vector = 4801 bp.
Plasmid including
receptor = 6000 bp.
HpaI
ScaI
StuI
ScaI
Ampicillin
resistance gene
StuI

3.7 Transient Transfection of COS-7 Cells

3.7.1 Origin of the COS-7 Cell Line

The COS-7 cell line was derived from monkey kidney cells transformed by the SV40 DNA tumour virus. The cells contain viral DNA encoding several viral proteins that stimulate proliferation of normally quiescent cells.

3.7.2 Cell Culture

COS-7 cells were cultured in the dedicated tissue culture suite using Class II hoods. Surfaces were swabbed with 70% ethanol and Tisept. All plasticware used was sealed in sterile packaging and only opened in the hoods. Waste was decontaminated by Presept or autoclaving. Cells were maintained in 162 cm^2 cell culture flasks at 37°C, 5% CO_2 in Complete Medium. Passaging was carried out twice per week, with cells being split approximately 1 in 20.

3.7.3 Transfection Reagent

SuperFect (Qiagen) is an activated-dendrimer transfection reagent, which assembles DNA into compact structures. The SuperFect-DNA complex has a net positive charge that interacts with negatively charged receptors. After internalisation, SuperFect buffers the lysosome once it fuses with the endosome. This causes pH inhibition of lysosomal nucleases. The DNA can then be transported to the nucleus.

3.7.4 Transfection Protocol

COS-7 cells were seeded in 100 mm^2 dishes at a density of 1.2 million cells/dish and maintained at 37°C, 5% CO_2 in 'Complete Medium'.

After 24 h, the cells were transiently transfected with GnRH receptor cDNA. Briefly, in 300 µl of Opti-MEM® (Life Technologies (GibcoBRL)), 30 µl of SuperFect was incubated with 10 µg of DNA to form a complex. After 10 min, this complex was added to each 100 mm^2 dish of COS-7 cells for 8 h, along with 3 ml of Complete Medium. The cells were then washed with PBS (Sigma-Aldrich) and incubated for a further 48 h in Complete Medium. The cells were either maintained in the 100 mm^2 dishes or transferred to 12 well plates, depending on the assay to be carried out.

3.7.5 Optimisation of Transfection Protocol

The protocol was optimised for quantity of SuperFect, cell density, incubation time with SuperFect and quantity of DNA. The protocol previously established in the laboratory was used as a starting point. The CRE-luciferase reporter gene assay was selected as a measure of relative transfection efficiency due to its speed and sensitivity.

CRE-Luciferase Reporter Gene Assay

The CRE-luciferase plasmid DNA included the luciferase gene whose promoter contained the cAMP response element (CRE). Luciferase is an enzyme whose activity results in luminescence. Over the time-scale involved in this assay, the luminescence can be considered proportional to the cAMP activity.

COS-7 cells were seeded and maintained in 100 mm^2 dishes. After 24 h, the cells were transiently transfected with 5 µg of CRE-luciferase DNA and 5 µg of receptor DNA (see Section 3.7.4). Following incubation with SuperFect, the cells were treated with trypsin and transferred to 12-well plates (1 dish of cells/plate). After a further 48 h, the cells were treated in triplicate with DMEM containing 0.1% BSA (Sigma-Aldrich) and a suitable concentration of GnRH agonist. Following incubation at 37°C for 4 h, the cells were washed with PBS and Promega Reporter Lysis Buffer added (250 µl/well). After shaking for 20 min, the cells were scraped and centrifuged for 1 min at 15000 rpm. 10 µl of cell extract was added to 50 µl of Promega Luciferase Assay Reagent in a luminometer tube. A luminometer was then used to measure the light emitted over 25 s.

Quantity of SuperFect

30 µl of SuperFect was routinely added to each 100 mm^2 dish. The effect of increasing the quantity of SuperFect was investigated. Figure 3.4 shows the luminescence produced using 30, 40 and 50 µl (relative to that produced using 30 µl).

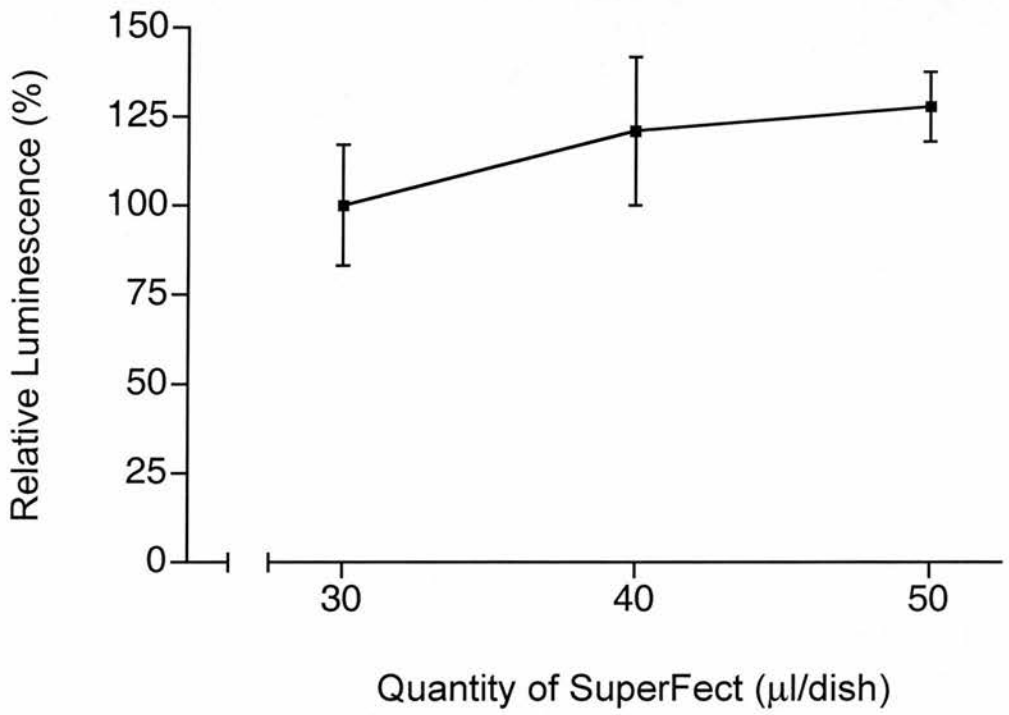


Figure 3.4. Effect of increasing quantity of SuperFect. Luminescence normalised to that produced using 30 µl/dish. Data produced in triplicate.

Increasing the quantity of SuperFect appeared to increase transfection efficiency. However, the increase was not great enough to justify the additional expense of increasing the quantity used routinely. Therefore, the practice of using 30 µl/100 mm^2 dish was continued.

Cell Density

COS-7 cells were routinely seeded in 100 mm^2 dishes at a density of 1.2 million cells/dish. The effect of changing this density was investigated. Figure 3.5 shows the luminescence produced using between 0.8 and 1.6 million cells/dish (relative to that produced using 1.2 million cells).

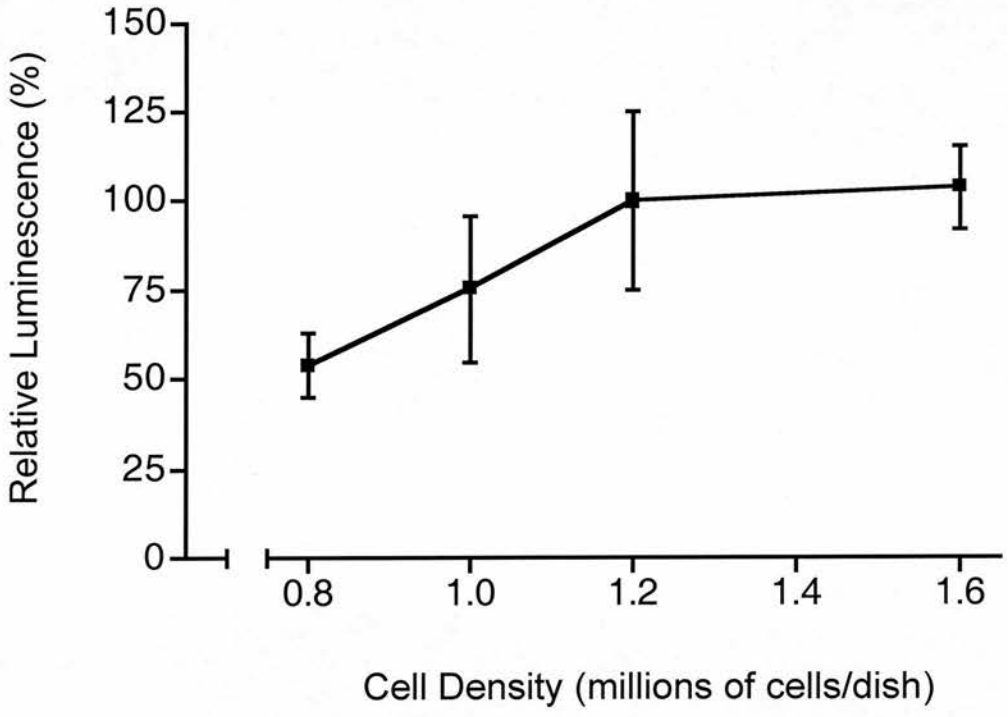


Figure 3.5. Effect of changing cell density. Luminescence normalised to that produced using 1.2 million cells/dish. Data produced in triplicate.

Decreasing cell density decreased transfection efficiency, however, increasing cell density had little effect. Therefore, the practice of using 1.2 million cells/100 mm^2 dish was continued.

Incubation Time with SuperFect

COS-7 cells were routinely incubated with SuperFect for 4 h. The effect of changing the incubation time was investigated. Figure 3.6 shows the luminescence produced following incubation with SuperFect for between 3 and 10 h (relative to that produced following incubation for 8 h).

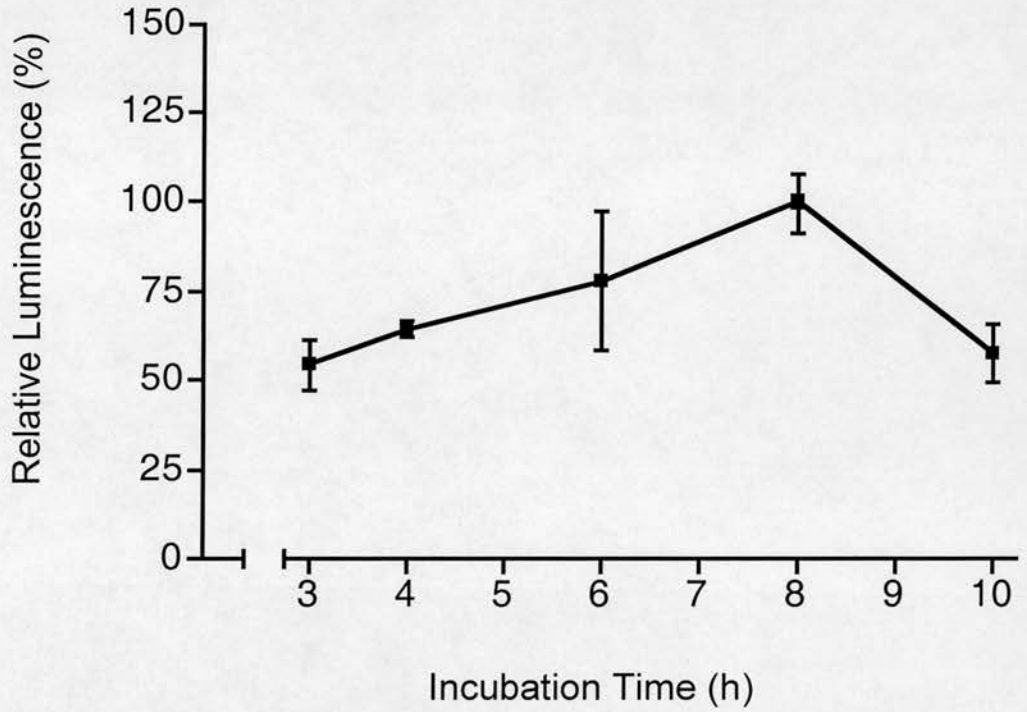


Figure 3.6. Effect of changing incubation time with SuperFect. Luminescence normalised to that produced using 8 h. Data produced in triplicate.

Increasing incubation time with SuperFect increases transfection efficiency up to 8 h, after which transfection efficiency decreases. This is probably due to cytotoxicity. Therefore, the studies carried out in this thesis used an incubation time with SuperFect of 8 h, rather than 4 h as practised previously.

Quantity of DNA

10 μg of DNA was routinely added to each 100 mm^2 dish. The effect of increasing the quantity of DNA was investigated. Figure 3.7 shows the luminescence produced using 10, 20 and 30 μg (relative to that produced using 10 μg).

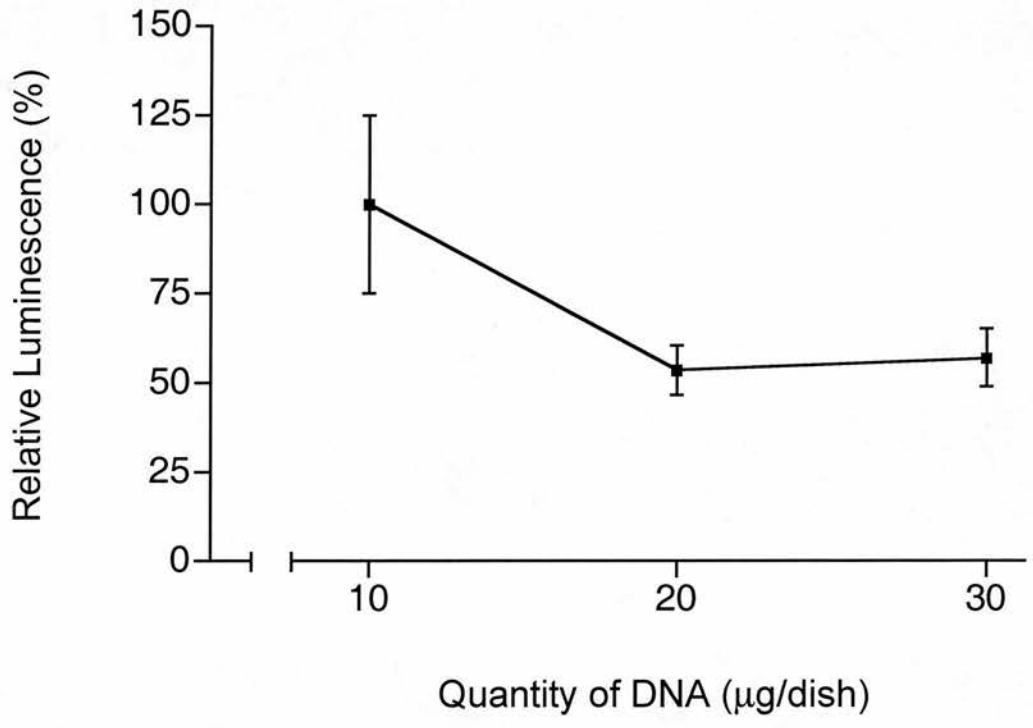


Figure 3.7. Effect of increasing quantity of DNA. Luminescence normalised to that produced using 10 μg/dish. Data produced in triplicate.

Increasing the quantity of DNA appeared to be detrimental to transfection efficiency. Therefore, the practice of using 10 μg/100 mm^2 dish was continued.

3.8 Radioligand Binding Assays

3.8.1 Iodination of [His^5,D-Tyr^6]-GnRH

This procedure used Iodo-Gen (Pierce), a water-insoluble oxidising agent. Briefly, 10 µl of ^{125}I (100 µCi/µl) was added to 20 µl of Iodo-Gen (2 µg/µl), 50 µl of PBS and 5 µl of [His^5,D-Tyr^6]-GnRH (1 mM). After 90 s, 500 µl of Iodination Running Buffer was added and the solution eluted through a column (10 mm diameter) containing Sephadex G-25 in Iodination Running Buffer (pre-washed with 5% BSA). The eluted radioligand was collected using a Gilson Model 201 fraction collector (Anachem). The iodinated peptide eluted after the ^{125}I peak due to adsorption on the gel filtration column.

3.8.2 Whole Cell Binding Assay

COS-7 cells were transiently transfected with GnRH receptor cDNA as described in Section 3.7.4. Following the incubation with SuperFect, the cells were treated with trypsin and transferred to 12 well plates. The cells were incubated for 48 h in Complete Medium at 37°C, 5% CO_2.

Competition binding assays were carried out using radiolabelled ^{125}I-[His^5,D-Tyr^6]-GnRH (approx. 120,000 cpm/tube). The high binding affinity of this tracer compared with conventional tracers was established previously (Flanagan et al., 1998). The cells were incubated for 4 h at 4°C in HEPES/DMEM containing 0.1% BSA, labelled ligand and varying concentrations of unlabelled GnRH analogues in duplicate. The cells were washed with ice-cold PBS and solubilised by shaking for 20 min at room temperature with 0.1 M NaOH. The solubilised radioactivity was pipetted into 12 mm plastic tubes and counted using a multigamma counter (Perkin Elmer (Wallac)).

3.8.3 Membrane Binding Assay

COS-7 cells were transiently transfected with GnRH receptor cDNA as described in Section 3.7.4. Following a 48 h incubation in the 100 mm^2 dishes, cells were scraped in PBS, pelleted and stored at -70°C.

The cell pellets were homogenised in ice-cold 'homogenisation buffer' using a Dounce tissue grinder (Jencons-PLS) and centrifuged at 15000 rpm for 10 min at 4°C. The crude membrane pellet was then resuspended in ice-cold 'assay buffer'. Competition binding assays were carried out using radiolabelled ^{125}I-[His^5,D-Tyr^6]-GnRH (approx. 120,000 cpm/tube). The membrane suspension was incubated overnight at 4°C with labelled ligand and varying concentrations of unlabelled GnRH analogues in triplicate. The suspensions were filtered through a membrane harvester (Brandel) onto Whatman GF/B filter paper (Merck) (pre-soaked in assay buffer containing 0.01% PEI) and washed three times with ice-cold assay buffer. Bound radioactivity was counted using a multigamma counter (Perkin Elmer (Wallac)).

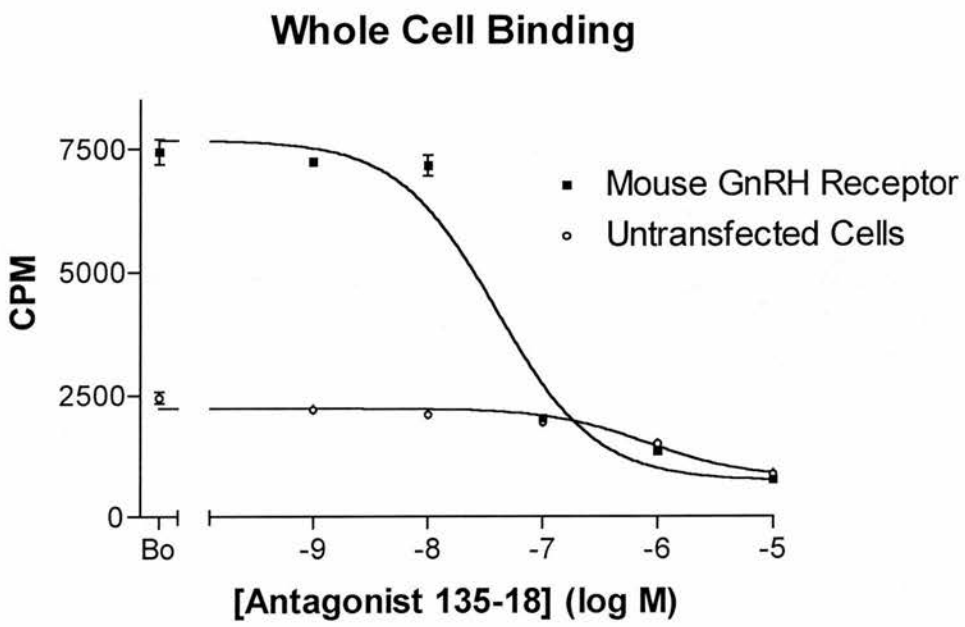
3.8.4 Selection of Binding Assay for Studies

Initial assays compared whole cell and membrane binding. Membrane binding was selected for the studies in this thesis because receptor number could be controlled and no change in the amount of radioligand bound was observed with cells transfected with vector only, even at ligand concentrations of 10 nM (Figure 3.8).

3.9 Data Reduction and Statistical Analysis

Binding curves and IC_{50} values were generated by Prism (GraphPad) graphing software using non-linear regression, assuming one site competition. Significant differences in wild type to mutant IC_{50} values were assessed using a two-tailed, unpaired Student's t-test with Welch's correction (does not assume equal variances).

A.



B.

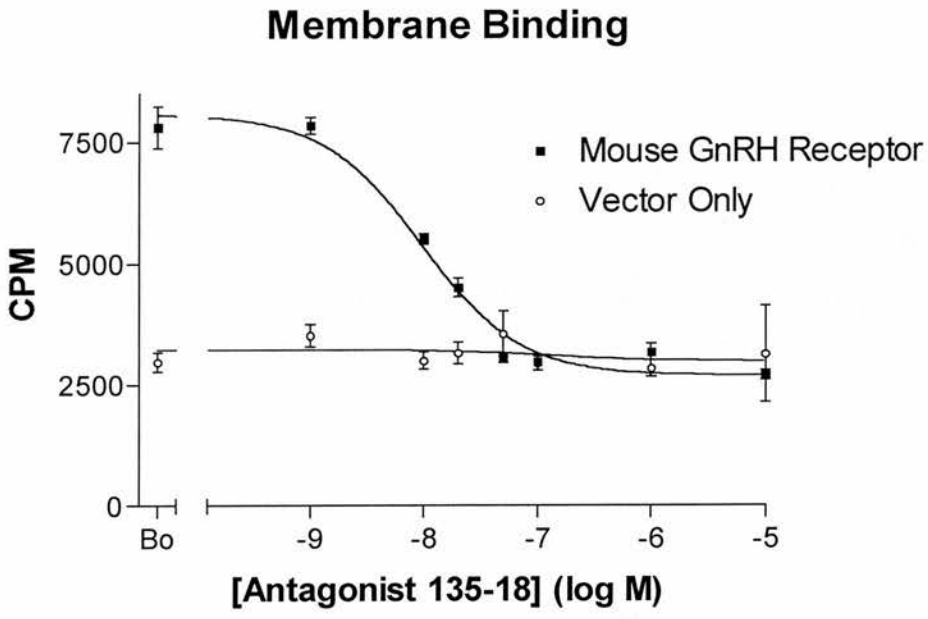


Figure 3.8. Antagonist 135-18 binding to whole COS-7 cells **(A)** and homogenised COS-7 cell membranes **(B)** with and without GnRH receptor cDNA

4 The Effect of Ligand Conformational Constraint on GnRH Receptor Binding

4.1 Introduction

Sixteen structural variants of GnRH have been identified (see Section 2.4). In jawed vertebrates these cluster into three groupings: GnRH I, GnRH II and GnRH III (Troskie et al., 1998; White et al., 1998). One of these, GnRH II, is totally conserved in structure from bony fish to man, suggesting that each individual amino acid is essential for biological activity (King and Millar, 1992).

The bioactive conformation of mGnRH acting on mammalian type I GnRH receptors was originally proposed to be largely determined by a β-II′ turn involving residues five to eight (Momany, 1976a). This conclusion was supported by a large body of evidence derived from a variety of approaches. Conformation-dependent mGnRH antisera that bind the N and C termini of mGnRH tolerate certain amino acid substitutions in the central region of the ligand, but not in other positions. This implied that mGnRH has a turn conformation resulting in closely apposed N and C termini (Millar et al., 1984).

Fluorescence measurements of Trp^3 at different pHs suggested that His^2 and Tyr^5 are in close proximity to Arg^8 in mGnRH (Shinitzky and Fridkin, 1976). This correlated with the proposed β-II′ turn conformation and indicated the possibility of intramolecular stabilising interactions between these residues. Further fluorescence studies supported these findings by comparing mGnRH and analogues with substitutions for Arg^8 (Milton et al., 1983). Chicken GnRH I (cGnRH I, [Gln^8]-GnRH) was found to lack such stabilising interactions, implying that a less structured conformation exists for this ligand.

The technique of conformational memories further supported the concept that a predominant β-II′ turn conformer of mGnRH accounts for biological activity (Guarnieri and Weinstein, 1996). Substitution of Arg^8 with Lys resulted in a loss of this structure and reduced binding affinity (Guarnieri and Weinstein, 1996; Mezei and Guarnieri, 1998).

NMR studies have provided direct structural evidence for mGnRH in a β type turn conformation consisting of three families (Maliekal et al., 1997). All possess the β type turn about Gly^6 and at least two hydrogen bonds: one between Ser^4 and

Arg^8, and another between $pGlu^1$ and Gly^{10}-NH_2. Hydrogen bonding of Arg^8 to either His^2 or Tyr^5 was also noted, supporting the conclusions from the fluorescence studies (Shinitzky and Fridkin, 1976; Milton et al., 1983). cGnRH I had different conformers grouped into four families. These also possessed several hydrogen bonds, but only that between $pGlu^1$ and Ser^4 was common to all four. This implied much greater flexibility of this molecule compared with mGnRH.

A series of cyclic GnRH antagonists were found to have a β-II′ turn conformation, further supporting the notion that this is the biologically relevant structure (Rivier et al., 2000a). However, since antagonists occupy different, but overlapping binding sites these observations do not necessarily support the structure of agonists. Indeed, in this study a potent cyclic antagonist was found to have a β-I′ turn at residues 6-7. This implies that, for antagonists at least, the specific turn type may not be important as long as it results in correct presentation of the backbone and side-chains critical for binding. Since agonists must satisfactorily bind and activate the receptor, they are likely to have more specific structural requirements.

D-amino acid substitution for Gly^6 (D-aa^6 substitution) is believed to stabilise the β-II′ turn conformation, thereby increasing affinity for the receptor (Monahan et al., 1973). Conformational energy analysis indicates that D-aa^6 substitution reduces the freedom for opening at position six so that the population of the bioactive conformer is increased (Momany, 1976b). A similar effect can be achieved by utilising a lactam ring between residues 6 and 7 (6,7 γ-lactam insertion) (Freidinger et al., 1980).

Although there is substantial evidence for the β-II′ turn conformation for active GnRH analogues at the mammalian type I receptor, it is uncertain whether constraint of non-mammalian GnRHs in this conformation enhances their activity at mammalian and non-mammalian receptors.

Mammalian type I and non-mammalian GnRH receptors have low sequence identity and structural features that suggest they may be configured differently. These include differences in intracellular domains (absence of C-terminal tail, longer intracellular loop 1 (ICL1) in mammalian type I receptors), extracellular disulphide bridges, and different interactions between residues in transmembrane domains 2 and 7 (Blomenrohr et al., 1997; Flanagan et al., 1999). In view of these differences that affect the overall three-dimensional structure of the receptor, the bioactive ligand conformational requirement may also differ (Sun et al., 2001).

The present study provides evidence that a major determinant of bioactive conformation at mammalian type I receptors, the β-II′ turn involving residues five to eight, is also necessary for high affinity binding at non-mammalian receptors. The

findings also indicate that, unlike the other GnRHs, the native GnRH II ligand is pre-configured in a bioactive conformation, which may account for its relatively high affinity for all GnRH receptors investigated and the conservation of its structure over 500 million years of evolution.

4.2 Materials and Methods

4.2.1 GnRH Analogues

mGnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), GnRH II ([His^5,Trp^7,Tyr^8]-GnRH), cGnRH I ([Gln^8]-GnRH), sGnRH ([Trp^7,Leu^8]-GnRH), [D-Ala^6]-GnRH, [D-Lys^6]-GnRH and [D-Trp^6]-GnRH were supplied by Bachem. [D-Ala^6]-cGnRH I, [D-Arg^6]-sGnRH, [D-Lys^6]-sGnRH, [D-Trp^6]-GnRH II, [D-Arg^6]-GnRH II, and [D-Lys^6]-GnRH II were gifts from the University of Cape Town, South Africa. [6,7 γ-lactam]-GnRH and [6,7 γ-lactam]-cGnRH I were gifts from R. Freidinger (Merck & Co., West Point, PA, U.S.A.).

4.2.2 GnRH Receptor cDNA

The human (Chi et al., 1993), mouse (Tsutsumi et al., 1992), chicken (Sun et al., 2001) and *Xenopus* II (B. Troskie, N. Illing and R. Millar, unpublished results) GnRH receptor cDNA constructs were gifts from the University of Cape Town, South Africa. The rat GnRH receptor was cloned by this laboratory (Eidne et al., 1992). The catfish GnRH receptor cDNA (Tensen et al., 1997) was a gift from Utrecht University, The Netherlands. The bullfrog II GnRH receptor cDNA was a gift from Chonnam National University, Republic of Korea (Wang et al., 2001). The bullfrog II receptor was originally classified by Wang and co-workers as Type III based on tissue distribution, however, it has greatest sequence homology for designated Type II receptors (B. Troskie, N. Illing and R. Millar, unpublished results) and will be considered as such in this thesis (see Section 2.5).

The mouse GnRH receptors, each having one of the conserved extracellular domain acidic residues mutated to its isosteric amide, were gifts from C. Flanagan (University of Cape Town, South Africa). They were produced as described previously (Flanagan et al., 1994).

4.2.3 Cell Culture and Transfection

COS-7 cells were cultured and transfected as described in Section 3.7.

4.2.4 Receptor Binding Assays

The procedure for receptor binding assays is described in Section 3.8.3. Maximum specific binding ranged between approximately 5000 and 10,000 cpm/tube with non-specific binding ranging between approximately 2000 and 4000 cpm/tube. No specific binding was detected with COS-7 cells transfected with vector only (see Section 3.8.4). Membrane concentration was adjusted to provide similar receptor numbers, with cells from two 100 mm^2 dishes being used for each binding curve, with the exception of the human, chicken and catfish receptors. The human and chicken receptors exhibited particularly low expression levels and so cells from four 100 mm^2 dishes were used for each curve. Conversely, the catfish receptor exhibited particularly high expression levels and so cells from one 100 mm^2 dish was used for each curve. Therefore, similar maximal specific radioligand binding was observed at all receptors.

4.2.5 Data Reduction and Statistical Analysis

Binding curves were generated and statistical analysis carried out using Prism graphing software (GraphPad) as described in Section 3.9.

4.3 Results

The mouse GnRH receptor binds mGnRH and GnRH II with highest affinity (Tables 4.1 and 4.2 and Figure 4.1). The chicken and catfish receptors bind GnRH II with highest affinity, followed by sGnRH, mGnRH and cGnRH I (Tables 4.1 and 4.2, and Figures 4.2 and 4.3). Substitution of a D-amino acid in position six of the ligand (D-aa^6) or the insertion of a γ-lactam moiety between residues six and seven (6,7 γ-lactam) significantly increases the binding affinity of mGnRH, cGnRH I and sGnRH acting on the mouse, chicken and catfish GnRH receptors (Tables 4.1 and 4.2).

Introduction of the aromatic D-Trp^6 residue into mGnRH increased the binding affinity at the mouse, chicken and catfish GnRH receptors by a substantial 74, 8.6 and 14-fold. The affinity of [D-Trp^6]-GnRH binding at the mouse receptor is significantly higher than that of [D-Ala^6]-GnRH and [6,7 γ-lactam]-GnRH ($p<0.05$). In contrast, substitution with the positively charged D-Lys^6 residue enhanced the binding affinity at these receptors by only 1.9, 3.6 and 2-fold.

GnRH II was unique amongst the natural GnRHs tested in binding all three species of GnRH receptor with relatively high affinity. The affinity for the non-mammalian receptors was particularly high. Substitution of a D-aa^6 has little or no effect on the binding affinity of the GnRH II ligand acting at the catfish and chicken receptors. At the mouse receptor, which has a much lower affinity for GnRH II, only D-Lys^6 substitution results in a substantial (8.4-fold) increase in affinity (Table 4.2).

The latter finding suggested that the D-Lys^6 in GnRH II might be interacting with one of the seven extracellular domain acidic residues that are conserved in mammalian type I GnRH receptors. Binding of [D-Lys^6]-GnRH II to mutant mouse GnRH receptors, in which each of these acidic residues was successively mutated to its isosteric amide (Flanagan et al., 1994), was compared with binding at the wild type receptor (Table 4.3 and Figure 4.4).

Table 4.2. IC_{50} data for analogues of sGnRH and GnRH II binding at mouse, chicken and catfish GnRH receptors

Agonist	Mouse Receptor			Chicken Receptor			Catfish Receptor		
	IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n
	nM			*nM*			*nM*		
sGnRH	1133.8 ± 298.5	1.0	4	7.6 ± 1.5	1.0	3	51.8 ± 11.3	1.0	4
[D-Arg^6]-sGnRH	170.0 ± 36.1	6.7 *	4	0.41 ± 0.15	18.8 *	3	10.3 ± 1.7	5.0 *	4
[D-Lys^6]-sGnRH	123.3 ± 24.4	9.2 *	3	0.36 ± 0.03	21.4 *	3	7.2 ± 1.8	7.2 *	4
GnRH II	127.9 ± 19.8	1.0	6	0.75 ± 0.21	1.0	7	1.3 ± 0.24	1.0	5
[D-Trp^6]-GnRH II	156.0 ± 28.5	0.8 [ns]	5	0.49 ± 0.06	1.5 [ns]	3	1.4 ± 0.52	0.9 [ns]	4
[D-Arg^6]-GnRH II	55.1 ± 10.9	2.3 *	5	0.47 ± 0.17	1.6 [ns]	4	0.58 ± 0.17	2.2 [ns]	4
[D-Lys^6]-GnRH II	15.3 ± 2.3	8.4 *	4	0.19 ± 0.03	4.0 *	3	0.71 ± 0.25	1.8 [ns]	3

[a] Mean ± S.E.M. of between three and seven experiments carried out in triplicate

[b] Fold increase in binding affinity relative to the wild type ligand

[ns], Not significantly different from wild type, $p>0.05$

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

Table 4.1. IC_{50} data for analogues of mGnRH and cGnRH I binding at mouse, chicken and catfish GnRH receptors

Agonist	Mouse Receptor			Chicken Receptor			Catfish Receptor		
	IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n
	nM			*nM*			*nM*		
mGnRH	174.2 ± 33.4	1.0	6	10.0 ± 1.1	1.0	4	633.8 ± 90.6	1.0	5
[D-Trp^6]-GnRH	2.4 ± 0.21	74.0 **	3	1.2 ± 0.39	8.6 *	3	45.3 ± 12.9	14.0 **	5
[D-Ala^6]-GnRH	23.8 ± 7.2	7.3 **	8	2.9 ± 1.2	3.4 **	6	89.6 ± 20.8	7.1 *	4
[6,7 γ-Lactam]-GnRH	32.1 ± 6.7	5.4 **	3	1.4 ± 0.26	7.3 *	3	113.1 ± 29.6	5.6 *	5
[D-Lys^6]-GnRH	89.9 ± 15.7	1.9 *	5	2.8 ± 1.3	3.6 *	4	318.0 ± 52.6	2.0 *	6
cGnRH I	4316.7 ± 216.2	1.0	3	81.9 ± 10.6	1.0	5	2505.0 ± 198.7	1.0	4
[D-Ala^6]-cGnRH I	1052.8 ± 249.6	4.1 ***	4	44.6 ± 9.2	1.8 *	5	618.0 ± 36.8	4.1 **	3
[6,7 γ-Lactam]-cGnRH I	880.7 ± 147.0	4.9 ***	3	16.6 ± 3.4	4.9 **	3	385.3 ± 50.7	6.5 **	3

[a] Mean ± S.E.M. of between three and eight experiments carried out in triplicate

[b] Fold increase in binding affinity relative to the wild type ligand

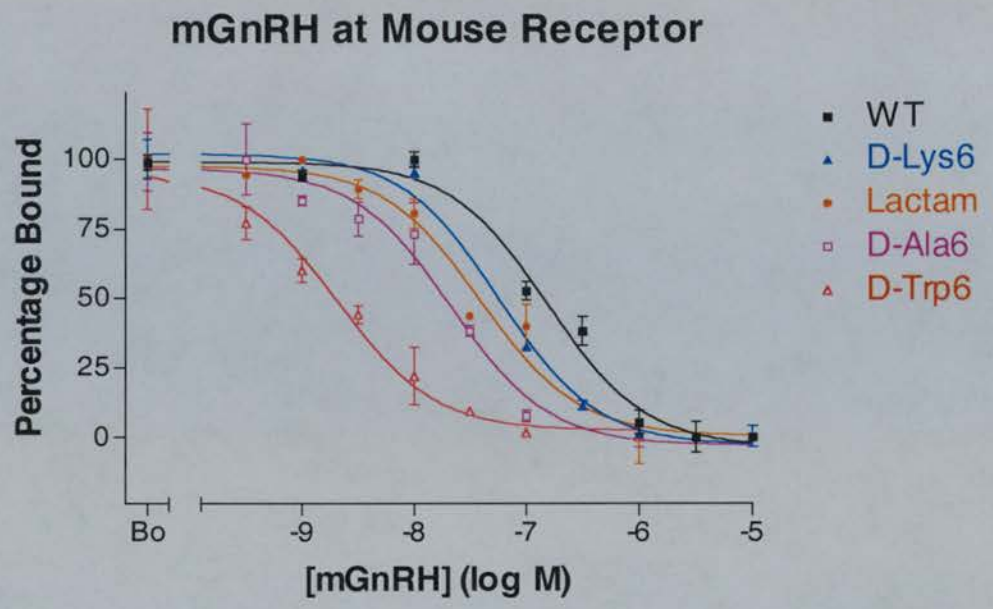
[ns], Not significantly different from wild type, $p>0.05$

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

**, Significantly different from wild type, $p<0.01$

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

A.



B.

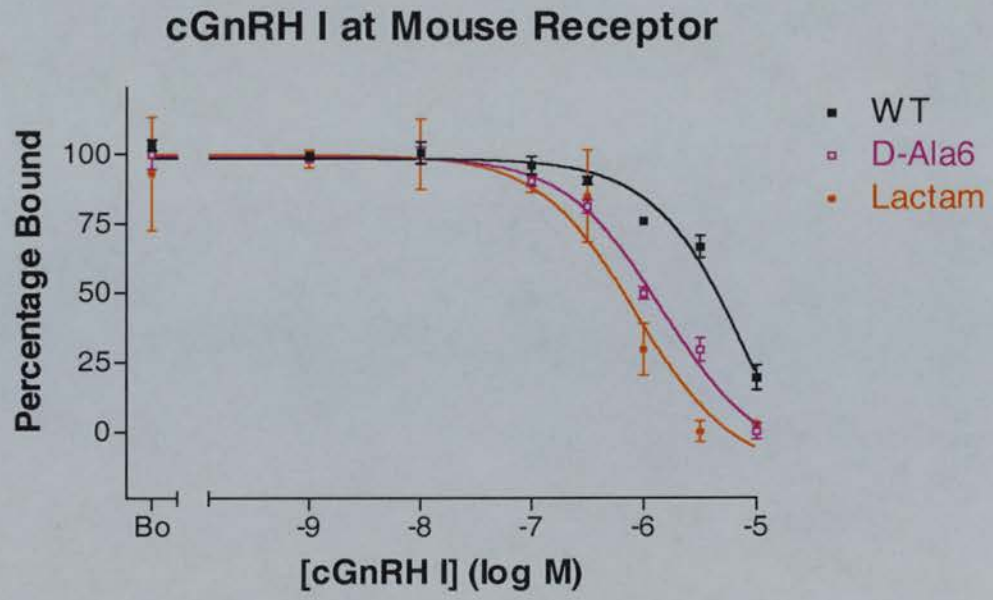
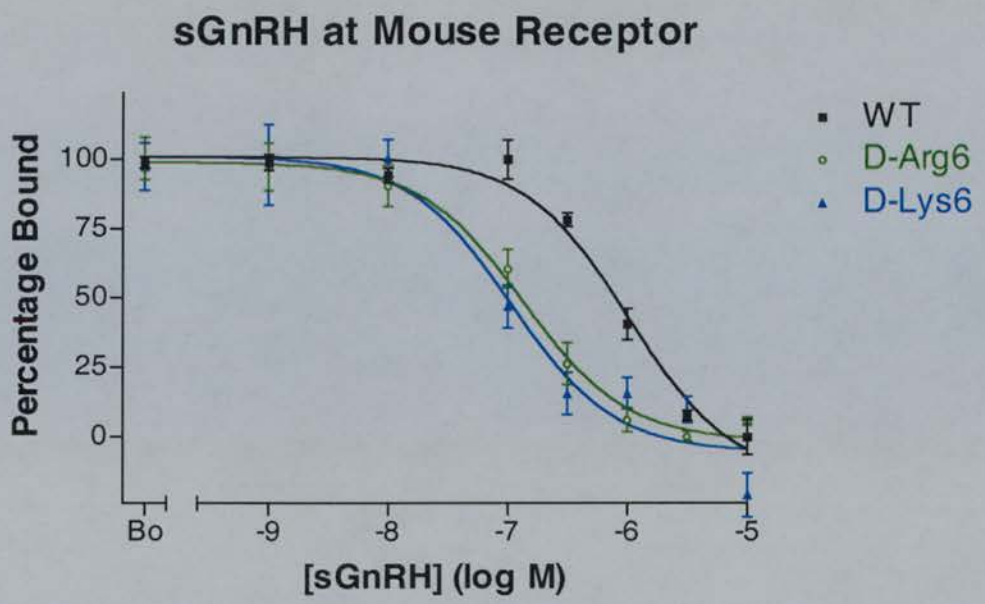


Figure 4.1. Binding of mGnRH analogues **(A)** and cGnRH I analogues **(B)** at the mouse GnRH receptor

Binding curves representative of between three and eight experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

C.



D.

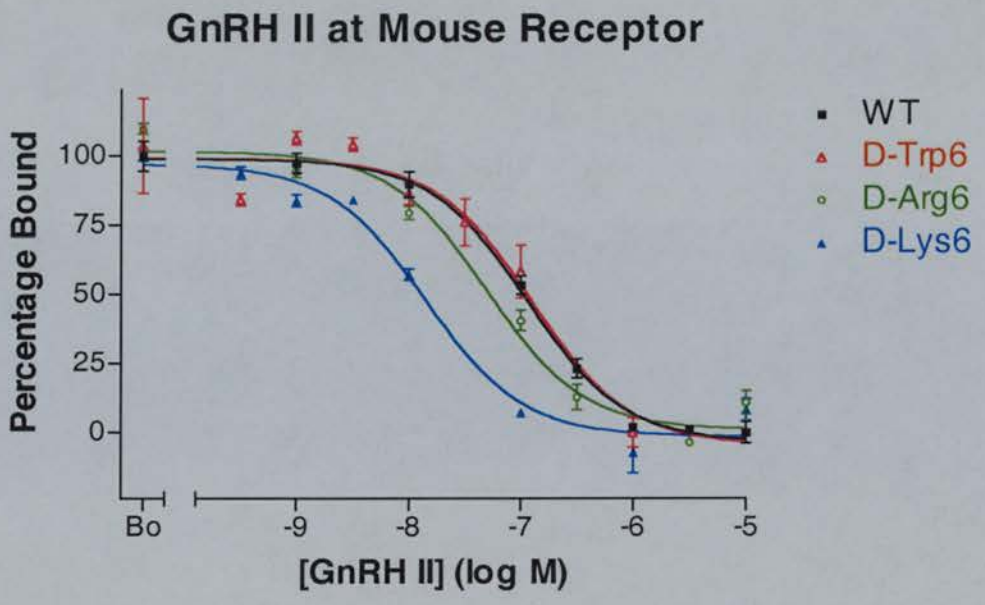
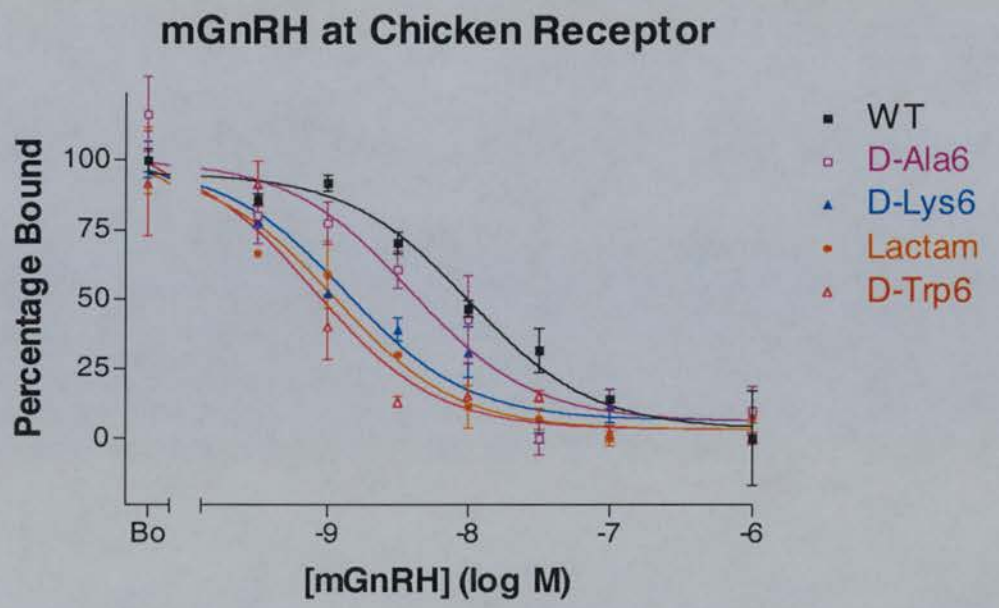


Figure 4.1 (Continued). Binding of sGnRH analogues **(C)** and GnRH II analogues **(D)** at the mouse GnRH receptor

A.



B.

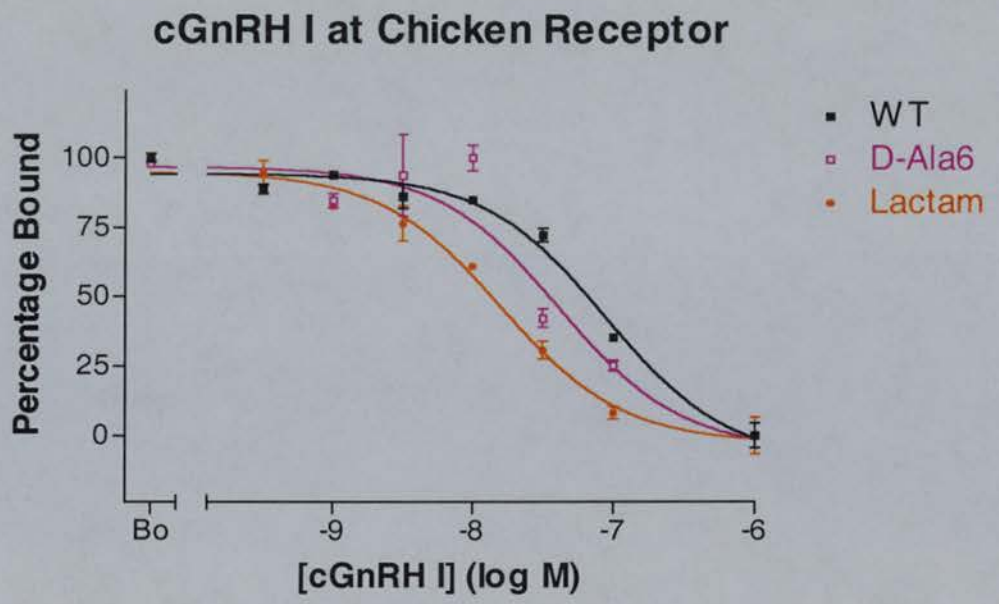
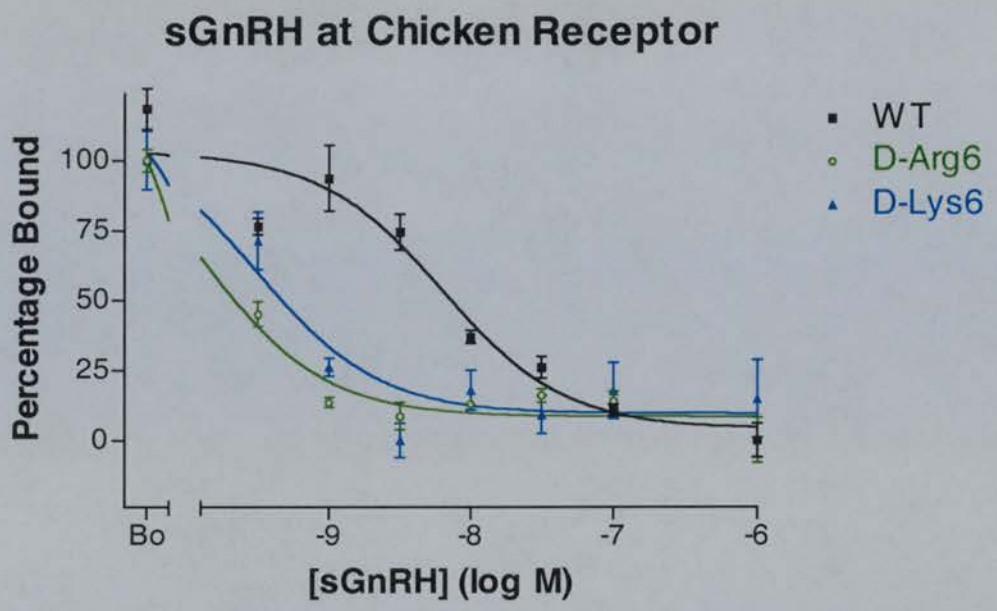


Figure 4.2. Binding of mGnRH analogues **(A)** and cGnRH I analogues **(B)** at the chicken GnRH receptor

Binding curves representative of between three and seven experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

C.



D.

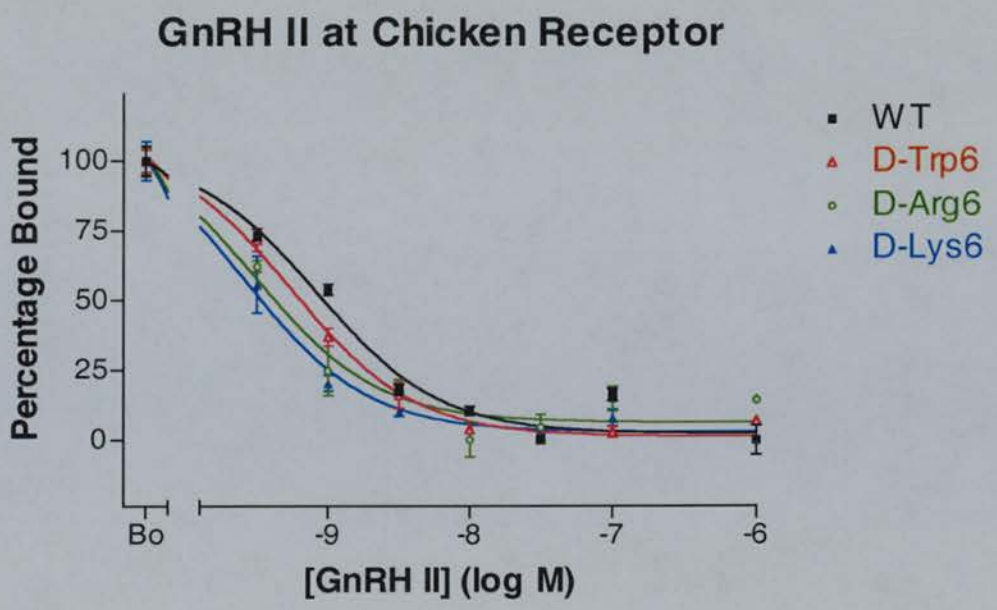
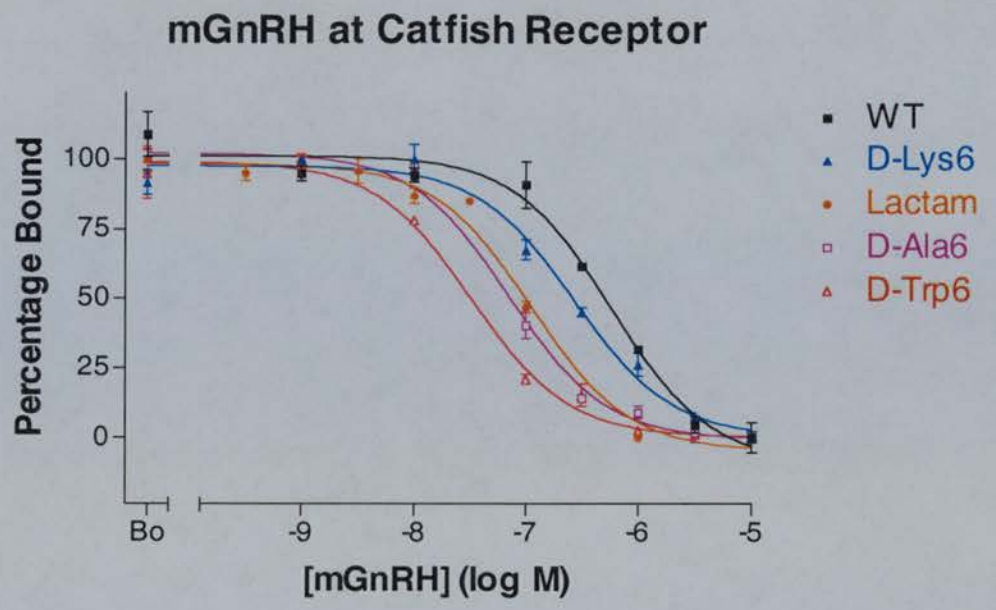


Figure 4.2 (Continued). Binding of sGnRH analogues **(C)** and GnRH II analogues **(D)** at the chicken GnRH receptor

A.



B.

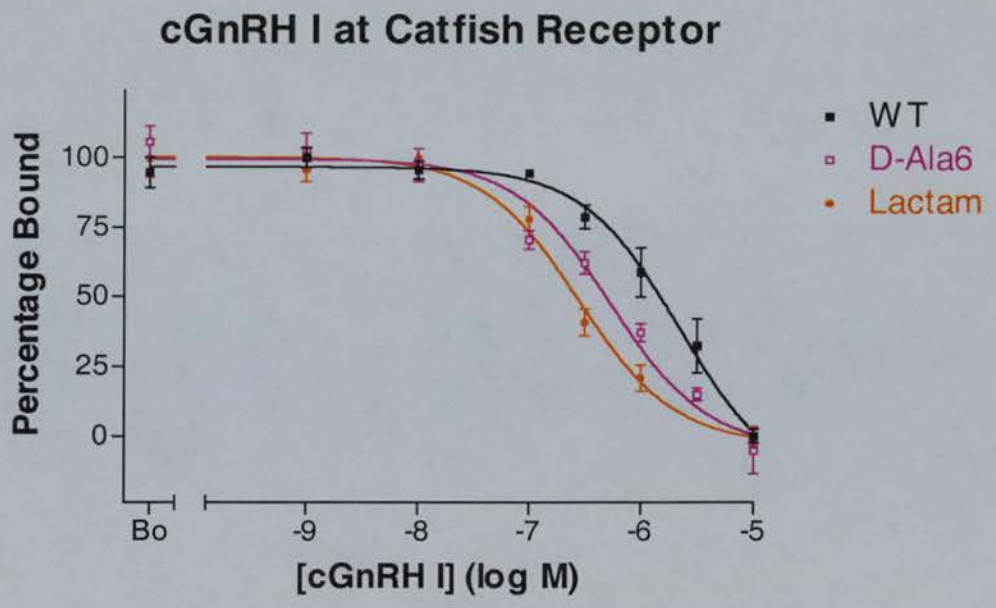
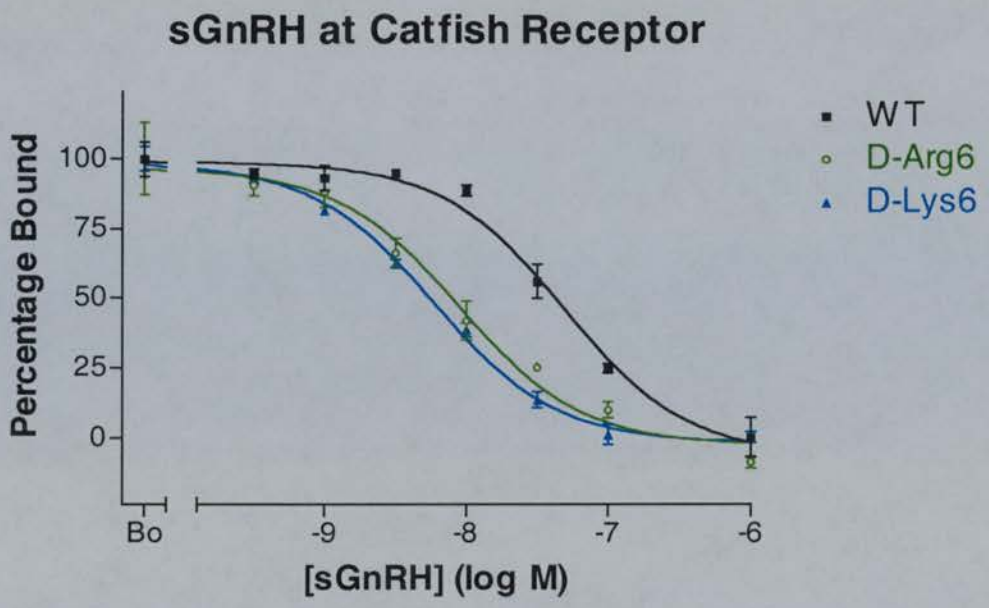


Figure 4.3. Binding of mGnRH analogues **(A)** and cGnRH I analogues **(B)** at the catfish GnRH receptor

Binding curves representative of between three and six experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

C.



D.

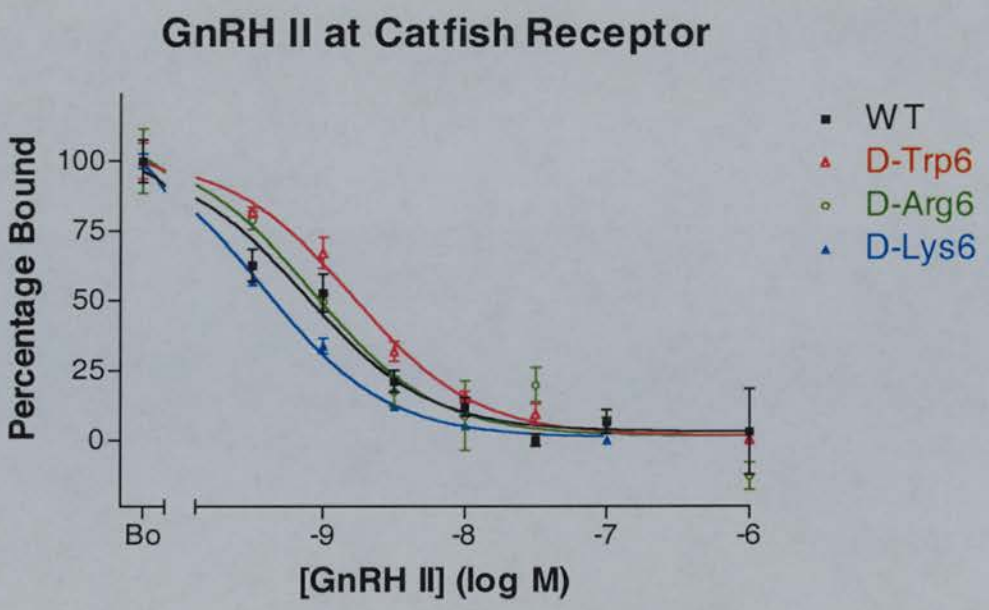


Figure 4.3 (Continued). Binding of sGnRH analogues **(C)** and GnRH II analogues **(D)** at the catfish GnRH receptor

Table 4.3. Agonist binding at point-mutated mouse GnRH receptors

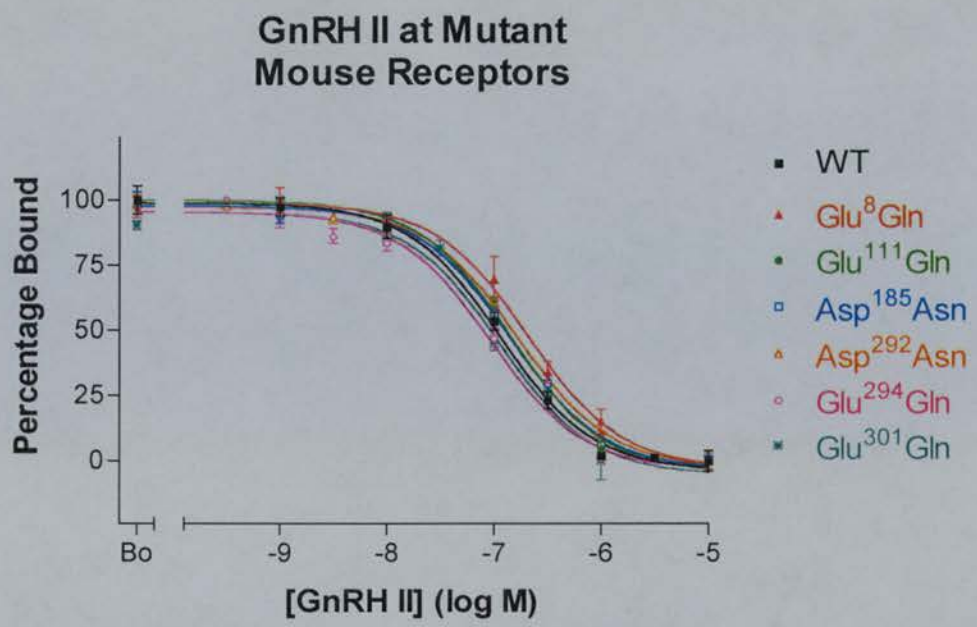
Radioligand binding assays were performed on homogenised membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} data (mean ± S.E.M.) generated by Prism graphing software (GraphPad) as described in Section 3.9.

Mutation in Mouse GnRH Receptor	GnRH II		[D-Lys^6]-GnRH II	
	IC_{50}[a]	n	IC_{50}[a]	n
	nM		*nM*	
Wild Type	127.9 ± 19.8	6	15.3 ± 2.3	4
Glu^{8}Gln	175.9 ± 37.6 [ns]	3	10.5 ± 3.5 [ns]	3
Glu^{111}Gln	113.0 ± 16.1 [ns]	3	37.0 ± 11.4 [ns]	3
Asp^{185}Asn	175.4 ± 17.5 [ns]	3	20.0 ± 2.1 [ns]	3
Asp^{292}Asn	185.3 ± 25.3 [ns]	3	25.7 ± 5.7 [ns]	3
Glu^{294}Gln	128.7 ± 32.6 [ns]	3	16.3 ± 3.1 [ns]	3
Glu^{301}Gln	130.4 ± 6.5 [ns]	3	9.3 ± 1.4 [ns]	3

[a] Mean ± S.E.M. of between three and six experiments carried out in triplicate

[ns], Not significantly different from wild type, $p>0.05$

A.



B.

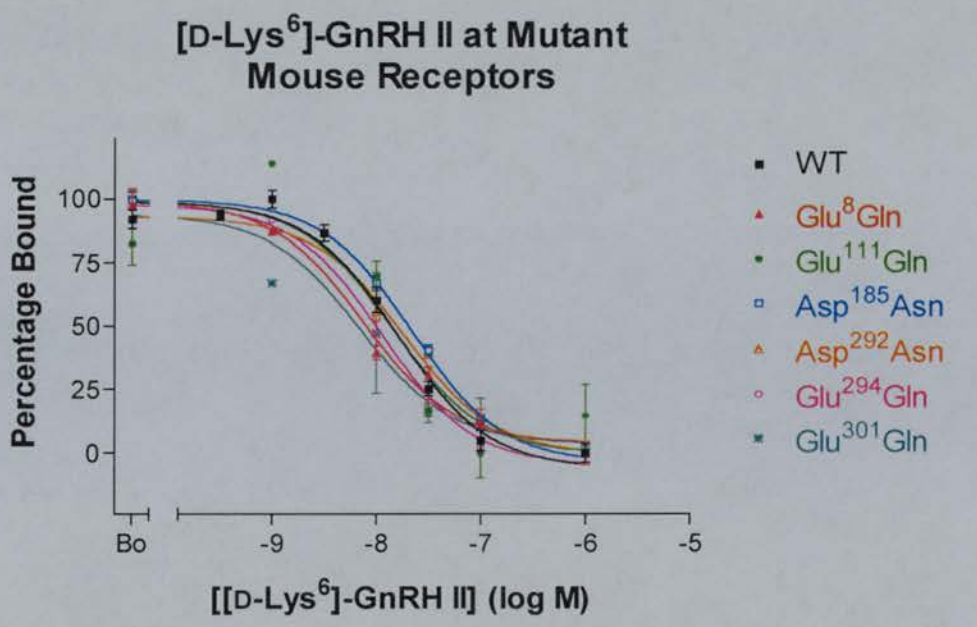


Figure 4.4. Binding of GnRH II **(A)** and [D-Lys^6]-GnRH II **(B)** at point-mutated mouse GnRH receptors

Binding curves representative of between three and six experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

In initial binding assays the $Glu^{8}Gln$, $Glu^{111}Gln$, $Asp^{185}Asn$, $Asp^{292}Asn$, $Glu^{294}Gln$ and $Glu^{301}Gln$ mutant receptors specifically bound radioligand, whereas the $Asp^{98}Asn$ mutant receptor did not. The latter result is expected as Asp^{98} in the mammalian type I receptor has been shown to interact with His^{2} in the GnRH ligand and mutations of this residue severely affect ligand binding (Flanagan et al., 2000). The mutant receptors that bound the labelled ligand were all found to have the same binding affinity as the wild type receptor for GnRH II and [D-Lys^{6}]-GnRH II (Table 4.3 and Figure 4.4). This indicates that an interaction of D-Lys^{6} with one of these residues was not responsible for the increased affinity of [D-Lys^{6}]-GnRH II.

D-Lys^{6} substitution substantially enhanced the binding affinity of GnRH II acting at the mammalian type I receptor, but not at the non-mammalian receptors. To examine this further, four more species of GnRH receptor were investigated: two mammalian type I (rat and human) and two non-mammalian type II (*Xenopus* II and bullfrog II). D-aa^{6} substitution did not significantly increase the binding affinity of the GnRH II ligand acting at the *Xenopus* II or bullfrog II receptors. D-Lys^{6} substitution substantially increased the binding affinity of the GnRH II ligand acting at the rat and human receptors (33.8 and 7.2-fold respectively), as noted for the mouse receptor. Another basic amino acid (D-Arg^{6}) substitution into GnRH II also substantially increased binding affinity at the rat receptor (Table 4.4 and Figure 4.5).

Table 4.4. IC_{50} data for analogues of GnRH II binding at rat, human, *Xenopus* II and bullfrog II GnRH receptors

Agonist	Rat			Human			*Xenopus* II			Bullfrog II		
	IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n
	nM			*nM*			*nM*			*nM*		
GnRH II	323.0 ± 33.1	1.0	3	135.6 ± 15.8	1.0	4	3.7 ± 1.3	1.0	6	0.91 ± 0.48	1.0	5
[D-Trp^6]-GnRH II	111.0 ± 15.3	2.9 *	3	106.6 ± 25.4	1.3 [ns]	3	1.4 ± 0.23	2.7 [ns]	3	0.45 ± 0.08	2.0 [ns]	3
[D-Arg^6]-GnRH II	35.2 ± 16.0	9.2 *	3	115.0 ± 19.4	1.2 [ns]	3	2.6 ± 1.5	1.4 [ns]	4	0.32 ± 0.10	2.8 [ns]	4
[D-Lys^6]-GnRH II	9.6 ± 1.1	33.8 *	3	19.0 ± 4.8	7.2 **	5	1.0 ± 0.21	3.6 [ns]	4	0.26 ± 0.01	3.4 [ns]	3

[a] Mean ± S.E.M. of between three and six experiments carried out in triplicate

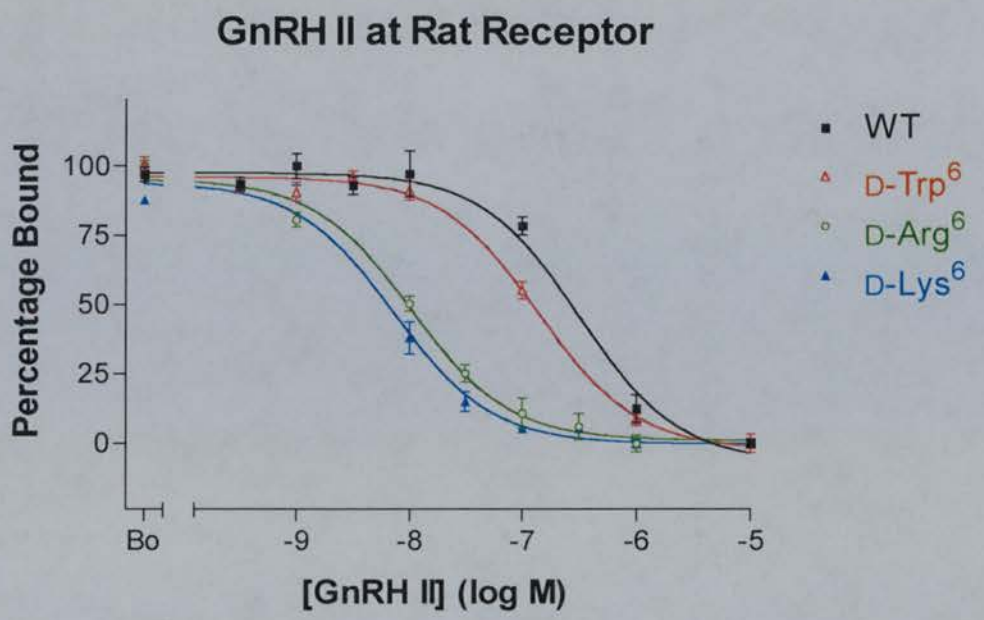
[b] Fold increase in binding affinity relative to the wild type ligand

[ns], Not significantly different from wild type, $p>0.05$

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

**, Significantly different from wild type, $p<0.01$

A.



B.

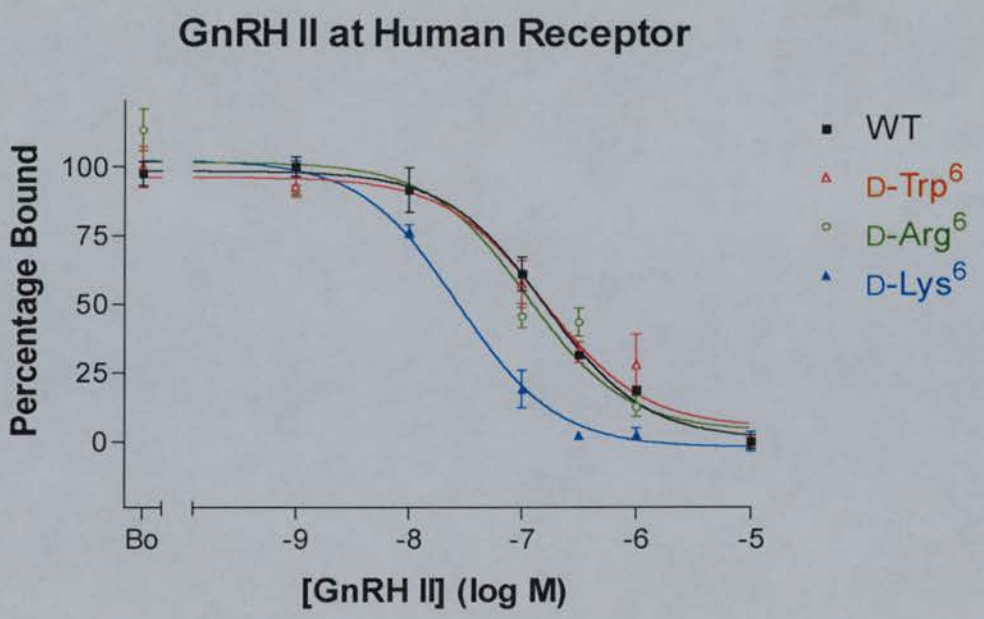
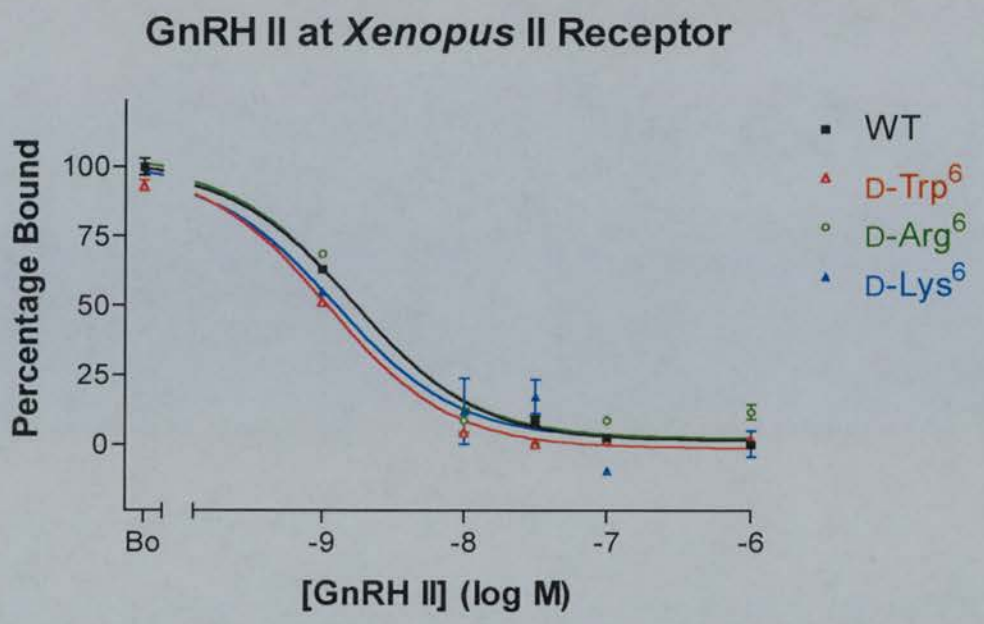


Figure 4.5. Binding of GnRH II analogues at the rat **(A)** and human **(B)** GnRH receptors

Binding curves representative of between three and six experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

C.



D.

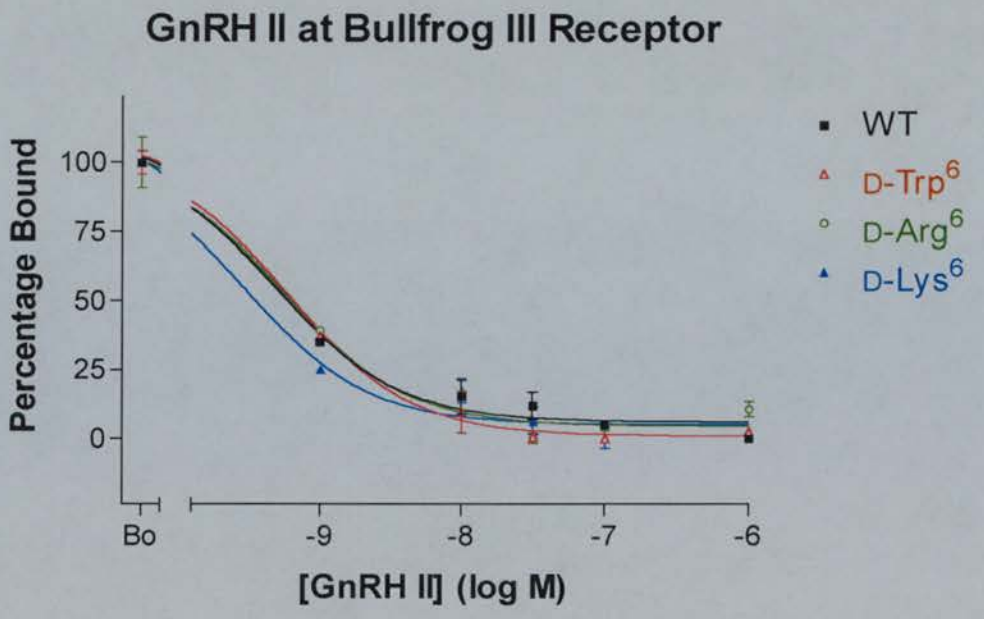


Figure 4.5 (Continued). Binding of GnRH II analogues at the *Xenopus* II **(C)** and bullfrog II **(D)** GnRH receptors

4.4 Discussion

There is now considerable accumulated evidence that mGnRH interacts with its receptor in a β-II′ turn conformation involving residues five to eight (Momany, 1976a; Shinitzky and Fridkin, 1976; Milton et al., 1983; Millar et al., 1984; Guarnieri and Weinstein, 1996; Maliekal et al., 1997; Mezei and Guarnieri, 1998). This conformation appears to be contributed by interactions between Ser^4 and Arg^8, as well as between $pGlu^1$ and Gly^{10}-NH_2 (Maliekal et al., 1997). Others have presented evidence for interactions of Arg^8 with His^2 and Tyr^5 (Shinitzky and Fridkin, 1976; Milton et al., 1983; Maliekal et al., 1997) contributing to the β-II′ turn conformation. An interaction of Arg^8 with an acidic residue in ECL3 of the receptor is also believed to contribute to the configuration of the ligand in the folded conformation (Flanagan et al., 1994; Fromme et al., 2001; Petry et al., 2002).

D-aa^6 substitution and 6,7 γ-lactam insertion further stabilises this conformation and enhances binding affinity (Monahan et al., 1973; Momany, 1976b; Freidinger et al., 1980; Karten and Rivier, 1986; Sealfon et al., 1997). Indeed, the D-aa^6 constraint can abrogate the need for the interaction between Arg^8 of mGnRH and the acidic residue in ECL3 of the receptor (Fromme et al., 2001). The present studies confirmed these concepts as D-Trp^6, D-Ala^6 and D-Lys^6 substitution and 6,7 γ-lactam insertion all significantly increased the binding affinity of mGnRH at the mouse GnRH receptor.

The potency of mGnRH increases with increasing hydrophobicity of the D-aa^6 (Karten and Rivier, 1986) and this is exemplified in the current study in which binding affinity successively increased in the series D-Trp^6 > D-Ala^6 > 6,7 γ-lactam > D-Lys^6. Since [D-Trp^6]-GnRH has a much higher affinity than the 6,7 γ-lactam analogue, it appears that the substitution with D-Trp^6 makes additional contributions to enhancement of affinity. Explanations for this include: possible hydrophobic interactions with the receptor; a stabilisation of the ligand conformation by intramolecular hydrophobic interactions or a reduction in flexibility due the size of these side-chains; and/or a reduction in the desolvation penalty upon binding to the receptor due to the more hydrophobic nature of the ligand (Mezei and Guarnieri, 1998). [D-Lys^6]-GnRH binds to the mouse and catfish receptors with significantly lower affinity than [6,7 γ-lactam]-GnRH implying that the basic lysine side-chain is detrimental to the binding of the GnRH ligand. The hydrophilic nature of this residue may disrupt one or more of the effects described above. Additionally, the positive charge of lysine may repel the positively charged Arg^8 and so affect ligand

conformation. D-Arg^6/D-Lys^6 substitution into sGnRH, which does not contain positively charged amino acids, resulted in a much greater increase in binding affinity in the mouse, chicken and catfish receptors. This concurs with the recorded effects of D-Arg^6 substitution in sGnRH at the goldfish receptor (Murthy et al., 1994; Illing et al., 1999).

Previous studies have shown that some non-mammalian receptors, unlike mammalian type I receptors, are not selective for Arg^8 containing ligands (Sun et al., 2001; Wang et al., 2001). In view of the role of Arg^8 in configuring the ligand, it was suggested that these receptors do not require GnRH to be configured in the β-II′ turn conformation (Millar and King, 1984). This interpretation was further supported by the observation that D-aa^6 substitution did not enhance binding at the cloned chicken receptor (Sun et al., 2001) and that NMR showed the N and C termini of cGnRH I were not closely apposed (Maliekal et al., 1997).

However, in the present study, D-aa^6 substitution clearly increased the binding affinity of mGnRH, cGnRH I and sGnRH at mouse, chicken and catfish receptors.

Although these observations appear to be contradictory, closer inspection reveals that the primary data concur. The interpretation that D-aa^6 substitution does not enhance binding affinity at the cloned chicken receptor was based on [D-Arg^6]-GnRH II having the same binding affinity as GnRH II and a GnRH analogue incorporating D-Ala^6 having the same affinity as mGnRH (Sun et al., 2001). The present study found a lack of enhancement for D-Arg^6 substitution into GnRH II, and that D-Ala^6 substitution into mGnRH produced a relatively small enhancement. Our data showing an increase in affinity resulting from D-Trp^6 substitution are supported by a study testing LH releasing activity using dispersed chicken anterior pituitary cells, in which D-Trp^6 analogues of cGnRH I and mGnRH were approximately 20-fold more potent than cGnRH I and mGnRH respectively (Millar and King, 1983). Furthermore, our data showing increased binding affinity of sGnRH at non-mammalian receptors following D-aa^6 substitution are supported by two studies that showed D-Arg^6 substitution in sGnRH enhanced binding affinity at the goldfish receptor (Murthy et al., 1994; Illing et al., 1999).

The NMR data show that the N and C termini of cGnRH I are not closely apposed, however, a turn conformation around Gly^6 was still identified (Maliekal et al., 1997). Conformational energy analysis indicates that cGnRH I can adopt the β-II′ turn conformation (Momany, 1976b). It is therefore likely that mGnRH and cGnRH I have similar conformations around Gly^6, but different conformations of the termini. This may explain how these ligands have such different affinities for the same receptor.

Studies using fluorescence (Milton et al., 1983) and NMR (Maliekal et al., 1997) have indicated a greater flexibility of the cGnRH I ligand compared with mGnRH. This implies that the non-mammalian receptors are able to stabilise the bioactive ligand conformation, either as a result of additional receptor contact sites, or as a result of a different spatial arrangement of conserved receptor contact sites as previously proposed (Sun et al., 2001). The latter concept is supported by the observation that non-mammalian receptors have a different conformation of ECL3 due to the altered positioning of a proline residue (Millar et al., 1997). This is examined further in Chapter 5.

Most D-aa^6 substitutions had limited effect on the binding affinity of GnRH II in contrast to that observed for mGnRH, cGnRH I and sGnRH. D-Trp^6 substitution, which gave the greatest increase in binding affinity of mGnRH (8.6 to 74-fold), did not substantially increase the binding affinity of GnRH II to any of the receptors (Tables 4.2 and 4.4), and none of the D-aa^6 substitutions substantially increased the binding affinity of GnRH II at the chicken, catfish, *Xenopus* II or bullfrog II GnRH receptors. Since GnRH II binds with high affinity to these receptors and D-aa^6 substitution does not substantially increase affinity, GnRH II would appear to be pre-configured in a bioactive conformation suitable for binding non-mammalian receptors.

Only D-Lys^6 substantially increased the binding affinity of GnRH II at the human and mouse GnRH receptors. In view of the failure of other D-aa^6 substitutions to increase binding affinity, it was considered that D-Lys^6 might provide an additional ligand-receptor interaction that does not occur with non-mammalian receptors. A candidate interaction is between the basic D-Lys^6 and an acidic residue. To address this possibility, extracellular domain acidic residues conserved in mammalian type I receptors were screened with GnRH II and [D-Lys^6]-GnRH II, using point-mutated mouse receptors. These mutations did not alter the binding affinity of either ligand (Table 4.3). Although there are other amino acid residues that can interact with a Lys side-chain (Ott et al., 2002), these have not been investigated. Instead, the possibility that D-Lys^6 substitution can contribute to the configuration of GnRH II was revisited.

Despite the evidence that GnRH II is pre-configured, its lower affinity at mammalian type I receptors (IC_{50} of 128 to 323 nM compared with 0.75 to 3.7 nM at non-mammalian receptors) suggests the interaction is not optimal. Therefore, the possibility that D-Lys^6 substitution may alter the conformation of GnRH II to improve its binding to mammalian type I receptors cannot be ruled out.

D-Arg^6 as well as D-Lys^6 substantially increased the binding affinity of GnRH II at the rat GnRH receptor. This concurs with previous findings using rat pituitary membranes (Millar et al., 1986). D-Lys^6 increased the binding affinity of GnRH II at the rat receptor much more (33.8-fold) than at the human (7.2-fold) and mouse receptors (8.4-fold). Closer inspection revealed that the binding affinity of GnRH II at the rat receptor (IC_{50} of 323 nM) was significantly lower than at either the human (IC_{50} of 135.6 nM) or mouse receptors (IC_{50} of 127.9 nM) ($p<0.05$) (Tables 4.2 and 4.4), however, the binding affinity of [D-Lys^6]-GnRH II was not significantly different at the three receptors. This is remarkable considering that rat and mouse GnRH receptors have 95% sequence homology (Eidne et al., 1992; Kaiser et al., 1992). It appears that a property of the rat receptor results in poor binding of GnRH II, but this is overcome by D-Lys^6 substitution. This accounts for the exceptional increase in binding affinity of GnRH II at the rat receptor following D-Lys^6 substitution. Further studies described in Chapter 6 show that a single residue in ECL2 is likely to be responsible for these different findings at the rat receptor compared with at the human and mouse receptors.

In conclusion, data has been obtained in support of the concept that, as for the mammalian type I receptors, the non-mammalian receptors have a preference for GnRH in the folded conformation involving a β-II′ turn for residues five to eight, which is enhanced in mGnRH, cGnRH I and sGnRH by D-aa^6 substitution. In contrast, the evolutionarily conserved GnRH II ligand appears to have a pre-configured β-II′ turn that accounts for its relatively high affinity for all GnRH receptors and a failure, in most instances, of any enhancement of binding affinity with D-aa^6 substitution. The surprising total conservation of GnRH II's primary structure from bony fish to man appears to have been a product of the coordinated evolutionary selection of amino acids contributing to binding, activation and configuration such that its structure cannot be improved by substitution with any natural amino acid at any position.

5 Extracellular Loop Interactions Differentially Affect mGnRH, GnRH II, Superagonist and Antagonist Binding

5.1 Introduction

GnRH receptors have been cloned from a variety of species (Sealfon et al., 1997; Millar, 2002) (see Section 2.5). Mammalian type I and non-mammalian GnRH receptors have different affinities for different structural variants of GnRH, such as mammalian GnRH (mGnRH) and GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) (Sun et al., 2001) that are expressed endogenously in the human (White et al., 1998). These differences in binding affinity are due to differences in ligand-receptor interactions or receptor conformation. The latter can influence affinity by altering the configuration of receptor contact sites or by affecting the ease with which a ligand can interact with these sites. By studying chimeric receptors that combine regions of mammalian type I and non-mammalian GnRH receptors, a greater understanding of how different domains influence ligand binding can be achieved.

The study described in Chapter 4 identified that, at mammalian type I receptors, [D-Trp^6]-GnRH and [D-Lys^6]-GnRH II are potent superagonist analogues of mGnRH and GnRH II respectively. That study also showed that all four ligands had significantly different binding affinities at the catfish receptor compared with mammalian type I receptors. In the present study, catfish GnRH receptor ECLs were substituted into the human GnRH receptor to identify structural domains underpinning these differences in affinities.

Antagonists interact with receptors in different orientations compared with agonists as they only need to bind the receptor and do not need to stabilise a particular conformation (Janovick et al., 1993; Assefa et al., 1999; Cui et al., 2000). Agonists, by definition, are required to stabilise the active conformation (see Section 2.2). If the 'antagonist' is actually an inverse agonist it will stabilise the inactive conformation. Antagonist 135-18 is of great interest because it acts as an antagonist at the human receptor, but as a partial agonist at the *Xenopus* I receptor and a full agonist at the chicken receptor (Sun et al., 2001; Ott et al., 2002). Therefore its

binding is able to stabilise the active conformations of the *Xenopus* I and chicken receptors, but not that of the human receptor. The manner in which the interaction of this ligand differs at mammalian type I compared with *Xenopus* I and chicken receptors promises to provide insights into the differences between the active and inactive receptor conformations. In the present study, Antagonist 135-18 was shown to have a significantly higher binding affinity at the human compared with at the catfish GnRH receptor. In addition to investigating the binding of the four agonists, this study endeavoured to identify structural domains underpinning the difference in affinity of Antagonist 135-18 at the human and catfish receptors.

A crucial locus for Family A GPCR configuration involves the residues in positions 2.50 of TMD2 and 7.49 of TMD7 (see Section 2.3.4). Mammalian type I GnRH receptors have $Asn^{2.50}$ and $Asp^{7.49}$. Mutation of $Asn^{2.50}$ to Asp in the mouse GnRH receptor resulted in very low receptor expression and no detectable ligand binding (Zhou et al., 1994; Flanagan et al., 1999). However, type II and non-mammalian GnRH receptors possess this $Asp^{2.50}$ and $Asp^{7.49}$ motif. In the catfish receptor, mutation of $Asp^{2.50}$ to Asn abolished ligand binding, probably as a result of incorrect receptor folding (Blomenrohr et al., 1997). These differences between mammalian type I and non-mammalian receptors at this locus suggest interactions in this microdomain differ, and that the TMD configuration of these receptors may also differ (Blomenrohr et al., 1997; Sun et al., 2001). If this is the case, the ECLs will also have different configurations as they are anchored to the TMDs.

Evidence has been presented for cysteine residues forming a disulphide bond between the N-terminus and ECL2 of mammalian type I GnRH receptors (Davidson et al., 1997). This disulphide bond is not present in mammalian type II and non-mammalian GnRH receptors as the cysteine residues are not conserved. Therefore, the configuration of the extracellular domains of these receptors is likely to differ significantly from that of the mammalian type I GnRH receptors.

Evidence has been provided for both mGnRH and GnRH II interacting with $Asp^{98(2.61)}$ (Flanagan et al., 2000) and $Asn^{102(2.65)}$ (Davidson et al., 1996) in the human GnRH receptor (Figure 2.8). $Lys^{3.32(121)}$ in the human receptor (Figure 2.8) is required for the binding of mGnRH, but not for antagonists (Zhou et al., 1995). In the catfish receptor, the homologous $Lys^{3.32(124)}$ is required for binding GnRH II, implying that mGnRH and GnRH II have similar interactions with this important locus of agonistic activity (Blomenrohr et al., 2001). As mGnRH and GnRH II interact with these same three conserved contact sites, the differences in affinities of these agonists at human and catfish receptors would seem to involve other, non-conserved contact sites, or be a product of different receptor configurations.

Asp/Glu$^{7.32}$ in ECL3 (Figure 2.8) appears to interact with Arg^{8} in mGnRH (Figure 2.7), but not with GnRH II (Flanagan et al., 1994; Fromme et al., 2001; Blomenrohr et al., 2002). In mammalian type I GnRH receptors, Asp/Glu$^{7.32}$ is followed by proline (S(D/E)P), whereas in non-mammalian type I GnRH receptors it is preceded by proline (P(D/E)Y). This difference may alter the orientation of the Asp/Glu side-chain, thereby affecting the interaction with Arg^{8} in mGnRH (Millar et al., 1997; Petry et al., 2002). The implication that in non-mammalian type I receptors this interaction does not occur (Millar et al., 1997) is now questionable, as Arg^{8} in mGnRH still appears to interact with Asp$^{7.32}$ in the catfish receptor (Blomenrohr et al., 2002). The present study investigates ligand-receptor interactions at the human and catfish GnRH receptors, including addressing this apparent contradiction. The two major questions to consider are: why does mGnRH bind at the human receptor with significantly higher affinity than at the catfish receptor, despite apparent conservation of these four ligand-receptor interactions; and why does GnRH II bind at the human receptor with significantly lower affinity than at the catfish receptor, despite both ligands interacting with Asp$^{2.61}$, Asn$^{2.65}$ and Lys$^{3.32}$, but not Asp$^{7.32}$?

5.2 Materials and Methods

5.2.1 Amino Acid Numbering Scheme

This scheme facilitates comparisons between G-protein coupled receptors and is described in section 3.1.

5.2.2 GnRH Analogues

mGnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), GnRH II ([His^{5},Trp^{7},Tyr^{8}]-GnRH) and [D-Trp^{6}]-GnRH were supplied by Bachem. [D-Lys^{6}]-GnRH II was a gift from the University of Cape Town, South Africa. Antagonist 135-18 ([Ac-D-Nal(2)^{1},D-4-Cl-Phe^{2},D-Pal(3)^{3},Ile^{5},D-Lys(iPr)^{6},Lys(iPr)^{8},D-Ala-NH_2^{10}]-GnRH) was a gift from R. Roeske (Indiana University, Indianapolis, U.S.A.).

5.2.3 Human and Catfish GnRH Receptor cDNA

The human GnRH receptor cDNA construct (Chi et al., 1993) was a gift from the University of Cape Town, South Africa. The catfish GnRH receptor cDNA (Tensen et al., 1997) was a gift from Utrecht University, The Netherlands.

5.2.4 Production of Chimeric GnRH Receptor cDNA

Techniques utilised for molecular cloning, as well as plasmid DNA production, purification and analysis, are described in Sections 3.2-3.5. The human GnRH receptor cDNA construct had previously been engineered to include a number of silent mutations, thereby introducing restriction sites at the TMD/ECL boundaries (Ott et al., 2002) (see Section 3.6). Oligonucleotide primers spanning these sites were designed and synthesised (Genosys) with each coding, partly for human GnRH receptor TMD, and partly for catfish GnRH receptor ECL (see Appendix III). DNA coding for the catfish GnRH receptor ECLs was generated by PCR using these primers in pairs, the proof-reading Deep Vent DNA polymerase (New England Biolabs), and the cloned catfish GnRH receptor cDNA as template. The products were digested with appropriate restriction endonucleases (Figure 3.2) and successively ligated into the engineered human GnRH receptor cDNA construct in place of the DNA coding for the analogous human ECLs. These constructs were used to transform One Shot™ Top 10 cells (Invitrogen) by heat-shock and plasmid DNA was extracted from the resultant colonies using the QIAprep Spin Miniprep Kit (Qiagen). To overcome the problem of additional restriction sites in the plasmid, the pBluescript vector (Stratagene) was used, with subcloning into the pZErO-2 vector (Invitrogen) for substitution of ECL1 (see Section 3.6). The DNA was initially screened by digesting with appropriate restriction enzymes. *Hsp*92 I digested the catfish receptor ECL1 cDNA, *Sal* I digested the catfish receptor ECL2 cDNA and *Nsp* I digested the catfish receptor ECL3 cDNA. None of these enzymes digested the engineered human receptor cDNA. The fragment sizes were confirmed by agarose gel electrophoresis. The constructs were then subcloned into the pcDNA-1/Amp vector (Invitrogen) for transfection into COS-7 cells. Receptor cDNA constructs were confirmed by automated sequencing using an ABI Prism 310 Genetic Analyser. A schematic representation of the human GnRH receptor shows the domains substituted by catfish receptor ECLs to produce the chimeric GnRH receptors (Figure 5.1).

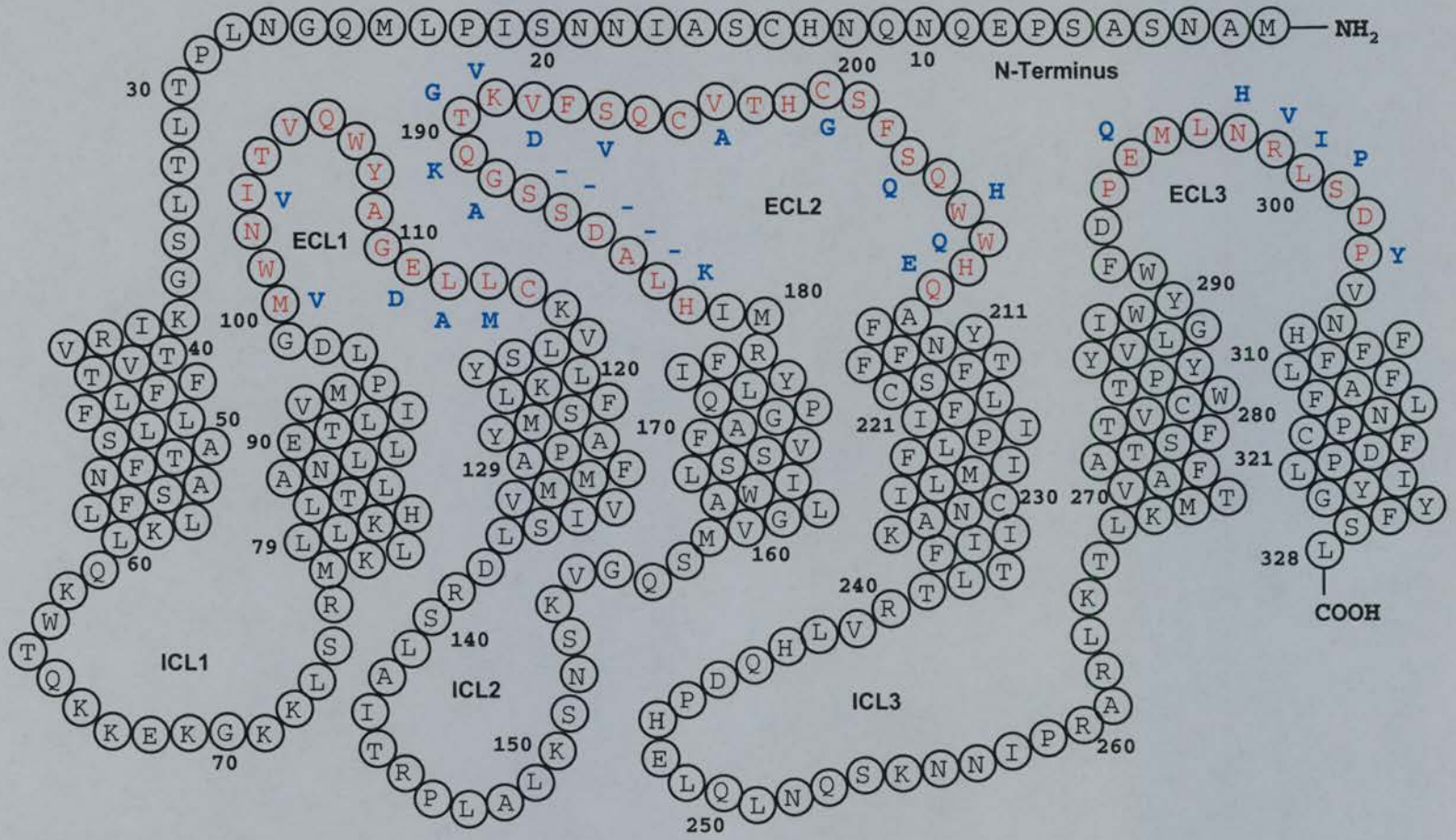


Figure 5.1.
Schematic representation of the human GnRH receptor, showing residues substituted with catfish receptor residues

Domains replaced to produce chimeric receptors indicated in red
Non-conserved catfish receptor ECL residues indicated in blue

5.2.5 Cell Culture and Transfection

COS-7 cells were cultured and transfected as described in Section 3.7.

5.2.6 Receptor Binding Assays

The procedure for receptor binding assays is described in Section 3.8.3. The membrane concentration was adjusted to provide similar receptor numbers. The cells from four 100 mm^2 dishes were used for each binding curve, with the exception of the human receptor containing catfish receptor ECLs 1 and 3, and the wild type catfish receptor. For the human receptor containing catfish receptor ECLs 1 and 3, cells from two 100 mm^2 dishes were used for each curve. For the wild type catfish receptor, cells from one 100 mm^2 dish was used for each curve.

Maximum specific binding ranged between approximately 5000 and 10,000 cpm/tube with non-specific binding ranging between approximately 2000 and 4000 cpm/tube. No specific binding was detected with COS-7 cells transfected with vector only.

5.2.7 Data Reduction and Statistical Analysis

Binding curves were generated and statistical analysis carried out using Prism graphing software (GraphPad) as described in Section 3.9.

5.3 Results

The binding affinity of mGnRH and [D-Trp^6]-GnRH at the wild type catfish GnRH receptor was 6.8 and 17.3-fold lower than that at the wild type human GnRH receptor respectively (Table 5.1). Incorporation of catfish receptor ECL3 into the human receptor with any combination of other ECLs resulted in a significant decrease in the binding affinity of mGnRH compared with that at the wild type human receptor. In contrast, a significant decrease in the binding affinity of [D-

Trp^6]-GnRH compared with that at the wild type human receptor was only observed following substitution with all three catfish ECLs. For both mGnRH and [D-Trp^6]-GnRH, triple ECL substitution reduced the binding affinity at the chimeric receptor to a level not significantly different from that observed at the wild type catfish receptor (Figure 5.2).

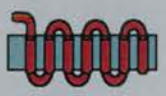
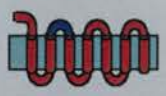
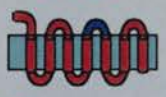
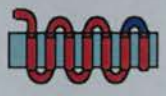
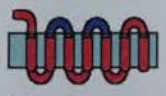
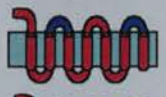
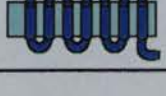
The binding affinity of GnRH II and [D-Lys^6]-GnRH II at the wild type catfish receptor was 104 and 27.1-fold higher than that at the wild type human receptor respectively (Table 5.2). For both GnRH II and [D-Lys^6]-GnRH II, in contrast to that observed for mGnRH and [D-Trp^6]-GnRH, the binding affinity at the chimeric receptor containing all three catfish ECLs was significantly different from that observed at the wild type catfish receptor ($p<0.05$). The binding affinity of GnRH II at the triple ECL-substituted chimeric receptor was just 3.8-fold higher than at the wild type human receptor. The binding affinity of [D-Lys^6]-GnRH II at the triple ECL-substituted chimeric receptor was not significantly different from that at the wild type human receptor (Figure 5.3). Thus, while introduction of the catfish receptor ECLs into the human receptor produces a phenotype for mGnRH and [D-Trp^6]-GnRH indistinguishable from the wild type catfish receptor, these changes do not completely convey catfish receptor binding characteristics with regard to GnRH II and [D-Lys^6]-GnRH II.

A significant increase in the binding affinity of GnRH II compared with that at the wild type human receptor was only observed at chimeric receptors containing both catfish ECLs 2 and 3 (Table 5.2). Surprisingly, a significant decrease in the binding affinity of [D-Lys^6]-GnRH II compared with that at the wild type human receptor was observed at chimeric receptors containing the catfish receptor ECL3 in combination with the human receptor ECL2. This particular combination of ECLs appears to be detrimental for the binding of [D-Lys^6]-GnRH II as its affinities at chimeric receptors containing both catfish receptor ECLs 2 and 3 were not significantly different from that observed at the wild type human receptor.

The binding affinity of Antagonist 135-18 at the wild type catfish GnRH receptor was 46.7-fold lower than that at the wild type human GnRH receptor (Table 5.3). As for GnRH II and [D-Lys^6]-GnRH II, and in contrast to that observed for mGnRH and [D-Trp^6]-GnRH, the binding affinity of Antagonist 135-18 at the chimeric receptor containing all three catfish ECLs was significantly different from that observed at the wild type catfish receptor ($p<0.05$). The affinity of Antagonist 135-18 for this triple ECL-substituted chimeric receptor was just 2.2-fold lower than at the wild type human receptor (Figure 5.4).

Table 5.1. Binding of mGnRH and [D-Trp^6]-GnRH at human-catfish chimeric GnRH receptors

Radioligand binding assays were performed on homogenised membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} data (mean ± S.E.M.) generated by Prism graphing software (GraphPad) as described in Section 3.9.

GnRH Receptor	Schematic	mGnRH			[D-Trp^6]-GnRH		
		IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n
		nM			*nM*		
Wild Type Human		93.8 ± 24.1	1.00	3	2.6 ± 1.1	1.00	6
Human + Catfish ECL1		110.2 ± 10.2	0.85 [ns]	3	11.8 ± 6.9	0.22 [ns]	4
Human + Catfish ECL2		120.5 ± 31.8	0.78 [ns]	4	1.8 ± 0.2	1.44 [ns]	4
Human + Catfish ECL3		273.1 ± 14.4	0.34 **	3	5.3 ± 0.5	0.49 [ns]	3
Human + Catfish ECL1+2		174.7 ± 10.5	0.54 [ns]	3	3.6 ± 0.6	0.72 [ns]	3
Human + Catfish ECL1+3		342.5 ± 18.7	0.27 **	3	4.8 ± 1.6	0.54 [ns]	3
Human + Catfish ECL2+3		313.6 ± 50.2	0.30 *	3	14.2 ± 4.8	0.18 [ns]	3
Human + Catfish ECL1+2+3		395.1 ± 61.6	0.24 *	3	35.3 ± 9.3	0.07 *	3
Wild Type Catfish		633.8 ± 90.6	0.15 **	5	45.3 ± 12.9	0.06 *	5

[a] Mean ± S.E.M. of between three and six experiments carried out in triplicate

[b] Fold increase in binding affinity relative to that at wild type human receptor

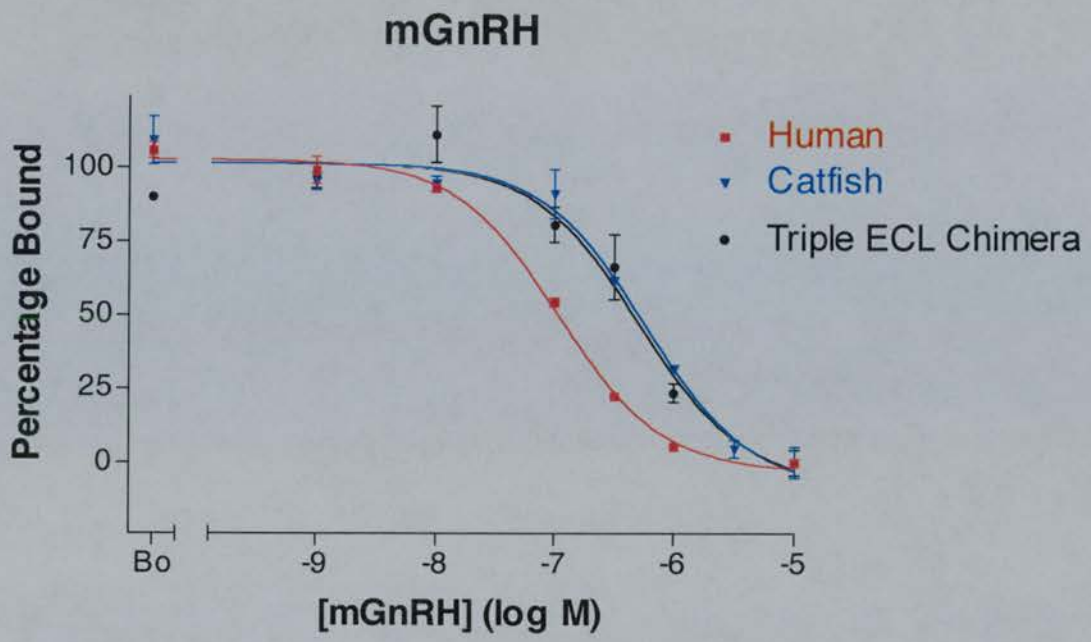
Human and catfish receptor amino acid sequences indicated in red and blue respectively

[ns] Not significantly different from affinity at wild type human receptor, $p>0.05$

* Significantly different from affinity at wild type human receptor, $p<0.05$

** Significantly different from affinity at wild type human receptor, $p<0.01$

A.



B.

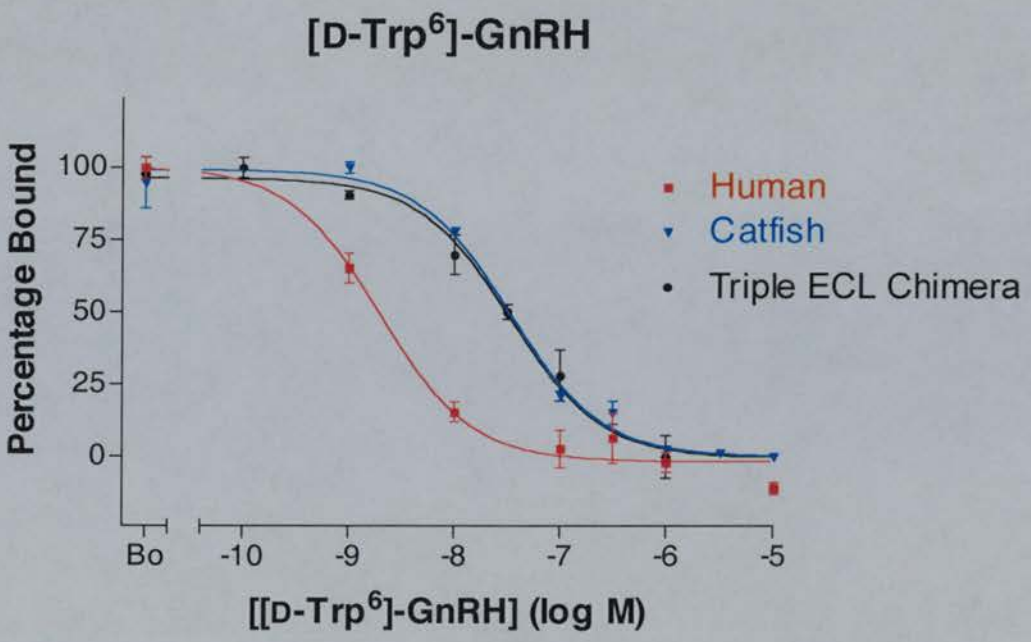
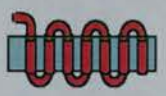
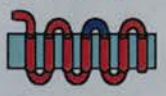
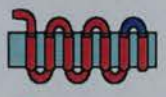
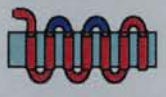


Figure 5.2. Binding of mGnRH **(A)** and [D-Trp^6]-GnRH **(B)** at the human and catfish GnRH receptors, and at the triple ECL-substituted chimeric GnRH receptor

Binding curves representative of between three and six experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

Table 5.2. Binding of GnRH II and [D-Lys^6]-GnRH II at human-catfish chimeric GnRH receptors

Radioligand binding assays were performed on homogenised membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} data (mean ± S.E.M.) generated by Prism graphing software (GraphPad) as described in Section 3.9.

GnRH Receptor	Schematic	GnRH II			[D-Lys^6]-GnRH II		
		IC_{50}[a]	Relative Affinity[b]	n	IC_{50}[a]	Relative Affinity[b]	n
		nM			*nM*		
Wild Type Human		135.6 ± 15.8	1.00	4	19.0 ± 4.8	1.00	5
Human + Catfish ECL1		145.1 ± 37.5	0.93 [ns]	4	40.0 ± 10.4	0.48 [ns]	4
Human + Catfish ECL2		138.4 ± 23.3	0.98 [ns]	3	18.1 ± 3.8	1.05 [ns]	3
Human + Catfish ECL3		157.0 ± 20.2	0.86 [ns]	3	45.3 ± 6.6	0.42 *	4
Human + Catfish ECL1+2		133.1 ± 37.9	1.02 [ns]	3	6.8 ± 1.2	2.80 [ns]	4
Human + Catfish ECL1+3		113.2 ± 21.5	1.20 [ns]	3	49.8 ± 5.5	0.38 *	3
Human + Catfish ECL2+3		54.2 ± 11.1	2.50 *	3	19.9 ± 1.9	0.95 [ns]	3
Human + Catfish ECL1+2+3		35.7 ± 7.8	3.80 **	3	10.7 ± 2.2	1.78 [ns]	3
Wild Type Catfish		1.3 ± 0.2	104 **	5	0.7 ± 0.3	27.1 *	3

[a] Mean ± S.E.M. of between three and five experiments carried out in triplicate

[b] Fold increase in binding affinity relative to that at wild type human receptor

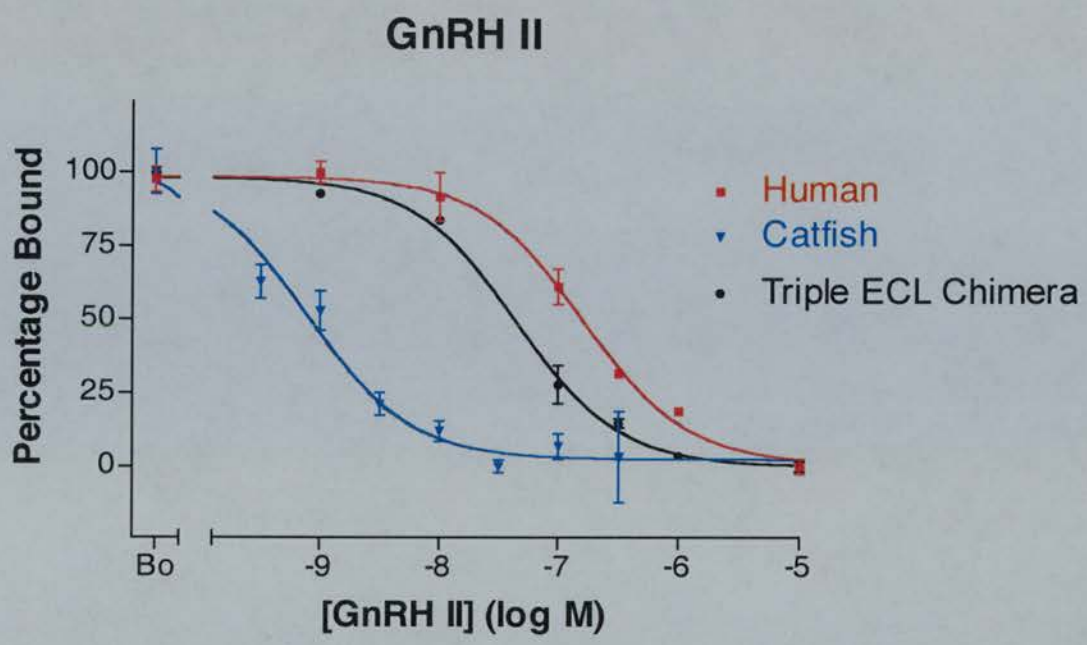
Human and catfish receptor amino acid sequences indicated in red and blue respectively

[ns] Not significantly different from affinity at wild type human receptor, $p>0.05$

* Significantly different from affinity at wild type human receptor, $p<0.05$

** Significantly different from affinity at wild type human receptor, $p<0.01$

A.



B.

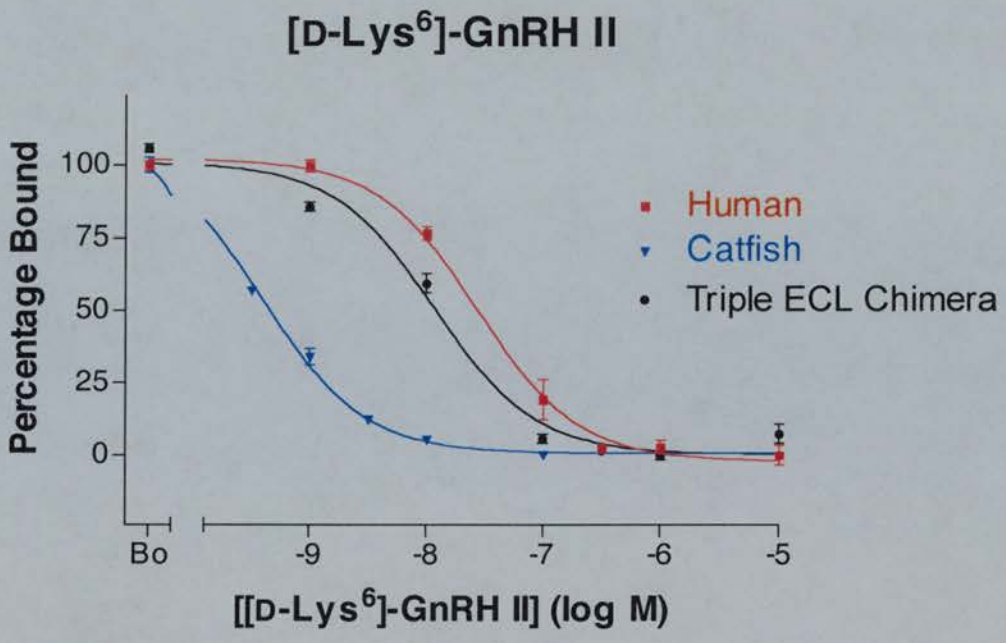
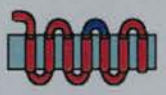
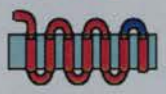
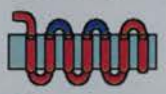
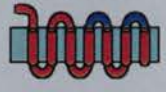
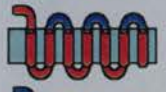


Figure 5.3. Binding of GnRH II **(A)** and [D-Lys^6]-GnRH II **(B)** at the human and catfish GnRH receptors, and at the triple ECL-substituted chimeric GnRH receptor

Binding curves representative of between three and five experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

Table 5.3. Binding of Antagonist 135-18 at human-catfish chimeric GnRH receptors

Radioligand binding assays were performed on homogenised membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} data (mean ± S.E.M.) generated by Prism graphing software (GraphPad) as described in Section 3.9.

		Antagonist 135-18		
GnRH Receptor	Schematic Diagram	IC_{50}[a]	Relative Affinity[b]	n
		nM		
Wild Type Human		8.1 ± 1.0	1.00	3
Human + Catfish ECL1		18.7 ± 9.3	0.43 [ns]	3
Human + Catfish ECL2		11.0 ± 0.1	0.74 [ns]	3
Human + Catfish ECL3		2.7 ± 0.2	3.00 *	3
Human + Catfish ECL1+2		43.1 ± 2.6	0.19 **	3
Human + Catfish ECL1+3		2.3 ± 0.4	3.52 *	3
Human + Catfish ECL2+3		9.1 ± 2.3	0.89 [ns]	3
Human + Catfish ECL1+2+3		18.1 ± 1.2	0.45 **	3
Wild Type Catfish		377.5 ± 65.3	0.02 *	3

[a] Mean ± S.E.M. of three experiments carried out in triplicate

[b] Fold increase in binding affinity relative to that at wild type human receptor

Human and catfish receptor amino acid sequences indicated in red and blue respectively

[ns] Not significantly different from affinity at wild type human receptor, $p>0.05$

* Significantly different from affinity at wild type human receptor, $p<0.05$

** Significantly different from affinity at wild type human receptor, $p<0.01$

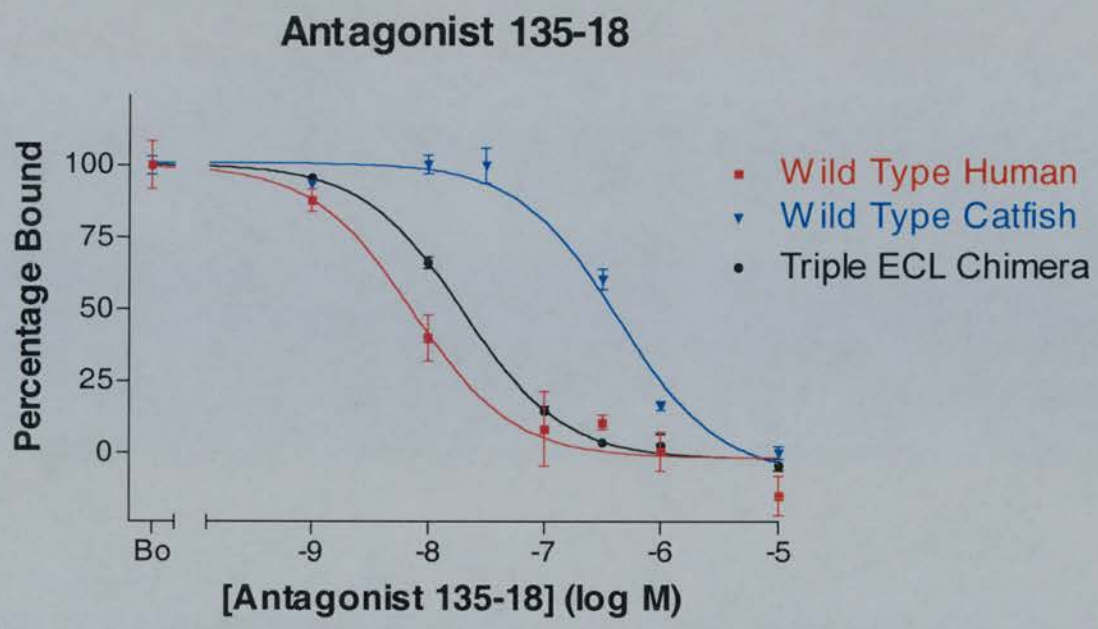


Figure 5.4. Binding of Antagonist 135-18 at the human and catfish GnRH receptors, and at the triple ECL-substituted chimeric GnRH receptor

Binding curves representative of three experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

A significant decrease in the binding affinity of Antagonist 135-18 compared with that at the wild type human receptor was only observed at chimeric receptors containing catfish receptor ECLs 1 and 2 (Table 5.3). Surprisingly, a significant increase in the binding affinity of Antagonist 135-18 compared with that at the wild type human receptor was observed at chimeric receptors containing the catfish receptor ECL3 in combination with the human receptor ECL2. This particular combination of ECLs appears to improve the binding of Antagonist 135-18 as its affinities at chimeric receptors containing both catfish receptor ECLs 2 and 3 were not significantly different from, or were significantly lower than, that observed at the wild type human receptor.

5.4 Discussion

A significant decrease in the binding affinity of mGnRH compared with that at the wild type human receptor was observed at the chimeric receptor containing the catfish receptor ECL3 (Table 5.1). This agrees with previous suggestions that the conformation of ECL3 is important for high affinity binding of mGnRH (Flanagan et al., 1994; Fromme et al., 2001). Asp/Glu$^{7.32}$ in mammalian type I receptors is believed to interact with Arg^{8} in mGnRH (Flanagan et al., 1994; Fromme et al., 2001). In the human receptor Asp$^{7.32}$ is followed by proline (SDP), whereas in the catfish receptor it is preceded by proline (PDY). This difference may alter the orientation of the aspartate side-chain, thereby affecting the interaction with Arg^{8} in mGnRH (Millar et al., 1997; Petry et al., 2002). A recent study suggests that Arg^{8} in mGnRH interacts with Asp$^{7.32}$ in the catfish receptor (Blomenrohr et al., 2002). Therefore, if the orientation of the aspartate side-chain in the catfish receptor is changed compared with in the human receptor, this does not appear to prevent interaction with Arg^{8} in mGnRH. Instead, the interaction may position the ligand less suitably for interaction with other contact sites compared with its positioning at the human receptor. The ligand would still interact with these other contact sites, but there would be an energetic penalty due to this alterative orientation. This would explain how a change in conformation of ECL3 reduces the binding affinity of mGnRH even though the Arg^{8} interaction still occurs.

mGnRH still had a significantly higher binding affinity at all single and double ECL-substituted chimeric receptors containing the catfish receptor ECL3 than at the wild type catfish receptor ($p<0.05$). However, it had an affinity at the triple ECL-substituted chimeric receptor that was not significantly different from that at the wild type catfish receptor (Figure 5.2). Therefore, in addition to the altered ECL3 conformation, the interaction of all three catfish ECLs appears to reduce the binding affinity of mGnRH.

In contrast to that observed with mGnRH, the binding affinity of [D-Trp^{6}]-GnRH was not changed significantly as a result of substituting ECL3. This also agrees with previous observations that conformational constraint of mGnRH overcomes the requirement for Arg^{8} to interact with Asp$^{7.32}$ for high affinity binding (Fromme et al., 2001). A significant decrease in the binding affinity of [D-Trp^{6}]-GnRH compared with that at the wild type human receptor was only observed following substitution with all three catfish receptor ECLs. As with mGnRH, [D-Trp^{6}]-GnRH had an affinity at this triple ECL-substituted chimeric receptor that was

not significantly different from that at the wild type catfish receptor (Figure 5.2). The interaction of all three catfish ECLs appears to reduce the binding affinity of mGnRH and [D-Trp^6]-GnRH, by altering the spatial arrangement of ECL contact sites, and/or by influencing the ease with which the ligand can interact with contact sites within the TMDs.

For GnRH II, [D-Lys^6]-GnRH II and Antagonist 135-18, in contrast to that observed for mGnRH and [D-Trp^6]-GnRH, triple ECL substitution had little effect on binding affinity (Figures 5.3 and 5.4). ECL substitutions may not restore the binding of GnRH II and [D-Lys^6]-GnRH II because if contact sites are introduced, they may be incorrectly configured. The spatial arrangement of the ECLs will, in part, be dictated by the relative positioning of the TMDs to which they are anchored. It has been suggested that mammalian type I and non-mammalian receptor TMDs have different configurations (Blomenrohr et al., 1997; Sun et al., 2001). Therefore, the three catfish receptor ECLs substituted into the human receptor may not be in the same spatial arrangement as in the wild type catfish receptor. Alternatively, additional GnRH II and [D-Lys^6]-GnRH II contact sites may exist within the TMDs or N-terminus.

It is surprising that the binding affinity of Antagonist 135-18 at the triple ECL-substituted chimeric receptor is only 2.2-fold lower than at the wild type human receptor, implying that this antagonist binds wholly to sites within the TMDs and N-terminus, or that it interacts with ECL contact sites that are conserved between human and catfish receptors, but configured differently due to the different positioning of the ECLs by the TMDs. The latter scenario may explain how Antagonist 135-18 acts as an antagonist at the human receptor, a partial agonist at the *Xenopus* I receptor and a full agonist at the chicken receptor (Sun et al., 2001; Ott et al., 2002). The binding of the ligand may result in stabilisation of distinct receptor conformations due to the different configuration of the conserved contact sites in the different receptors. Lys$^{3.32}$ is believed to be crucial for agonist binding, but not for antagonist binding (Zhou et al., 1995). Perhaps the different configuration of the conserved ECL contact sites alters the ability of Antagonist 135-18 to interact with such TMD contact sites that differentiate between agonists and antagonists.

This study has highlighted differences between the binding of mGnRH, GnRH II, superagonists and Antagonist 135-18 at GnRH receptors. The different affinities of mGnRH and [D-Trp^6]-GnRH for the human receptor compared with the catfish receptor can be explained by the different ECLs. This is not the case for GnRH II, [D-Lys^6]-GnRH II or Antagonist 135-18, implying that these ligands form different interactions with GnRH receptors compared with those formed by mGnRH

and [D-Trp^6]-GnRH. This is unsurprising for Antagonist 135-18, as antagonists have been shown to form different receptor interactions compared with agonists (Janovick et al., 1993; Assefa et al., 1999). However, Asp$^{2.61}$, Asn$^{2.65}$ and Lys$^{3.32}$, which are all conserved between the human and catfish receptors, are believed to interact with both mGnRH and GnRH II (Zhou et al., 1995; Davidson et al., 1996; Flanagan et al., 2000; Blomenrohr et al., 2001). Therefore, mGnRH and GnRH II would appear to have different ligand conformations, form different additional ligand-receptor interactions, and/or interact with conserved contact sites that are in different spatial arrangements in human and catfish receptors.

The study described in Chapter 4 suggested that residues five to eight of GnRH II are pre-configured for interaction with non-mammalian receptors, but that D-Lys^6 substitution may alter this conformation to improve binding at mammalian type I receptors. The N and C-termini may be configured differently in mGnRH compared with GnRH II. NMR studies of mGnRH and cGnRH I ([Gln^8]-GnRH) show these two ligands having similar turn conformations around Gly^6, but very different conformations of the N and C-termini (Maliekal et al., 1997). cGnRH I differs from mGnRH by a single residue. Therefore, it is conceivable that GnRH II, which has three residues different from mGnRH, also has different N and C-terminal conformations. As discussed above, mammalian type I and non-mammalian receptors may have different configurations. Therefore, I suggest that the conformation of mGnRH selects for the human receptor and that of GnRH II selects for the differently configured catfish receptor. It has been suggested that, as Arg^8 in mGnRH interacts with Asp$^{7.32}$ in the catfish receptor, the reason for mGnRH binding to the human receptor with higher affinity is due to the side-chains of Tyr^5 and Leu^7 fitting better than in the catfish receptor (Blomenrohr et al., 2002). However, the study described in Chapter 4 shows that sGnRH ([Trp^7,Leu^8]-GnRH) binds with low affinity to a mammalian type I receptor (IC_{50} of 1134 nM at mouse receptor) and with higher affinity to the catfish receptor (IC_{50} of 52 nM). Therefore, it would appear that Tyr^5 is tolerated by the catfish receptor (as it is present in sGnRH). Trp^7 seems to confer high affinity binding to ligands acting at the catfish receptor, whereas the conformation of ECL3 appears responsible for the high binding affinity of [Arg^8]-containing ligands at the mammalian type I receptors.

Although ECL substitution did not account for the difference in binding affinities of GnRH II, [D-Lys^6]-GnRH II and Antagonist 135-18 at the human receptor compared with the catfish receptor, there were significant changes in the binding affinity of these ligands at chimeric receptors compared with that at the wild type human receptor (Tables 5.2 and 5.3). A significant increase in the binding

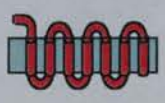
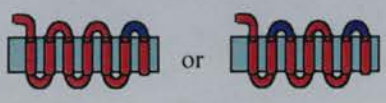
affinity of GnRH II compared with that at the wild type human receptor was observed at chimeric receptors containing both catfish ECLs 2 and 3. This implies that the interaction of these two ECLs alters the spatial arrangement of ECL contact sites, and/or influences the ease with which the ligand can access contact sites within the TMDs. Models of the human, rat, mouse and catfish GnRH receptors show TMDs 5 and 6 in close proximity, implying that the C-terminus of ECL2 and the N-terminus of ECL3 are also in close proximity (Zhou et al., 1994; Chauvin et al., 2000; Hoffmann et al., 2000; Blomenrohr et al., 2001; Blomenrohr et al., 2002). Furthermore, a number of residues in this region affect receptor conformation and/or ligand binding, namely $Trp^{5.33}$, $Tyr^{5.38}$, $Asn^{5.39}$, $Phe^{5.41}$, $Thr^{5.42}$ and $Trp^{6.48}$ (Chauvin et al., 2000; Hoffmann et al., 2000). Therefore, the present study supports previous suggestions that this region forms part of the ligand binding pocket (Chauvin et al., 2000; Hoffmann et al., 2000), and implies that the interactions between ECLs 2 and 3 in the human receptor are less conducive to GnRH II binding than in the catfish receptor.

The binding affinity of [D-Lys^6]-GnRH II at the triple ECL-substituted chimeric receptor was not significantly different from that at the wild type human receptor. D-Lys^6 appears to alter the conformation of GnRH II, or form an additional interaction with the human receptor, so that triple catfish ECL substitution does not significantly improve binding. If the interaction of ECLs 2 and 3 in the human receptor is less conducive to the binding of GnRH II, D-Lys^6 appears to overcome this. It would then be a further example of conformational constraint around position six of the ligand overcoming changes in ECL conformation, as observed with conformationally-constrained GnRH analogues overcoming the requirement for Arg^8 to interact with ECL3 (Fromme et al., 2001). Catfish ECL substitution was expected to increase the binding affinity of [D-Lys^6]-GnRH II. Therefore, a surprising observation is that the ECL configuration resulting from the artificial interaction between human ECL2 and catfish ECL3 appears to reduce the binding affinity of [D-Lys^6]-GnRH II at chimeric receptors compared with that at the wild type human receptor. This also implies that [D-Lys^6]-GnRH II has a different conformation to GnRH II or that the D-Lys^6 forms an additional interaction with the receptor, which is disrupted by this particular ECL interaction.

The significant decrease in the binding affinity of Antagonist 135-18 at chimeric receptors containing catfish ECLs 1 and 2 compared with that at the wild type human receptor, implies that the interaction of these two ECLs alters the spatial arrangement of ECL contact sites and/or influences the ease with which the ligand can access contact sites within the TMDs. This interaction does not appear to affect

agonist binding, again illustrating that Antagonist 135-18 has a different binding mode compared to the agonists. The artificial interaction between human ECL2 and catfish ECL3 results in a surprising observation with Antagonist 135-18. Instead of decreasing binding affinity for Antagonist 135-18, this interaction appears to increase binding affinity at chimeric receptors compared with that at the wild type human receptor. It would appear that this receptor conformation is very well suited to the binding of Antagonist 135-18, which binds at the wild type human receptor with higher affinity than at the wild type catfish receptor (Table 5.4). On the other hand, it is particularly unsuitable for binding [D-Lys^6]-GnRH II, which binds at the wild type catfish receptor with higher affinity than at the wild type human receptor (Table 5.4). These observations provide evidence that Antagonist 135-18 and [D-Lys^6]-GnRH II select for different receptor configurations and that, as observed for GnRH II, the interaction of ECLs 2 and 3 influences configuration of the binding sites.

Table 5.4. Antagonist 135-18 and [D-Lys^6]-GnRH II differentiate between three apparently different configurations of GnRH receptor

	Configuration A:	Configuration B:	Configuration C:
	Wild Type Catfish GnRH Receptor	Wild Type Human GnRH Receptor	Chimeric GnRH Receptors containing Human Receptor ECL2 and Catfish Receptor ECL3
			
Relative Affinity:			
Antagonist 135-18	+	+++	++++
[D-Lys^6]-GnRH II	+++++	+++	++

Human and catfish receptor amino acid sequences indicated in red and blue respectively

Relative ligand binding affinity:

+, IC_{50} > 125 nM

++, IC_{50} of 25-125 nM

+++, IC_{50} of 5-25 nM

++++, IC_{50} of 1-5 nM

+++++, IC_{50} < 1 nM

In conclusion, it would appear that mGnRH, GnRH II, superagonists and Antagonist 135-18 interact with GnRH receptors in distinct conformations that are influenced differently by ECL interactions, particularly between ECLs 2 and 3. The introduction of all three catfish receptor ECLs into the human receptor produces a phenotype for mGnRH and [D-Trp^6]-GnRH indistinguishable from the wild type catfish receptor, however, these changes do not completely convey catfish receptor binding characteristics with regard to GnRH II, [D-Lys^6]-GnRH II or Antagonist 135-18. Additional receptor contact sites for these ligands may exist within the N-terminus/TMDs, and/or the three catfish receptor ECLs substituted into the human receptor are not in the same spatial arrangement as in the wild type catfish receptor.

Human and catfish receptors appear to have distinct conformations that select for different ligands. With increasing evidence of multiple GnRH ligands and multiple GnRH receptors within each species (Powell et al., 1996; Tensen et al., 1997; Troskie et al., 1998; White et al., 1998; Illing et al., 1999; Millar et al., 2001; Wang et al., 2001), the importance of this selectivity is beginning to be realised. This study illustrates that point-mutation studies on G-protein coupled receptors, while being extremely valuable, cannot in isolation explain differences between the binding affinities of different receptors. Increased knowledge of ligand and receptor conformations is crucial for understanding how the ligand interacts with the receptor and, in the case of agonists, stabilises the active receptor conformation.

6 The Effect of Extracellular Loops 2 and 3 on GnRH Analogue Binding

6.1 Introduction

As discussed in Chapter 5, models of GnRH receptors show the outer regions of TMDs 5 and 6 in close proximity, implying that the C-terminus of ECL2 and the N-terminus of ECL3 are also in close proximity (Zhou et al., 1994; Chauvin et al., 2000; Hoffmann et al., 2000; Blomenrohr et al., 2001; Blomenrohr et al., 2002). Furthermore, a number of residues in this region of TMD5/TMD6 affect receptor conformation and/or ligand binding, namely $Trp^{5.33}$, $Tyr^{5.38}$, $Asn^{5.39}$, $Phe^{5.41}$, $Thr^{5.42}$ and $Trp^{6.48}$ (Chauvin et al., 2000; Hoffmann et al., 2000). Therefore, it has been suggested that this region of TMD5/TMD6 forms part of the ligand binding pocket (Chauvin et al., 2000; Hoffmann et al., 2000).

It is apparent from the study of human-catfish chimeric GnRH receptors that the interactions of ECLs influence ligand binding, either by altering the spatial arrangement of ECL contact sites, and/or by influencing the ease with which the ligand can interact with contact sites within the TMDs (see Chapter 5). Interactions between ECLs 2 and 3 were found to affect the binding affinity of GnRH II, [D-Lys^6]-GnRH II and Antagonist 135-18 at these chimeric GnRH receptors.

This chapter focuses on the putative interaction between ECLs 2 and 3 in greater detail. The study described in Chapter 4 showed that GnRH II had a significantly lower binding affinity at the rat receptor compared with at the human and mouse receptors, an unexpected finding considering that rat and mouse GnRH receptors have 95% sequence homology (Eidne et al., 1992; Kaiser et al., 1992). The present study proposes that a single residue in ECL2, namely $Glu^{5.35}$, is likely to be responsible for these different affinities. The negatively charged $Glu^{5.35}$ is proposed to repel one of the negatively charged residues ($Asp^{6.61}$ or $Glu^{6.63}$) in the N-terminus of ECL3, thereby altering receptor configuration.

The human receptor has a histidine residue in the C-terminus of ECL2 and two acidic residues in the N-terminus of ECL3, whereas the chicken receptor has the opposite (residue positions indicated in red in Figure 6.1).

A.

	C-Terminus of ECL2	TMD5
Sheep	FSQCVTHCSFPQWWHQA	FYNFFTFSCLFII
Cow	FSQCVTHCSFPQWWHQA	FYNFFTFSCLFII
Dog	FPQCVTHCSFPQWWHQA	FYNFFTFSCLFII
Bonnet macaque	FSQCVTHCSFPQWWHQA	FYNFFTFSCLFII
Human	FSQCVTHCSFSQWWHQA	FYNFFTFSCLFII
Mouse	FSQCVTHCSFPQWWHQA	FYNFFTFGCLFII
Rat	FSQCVTHCSFPQWWHEA	FYNFFTFSCLFII
Striped bass	FTQCTTRGSFVTHWHET	AYNMFTFSCLFLL
Catfish	FVQCATHGSFQQHWQET	AYNMFHFVTLYVF
Bullfrog	FTQCATHGSFAQHWQET	AYNMFTFCTLFVT
Xenopus	FTQCATHGSFTQHWQET	AYNMFTFCTLFVT
Chicken	FTQCVTHGSFRAHWEET	VYNMFTFTTLYIT
Xenopus (II)	FVQCATVGSFQAHWQET	IYNMFTFFCLFLL
Bullfrog (II)	FVQCATVGSFKAHWLET	LYNMFTFCCLFLL
	↑ ↑↑ ↑	

B.

	TMD6	N-Terminus of ECL3
Sheep	CWTPYYVLGIWY	WFDPDMVN
Cow	CWTPYYVLGIWY	WFDPDMVN
Dog	CWTPYYVLGIWY	WFDPEMLN
Bonnet macaque	CWTPYYVLGIWY	WFDPEMLN
Human	CWTPYYVLGIWY	WFDPEMLN
Mouse	CWTPYYVLGIWY	WFDPEMLN
Rat	CWTPYYVLGIWY	WFDPEMLN
Striped bass	CWTPYYLLGLWY	WFFPDDLE
Catfish	CWTPYYLLGIWY	WFQPQMLH
Bullfrog	CWTPYYLLGIWY	WFQPEMIY
Xenopus	CWTPYYLLGLWY	WFQPEMIN
Chicken	CWTPYYLLGLWY	WFHPAMIQ
Xenopus (II)	CWTPYYLLGIWY	WFSPEMLT
Bullfrog (II)	CWTPYYLLGIWY	WFSPEMLT

Figure 6.1. Partial amino acid sequences of different GnRH receptors: C-terminus of ECL2/outer region of TMD5 **(A)**; outer region of TMD6/N-terminus of ECL3 **(B)**.

Human, rat and chicken GnRH receptor partial amino acid sequences indicated in blue

Loci investigated in the present study indicated in red

Loci previously found to affect GnRH receptor conformation and/or ligand binding indicated in green

Loci previously found to confer agonism to Antagonist 135-18 indicated in orange

↑, Residues in the C-terminus of ECL2 that differ between *Xenopus* and chicken GnRH receptors

It was hypothesised that, by studying different combinations of substitutions at these positions in human-chicken chimeric receptors, insights would be gained as to their role in ligand binding. Five such chimeric receptors (human receptor with $His^{207(5.34)}Glu/Gln^{208(5.35)}Glu$; human receptor containing chicken receptor ECL2; human receptor containing chicken receptor ECL3; human receptor with $His^{207(5.34)}Glu/Gln^{208(5.35)}Glu$ and containing chicken receptor ECL3; and human receptor containing chicken receptor ECLs 2 and 3) were used to demonstrate that ECLs 2 and 3 play an important role in ligand binding and/or receptor expression.

6.2 Materials and Methods

6.2.1 Amino Acid Numbering Scheme

This scheme facilitates comparisons between G-protein coupled receptors and is described in section 3.1.

6.2.2 GnRH Analogues

mGnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) and [D-Trp^6]-GnRH were supplied by Bachem. [D-Lys^6]-GnRH II was a gift from the University of Cape Town, South Africa. Antagonist 135-18 ([Ac-D-Nal(2)^1,D-4-Cl-Phe^2,D-Pal(3)^3,Ile^5,D-Lys(iPr)^6,Lys(iPr)^8,D-Ala-$NH_2{}^{10}$]-GnRH) was a gift from R. Roeske (Indiana University, Indianapolis, U.S.A.).

6.2.3 GnRH Receptor cDNA Supplied to the Study

Human (Chi et al., 1993) and chicken (Sun et al., 2001) GnRH receptor cDNA constructs were gifts from the University of Cape Town, South Africa. The rat GnRH receptor was cloned by this laboratory (Eidne et al., 1992). The human receptor cDNA with $Gln^{5.35}$ mutated to Glu (Ott et al., 2002) and the human receptor/chicken receptor ECL2 cDNA (Sun et al., 2001) were produced previously

by T. Ott. The human receptor/chicken receptor ECL3 cDNA was modified from a construct produced by B. Fromme (University of Cape Town, South Africa).

6.2.4 Production of Chimeric GnRH Receptor cDNA

Techniques utilised for molecular cloning, as well as plasmid DNA production, purification and analysis, are described in Sections 3.2-3.5. The human GnRH receptor cDNA construct had previously been engineered to include a number of silent mutations, thereby introducing restriction sites at the TMD/ECL boundaries (Ott et al., 2002). All constructs were generated in the pBluescript vector (Stratagene) and subsequently subcloned into the pcDNA1/Amp vector (Invitrogen) for transfection into COS-7 cells (see Section 3.6).

For production of the human receptor containing the $His^{5.34}Glu$ and $Gln^{5.35}Glu$ mutations, an antisense oligonucleotide primer was designed and synthesised (Genosys) that spanned the *Stu* I restriction site and the sequence coding for the two mutations (see Appendix III). A PCR fragment was generated using this primer, the T3 primer (see Appendix III), the proof-reading Deep Vent DNA polymerase (New England Biolabs) and the engineered human GnRH receptor cDNA as template. The PCR fragment was digested with *Stu* I and *Eco*R I, and ligated into the engineered human GnRH receptor cDNA construct that had been digested with the same enzymes. This was used to transform One Shot™ Top 10 cells (Invitrogen) by heat-shock and plasmid DNA was extracted from the resultant colonies using the QIAprep Spin Miniprep Kit (Qiagen). The DNA was initially screened by digesting with *Stu* I and *Pfl*M I and the fragment sizes confirmed by agarose gel electrophoresis. Subsequently, DNA was confirmed by automated sequencing using an ABI Prism 310 Genetic Analyser.

The human receptor/chicken receptor ECL3 cDNA construct was modified by digesting with *Hpa* I and *Bsr*G I, followed by ligation of the isolated receptor cDNA fragment into the engineered human receptor cDNA. The cDNA encoding the human receptor with $His^{5.34}Glu$, $Gln^{5.35}Glu$/chicken receptor ECL3 was generated by digesting the human receptor/chicken receptor ECL3 cDNA with *Stu* I and *Xho* I, followed by ligation of the isolated receptor cDNA fragment into the $His^{5.34}Glu$ and $Gln^{5.35}Glu$ mutated human receptor cDNA. Following transformation and plasmid DNA extraction, both of these constructs were screened by digesting with *Ava* I, which cuts chicken receptor ECL3 cDNA, but not human receptor ECL3 cDNA. Subsequently, the DNA sequence was confirmed by automated sequencing.

The human receptor/chicken receptor ECLs 2 and 3 cDNA was generated by digesting the human receptor/chicken receptor ECL2 cDNA with *Stu* I and *Eco*R I, followed by ligation of the isolated receptor cDNA fragment into the human receptor/chicken receptor ECL3 cDNA. Following transformation and plasmid DNA extraction, this construct was screened by digesting with *Dra* III, which cuts chicken receptor ECL2 cDNA, but not human receptor ECL2 cDNA. The DNA sequence was confirmed by automated sequencing. A schematic representation of the human GnRH receptor showing the mutated residues is shown in Figure 6.2.

6.2.5 Cell Culture and Transfection

COS-7 cells were cultured and transfected as described in Section 3.7.

6.2.6 Receptor Binding Assays

The procedure for receptor binding assays is described in Section 3.8.3. For the human and rat receptor study, the membrane concentration was adjusted to provide similar receptor numbers. This was achieved by using cells from four 100 mm^2 dishes for each binding assay using wild type and mutated human receptors, while cells from two 100 mm^2 dishes were used for each assay using the wild type rat receptor. Maximum specific binding was similar for all receptors, ranging between approximately 5000 and 10,000 cpm/tube. For the human-chicken chimeric receptor study, cells from four 100 mm^2 dishes were used for each binding curve. The human receptor containing the chicken receptor ECL2 exhibited maximal specific binding ranging between approximately 8000 and 22,000 cpm/tube, with wild type human and chicken receptors exhibiting between approximately 5000 and 10,000 cpm/tube. For both studies, non-specific binding ranged between approximately 2000 and 4000 cpm/tube and no specific binding was detected with COS-7 cells transfected with vector only (see Section 3.8.4).

6.2.7 Data Reduction and Statistical Analysis

Binding curves were generated and statistical analysis carried out using Prism graphing software (GraphPad) as described in Section 3.9.

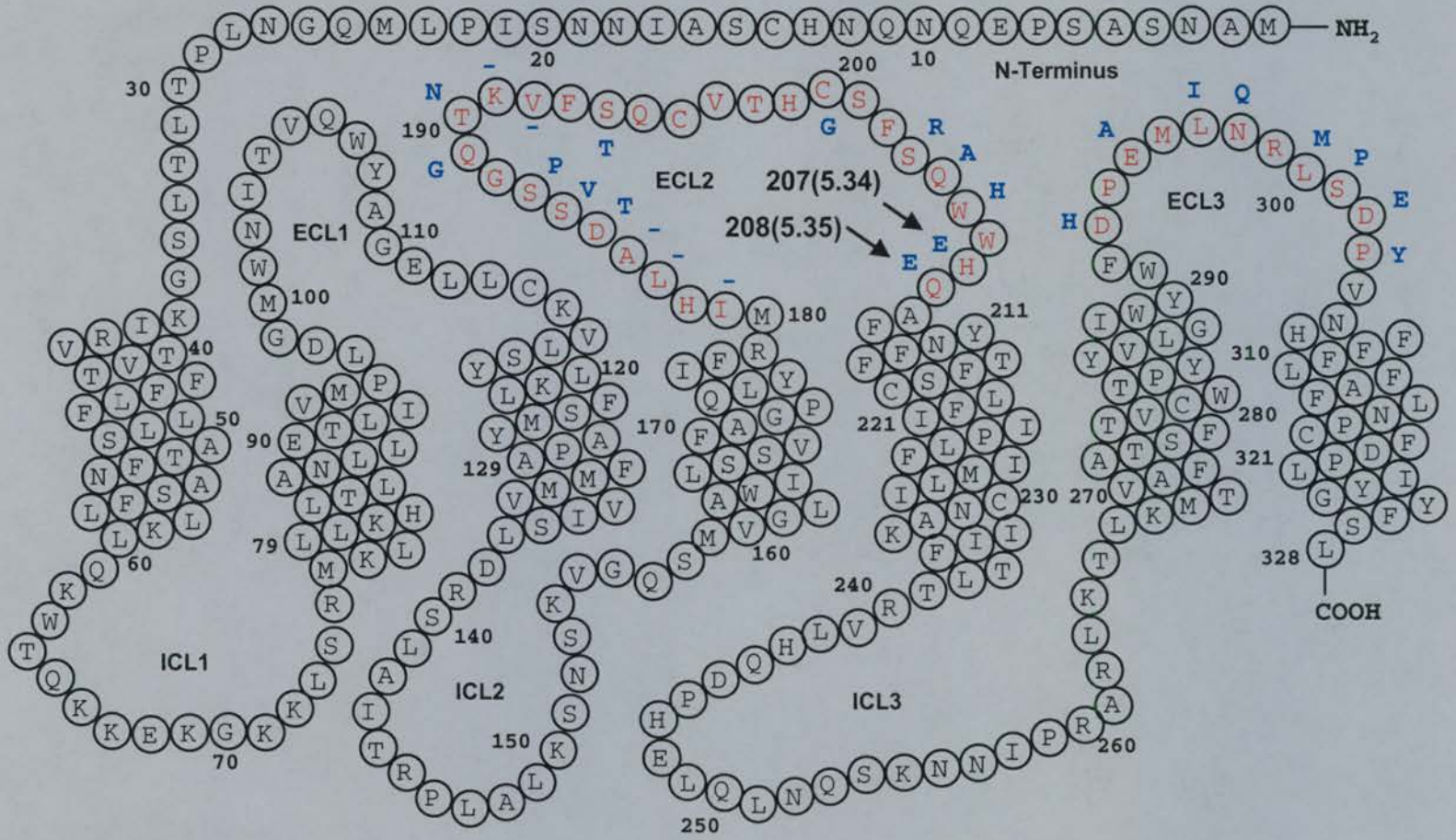


Figure 6.2.
Schematic representation of the human GnRH receptor, showing residues substituted with chicken receptor residues

Domains replaced to produce chimeric receptors indicated in red
Non-conserved chicken receptor ECL residues indicated in blue

6.3 Results

Mammalian type I GnRH receptors have conserved $His^{5.34}$ (H) and $Gln^{5.35}$ (Q) residues in the C-terminus of ECL2, as well as conserved $Asp^{6.61}$ (D) and $Asp/Glu^{6.63}$ (D/E) residues in the N-terminus of ECL3 (indicated in red in Figure 6.1). The rat GnRH receptor is an interesting exception, having the negatively charged $Glu^{5.35}$ (E) instead of the neutral Gln (Q). Substitution of D-Arg^6 or D-Lys^6 into GnRH II enhanced binding at the rat receptor more than at the mouse or human receptors (see Chapter 4). Comparison of the amino acid sequences of the rat and mouse GnRH receptors shows that there are only 17 amino acids not conserved between these species, only 6 of which are in extracellular domains (Figure 6.3). $Glu^{5.35}$ was selected as the prime candidate for differentiating the rat receptor from the mouse and human receptors, with respect to GnRH II binding (indicated in blue in Figure 6.3).

The binding affinity of GnRH II at the wild type rat GnRH receptor was significantly lower than at the wild type human GnRH receptor ($p<0.05$) (see Chapter 4). When $Gln^{5.35}$ was mutated to Glu, as in the rat receptor, the affinity of GnRH II at the human receptor was indistinguishable from that at the rat receptor (Table 6.1 and Figure 6.4). In contrast, the binding affinities of [D-Lys^6]-GnRH II at the wild type rat and mutant human receptors were not significantly different from that at the wild type human receptor.

The lower binding affinity of mGnRH at the wild type rat receptor compared with that at the wild type human receptor was barely significant ($p=0.0499$), and the affinity at the mutant human receptor was not significantly different from that at either wild type receptor (Table 6.1 and Figure 6.4). The binding affinities of [D-Trp^6]-GnRH were not significantly different at the three receptors, as was the case for Antagonist 135-18. It would appear that the residue in position 5.35 influences the binding of GnRH II, but not [D-Lys^6]-GnRH II, mGnRH, [D-Trp^6]-GnRH or Antagonist 135-18.

Figure 6.3

Amino acid sequence alignment of the rat (top) and mouse (bottom) GnRH receptors

•, Conserved residue

|, Non-conserved residue

Putative extracellular domains indicated in red

Glu/Gln$^{5.35}$ indicated in blue

Table 6.1
Ligand binding at the human, human $Gln^{208(5.35)}Glu$ mutant and rat GnRH receptors

Radioligand binding assays were performed on homogenised membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} data (mean ± S.E.M.) generated by Prism graphing software (GraphPad) as described in Section 3.9.

	Wild Type Human		Human $Gln^{208(5.35)}Glu$		Wild Type Rat	
Ligand	IC_{50}[a]	n	IC_{50}[a]	n	IC_{50}[a]	n
	nM		*nM*		*nM*	
GnRH II	135.6 ± 15.8	4	350.1 ± 15.5 ***	3	323.0 ± 33.1 *	3
[D-Lys^6]-GnRH II	19.0 ± 4.8	5	20.0 ± 2.0 [ns]	3	9.6 ± 1.1 [ns]	3
mGnRH	93.8 ± 24.1	3	115.1 ± 12.8 [ns]	3	243.8 ± 40.5 *	3
[D-Trp^6]-GnRH	2.6 ± 1.1	6	0.53 ± 0.12 [ns]	3	2.0 ± 1.2 [ns]	3
Antagonist 135-18	8.1 ± 1.0	3	22.6 ± 9.1 [ns]	3	21.9 ± 5.8 [ns]	3

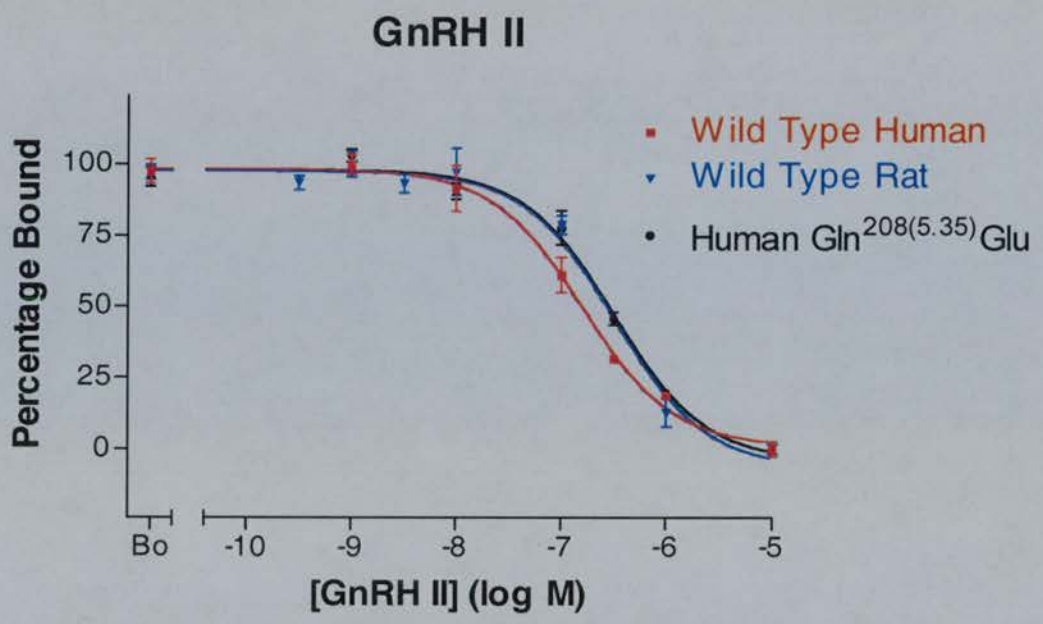
[a] Mean ± S.E.M. of between three and six experiments carried out in triplicate

[ns], Not significantly different from wild type human, $p>0.05$

*, Significantly different from wild type human, $p<0.05$

***, Significantly different from wild type human, $p<0.001$

A.



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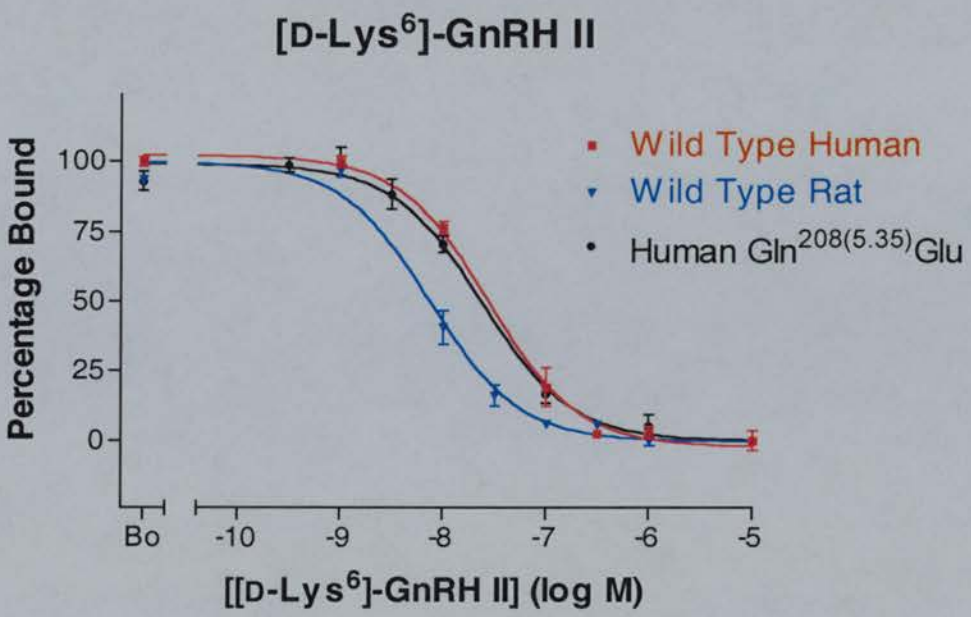
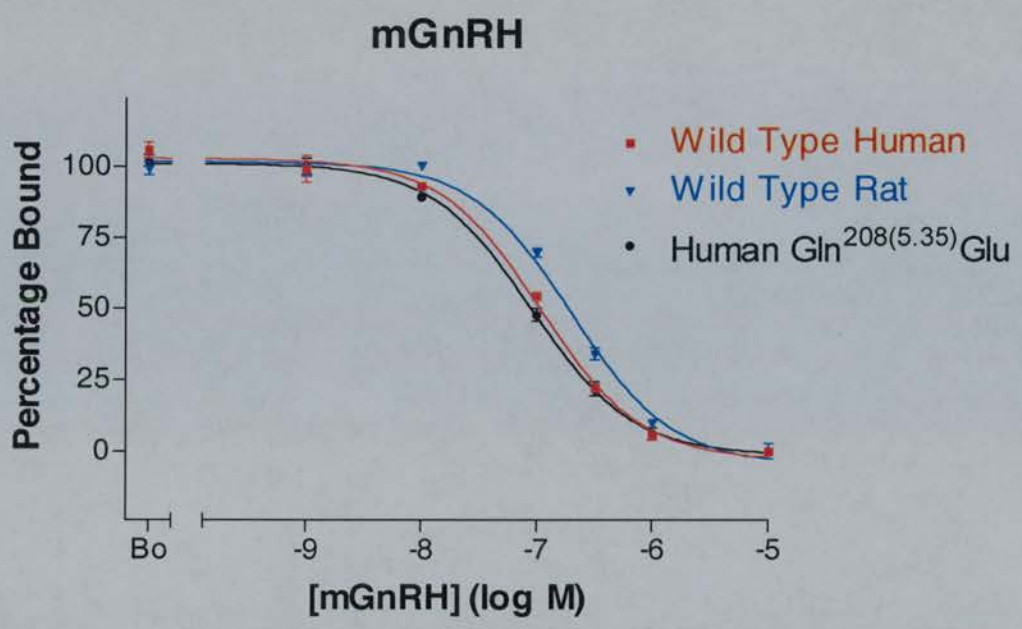


Figure 6.4. Binding of GnRH II **(A)** and [D-Lys^6]-GnRH II **(B)** at the human, human Gln$^{208(5.35)}$Glu mutant and rat GnRH receptors

Binding curves representative of between three and six experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

C.



D.

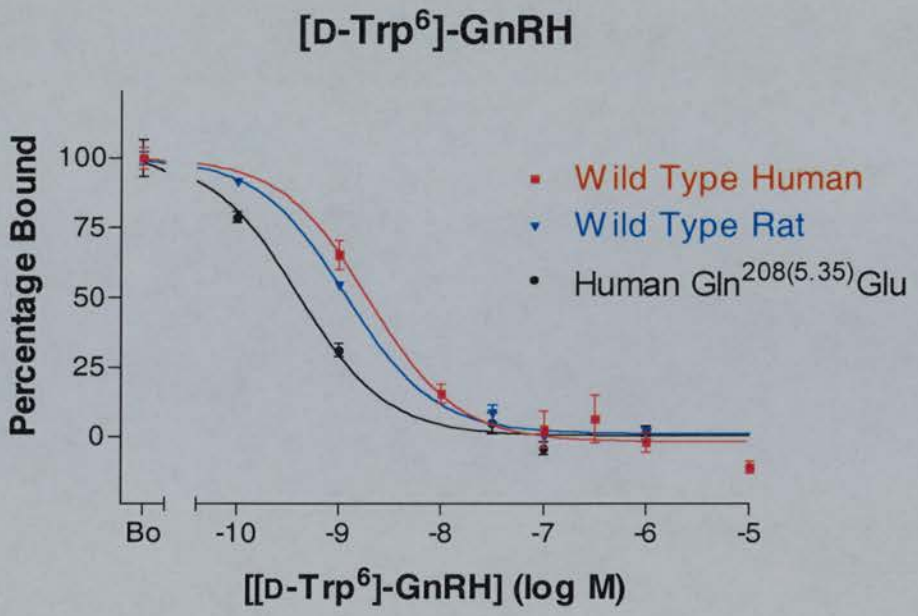


Figure 6.4 (Continued). Binding of mGnRH **(C)** and [D-Trp^6]-GnRH **(D)** at the human, human Gln$^{208(5.35)}$Glu mutant and rat GnRH receptors

E.

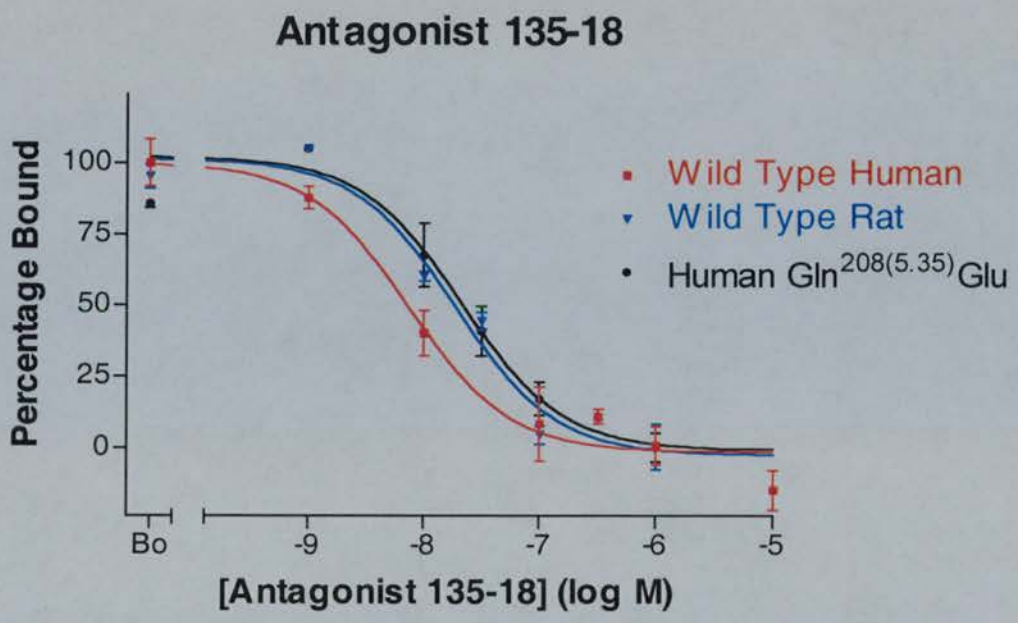


Figure 6.4 (Continued). Binding of Antagonist 135-18 **(E)** at the human, human $Gln^{208(5.35)}Glu$ mutant and rat GnRH receptors

The chicken GnRH receptor has two acidic residues in the C-terminus of ECL2 ($Glu^{5.34}$ (E) and $Glu^{5.35}$ (E)), as well as $His^{6.61}$ (H) in the N-terminus of ECL3 (indicated in red in Figure 6.1). This is the opposite arrangement to most mammalian type I GnRH receptors.

Mutation of $Gln^{5.35}$ to Glu in the human receptor significantly decreases the binding affinity of GnRH II (2.6-fold lower than at the wild type human receptor), but not [D-Lys^6]-GnRH II (Table 6.1). The double mutation of $His^{5.34}$ to Glu and $Gln^{5.35}$ to Glu results in levels of specific radioligand binding too low to provide reproducible data (n=3) (Table 6.2). Surprisingly, substitution of the entire chicken receptor ECL2 into the human receptor resulted in apparently higher specific binding (approximately 8000-22,000 cpm/tube) than at either the wild type human or chicken receptors (approximately 5000-10,000 cpm/tube). The binding affinity of GnRH II at this chimeric receptor was even lower than at the $Gln^{5.35}Glu$ mutant receptor (5.1-fold lower than at the wild type human receptor), although the affinity of [D-Lys^6]-GnRH II was again not significantly different from that at the wild type human receptor (Table 6.2 and Figure 6.5).

The substitution of the chicken receptor ECL3 into the human receptor again resulted in levels of specific radioligand binding too low to provide reproducible data (n=3) (Table 6.2). Substitution of $His^{5.34}Glu/Gln^{5.35}Glu$ or the chicken receptor ECL2 in addition to the chicken receptor ECL3 did not restore specific radioligand binding.

Table 6.2. Agonist binding at human-chicken chimeric GnRH receptors

Radioligand binding assays were performed on homogenised membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} data (mean ± S.E.M.) generated by Prism graphing software (GraphPad) as described in Section 3.9.

Mutation in Human GnRH Receptor	GnRH II		[D-Lys^6]-GnRH II	
	IC_{50}[a]	n	IC_{50}[a]	n
	nM		*nM*	
Wild Type Human Receptor[b]	135.6 ± 15.8	4	19.0 ± 4.8	5
$Gln^{208(5.35)}Glu$[b]	350.1 ± 15.5 ***	3	20.0 ± 2.0 [ns]	3
$His^{207(5.34)}Glu/Gln^{208(5.35)}Glu$	N.D.	3	N.D.	3
Chicken ECL2	693.4 ± 74.7 *	3	44.8 ± 10.7 [ns]	4
Chicken ECL3	N.D.	3	N.D.	3
$His^{207(5.34)}Glu/Gln^{208(5.35)}Glu$/Chicken ECL3	N.D.	3	N.D.	3
Chicken ECLs 2 and 3	N.D.	3	N.D.	3
Wild Type Chicken Receptor	0.75 ± 0.21 **	7	0.19 ± 0.03 *	3

[a] Mean ± S.E.M. of between three and seven experiments carried out in triplicate

[b] Data shown in Table 6.1: included in this table for comparison

N.D., Not determined: specific binding too low to provide reproducible data (n=3)

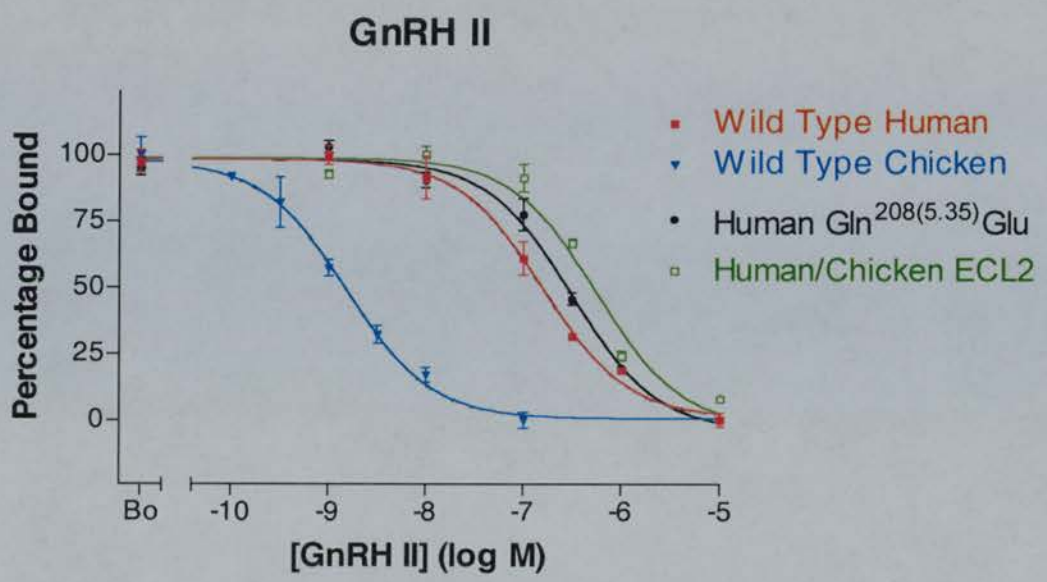
[ns], Not significantly different from wild type human, $p>0.05$

*, Significantly different from wild type human, $p<0.05$

**, Significantly different from wild type human, $p<0.01$

***, Significantly different from wild type human, $p<0.001$

A.



B.

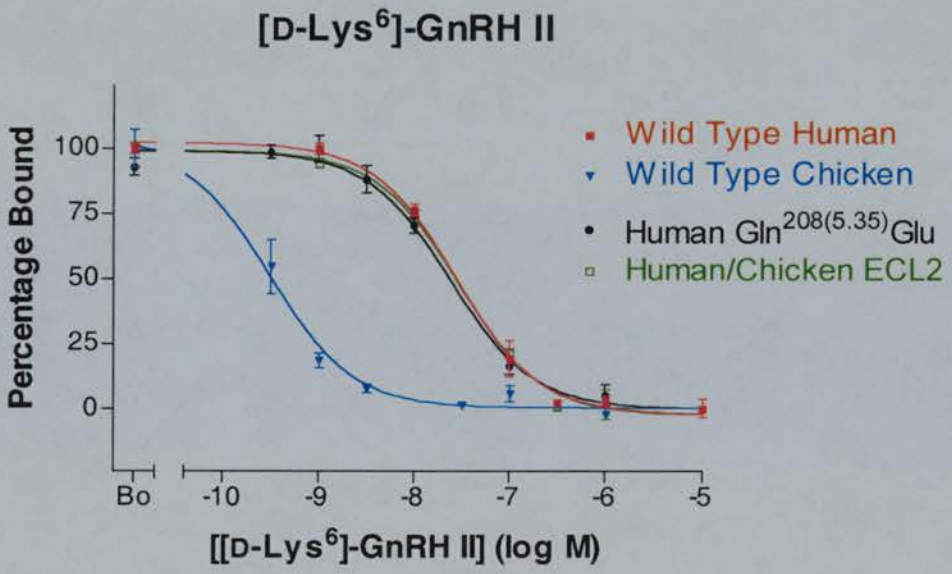


Figure 6.5.

Binding of GnRH II and [D-Lys^6]-GnRH II at human-chicken chimeric GnRH receptors compared with at the wild type human and chicken GnRH receptors

Binding curves representative of between three and six experiments from which the IC_{50} data were generated by Prism graphing software (GraphPad) as described in Section 3.9.

6.4 Discussion

D-Arg^{6} substantially increased the binding affinity of GnRH II at the rat receptor, but not at the mouse or human receptors. D-Lys^{6} increased the binding affinity of GnRH II at the rat receptor much more (33.8-fold) than at the human (7.2-fold) and mouse receptors (8.4-fold) (see Chapter 4). A search for amino acids different in the rat receptor from the mouse and human receptors revealed the presence of a glutamate residue in ECL2 of the rat receptor in the homologous position to a glutamine residue in the mouse and human receptors (Figures 6.1 and 6.2). The mutation of Gln$^{5.35}$ to Glu in the human receptor reduced the binding affinity of GnRH II to that at the rat receptor and D-Lys^{6} substitution increased binding affinity by a similar amount to that found with the rat receptor. Either Gln$^{5.35}$ was able to interact with GnRH II in a manner not possible by Glu in this position, or the introduction of a negative charge was detrimental to GnRH II binding. As GnRH II does not contain a negatively charged residue to repel Glu$^{5.35}$, an intramolecular charge repulsion that alters receptor configuration should be considered. Models of the human, rat, mouse and catfish GnRH receptors imply that the C-terminus of ECL2 is in close proximity to the N-terminus of ECL3 (Zhou et al., 1994; Chauvin et al., 2000; Hoffmann et al., 2000; Blomenrohr et al., 2001; Blomenrohr et al., 2002). In mammalian type I GnRH receptors, the N-terminus of ECL3 contains two acidic residues: Asp$^{6.61}$ and Glu/Asp$^{6.63}$ (indicated in red in Figure 6.1). Charge repulsion between either of these residues and Glu$^{5.35}$ could explain the low binding of GnRH II at the rat receptor, as the resultant change in spatial arrangement of ECLs 2 and 3 could cause distortion of GnRH II binding site configuration and/or influence the ease with which GnRH II can interact with contact sites within the TMDs.

The concept that such a change in ECL spatial arrangement could affect ligand binding is supported by the identification of a number of residues in this region that affect receptor conformation and/or ligand binding (Chauvin et al., 2000; Hoffmann et al., 2000) (indicated in green in Figure 6.1). This putative repulsion between ECLs 2 and 3 appears to affect the binding of GnRH II, but not [D-Lys^{6}]-GnRH II, again implying that [D-Lys^{6}]-GnRH II has a different conformation to GnRH II or that the D-Lys^{6} forms an additional interaction with the receptor (see Chapters 4 and 5).

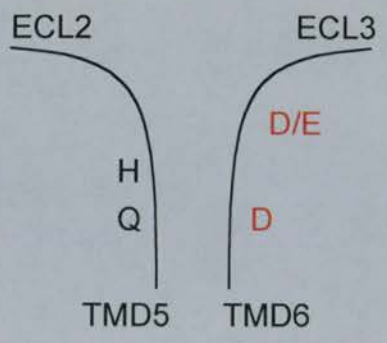
The results for mGnRH were less conclusive. The binding affinity of mGnRH at the rat receptor was significantly lower than at the human receptor, but

only marginally (p=0.0499). However, the affinity of mGnRH at the point-mutated human receptor was not significantly different from that at either the human or rat wild type receptors. [D-Trp^6]-GnRH bound to both rat and human receptors with similar affinities and so it was unsurprising that the affinity of [D-Trp^6]-GnRH at the point-mutated human receptor was similar to that at both human and rat wild type receptors. Similarly, Antagonist 135-18 bound to all three receptors with similar affinities, implying that the putative change in spatial arrangement of ECLs 2 and 3 did not influence binding of this ligand. This is perhaps unsurprising as, like [D-Trp^6]-GnRH and [D-Lys^6]-GnRH II, it is also conformationally-constrained by a D-amino acid in position 6 (D-Lys(iPr)).

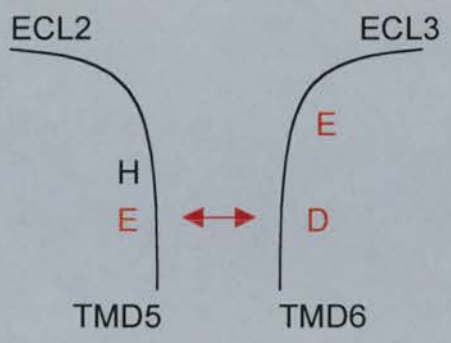
It appears that the acidic residues in this region of both mammalian type I and non-mammalian GnRH receptors are arranged so that they do not repel each other. The exception appears to be the rat receptor, in which putative repulsion of acidic residues in ECLs 2 and 3 appears detrimental to GnRH II binding (Figure 6.6). Residues in close proximity often have reciprocal arrangements in different receptors, an example of which is Asp$^{2.50}$ and Asn$^{7.49}$ in most G-protein coupled receptors instead of Asn$^{2.50}$ and Asp$^{7.49}$ in mammalian type I GnRH receptors (Zhou et al., 1994; Flanagan et al., 1999). The reciprocal arrangement of His and two acidic residues in ECLs 2 and 3 of human and chicken GnRH receptors supports the proposal that these regions are in close proximity.

The hypothesis that residues in ECLs 2 and 3 interact with (and potentially repel) each other thereby affecting ligand binding was tested using human-chicken chimeric receptors. The mutation of Gln$^{208(5.35)}$ to Glu in the human receptor was detrimental to the binding of GnRH II. It was therefore envisaged that the double mutation of His$^{207(5.34)}$Glu/Gln$^{208(5.35)}$Glu in the human receptor, or the substitution of chicken receptor ECL2 in the human receptor, would be even more detrimental. To test whether this was due to an interaction between ECLs, ECL3 was substituted for that of the chicken receptor in combination with His$^{207(5.34)}$Glu/Gln$^{208(5.35)}$Glu or chicken receptor ECL2, the hypothesis being that this would produce the reciprocal arrangement of residues and restore ligand binding. Substitution of the chicken receptor ECL2 into the human receptor resulted in an even greater decrease in the binding affinity of GnRH II than the Gln$^{207(5.35)}$Glu mutation, concurrent with the hypothesis. The binding affinity of GnRH II at the wild type chicken receptor was 181-fold higher than at the wild type human receptor. It would appear that artificial interactions between the chicken receptor ECL2 and the remainder of the human receptor are responsible for the lower binding affinity of GnRH II at the human receptor containing the chicken receptor ECL2.

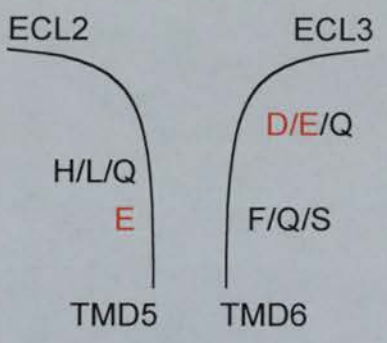
A. Mammalian type I GnRH Receptors, except Rat GnRH Receptor:



B. Rat GnRH Receptor:



C. Non-mammalian GnRH Receptors, except Chicken GnRH Receptor:



D. Chicken GnRH Receptor:

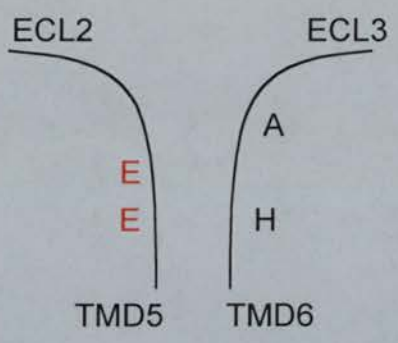


Figure 6.6. Schematic diagrams showing possible arrangements of ECLs 2 and 3

Mammalian type I GnRH receptors except the rat GnRH receptor ***(A)*** *have two acidic residues in the N-terminus of ECL3, but none in the C-terminus of ECL2. The rat GnRH receptor* ***(B)*** *has acidic residues in both the C-terminus of ECL2 and the N-terminus of ECL3. These residues are proposed to repel each other, thereby altering receptor configuration. Non-mammalian GnRH receptors, except the chicken GnRH receptor* ***(C)*** *have acidic residues in both the C-terminus of ECL2 and the N-terminus of ECL3. However, these residues appear to be arranged so that they do not repel each other. The chicken GnRH receptor* ***(D)*** *has two acidic residues in the C-terminus of ECL2 but none in the N-terminus of ECL3.*

Acidic residues indicated in red

↔, indicates putative charge repulsion

The human receptor with the $His^{207(5.34)}Glu/Gln^{208(5.35)}Glu$ double mutation bound radioligand too poorly to provide reproducible data. Preliminary findings by A. Pawson indicate that this receptor produces agonist-induced inositol phosphate (IP) turnover and therefore appears to be expressed at the cell membrane. It would seem that the $His^{207(5.34)}Glu/Gln^{208(5.35)}Glu$ mutation in the human receptor reduces receptor expression without abolishing it, or disrupts binding of ^{125}I-[His^5,D-Tyr^6]-GnRH. This is surprising considering that substitution of the entire chicken receptor ECL2 into the human receptor seems to result in higher specific radioligand binding than at the wild type receptor (approximately 8000-22,000 cpm/tube compared with 5000-10,000 cpm/tube). Perhaps the conformation of the chicken receptor ECL2 presents the two acidic residues differently so that they reduce the binding of GnRH II, but not that of conformationally-constrained GnRH analogues, as was the case with the $Gln^{208(5.35)}Glu$ mutation. Indeed, [D-Lys^6]-GnRH II was found to bind at the chicken receptor ECL2-substituted receptor with an affinity not significantly different from that at the wild type human receptor, supporting this concept.

Previous studies have shown that substituting non-mammalian GnRH receptor ECL2s into the human receptor influences ligand binding. Substitution of chicken receptor ECL2 into the human receptor conferred partial agonism to Antagonist 135-18, which was an antagonist at the wild type human receptor and a full agonist at the wild type chicken receptor (Sun et al., 2001). Furthermore, substitution of the *Xenopus* type I GnRH receptor ECL2 into the human receptor conferred full agonism to Antagonist 135-18, with partial agonism being conferred by substitution with portions of ECL2 (Ott et al., 2002). This study went on to identify a double mutation required for Antagonist 135-18 to be recognised as an agonist at the human receptor, namely $Val^{5.24}Ala$ and $Trp^{5.32}His$ (indicated in orange in Figure 6.1), implicating an interaction between $His^{5.32}$ in the receptor and D-$Lys(iPr)^6$ in Antagonist 135-18 through a charge-supported hydrogen bond. The mutation of $Trp^{5.32}$ to His on its own was not sufficient, suggesting that a particular conformation of the ECL is required to present this residue for ligand interaction. Interestingly, the wild type chicken receptor has $Val^{5.24}$ and therefore would be expected not to confer agonism to Antagonist 135-18. As this is not the case (Sun et al., 2001), the conformation of ECL2 in the chicken receptor is likely to be different from that in the *Xenopus* I receptor. The only differences between these receptors in the C-terminus of ECL2 are indicated by arrows in Figure 6.1. It is interesting to note that a positively-charged residue, arginine, is unique to the chicken receptor in position 5.30, which is also unique in having two negatively-charged residues in this region. Perhaps the interaction of these residues configures ECL2 differently in the

chicken receptor compared with the *Xenopus* I receptor, and indeed the human receptor.

Substitution of the chicken receptor ECL3 into the human receptor again resulted in specific radioligand binding too low to provide reproducible data. It was hoped that substitution of $His^{5.34}Glu/Gln^{5.35}Glu$ or chicken receptor ECL2 in addition to chicken receptor ECL3 would restore function as appears to be the case when the chicken receptor ECL2 is substituted instead of just $His^{5.34}Glu/Gln^{5.35}Glu$. Unfortunately this was not the case as specific radioligand binding remained low, although preliminary data produced by A. Pawson measuring IP turnover again indicates that these receptors are expressed at the cell membrane.

It is unclear why substitution of the chicken receptor ECL3 has this effect, particularly as substitution of the catfish receptor ECL3 into the human receptor is well tolerated (see Chapter 5). It would seem that the chicken receptor has a number of differences compared with other non-mammalian type I receptors, such as *Xenopus* I and catfish. It is even more surprising that the combination of chicken receptor ECLs 2 and 3 did not restore function. The concept that mammalian type I and non-mammalian GnRH receptors may have different configurations of the TMDs resulting in different ECL spatial arrangements was discussed in Chapter 5. Substitution of both chicken receptor ECLs 2 and 3 into the human receptor may not restore function because these loops are presented differently compared with in the wild type chicken receptor.

In conclusion, this study has shown that certain residues in the C-terminus of ECL2 affect ligand binding and/or receptor expression, namely those in positions 5.34 and 5.35. Indeed, the presence of $Glu^{5.35}$ in the rat receptor is likely to be the reason for it binding GnRH II with low affinity, differentiating it from the mouse and human receptors. It is suggested that residues in these positions are in sufficiently close proximity to residues in the N-terminus of ECL3 for charge repulsion to occur, thereby influencing receptor configuration.

7 Concluding Discussion

This thesis has obtained data in support of the concept that, as for the mammalian type I GnRH receptors, the non-mammalian GnRH receptors have a preference for GnRH in the folded conformation involving a β-II′ turn for residues five to eight, which is enhanced in mGnRH, cGnRH I and sGnRH by D-aa^{6} substitution. In contrast, the evolutionarily conserved GnRH II ligand appears to have a pre-configured β-II′ turn that accounts for its relatively high affinity for all GnRH receptors and a failure, in most instances, of any enhancement of binding affinity with D-aa^{6} substitution.

Future research should include the use of more techniques, such as NMR and conformational memories, to gain further insights into the bioactive conformation of GnRH II. Three pertinent questions are: do these techniques confirm the findings of this thesis; how do the N and C terminal configurations compare to those of mGnRH and cGnRH I; and if GnRH II does indeed possess a pre-configured β-II′ turn for residues five to eight as this thesis suggests, what are the intramolecular interactions responsible for the stabilisation of this structure?

As part of the characterisation of newly cloned mammalian type II GnRH receptors, the effect of conformational constraint on the binding of GnRH analogues should be assessed. In particular, does [D-Lys^{6}]-GnRH II bind to these receptors with higher affinity than GnRH II as at mammalian type I receptors, or is the β-II′ turn involving residues five to eight of GnRH II optimally pre-configured for interaction with these receptors as it appears to be for non-mammalian receptors?

The study of human-catfish chimeric GnRH receptors found that mGnRH, GnRH II, superagonists and Antagonist 135-18 interact with GnRH receptors in distinct conformations that are influenced differently by ECL interactions, particularly between ECLs 2 and 3. The findings confirmed the importance of ECL3 conformation in the binding of mGnRH, despite recent indications that Asp$^{7.32}$ in the catfish receptor interacts with Arg^{8} in mGnRH (Blomenrohr et al., 2002).

Future studies to continue the research on human-catfish chimeric receptors should investigate binding to the N-terminus using two further chimeric receptors: a human receptor containing the catfish receptor N-terminus; and a human receptor containing all of the catfish receptor extracellular domains. The results should either implicate the N-terminus as an important determinant of ligand-receptor interactions, or eliminate it as such. The latter finding would leave two possible reasons why triple catfish receptor ECL substitution into the human receptor had little effect on

the binding affinity of GnRH II, [D-Lys^6]-GnRH II or Antagonist 135-18: additional receptor contact sites exist within the TMDs and/or the three catfish receptor ECLs substituted into the human receptor are not in the same spatial arrangement as in the wild type catfish receptor.

In the last few years, a number of point-mutation studies have identified various GnRH receptor residues that indirectly influence ligand binding through effects on receptor conformation (Millar, 2002). Important microdomains have been identified, such as the interaction between positions 2.50 and 7.49 (Flanagan et al., 1999). This thesis identifies both ECL conformation and interactions between ECLs as important for ligand binding. In particular, the findings indicate that a single residue, namely Glu/Gln$^{5.35}$ in ECL2, may be responsible for the lower affinity of GnRH II for rat compared with human and mouse GnRH receptors. This residue is proposed to influence configuration of GnRH II binding sites due to charge repulsion between ECLs 2 and 3. This illustrates that, while point-mutation studies on G-protein coupled receptors are extremely valuable, they cannot in isolation explain how ligands interact with their cognate receptors. It is critical to understand how the ligand and receptor are configured for interaction with each other. Understanding how contact sites are presented for interaction is as important as knowing which residues actually interact.

The research into interactions between ECLs 2 and 3 discussed in Chapter 6 should be continued by investigating intracellular coupling to signalling pathways. The preliminary findings by A. Pawson show that human-chicken chimeric receptors can initiate downstream signalling events that are agonist dependent, despite radioligand binding being too low to provide reproducible receptor binding data. If such studies show that the interaction between these ECLs alters the EC_{50}, this may be due to altered ligand binding, but could also be due to changes in coupling efficiency. A more direct approach would be to carry out binding studies using a radiolabelled antagonist that binds to contact sites not affected by partial disruption of the agonist binding pocket, while retaining the ability to compete with ligands still able to occupy the agonist binding pocket. However, such an antagonist would also need to bind the poorly expressed human GnRH receptor with high affinity. The radiolabelled agonist used in the present study was selected due to its unusually high affinity for the human receptor (Flanagan et al., 1998).

A second follow-up study to investigate how interactions between ECLs 2 and 3 influence ligand binding involves the identification of residues in the extracellular end of TMD5 that differ between the human and chicken receptors (the extracellular end of TMD6 is completely conserved between mammalian and non-

mammalian GnRH receptors). Point-mutated human and chicken receptors should then be produced to examine the role of the non-conserved residues. An example is the residue in position 5.36 at the boundary of ECL2 and TMD5 (Figure 6.1). The residue is an alanine in all mammalian type I receptors and a threonine in all non-mammalian receptors. A threonine residue has the capacity to form hydrogen bonds, whereas an alanine residue does not. Additionally, the residue one helical turn below position 5.36 in the human receptor is $Asn^{212(5.39)}$ (Figure 2.8), which is believed to be involved in the ligand binding pocket (Hoffmann et al., 2000). A second possibility is a residue adjacent to position 5.39. $Met^{5.40}$ in TMD5 is conserved in non-mammalian receptors, but is Phe in mammalian type I receptors.

A few years ago, GnRH receptors were divided into mammalian and non-mammalian receptors. Following the cloning of three mammalian type II GnRH receptors (Millar et al., 2001; Neill et al., 2001), it is now clear that mammalian type I and type II receptors have distinct structures and pharmacological profiles. Indeed, mammalian type II receptors share many characteristics with non-mammalian receptors, not least the presence of a C-terminal tail and the selectivity for GnRH II over mGnRH (Millar et al., 2001; Neill et al., 2001).

This thesis suggests that the 'non-mammalian' group will inevitably be sub-divided as our understanding of GnRH receptor structure and function increases. Division into types I, II and III based on sequence homology is only the first step. This thesis indicates that type I GnRH receptors from birds, such as the chicken, are distinct from type I GnRH receptors from fish and amphibians, such as the catfish and *Xenopus* I.

mGnRH and its cognate receptor have a critical role in the control of human reproduction, with GnRH receptor agonists and antagonists having a wide range of important clinical applications. The identification of new and improved agonists and antagonists promises to have enormous health benefits, and the key to unlocking this potential lies in a better understanding of how GnRH interacts with its cognate receptor. This thesis has contributed to this improved understanding, by providing novel insights into structural determinants of GnRH ligand-receptor interactions.

Bibliography

Adams BA, Vickers ED, Warby C, Park M, Fischer WH, Grey CA, Rivier JE and Sherwood NM (2002) Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, Coregonus clupeaformis. *Biol Reprod* **67**:232-239.

Albert AD and Litman BJ (1978) Independent structural domains in the membrane protein bovine rhodopsin. *Biochemistry* **17**:3893-900.

Allman K, Page KM, Curtis CA and Hulme EC (2000) Scanning mutagenesis identifies amino acid side chains in transmembrane domain 5 of the M(1) muscarinic receptor that participate in binding the acetyl methyl group of acetylcholine. *Mol Pharmacol* **58**:175-84.

Alok D, Hassin S, Sampath Kumar R, Trant JM, Yu K and Zohar Y (2000) Characterization of a pituitary GnRH-receptor from a perciform fish, Morone saxatilis: functional expression in a fish cell line. *Mol Cell Endocrinol* **168**:65-75.

Amory JK and Bremner W (2001) Endocrine regulation of testicular function in men: implications for contraceptive development. *Molecular and Cellular Endocrinology* **182**:175-179.

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

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

Arora KK, Cheng Z and Catt KJ (1997) Mutations of the conserved DRS motif in the second intracellular loop of the gonadotropin releasing hormone receptor affect expression, activation and internalization. *Mol Endocrinol* **11**:1203-1212.

Assefa D, Pawson AJ, McArdle CA, Millar RP, Flanagan CA, Roeske R and Davidson JS (1999) A new photoreactive antagonist cross-links to the N-terminal domain of the gonadotropin-releasing hormone receptor. *Mol Cell Endocrinol* **156**:179-188.

Attwood TK, Eliopoulos EE and Findlay JB (1991) Multiple sequence alignment of protein families showing low sequence homology: a methodological approach using database pattern-matching discriminators for G-protein-linked receptors. *Gene* **98**:153-9.

Awara WM, Guo CH and Conn PM (1996) Effects of Asn318 and Asp87Asn318 mutations on signal transduction by the gonadotropin-releasing hormone receptor and receptor regulation. *Endocrinology* **137**:655-62.

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

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

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

Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci* **25**:366-428.

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

Baniak EL, 2nd, Rivier JE, Struthers RS, Hagler AT and Gierasch LM (1987) Nuclear magnetic resonance analysis and conformational characterization of a cyclic decapeptide antagonist of gonadotropin-releasing hormone. *Biochemistry* **26**:2642-56.

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

Barbieri RL (1992) Clinical applications of GnRH and its analogues. *Trends Endocrinol Metab* **3**:30-34.

Barnes MR, Duckworth DM and Beeley LJ (1998) Frizzled proteins constitute a novel family of G protein-coupled receptors, most closely related to the secretin family. *Trends Pharmacol Sci* **19**:399-400.

Beck M, Sakmar TP and Siebert F (1998) Spectroscopic evidence for interaction between transmembrane helices 3 and 5 in rhodopsin. *Biochemistry* **37**:7630-9.

Beinborn M, Lee YM, McBride EW, Quinn SM and Kopin AS (1993) A single amino acid of the cholecystokinin-B/gastrin receptor determines specificity for non-peptide antagonists. *Nature* **362**:348-50.

Bennett WS, Jr. and Steitz TA (1978) Glucose-induced conformational change in yeast hexokinase. *Proc Natl Acad Sci U S A* **75**:4848-52.

Bihoreau C, Monnot C, Davies E, Teutsch B, Bernstein KE, Corvol P and Clauser E (1993) Mutation of Asp74 of the rat angiotensin II receptor confers changes in antagonist affinities and abolishes G-protein coupling. *Proc Natl Acad Sci U S A* **90**:5133-7.

Blomenrohr M, Bogerd J, Leurs R, Schulz RW, Tensen CP, Zandbergen MA and Goos HJ (1997) Differences in structure-function relations between nonmammalian and mammalian gonadotropin-releasing hormone receptors. *Biochem Biophys Res Commun* **238**:517-22.

Blomenrohr M, Kuhne R, Hund E, Leurs R, Bogerd J and ter Laak T (2001) Proper receptor signalling in a mutant catfish gonadotropin-releasing hormone receptor lacking the highly conserved Asp(90) residue. *FEBS Lett* **501**:131-4.

Blomenrohr M, ter Laak T, Kuhne R, Beyermann M, Hund E, Bogerd J and Leurs R (2002) Chimaeric gonadotropin-releasing hormone (GnRH) peptides with improved affinity for the catfish (Clarias gariepinus) GnRH receptor. *Biochem J* **361**:515-23.

Brooks J, Taylor PL, Saunders PT, Eidne KA, Struthers WJ and McNeilly AS (1993) Cloning and sequencing of the sheep pituitary gonadotropin-releasing hormone receptor and changes in expression of its mRNA during the estrous cycle. *Mol Cell Endocrinol* **94**:R23-7.

Brown P and McNeilly AS (1999) Transcriptional regulation of pituitary gonadotrophin subunit genes. *Rev Reprod* **4**:117-24.

Burgus R, Butcher M, Amoss M, Ling N, Monahan M, Rivier J, Fellows R, Blackwell R, Vale W and Guillemin R (1972) Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF). *Proc Natl Acad Sci U S A* **69**:278-82.

Byrne B, McGregor A, Taylor PL, Sellar R, Rodger FE, Fraser HM and Eidne KA (1999) Isolation and characterisation of the marmoset gonadotrophin releasing hormone receptor: Ser(140) of the DRS motif is substituted by Phe. *J Endocrinol* **163**:447-56.

Carolsfeld J, Powell JF, Park M, Fischer WH, Craig AG, Chang JP, Rivier JE and Sherwood NM (2000) Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. *Endocrinology* **141**:505-12.

Cascieri MA, Chicchi GG, Freidinger RM, Colton CD, Perlow DS, Williams B, Curtis NR, McKnight AT, Maguire JJ, Veber DF and et al. (1986) Conformationally constrained tachykinin analogs which are selective ligands for the eledoisin binding site. *Mol Pharmacol* **29**:34-8.

Cedars MI, Lu JK, Meldrum DR and Judd HL (1990) Treatment of endometriosis with a long-acting gonadotropin-releasing hormone agonist plus medroxyprogesterone acetate. *Obstet Gynecol* **75**:641-5.

Chang JK, Sievertsson H, Currie BL, Bogentoft C, Folkers K and Bowers CY (1972) Synthesis of the luteinizing-releasing hormone of the hypothalamus and the 8-lysine analog. *J Med Chem* **15**:623-7.

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

Chen S, Xu M, Lin F, Lee D, Riek P and Graham RM (1999) Phe310 in transmembrane VI of the alpha1B-adrenergic receptor is a key switch residue involved in activation and catecholamine ring aromatic bonding. *J Biol Chem* **274**:16320-30.

Cheung TC and Hearn JP (2002) Cloning and cellular localization of the wallaby gonadotropin-releasing hormone receptor and its variant molecules, in *84th Annual Meeting of the Endocrine Society* Abstract P1-115, San Francisco, California, USA.

Chi L, Zhou W, Prikhozhan A, Flanagan C, Davidson JS, Golembo M, Illing N, Millar RP and Sealfon SC (1993) Cloning and characterization of the human GnRH receptor. *Mol Cell Endocrinol* **91**:R1-6.

Chidiac P, Hebert TE, Valiquette M, Dennis M and Bouvier M (1994) Inverse agonist activity of beta-adrenergic antagonists. *Mol Pharmacol* **45**:490-9.

Chung FZ, Wang CD, Potter PC, Venter JC and Fraser CM (1988) Site-directed mutagenesis and continuous expression of human beta-adrenergic receptors. Identification of a conserved aspartate residue involved in agonist binding and receptor activation. *J Biol Chem* **263**:4052-5.

Colson AO, Perlman JH, Smolyar A, Gershengorn MC and Osman R (1998) Static and dynamic roles of extracellular loops in G-protein-coupled receptors: a mechanism for sequential binding of thyrotropin-releasing hormone to its receptor. *Biophys J* **74**:1087-100.

Conklin BR and Bourne HR (1993) Structural elements of G alpha subunits that interact with G beta gamma, receptors, and effectors. *Cell* **73**:631-41.

Conn PJ and Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol* **37**:205-37.

Conn PM and Crowley WF, Jr. (1994) Gonadotropin-releasing hormone and its analogs. *Annu Rev Med* **45**:391-405.

Cook JV, McGregor A, Lee T, Milligan G and Eidne KA (1996) A disulfide bonding interaction role for cysteines in the extracellular domain of the thyrotropin-releasing hormone receptor. *Endocrinology* **137**:2851-8.

Cook JV and Eidne KA (1997) An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation. *Endocrinology* **138**:2800-6.

Coy DH, Coy EJ, Schally AV, Vilchez-Martinez J, Hirotsu Y and Arimura A (1974) Synthesis and biological properties of (D-Ala-6, des-Gly-NH2-10)-LH-RH ethylamide, a peptide with greatly enhanced LH- and FSH- releasing activity. *Biochem Biophys Res Commun* **57**:335-40.

Coy DH, Vilchez-Martinez JA, Coy EJ and Schally AV (1976) Analogs of luteinizing hormone-releasing hormone with increased biological activity produced by D-amino acid substitutions in position 6. *J Med Chem* **19**:423-5.

Cui J, Smith RG, Mount GR, Lo JL, Yu J, Walsh TF, Singh SB, DeVita RJ, Goulet MT, Schaeffer JM and Cheng K (2000) Identification of Phe313 of the gonadotropin-releasing hormone (GnRH) receptor as a site critical for the binding of nonpeptide GnRH antagonists. *Mol Endocrinol* **14**:671-81.

Davidson FF, Loewen PC and Khorana HG (1994) Structure and function in rhodopsin: replacement by alanine of cysteine residues 110 and 187, components of a conserved disulfide bond in rhodopsin, affects the light-activated metarhodopsin II state. *Proc Natl Acad Sci U S A* **91**:4029-33.

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

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

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

de Ziegler D, Cedars MI, Randle D, Lu JK, Judd HL and Meldrum DR (1987) Suppression of the ovary using a gonadotropin releasing-hormone agonist prior to stimulation for oocyte retrieval. *Fertil Steril* **48**:807-10.

Dean MK, Higgs C, Smith RE, Bywater RP, Snell CR, Scott PD, Upton GJG, Howe TJ and Reynolds CA (2001) Dimerization of G-protein-coupled receptors. *J Med Chem* **44**:4595-4614.

Devi LA and Brady LS (2000) Dimerization of G-protein coupled receptors. *Neuropsychopharmacology* **23**:S3-4.

Dixon RA, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, Frielle T, Bolanowski MA, Bennett CD, Rands E, Diehl RE and et al. (1986) Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* **321**:75-9.

Dixon RA, Sigal IS, Rands E, Register RB, Candelore MR, Blake AD and Strader CD (1987) Ligand binding to the beta-adrenergic receptor involves its rhodopsin-like core. *Nature* **326**:73-7.

Dixon RA, Hill WS, Candelore MR, Rands E, Diehl RE, Marshall MS, Sigal IS and Strader CD (1989) Genetic analysis of the molecular basis for beta-adrenergic receptor subtype specificity. *Proteins* **6**:267-74.

Dohlman HG, Caron MG, DeBlasi A, Frielle T and Lefkowitz RJ (1990) Role of extracellular disulfide-bonded cysteines in the ligand binding function of the beta 2-adrenergic receptor. *Biochemistry* **29**:2335-42.

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

Dutta AS, Furr BJ, Giles MB, Valcaccia B and Walpole AL (1978) Potent agonist and antagonist analogues of luliberin containing an azaglycine residue in position 10. *Biochem Biophys Res Commun* **81**:382-90.

Eidne KA, Sellar RE, Couper G, Anderson L and Taylor PL (1992) Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. *Mol Cell Endocrinol* **90**:R5-9.

Emons G and Schally AV (1994) The use of luteinizing hormone releasing hormone agonists and antagonists in gynaecological cancers. *Hum Reprod* **9**:1364-79.

Erchegyi J, Coy DH, Nekola MV, Coy EJ, Schally AV, Mezo I and Teplan I (1981) Luteinizing hormone-releasing hormone analogs with increased anti-ovulatory activity. *Biochem Biophys Res Commun* **100**:915-20.

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240**:889-95.

Filicori M (1994) Gonadotrophin-releasing hormone agonists. A guide to use and selection. *Drugs* **48**:41-58.

Findlay J and Eliopoulos E (1990) Three-dimensional modelling of G protein-linked receptors. *Trends Pharmacol Sci* **11**:492-9.

Flanagan CA, Becker, II, Davidson JS, Wakefield IK, Zhou W, Sealfon SC and Millar RP (1994) Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone. *J Biol Chem* **269**:22636-41.

Flanagan CA, Millar RP and Illing N (1997) Advances in understanding gonadotrophin-releasing hormone receptor structure and ligand interactions. *Rev Reprod* **2**:113-20.

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**:4115-9.

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

Flanagan CA, Rodic V, Konvicka K, Yuen T, Chi L, Rivier JE, Millar RP, Weinstein H and Sealfon SC (2000) Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction. *Biochemistry* **39**:8133-41.

Flower DR (1999) Modelling G-protein-coupled receptors for drug design. *Biochim Biophys Acta* **1422**:207-34.

Fong TM, Huang RR and Strader CD (1992) Localization of agonist and antagonist binding domains of the human neurokinin-1 receptor. *J Biol Chem* **267**:25664-7.

Fong TM, Cascieri MA, Yu H, Bansal A, Swain C and Strader CD (1993) Amino-aromatic interaction between histidine 197 of the neurokinin-1 receptor and CP 96345. *Nature* **362**:350-3.

Franke RR, Konig B, Sakmar TP, Khorana HG and Hofmann KP (1990) Rhodopsin mutants that bind but fail to activate transducin. *Science* **250**:123-5.

Fraser CM, Chung FZ, Wang CD and Venter JC (1988) Site-directed mutagenesis of human beta-adrenergic receptors: substitution of aspartic acid-130 by asparagine produces a receptor with high-affinity agonist binding that is uncoupled from adenylate cyclase. *Proc Natl Acad Sci U S A* **85**:5478-82.

Fraser CM, Wang CD, Robinson DA, Gocayne JD and Venter JC (1989) Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol Pharmacol* **36**:840-7.

Fraser HM (1993) GnRH analogues for contraception. *Br Med Bull* **49**:62-72.

Freidinger RM, Veber DF, Perlow DS, Brooks JR and Saperstein R (1980) Bioactive conformation of luteinizing hormone-releasing hormone: evidence from a conformationally constrained analog. *Science* **210**:656-8.

Friedman AJ, Daly M, Juneau-Norcross M, Rein MS, Fine C, Gleason R and Leboff M (1993) A prospective, randomized trial of gonadotropin-releasing hormone agonist plus estrogen-progestin or progestin "add-back" regimens for women with leiomyomata uteri. *J Clin Endocrinol Metab* **76**:1439-45.

Frielle T, Daniel KW, Caron MG and Lefkowitz RJ (1988) Structural basis of beta-adrenergic receptor subtype specificity studied with chimeric beta 1/beta 2-adrenergic receptors. *Proc Natl Acad Sci U S A* **85**:9494-8.

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. *Mol Pharmacol* **60**:1280-7.

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

Gales C, Kowalski-Chauvel A, Dufour MN, Seva C, Moroder L, Pradayrol L, Vaysse N, Fourmy D and Silvente-Poirot S (2000) Mutation of Asn-391 within the conserved NPXXY motif of the cholecystokinin B receptor abolishes Gq protein activation without affecting its association with the receptor. *J Biol Chem* **275**:17321-7.

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

Gaudin P, Couvineau A, Maoret JJ, Rouyer-Fessard C and Laburthe M (1995) Mutational analysis of cysteine residues within the extracellular domains of the human vasoactive intestinal peptide (VIP) 1 receptor identifies seven mutants that are defective in VIP binding. *Biochem Biophys Res Commun* **211**:901-8.

Gershengorn MC and Osman R (2001) Minireview: Insights into G protein-coupled receptor function using molecular models. *Endocrinology* **142**:2-10.

Gether U, Johansen TE, Snider RM, Lowe JA, 3rd, Nakanishi S and Schwartz TW (1993a) Different binding epitopes on the NK1 receptor for substance P and non-peptide antagonist. *Nature* **362**:345-8.

Gether U, Yokota Y, Emonds-Alt X, Breliere JC, Lowe JA, 3rd, Snider RM, Nakanishi S and Schwartz TW (1993b) Two nonpeptide tachykinin antagonists act through epitopes on corresponding segments of the NK1 and NK2 receptors. *Proc Natl Acad Sci U S A* **90**:6194-8.

Gether U, Lin S and Kobilka BK (1995) Fluorescent labeling of purified beta 2 adrenergic receptor. Evidence for ligand-specific conformational changes. *J Biol Chem* **270**:28268-75.

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

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

Gharib SD, Wierman ME, Shupnik MA and Chin WW (1990) Molecular biology of the pituitary gonadotropins. *Endocr Rev* **11**:177-99.

Govaerts C, Lefort A, Costagliola S, Wodak SJ, Ballesteros JA, Van Sande J, Pardo L and Vassart G (2001) A conserved Asn in transmembrane helix 7 is an on/off switch in the activation of the thyrotropin receptor. *J Biol Chem* **276**:22991-9.

Goy MF (1991) cGMP: the wayward child of the cyclic nucleotide family. *Trends Neurosci* **14**:293-9.

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

Halvorson LM, Kaiser UB and Chin WW (1999) The protein kinase C system acts through the early growth response protein 1 to increase LHbeta gene expression in synergy with steroidogenic factor-1. *Mol Endocrinol* **13**:106-16.

Hauser F, Sondergaard L and Grimmelikhuijzen CJ (1998) Molecular cloning, genomic organization and developmental regulation of a novel receptor from Drosophila melanogaster structurally related to gonadotropin-releasing hormone receptors for vertebrates. *Biochem Biophys Res Commun* **249**:822-8.

Henderson R and Unwin PN (1975) Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* **257**:28-32.

Henderson R, Baldwin JM, Ceska TA, Zemlin F, Beckmann E and Downing KH (1990) Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J Mol Biol* **213**:899-929.

Hillier SG (1991) Regulatory functions for inhibin and activin in human ovaries. *J Endocrinol* **131**:171-5.

Ho BY, Karschin A, Branchek T, Davidson N and Lester HA (1992) The role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT1A receptor: a site-directed mutation study. *FEBS Lett* **312**:259-62.

Ho TL, Nestor JJ, Jr., McCrae GI and Vickery BH (1984) Hydrophobic, aza-glycine analogues of luteinizing hormone-releasing hormone. *Int J Pept Protein Res* **24**:79-84.

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

Hoflack J, Trumpp-Kallmeyer S and Hibert M (1994) Re-evaluation of bacteriorhodopsin as a model for G protein-coupled receptors. *Trends Pharmacol Sci* **15**:7-9.

Horn F, Weare J, Beukers MW, Horsch S, Bairoch A, Chen W, Edvardsen O, Campagne F and Vriend G (1998) GPCRDB: an information system for G protein-coupled receptors. *Nucleic Acids Res* **26**:275-9.

Huang RR, Yu H, Strader CD and Fong TM (1994) Localization of the ligand binding site of the neurokinin-1 receptor: interpretation of chimeric mutations and single-residue substitutions. *Mol Pharmacol* **45**:690-5.

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

Hur EM and Kim KT (2002) G protein-coupled receptor signalling and cross-talk. Achieving rapidity and specificity. *Cell Signal* **14**:397-405.

Illing N, Jacobs GF, Becker, II, Flanagan CA, Davidson JS, Eales A, Zhou W, Sealfon SC and Millar RP (1993) Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity among mammalian receptors. *Biochem Biophys Res Commun* **196**:745-51.

Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE and Millar RP (1999) Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (Carassius auratus). *Proc Natl Acad Sci U S A* **96**:2526-2531.

Jakubik J, El-Fakahany EE and Tucek S (2000) Evidence for a tandem two-site model of ligand binding to muscarinic acetylcholine receptors. *J Biol Chem* **275**:18836-44.

Janovick JA, Haviv F, Fitzpatrick TD and Conn PM (1993) Differential Orientation of a Gnrh Agonist and Antagonist in the Pituitary Gnrh Receptor. *Endocrinology* **133**:942-5.

Javitch JA, Ballesteros JA, Weinstein H and Chen J (1998) A cluster of aromatic residues in the sixth membrane-spanning segment of the dopamine D2 receptor is accessible in the binding-site crevice. *Biochemistry* **37**:998-1006.

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

Jimenez-Linan M, Rubin BS and King JC (1997) Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. *Endocrinology* **138**:4123-30.

Johnson MH and Everitt BJ (1995) *Essential Reproduction*. Blackwell Science, Oxford, UK.

Josefsson LG (1999) Evidence for kinship between diverse G-protein coupled receptors. *Gene* **239**:333-40.

Kaiser UB, Zhao D, Cardona GR and Chin WW (1992) Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. *Biochem Biophys Res Commun* **189**:1645-1652.

Kakar SS, Musgrove LC, Devor DC, Sellers JC and Neill JD (1992) Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. *Biochem Biophys Res Commun* **189**:289-95.

Kakar SS, Rahe CH and Neill JD (1993) Molecular cloning, sequencing, and characterizing the bovine receptor for gonadotropin releasing hormone (GnRH). *Domest Anim Endocrinol* **10**:335-42.

Karlin A (1993) Structure of nicotinic acetylcholine receptors. *Curr Opin Neurobiol* **3**:299-309.

Karnik SS and Khorana HG (1990) Assembly of functional rhodopsin requires a disulfide bond between cysteine residues 110 and 187. *J Biol Chem* **265**:17520-4.

Karsch FJ and Evans NP (1996) Feedback actions of estradiol on GnRH secretion during the follicular phase of the estrous cycle. *Acta Neurobiol Exp (Warsz)* **56**:715-25.

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

Kaynard AH, Malpaux B, Robinson JE, Wayne NL and Karsch FJ (1988) Importance of pituitary and neural actions of estradiol in induction of the luteinizing hormone surge in the ewe. *Neuroendocrinology* **48**:296-303.

Khorana HG (1992) Rhodopsin, photoreceptor of the rod cell. An emerging pattern for structure and function. *J Biol Chem* **267**:1-4.

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

King JA and Millar RP (1992) Evolution of Gonadotropin-Releasing Hormones. *Trends Endocrinol Metab* **3**:339-346.

King JA, Fidler A, Lawrence S, Adam T, Millar RP and Katz A (2000) Cloning and expression, pharmacological characterization, and internalization kinetics of the pituitary GnRH receptor in a metatherian species of mammal. *Gen Comp Endocrinol* **117**:439-48.

King JC, Anthony EL, Fitzgerald DM and Stopa EG (1985) Luteinizing hormone-releasing hormone neurons in human preoptic/hypothalamus: differential intraneuronal localization of immunoreactive forms. *J Clin Endocrinol Metab* **60**:88-97.

Kitanovic S, Yuen T, Flanagan CA, Ebersole BJ and Sealfon SC (2001) Insertional mutagenesis of the arginine cage domain of the gonadotropin-releasing hormone receptor. *Mol Endocrinol* **15**:390-7.

Kobilka BK, Kobilka TS, Daniel K, Regan JW, Caron MG and Lefkowitz RJ (1988) Chimeric alpha 2-,beta 2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science* **240**:1310-6.

Koerber SC, Rizo J, Struthers RS and Rivier JE (2000) Consensus bioactive conformation of cyclic GnRH antagonists defined by NMR and molecular modeling. *J Med Chem* **43**:819-28.

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

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

Kovacs M, Seprodi J, Vincze B, Teplan I and Flerko B (2002) Lamprey gonadotropin-releasing hormone-III (GnRH III) is a weak agonist for the mammalian GnRH receptor, in *84th Annual Meeting of the Endocrine Society* Abstract P1-118, San Francisco, California, USA.

Laakkonen L, Li W, Perlman JH, Guarnieri F, Osman R, Moeller KD and Gershengorn MC (1996) Restricted analogues provide evidence of a biologically active conformation of thyrotropin-releasing hormone. *Mol Pharmacol* **49**:1092-6.

Laburthe M, Couvineau A, Gaudin P, Maoret JJ, Rouyer-Fessard C and Nicole P (1996) Receptors for VIP, PACAP, secretin, GRF, glucagon, GLP-1, and other members of their new family of G protein-linked receptors: structure-function relationship with special reference to the human VIP-1 receptor. *Ann N Y Acad Sci* **805**:94-109; discussion 110-1.

LaRosa GJ, Thomas KM, Kaufmann ME, Mark R, White M, Taylor L, Gray G, Witt D and Navarro J (1992) Amino terminus of the interleukin-8 receptor is a major determinant of receptor subtype specificity. *J Biol Chem* **267**:25402-6.

Lefkowitz RJ, Cotecchia S, Samama P and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14**:303-7.

Lemay A (1989) Clinical appreciation of LHRH analogue formulations. *Horm Res* **32 Suppl 1**:93-101; discussion 102.

Leyendecker G, Waibel-Treber S and Wildt L (1990) The central control of follicular maturation and ovulation in the human, in *Oxford reviews of reproductive biology* (Milligan SR ed) pp 93-146, Oxford University Press, Oxford, UK.

Liapakis G, Ballesteros JA, Papachristou S, Chan WC, Chen X and Javitch JA (2000) The forgotten serine. A critical role for Ser-203$^{5.42}$ in ligand binding to and activation of the beta 2-adrenergic receptor. *J Biol Chem* **275**:37779-88.

Ling N and Vale W (1975) Analogs of luteinizing hormone releasing factor (LRF). Synthesis and biological activity of ((Nalpha-Me)Leu-7)LRF and (D-Ala-6,(Nalpha-Me)Leu-7)LRF. *Biochem Biophys Res Commun* **63**:801-6.

Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE and Sherwood NM (1992) Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc Natl Acad Sci U S A* **89**:6373-7.

Madigou T, Mananos-Sanchez E, Hulshof S, Anglade I, Zanuy S and Kah O (2000) Cloning, tissue distribution, and central expression of the gonadotropin-releasing hormone receptor in the rainbow trout (Oncorhynchus mykiss). *Biol Reprod* **63**:1857-66.

Maliekal JC, Jackson GE, Flanagan CA and Millar RP (1997) Solution conformations of gonadotropin releasing hormone (GnRH) and [Gln(8)]GnRH. *S Afr J Chem* **50**:217-219.

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

Marullo S, Emorine LJ, Strosberg AD and Delavier-Klutchko C (1990) Selective binding of ligands to beta 1, beta 2 or chimeric beta 1/beta 2-adrenergic receptors involves multiple subsites. *Embo J* **9**:1471-6.

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

Meng EC and Bourne HR (2001) Receptor activation: what does the rhodopsin structure tell us? *Trends Pharmacol Sci* **22**:587-93.

Mezei M and Guarnieri F (1998) Computer simulation studies of the fully solvated wild-type and mutated GnRH in extended and beta-turn conformations. *J Biomol Struct Dyn* **16**:723-32.

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

Millar RP and King JA (1983) Synthesis and biological activity of [D-Trp6] chicken luteinizing hormone-releasing hormone. *Peptides* **4**:425-429.

Millar RP and King JA (1984) Structure-activity relations of LH-RH in birds. *J Exp Zool* **232**:419-423.

Millar RP, Tobler CJ, King JA and Arimura A (1984) Region-specific antisera in molecular biology of neuropeptides: application in quantitation, structural characterisation and metabolism of luteinizing hormone-releasing hormone., in *Molecular biology approach to the neurosciences* (Soreq H ed) pp 221-230, Wiley, New York.

Millar RP, Milton RC, Follett BK and King JA (1986) Receptor binding and gonadotropin-releasing activity of a novel chicken gonadotropin-releasing hormone ([His5, Trp7, Tyr8]GnRH) and a D-Arg6 analog. *Endocrinology* **119**:224-31.

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

Millar RP, Troskie B, Sun YM, Ott T, Wakefield I, Myburgh D, Pawson A, Davidson JS, Flanagan C, Katz A, Hapgood J, Illing N, Weinstein H, Sealfon SC, Peter RE, Terasawa E and King JA (1997) Plasticity in the structural and functional evolution of GnRH: a peptide for all seasons, in *Advances in Comparative Endocrinology. Proceedings of the XIII International Congress of Comparative Endocrinology* (Kawashima S and Kikuyama S eds) pp 15-27, Monduzzi Editore, Bologna, Italy.

Millar RP, Zhu YF, Chen C and Struthers RS (2000) Progress towards the development of non-peptide orally-active gonadotropin-releasing hormone (GnRH) antagonists: therapeutic implications. *Br Med Bull* **56**:761-72.

Millar RP (2002) Gonadotropin-releasing hormones and their receptors, in *Reproductive medicine: molecular cellular and genetic fundamentals* (Fauser BCJM ed), Parthenon Publishing, Lancaster. **In Press**

Miller WR (1991) Oestrogens and breast cancer: biological considerations. *Br Med Bull* **47**:470-83.

Milton RCdL, King JA, Badminton MN, Tobler CJ, Lindsey GG, Fridkin M and Millar RP (1983) Comparative structure-activity studies on mammalian [Arg8] LH-RH and chicken [Gln8] LH-RH by fluorimetric titration. *Biochem Biophys Res Commun* **111**:1082-1088.

Mitchell R, McCulloch D, Lutz E, Johnson M, MacKenzie C, Fennell M, Fink G, Zhou W and Sealfon SC (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature* **392**:411-4.

Miyamoto K, Hasegawa Y, Igarashi M, Chino N, Sakakibara S, Kangawa K and Matsuo H (1983) Evidence that chicken hypothalamic luteinizing hormone-releasing hormone is [Gln8]-LH-RH. *Life Sci* **32**:1341-7.

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

Miyazono K, Kusanagi K and Inoue H (2001) Divergence and convergence of TGF-beta/BMP signaling. *J Cell Physiol* **187**:265-76.

Mizobe T, Maze M, Lam V, Suryanarayana S and Kobilka BK (1996) Arrangement of transmembrane domains in adrenergic receptors. Similarity to bacteriorhodopsin. *J Biol Chem* **271**:2387-9.

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

Momany FA (1976b) Conformational energy analysis of the molecule, luteinizing hormone- releasing hormone. 2. Tetrapeptide and decapeptide analogues. *J Am Chem Soc* **98**:2996-3000.

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

Montaner AD, Park MK, Fischer WH, Craig AG, Chang JP, Somoza GM, Rivier JE and Sherwood NM (2001) Primary structure of a novel gonadotropin-releasing hormone in the brain of a teleost, Pejerrey. *Endocrinology* **142**:1453-60.

Moro S, Hoffmann C and Jacobson KA (1999) Role of the extracellular loops of G protein-coupled receptors in ligand recognition: a molecular modeling study of the human P2Y1 receptor. *Biochemistry* **38**:3498-507.

Murthy CK, Turner RJ, Nestor JJJ, Rivier JE and Peter RE (1994) A new gonadotropin-releasing hormone (GnRH) superagonist in goldfish: influence of dialkyl-D-homoarginine at position 6 on gonadotropin-II and growth hormone release. *Regul Pept* **53**:1-15.

Nagayama Y, Wadsworth HL, Chazenbalk GD, Russo D, Seto P and Rapoport B (1991) Thyrotropin-luteinizing hormone/chorionic gonadotropin receptor extracellular domain chimeras as probes for thyrotropin receptor function. *Proc Natl Acad Sci U S A* **88**:902-5.

Naor Z, Harris D and Shacham S (1998) Mechanism of GnRH receptor signaling: combinatorial cross-talk of Ca2+ and protein kinase C. *Front Neuroendocrinol* **19**:1-19.

Nathans J and Hogness DS (1983) Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin. *Cell* **34**:807-14.

Neill JD, Duck LW, Sellers JC and Musgrove LC (2001) A gonadotropin-releasing hormone (GnRH) receptor specific for GnRH II in primates. *Biochem Biophys Res Commun* **282**:1012-8.

Nestor JJJ, Ho TL, Tahilramani R, Horner BL, Simpson RA, Jones GH, McRae GI and Vickery BH (1984) LHRH agonists and antagonists containing very hydrophobic amino acids, in *LHRH and its analogs* (Vickery BH, Nestor JJJ and Hafez ESE eds) pp 23-33, MTP Press, Lancaster, UK.

Neve KA, Cox BA, Henningsen RA, Spanoyannis A and Neve RL (1991) Pivotal role for aspartate-80 in the regulation of dopamine D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol Pharmacol* **39**:733-9.

Ngamvongchon S, Sherwood NM, Warby CM and Rivier JE (1992) Gonadotropin-releasing hormone from thai catfish: chromatographic and physiological studies. *Gen Comp Endocrinol* **87**:266-74.

O'Dowd BF, Hnatowich M, Caron MG, Lefkowitz RJ and Bouvier M (1989) Palmitoylation of the human beta 2-adrenergic receptor. *J Biol Chem* **264**:7564-7569.

Ohta K, Hayashi H, Mizuguchi H, Kagamiyama H, Fujimoto K and Fukui H (1994) Site-directed mutagenesis of the histamine H1 receptor: roles of aspartic acid107, asparagine198 and threonine194. *Biochem Biophys Res Commun* **203**:1096-101.

Ohyama K, Yamano Y, Chaki S, Kondo T and Inagami T (1992) Domains for G-protein coupling in angiotensin II receptor type I: studies by site-directed mutagenesis. *Biochem Biophys Res Commun* **189**:677-83.

Ohyama K, Yamano Y, Sano T, Nakagomi Y, Hamakubo T, Morishima I and Inagami T (1995) Disulfide bridges in extracellular domains of angiotensin II receptor type IA. *Regul Pept* **57**:141-7.

Okubo K, Amano M, Yoshiura Y, Suetake H and Aida K (2000a) A novel form of gonadotropin-releasing hormone in the medaka, Oryzias latipes. *Biochem Biophys Res Commun* **276**:298-303.

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

Okubo K, Nagata S, Ko R, Kataoka H, Yoshiura Y, Mitani H, Kondo M, Naruse K, Shima A and Aida K (2001) Identification and characterization of two distinct GnRH receptor subtypes in a teleost, the medaka Oryzias latipes. *Endocrinology* **142**:4729-39.

Oliveira L, Paiva AC, Sander C and Vriend G (1994) A common step for signal transduction in G protein-coupled receptors. *Trends Pharmacol Sci* **15**:170-2.

Ott TR, Troskie BE, Roeske RW, Illing N, Flanagan CA and Millar RP (2002) Two Mutations in Extracellular Loop 2 of the Human GnRH Receptor Convert an Antagonist to an Agonist. *Mol Endocrinol* **16**:1079-1088.

Padmanabhan V, McFadden K, Mauger DT, Karsch FJ and Midgley AR, Jr. (1997) Neuroendocrine control of follicle-stimulating hormone (FSH) secretion. I. Direct evidence for separate episodic and basal components of FSH secretion. *Endocrinology* **138**:424-32.

Padmanabhan V and McNeilly AS (2001) Is there an FSH-releasing factor? *Reproduction* **121**:21-30.

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

Pardo L, Ballesteros JA, Osman R and Weinstein H (1992) On the use of the transmembrane domain of bacteriorhodopsin as a template for modeling the three-dimensional structure of guanine nucleotide-binding regulatory protein-coupled receptors. *Proc Natl Acad Sci U S A* **89**:4009-12.

Parent JL, Gouill CL, Escher E, Rola-Pleszczynski M and Stakova J (1996) Identification of transmembrane domain residues determinant in the structure-function relationship of the human platelet-activating factor receptor by site-directed mutagenesis. *J Biol Chem* **271**:23298-303.

Pebay-Peyroula E, Rummel G, Rosenbusch JP and Landau EM (1997) X-ray structure of bacteriorhodopsin at 2.5 angstroms from microcrystals grown in lipidic cubic phases. *Science* **277**:1676-81.

Perez HD, Holmes R, Vilander LR, Adams RR, Manzana W, Jolley D and Andrews WH (1993) Formyl peptide receptor chimeras define domains involved in ligand binding. *J Biol Chem* **268**:2292-5.

Perlman JH, Wang W, Nussenzveig DR and Gershengorn MC (1995) A disulfide bond between conserved extracellular cysteines in the thyrotropin-releasing hormone receptor is critical for binding. *J Biol Chem* **270**:24682-5.

Perlman JH, Colson AO, Jain R, Czyzewski B, Cohen LA, Osman R and Gershengorn MC (1997a) Role of the extracellular loops of the thyrotropin-releasing hormone receptor: evidence for an initial interaction with thyrotropin-releasing hormone. *Biochemistry* **36**:15670-6.

Perlman JH, Colson AO, Wang W, Bence K, Osman R and Gershengorn MC (1997b) Interactions between conserved residues in transmembrane helices 1, 2, and 7 of the thyrotropin-releasing hormone receptor. *J Biol Chem* **272**:11937-42.

Perrin MH, Bilezikjian LM, Hoeger C, Donaldson CJ, Rivier J, Haas Y and Vale WW (1993) Molecular and functional characterization of GnRH receptors cloned from rat pituitary and a mouse pituitary tumor cell line. *Biochem Biophys Res Commun* **191**:1139-44.

Petry R, Craik D, Haaima G, Fromme B, Klump H, Kiefer W, Palm D and Millar R (2002) Secondary structure of the third extracellular loop responsible for ligand selectivity of a mammalian gonadotropin-releasing hormone receptor. *J Med Chem* **45**:1026-1034.

Pollock NJ, Manelli AM, Hutchins CW, Steffey ME, MacKenzie RG and Frail DE (1992) Serine mutations in transmembrane V of the dopamine D1 receptor affect ligand interactions and receptor activation. *J Biol Chem* **267**:17780-6.

Powell J, Zohar Y, Elizur A, Park M, Fischer W, Craig A, Rivier J, Lovejoy D and Sherwood N (1994) Three Forms of Gonadotropin-Releasing Hormone Characterized from Brains of One Species. *PNAS* **91**:12081-12085.

Powell JFF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO and Sherwood NM (1996) Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc Natl Acad Sci USA* **93**:10461-10464.

Probst WC, Snyder LA, Schuster DI, Brosius J and Sealfon SC (1992) Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol* **11**:1-20.

Rang HP, Dale MM and Ritter JM (1999) *Pharmacology*. Churchill Livingstone, Edinburgh, UK.

Reinhart J, Mertz LM and Catt KJ (1992) Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. *J Biol Chem* **267**:21281-4.

Rios CD, Jordan BA, Gomes I and Devi LA (2001) G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol Ther* **92**:71-87.

Rivier J, Kupryszewski G, Varga J, Porter J, Rivier C, Perrin M, Hagler A, Struthers S, Corrigan A and Vale W (1988) Design of potent cyclic gonadotropin releasing hormone antagonists. *J Med Chem* **31**:677-82.

Rivier JE, Jiang G, Struthers RS, Koerber SC, Porter J, Cervini LA, Kirby DA, Craig AG and Rivier CL (2000a) Design of potent dicyclic (1-5/4-10) gonadotropin releasing hormone (GnRH) antagonists. *J Med Chem* **43**:807-18.

Rivier JE, Porter J, Cervini LA, Lahrichi SL, Kirby DA, Struthers RS, Koerber SC and Rivier CL (2000b) Design of monocyclic (1-3) and dicyclic (1-3/4-10) gonadotropin releasing hormone (GnRH) antagonists. *J Med Chem* **43**:797-806.

Rivier JE, Struthers RS, Porter J, Lahrichi SL, Jiang G, Cervini LA, Ibea M, Kirby DA, Koerber SC and Rivier CL (2000c) Design of potent dicyclic (4-10/5-8) gonadotropin releasing hormone (GnRH) antagonists. *J Med Chem* **43**:784-96.

Roberson MS, Misra-Press A, Laurance ME, Stork PJ and Maurer RA (1995) A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone alpha-subunit promoter by gonadotropin-releasing hormone. *Mol Cell Biol* **15**:3531-9.

Robison RR, White RB, Illing N, Troskie BE, Morley M, Millar RP and Fernald RD (2001) Gonadotropin-releasing hormone receptor in the teleost Haplochromis burtoni: structure, location, and function. *Endocrinology* **142**:1737-43.

Rose PM, Krystek SR, Jr., Patel PS, Liu EC, Lynch JS, Lach DA, Fisher SM and Webb ML (1995) Aspartate mutation distinguishes ETA but not ETB receptor subtype-selective ligand binding while abolishing phospholipase C activation in both receptors. *FEBS Lett* **361**:243-9.

Rosenthal W, Antaramian A, Gilbert S and Birnbaumer M (1993) Nephrogenic diabetes insipidus. A V2 vasopressin receptor unable to stimulate adenylyl cyclase. *J Biol Chem* **268**:13030-3.

Roth BL, Shoham M, Choudhary MS and Khan N (1997) Identification of conserved aromatic residues essential for agonist binding and second messenger production at 5-hydroxytryptamine2A receptors. *Mol Pharmacol* **52**:259-66.

Sakmar TP (2002) Structure of rhodopsin and the superfamily of seven-helical receptors: the same and not the same. *Curr Opin Cell Biol* **14**:189-95.

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

Santoro N (1990) Efficacy and safety of intravenous pulsatile gonadotropin-releasing hormone: Lutrepulse for injection. *Am J Obstet Gynecol* **163**:1759-64.

Santra S, Rao VS, Shanker YG and Rao AJ (2000) Cloning and characterization of bonnet monkey GnRH receptor. *Mol Hum Reprod* **6**:415-421.

Saunders BD, Sabbagh E, Chin WW and Kaiser UB (1998) Differential use of signal transduction pathways in the gonadotropin-releasing hormone-mediated regulation of gonadotropin subunit gene expression. *Endocrinology* **139**:1835-43.

Savarese TM, Wang CD and Fraser CM (1992) Site-directed mutagenesis of the rat m1 muscarinic acetylcholine receptor. Role of conserved cysteines in receptor function. *J Biol Chem* **267**:11439-48.

Saviano G, Temussi PA, Motta A, Maggi CA and Rovero P (1991) Conformation-activity relationship of tachykinin neurokinin A (4-10) and of some [Xaa8] analogues. *Biochemistry* **30**:10175-81.

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

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

Schlessinger J and Ullrich A (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron* **9**:383-91.

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

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

Sherwood N, Eiden L, Brownstein M, Spiess J, Rivier J and Vale W (1983) Characterization of a teleost gonadotropin-releasing hormone. *Proc Natl Acad Sci U S A* **80**:2794-8.

Sherwood NM, Sower SA, Marshak DR, Fraser BA and Brownstein MJ (1986) Primary structure of gonadotropin-releasing hormone from lamprey brain. *J Biol Chem* **261**:4812-9.

Shi H and Segaloff DL (1995) A role for increased lutropin/choriogonadotropin receptor (LHR) gene transcription in the follitropin-stimulated induction of the LHR in granulosa cells. *Mol Endocrinol* **9**:734-44.

Shinitzky M and Fridkin M (1976) Structural features of luliberin (luteinising hormone-releasing factor) inferred from fluorescence measurements. *Biochim Biophys Acta* **434**:137-143.

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

Slusarski DC, Corces VG and Moon RT (1997) Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**:410-3.

Soppa J (1994) Two hypotheses--one answer. Sequence comparison does not support an evolutionary link between halobacterial retinal proteins including bacteriorhodopsin and eukaryotic G-protein-coupled receptors. *FEBS Lett* **342**:7-11.

Sower SA, Chiang YC, Lovas S and Conlon JM (1993) Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* **132**:1125-31.

Spicer DV, Shoupe D and Pike MC (1991) GnRH agonists as contraceptive agents: predicted significantly reduced risk of breast cancer. *Contraception* **44**:289-310.

Spiegel AM, Shenker A and Weinstein LS (1992) Receptor-effector coupling by G proteins: implications for normal and abnormal signal transduction. *Endocr Rev* **13**:536-65.

Stojilkovic SS and Catt KJ (1992) Calcium oscillations in anterior pituitary cells. *Endocr Rev* **13**:256-80.

Stojilkovic SS, Reinhart J and Catt KJ (1994) Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. *Endocr Rev* **15**:462-99.

Strader CD, Sigal IS, Register RB, Candelore MR, Rands E and Dixon RA (1987) Identification of residues required for ligand binding to the beta-adrenergic receptor. *Proc Natl Acad Sci U S A* **84**:4384-8.

Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS and Dixon RA (1988) Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263**:10267-71.

Strader CD, Candelore MR, Hill WS, Sigal IS and Dixon RA (1989) Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J Biol Chem* **264**:13572-8.

Strader CD, Gaffney T, Sugg EE, Candelore MR, Keys R, Patchett AA and Dixon RA (1991) Allele-specific activation of genetically engineered receptors. *J Biol Chem* **266**:5-8.

Strader CD, Fong TM, Tota MR, Underwood D and Dixon RA (1994) Structure and function of G protein-coupled receptors. *Annu Rev Biochem* **63**:101-32.

Strader CD, Fong TM, Graziano MP and Tota MR (1995) The family of G-protein-coupled receptors. *Faseb J* **9**:745-54.

Strahl BD, Huang HJ, Sebastian J, Ghosh BR and Miller WL (1998) Transcriptional activation of the ovine follicle-stimulating hormone beta-subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. *Endocrinology* **139**:4455-65.

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. *J Biol Chem* **276**:7754-61.

Suzuki N, Cho N, Furuya S, Harada M, Urushibara T, Takekawa S, Horinouchi A, Clark E, Onda H, Boyce M and Fujino M (2002) TAK-013: A novel, potent, and orally active nonpeptide antagonist for the human gonadotropin-releasing hormone receptor, in *84th Annual Meeting of the Endocrine Society* Abstract OR7-2, San Francisco, California, USA.

Tao Q and Abood ME (1998) Mutation of a highly conserved aspartate residue in the second transmembrane domain of the cannabinoid receptors, CB1 and CB2, disrupts G-protein coupling. *J Pharmacol Exp Ther* **285**:651-8.

Teller DC, Okada T, Behnke CA, Palczewski K and Stenkamp RE (2001) Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* **40**:7761-72.

Tensen C, Okuzawa K, Blomenrohr M, Rebers F, Leurs R, Bogerd J, Schulz R and Goos H (1997) Distinct efficacies for two endogenous ligands on a single cognate gonadoliberin receptor. *Eur J Biochem* **243**:134-40.

Troskie B, Illing N, Rumbak E, Sun YM, Hapgood J, Sealfon S, Conklin D and Millar R (1998) Identification of three putative GnRH receptor subtypes in vertebrates. *Gen Comp Endocrinol* **112**:296-302.

Troskie BE, Hapgood JP, Millar RP and Illing N (2000) Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotropin-releasing hormone receptor expressed in the pituitary and midbrain of Xenopus laevis. *Endocrinology* **141**:1764-71.

Tse A, Tse FW, Almers W and Hille B (1993) Rhythmic exocytosis stimulated by GnRH-induced calcium oscillations in rat gonadotropes. *Science* **260**:82-4.

Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B and Sealfon SC (1992) Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. *Mol Endocrinol* **6**:1163-9.

Ulloa-Aguirre A, Stanislaus D, Janovick JA and Conn PM (1999) Structure-activity relationships of G protein-coupled receptors. *Arch Med Res* **30**:420-35.

Unger VM, Hargrave PA, Baldwin JM and Schertler GF (1997) Arrangement of rhodopsin transmembrane alpha-helices. *Nature* **389**:203-6.

Vale W, Grant G, Rivier J, Monahan M, Amoss M, Blackwell R, Burgus R and Guillemin R (1972) Synthetic polypeptide antagonists of the hypothalamic luteinizing hormone releasing factor. *Science* **176**:933-4.

van Rhee AM and Jacobson KA (1996) Molecular architecture of G protein-coupled receptors. *Drug Dev Res* **37**:1-38.

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

Wang CD, Gallaher TK and Shih JC (1993) Site-directed mutagenesis of the serotonin 5-hydroxytrypamine2 receptor: identification of amino acids necessary for ligand binding and receptor activation. *Mol Pharmacol* **43**:931-40.

Wang L, Bogerd J, Choi HS, Seong JY, Soh JM, Chun SY, Blomenrohr M, Troskie BE, Millar RP, Yu WH, McCann SM and Kwon HB (2001) Three distinct types of GnRH receptor characterized in the bullfrog. *Proc Natl Acad Sci U S A* **98**:361-6.

Ward SD, Curtis CA and Hulme EC (1999) Alanine-scanning mutagenesis of transmembrane domain 6 of the M(1) muscarinic acetylcholine receptor suggests that Tyr381 plays key roles in receptor function. *Mol Pharmacol* **56**:1031-41.

Weck J, Fallest PC, Pitt LK and Shupnik MA (1998) Differential gonadotropin-releasing hormone stimulation of rat luteinizing hormone subunit gene transcription by calcium influx and mitogen-activated protein kinase-signaling pathways. *Mol Endocrinol* **12**:451-7.

Weesner GD and Matteri RL (1994) Rapid communication: nucleotide sequence of luteinizing hormone-releasing hormone (LHRH) receptor cDNA in the pig pituitary. *J Anim Sci* **72**:1911.

Wess J, Bonner TI and Brann MR (1990) Chimeric m2/m3 muscarinic receptors: role of carboxyl terminal receptor domains in selectivity of ligand binding and coupling to phosphoinositide hydrolysis. *Mol Pharmacol* **38**:872-7.

Wess J, Gdula D and Brann MR (1991) Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. *Embo J* **10**:3729-34.

Wess J (1998) Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol Ther* **80**:231-64.

Wetsel WC, Liposits Z, Seidah NG and Collins S (1995) Expression of candidate Pro-GnRH processing enzymes in rat hypothalamus and an immortalized hypothalamic neuronal cell line. *Neuroendocrinology* **62**:166-177.

White RB, Eisen JA, Kasten TL and Fernald RD (1998) Second gene for gonadotropin-releasing hormone in humans. *Proc Natl Acad Sci U S A* **95**:305-309.

White SA, Kasten TL, Bond CT, Adelman JP and Fernald RD (1995) Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. *Proc Natl Acad Sci U S A* **92**:8363-7.

Yabe Y, Miura C, Horikoshi H and Baba Y (1976) Analogues of luteinizing hormone-releasing hormone with modification in position 3. *Chem Pharm Bull (Tokyo)* **24**:3149-57.

Yamano Y, Ohyama K, Chaki S, Guo DF and Inagami T (1992) Identification of amino acid residues of rat angiotensin II receptor for ligand binding by site directed mutagenesis. *Biochem Biophys Res Commun* **187**:1426-31.

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

Yoo MS, Kang HM, Choi HS, Kim JW, Troskie BE, Millar RP and Kwon HB (2000) Molecular cloning, distribution and pharmacological characterization of a novel gonadotropin-releasing hormone ([Trp8] GnRH) in frog brain. *Mol Cell Endocrinol* **164**:197-204.

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

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

Appendix I

Composition of Reagents

Reagents listed in order of first reference in text.

Ref.:	Reagent:	Composition:
3.2.3	Ready-To-Go™ T4 DNA ligase	Minimum of 6 Weiss units of FPLC*pure*™ T4 DNA ligase, 66mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM spermidine, 10 mM DTT and stabilisers
3.3.1	Presept	50% Sodium Dichloroisocyanurate
3.3.1	LB Broth medium	1% tryptone, 0.5% yeast extract and 1% NaCl
3.3.1	LB Agar medium	1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% Agar
3.3.3	SOC medium	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose
3.3.4	Wizard® cell resuspension solution	50 mM Tris-HCl, (pH 7.5), 10 mM EDTA and 100 μg/ml RNase A.
3.3.4	Wizard® cell lysis solution	0.2 M NaOH and 1% SDS
3.3.4	Wizard® neutralisation solution	1.32 M potassium acetate (pH 4.8)

Ref.:	Solution:	Composition:
3.3.4	Wizard® column wash solution	80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 μM EDTA and 55% ethanol
3.3.4	TE buffer	10 mM Tris-HCl (pH 8.0) and 1 mM EDTA
3.4.4	TAE buffer	40 mM Tris-acetate and 1 mM EDTA
3.5.3	Sequencing loading buffer	250 μl formamide and 50 μl 50 mM EDTA
3.5.3	ABI Ready Reaction Mix	Dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth* pyrophosphatase, $MgCl_2$ and buffer (concentrations undisclosed)
3.5.3	Half Term buffer	200 mM Tris HCl (pH 9.0) and 5 mM $MgCl_2$
3.7.2	Tisept	Chlorhexidine gluconate 0.015% w/v and cetrimide Ph. Eur. 0.15% w/v
3.7.2	Cell culture 'Complete Medium'	Dulbecco's modified Eagle's medium containing: 10% fetal calf serum, 0.3 mg/ml glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin
3.7.4	Opti-MEM®	Includes: L-glutamine, sodium bicarbonate (2.4 g/l), HEPES, sodium pyruvate, hypoxanthine, thymidine, trace elements, growth factors and Phenol Red (1.1 mg/l)
3.7.5	Dulbecco's modified Eagle's medium	Includes: glucose (4.5 mg/ml), L-glutamine and sodium bicarbonate

Ref.:	Solution:	Composition:
3.8.1	Iodo-Gen® Iodination Reagent	1,3,4,6-tetrachloro-3α-6α-diphenylglycouril
3.8.1	Iodination Running Buffer	0.06% glacial acetic acid and 0.1% BSA
3.8.2	HEPES/DMEM	DMEM including glucose (4.5 mg/ml) HEPES (25 mM) and sodium bicarbonate, but no L-glutamine
3.8.3	Membrane Binding Homogenisation Buffer	20 mM Tris (pH 7.2) and 2 mM $MgCl_2$
3.8.3	Membrane Binding Assay Buffer	40 mM Tris (pH 7.4), 2 mM $MgCl_2$ and 0.1% BSA

Appendix II

Addresses of Suppliers

Suppliers are listed in alphabetical order.

Supplier:	**Location:**
ABI (PE Applied Biosystems)	Warrington, UK
Amersham Pharmacia	Little Chalfont, UK
Anachem	Luton, UK
Bachem	Saffron Walden, UK
Biosoft	Cambridge, UK
Brandel	St. Albans, UK
Genosys	Cambridge, UK
GraphPad	San Diego, USA
Invitrogen	Groningen, The Netherlands
Jencons-PLS	Leighton Buzzard, UK
Life Technologies (GibcoBRL)	Paisley, UK
Merck	Lutterworth, UK
New England Biolabs	Hitchin, UK
Perkin Elmer (Wallac)	Cambridge, UK
Pierce	Chester, UK
Promega	Southampton, UK
Qiagen	Crawley, UK
Sigma-Aldrich	Poole, UK
Stratagene	Cambridge, UK

Appendix III

Sequences of Oligonucleotide Primers

Primers were synthesised by Genosys and are listed in order of first reference in text.

Primers for General Subcloning and Sequencing

(See Sections 3.5.3, 5.2.4, 6.2.4 and Figure 3.3):

T7: 5'-TAATACGACTCACTATAGGG-3'

T3: 5'-AATTAACCCTCACTAAAGGG-3'

SP6: 5'-ATTTAGGTGACACTATAG-3'

Internal Primers for Sequencing

Two internal sense primers annealing to the engineered human GnRH receptor cDNA were used for sequencing, in addition to those listed above (see Section 3.5.3). These primers were provided by T. Ott:

96-359 (Intracellular end of TMD2): 5'-AAAGCTCTCGCGAATGAAGC-3'

96-360 (Extracellular end of TMD3): 5'-TCTGCAAAGTACTCAGTTATC-3'

Primers for the Human-Catfish Chimeric GnRH Receptor cDNA

The human GnRH receptor cDNA construct had previously been engineered to include a number of silent mutations, thereby introducing restriction sites at the TMD/ECL boundaries (Ott et al., 2002) (see Section 3.6). Each of the primers spanned one of these

sites and the sequence coded, partly for human GnRH receptor TMD, and partly for catfish GnRH receptor ECL (see Section 5.2.4). The catfish receptor ECLs were produced by PCR using the following primers in pairs:

Pair 1:
TMD2/ECL1 (sense):
5'-CATGCCACTGGATGGGGTGTGGAATGTGAC-3'

ECL1/TMD3 (antisense):
5'-ACTGAGTACTTTGCACATGGCGTCTC-3'

Pair 2:
TMD4/ECL2 (sense):
5'-CAGTTGTACATCTTCAGGATGATTAAGGCCAAAGG-3'

ECL2/TMD5 (antisense):
5'-TAAAAGGCCTCCTGCCAGTGCTGT-3'

Pair 3:
TMD6/ECL3 (sense):
5'-CTACTACGTACTAGGCATTTGGTATTGGTTTGATCCACAGATGCTGCA-3'

ECL3/TMD7 (antisense):
5'-AGTGGTTAACATAATCAGGGATCA-3'

Primer for the Human His$^{207(5.34)}$Glu/Gln$^{208(5.35)}$Glu mutant GnRH Receptor cDNA

The following is the antisense primer spanning the *Stu* I restriction site and the sequence coding for the His$^{207(5.34)}$Glu and Gln$^{208(5.35)}$Glu mutations in the engineered human GnRH receptor cDNA sequence (see Section 6.2.4):

5'-TAAAAGGCCTCCTCCCACCATTGTG-3'

Appendix IV

Publications and Presentations

Publication:

Pfleger KDG, Bogerd J and Millar RP (2002) Conformational constraint of mammalian, chicken and salmon GnRHs, but not GnRH II, enhances binding at mammalian and non-mammalian receptors: evidence for pre-configuration of GnRH II. *Mol Endocrinol* **16:**2155-62.

Conference Oral Communication:

Pfleger KDG, Ott TR and Millar RP (2001) Ligand binding to mammalian and non-mammalian GnRH receptors apparently involves similar receptor contact sites in different spatial arrangements, in *192nd Meeting of the Society for Endocrinology* Abstract OC27, BioScientifica, London, UK.

Conference Poster Presentations:

Pfleger KDG and Millar RP (2001) D-Amino acid incorporation in position 6 of GnRH II does not enhance binding affinity: evidence for conformational constraint, in *83rd Annual Meeting of the Endocrine Society* Abstract P3-476, Denver, Colorado, USA.

Pfleger KDG and Millar RP (2001) The use of chimeric receptors to investigate the role of extracellular loops in the binding of agonists to the GnRH receptor, in *Drug Discovery 2001* p 8, Pfizer Global Research and Development, Sandwich, Kent, UK.

Pfleger KDG, Miller NA, Ott TR and Millar RP (2002) Extracellular loop (ECL) interactions differentially affect GnRH, GnRH II, superagonist and antagonist binding to GnRH receptors, in *84th Annual Meeting of the Endocrine Society* Abstract P1-117, San Francisco, California, USA.

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Conformational Constraint of Mammalian, Chicken, and Salmon GnRHs, But Not GnRH II, Enhances Binding at Mammalian and Nonmammalian Receptors: Evidence for Preconfiguration of GnRH II

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Mammalian GnRH (mGnRH) is believed to interact with mGnRH type I receptors in a β-II′ turn conformation involving residues 5–8. This conformation can be constrained by substitution of a D-amino acid at position 6 or by a lactam ring involving residues 6 and 7, thereby increasing receptor binding affinity. It has been proposed that this is not the case for non-mGnRH receptors. However, we show that this conformational constraint increases the binding affinity of mammalian, chicken, and salmon GnRH for the chicken and catfish receptors, as well as for the mouse receptor. Therefore, we conclude that the β-II′ turn conformation enhances ligand binding for non-mGnRH as well as mGnRH type I receptors. In contrast, most substitutions of a D-amino acid in position 6 have limited effect on binding affinity for GnRH II. We suggest that this ligand is preconfigured through intramolecular interactions, which accounts for its high binding affinity and total conservation of primary structure over 500 million years of evolution. (*Molecular Endocrinology* 16: 2155–2162, 2002)

MAMMALIAN GnRH (mGnRH) is a decapeptide released from the hypothalamus to interact with cognate receptors and regulate LH and FSH release from pituitary gonadotroph cells (1). Fifteen structural variants of GnRH have been identified (2, 3). In jawed vertebrates these cluster into three groupings: GnRH I, GnRH II, and GnRH III (4, 5). One of these, GnRH II, is totally conserved in structure from bony fish to man, suggesting that each individual amino acid is essential for biological activity (6). We propose that the N and C termini, which are conserved between all GnRHs, are involved in receptor binding and activation as for mGnRH, whereas the central residues are crucial for the configuration of GnRH II and appropriate presentation of the N and C termini.

The bioactive conformation of mGnRH acting on mGnRH type I receptors was originally proposed to be largely determined by a β-II′ turn involving residues 5–8 (7). This conclusion was supported by a large body of evidence derived from a variety of approaches. Conformation-dependent mGnRH antisera that bind the N and C termini of mGnRH tolerate certain amino acid substitutions in the central region of the ligand, but not in other positions. This implied that mGnRH has a turn conformation resulting in closely apposed N and C termini (8).

Fluorescence measurements of Trp^3 at different pH values suggested that His^2 and Tyr^5 are in close proximity to Arg^8 in mGnRH (9). This correlated with the proposed β-II′ turn conformation and indicated the possibility of intramolecular stabilizing interactions between these residues. Further fluorescence studies supported these findings by comparing mGnRH and analogs with substitutions for Arg^8 (10). Chicken GnRH I (cGnRH I, [Gln^8]-GnRH) was found to lack such stabilizing interactions, implying that a less structured conformation exists for this ligand.

The technique of conformational memories further supported the concept that a predominant β-II′ turn conformer of mGnRH accounts for biological activity (11). Substitution of Arg^8 with Lys resulted in a loss of this structure and reduced binding affinity (11, 12).

Nuclear magnetic resonance (NMR) studies have provided direct structural evidence for mGnRH in a β-type turn conformation consisting of three families (13). All possess the β-type turn about Gly^6 and at least two hydrogen bonds: one between Ser^4 and Arg^8 and another between $pGlu^1$ and Gly^{10}-NH_2. Hydrogen bonding of Arg^8 to either His^2 or Tyr^5 was also noted, supporting the conclusions from the fluorescence studies (9, 10). cGnRH I had different conformers grouped into four families. These also possessed several hydrogen bonds, but only that between $pGlu^1$ and Ser^4 was common to all four. This implied much greater flexibility of this molecule compared with mGnRH.

A series of cyclic GnRH antagonists were found to have a β-II′ turn conformation, further supporting the notion that this is the biologically relevant structure (14). However, because antagonists occupy different,

Abbreviations: cGnRH, Chicken GnRH; mGnRH, mammalian GnRH; NMR, nuclear magnetic resonance; sGnRH, salmon GnRH.

but overlapping, binding sites, these observations do not necessarily support the structure of agonists. Indeed, in this study a potent cyclic antagonist was found to have a β-I′ turn at residues 6–7. This implies that, for antagonists at least, the specific turn type may not be important as long as it results in correct presentation of the backbone and side chains critical for binding. Because agonists must satisfactorily bind and activate the receptor, they are likely to have more specific structural requirements.

D-Amino acid substitution for Gly^6 (D-aa^6 substitution) is believed to stabilize the β-II′ turn conformation, thereby increasing affinity for the receptor (15). Conformational energy analysis indicates that D-aa^6 substitution reduces the freedom for opening at position 6 so that the population of the bioactive conformer is increased (16). A similar effect can be achieved by utilizing a lactam ring between residues 6 and 7 (6,7 γ-lactam insertion) (17).

Although there is substantial evidence for the β-II′ turn conformation for active GnRH analogs at the mGnRH type I receptor, it is uncertain whether constraint of nonmammalian GnRHs in this conformation enhances their activity at mammalian and nonmammalian receptors.

mGnRH type I and non-mGnRH receptors have low sequence identity and structural features that suggest they may be configured differently. These include differences in intracellular domains (absence of C-terminal tail, longer intracellular loop 1 in mGnRH type I receptors), extracellular disulfide bridges, and different interactions between residues in transmembrane domains 2 and 7 (18, 19). In view of these differences that affect the overall three-dimensional structure of the receptor, the bioactive ligand conformational requirement may also differ (20).

The present study provides evidence that a major determinant of bioactive conformation at mGnRH type I receptors, the β-II′ turn involving residues 5–8, is also necessary for high-affinity binding at non-mGnRH receptors. The findings also indicate that, unlike the other GnRHs, the native GnRH II ligand is preconfigured in a bioactive conformation, which may account for its relatively high affinity for all GnRH receptors investigated and the conservation of its structure over 500 million yr of evolution.

RESULTS

The mouse GnRH receptor binds mGnRH and GnRH II with highest affinity (Table 1). The catfish and chicken receptors bind GnRH II with highest affinity, followed by salmon GnRH (sGnRH), mGnRH, and cGnRH I (Table 1). Substitution of a D-amino acid in position 6 of the ligand (D-aa^6) or the insertion of a γ-lactam moiety between residues 6 and 7 (6, 7 γ-lactam) significantly

Table 1. Summary of Ligand Binding to Mouse, Chicken, and Catfish GnRH Receptors

Ligand	Mouse Receptor		Chicken Receptor		Catfish Receptor	
	IC_{50}[a] (nM)	Relative Affinity[b]	IC_{50}[a] (nM)	Relative Affinity[b]	IC_{50}[a] (nM)	Relative Affinity[b]
mGnRH	174.2 ± 33.4		10.0 ± 1.1		633.8 ± 90.6	
[D-Trp^6]-GnRH	2.4 ± 0.21	74.0[c]	1.2 ± 0.39	8.6[d]	45.3 ± 12.9	14.0[c]
[D-Ala^6]-GnRH	23.8 ± 7.2	7.3[c]	2.9 ± 1.2	3.4[c]	89.6 ± 20.8	7.1[d]
[6,7 γ-Lactam]-GnRH	32.1 ± 6.7	5.4[c]	1.4 ± 0.26	7.3[d]	113.1 ± 29.6	5.6[d]
[D-Lys^6]-GnRH	89.9 ± 15.7	1.9[d]	2.8 ± 1.3	3.6[d]	318.0 ± 52.6	2.0[d]
cGnRH I	4316.7 ± 216.2		81.9 ± 10.6		2505.0 ± 198.7	
[D-Ala^6]-cGnRH I	1052.8 ± 249.6	4.1[e]	44.6 ± 9.2	1.8[d]	618.0 ± 36.8	4.1[c]
[6,7 γ-Lactam]-cGnRH I	880.7 ± 147.0	4.9[e]	16.6 ± 3.4	4.9[c]	385.3 ± 50.7	6.5[c]
sGnRH	1133.8 ± 298.5		7.6 ± 1.5		51.8 ± 11.3	
[D-Arg^6]-sGnRH	170.0 ± 36.1	6.7[d]	0.41 ± 0.15	18.8[d]	10.3 ± 1.7	5.0[d]
[D-Lys^6]-sGnRH	123.3 ± 24.4	9.2[d]	0.36 ± 0.03	21.4[d]	7.2 ± 1.8	7.2[d]
GnRH II	127.9 ± 19.8		0.75 ± 0.21		1.3 ± 0.24	
[D-Trp^6]-GnRH II	156.0 ± 28.5	0.8[f]	0.49 ± 0.06	1.5[f]	1.4 ± 0.52	0.9[f]
[D-Arg^6]-GnRH II	55.1 ± 10.9	2.3[d]	0.47 ± 0.17	1.6[f]	0.58 ± 0.17	2.2[f]
[D-Lys^6]-GnRH II	15.3 ± 2.3	8.4[d]	0.19 ± 0.03	4.0[d]	0.71 ± 0.25	1.8[f]

Radioligand binding assays were performed on homogenized membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} values were calculated as described in *Materials and Methods*.

[a] Mean ± SEM of between three and eight experiments carried out in triplicate.

[b] Fold increase in binding affinity relative to the wild-type ligand.

[c] Significantly different from wild type, $P < 0.01$.

[d] Significantly different from wild type, $P < 0.05$.

[e] Significantly different from wild type, $P < 0.001$.

[f] Not significantly different from wild type, $P > 0.05$.

increases the binding affinity of mGnRH, cGnRH I, and sGnRH acting on the mouse, chicken, and catfish GnRH receptors (Table 1).

Introduction of the aromatic D-Trp^6 residue into mGnRH increased the binding affinity at the mouse, chicken, and catfish GnRH receptors by a substantial 74-, 8.6-, and 14-fold. The affinity of [D-Trp^6]-GnRH binding at the mouse receptor is significantly higher than that of [D-Ala^6]-GnRH and [6,7 γ-lactam]-GnRH ($P < 0.05$). In contrast, substitution with the positively charged D-Lys^6 residue enhanced the binding affinity at these receptors by only 1.9-, 3.6-, and 2-fold.

GnRH II was unique among the natural GnRHs tested in binding all three species of GnRH receptor with relatively high affinity. The affinity for the non-mammalian receptors was particularly high. Substitution of a D-aa^6 has little or no effect on the binding affinity of the GnRH II ligand acting at the catfish and chicken receptors. At the mouse receptor, which has a much lower affinity for GnRH II, only D-Lys^6 substitution results in a substantial (8.4-fold) increase in affinity (Table 1).

The latter finding suggested that the D-Lys^6 in GnRH II may be interacting with one of the seven extracellular domain acidic residues that are conserved in mGnRH type I receptors. Binding of [D-Lys^6]-GnRH to mutant mouse GnRH receptors, in which each of these acidic residues was successively mutated to its isosteric amide (21), was compared with wild-type receptors. In initial binding assays the Glu^{8}Gln, Glu^{111}Gln, Asp^{185}Asn, Asp^{292}Asn, Glu^{294}Gln, and Glu^{301}Gln mutant receptors specifically bound radioligand, whereas the Asp^{98} Asn mutant receptor did not (data not shown). The latter result is expected as the Asp^{98} residue in the mGnRH type I receptor has been shown to interact with the His^2 residue in the GnRH ligand, and mutations of this residue severely affect ligand binding (22). The mutant receptors that bound the labeled ligand were all found to have the same binding affinity as the wild-type receptor for GnRH II and [D-Lys^6]-GnRH II (Table 2). This indicates that an interaction of D-Lys^6 with one of these residues was not responsible for the increased affinity of [D-Lys^6]-GnRH II.

D-Lys^6 substitution substantially enhanced the binding affinity of GnRH II acting at the mGnRH type I receptor, but not at the non-mGnRH receptors. To examine this further, four more species of GnRH receptor were investigated: two mGnRH type I receptors (rat and human) and two non-mGnRH receptors (*Xenopus* II and bullfrog III). D-aa^6 Substitution did not significantly increase the binding affinity of the GnRH II ligand acting at the *Xenopus* II or bullfrog III receptors (Table 3). D-Lys^6 substitution substantially increased the binding affinity of the GnRH II ligand acting at the rat and human receptors (33.8- and 7.2-fold, respectively), as noted for the mouse receptor. Another basic amino acid (D-Arg^6) substitution into GnRH II also increases binding affinity at the rat receptor.

Table 2. Summary of Ligand Binding to Point-Mutated Mouse GnRH Receptors

Mutation in Mouse GnRH Receptor	GnRH II	[D-Lys^6]-GnRH II
	IC_{50}[a] (nM)	IC_{50}[a] (nM)
Wild type	127.9 ± 19.8	15.3 ± 2.3
Glu^{8}Gln	175.9 ± 37.6[b]	10.5 ± 3.5[b]
Glu^{111}Gln	113.0 ± 16.1[b]	37.0 ± 11.4[b]
Asp^{185}Asn	175.4 ± 17.5[b]	20.0 ± 2.1[b]
Asp^{292}Asn	185.3 ± 25.3[b]	25.7 ± 5.7[b]
Glu^{294}Gln	128.7 ± 32.6[b]	16.3 ± 3.1[b]
Glu^{301}Gln	130.4 ± 6.5[b]	9.3 ± 1.4[b]

Radioligand binding assays were performed on homogenized membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} values were calculated as described in *Materials and Methods.*

[a] Mean ± SEM of between three and six experiments carried out in triplicate.

[b] Not significantly different from wild type, $P > 0.05$.

Substitution of a basic D-aa^6 into GnRH II enhances binding at the rat receptor more than at the mouse or human receptors. Comparison of the amino acid sequences of the human, mouse, and rat GnRH receptor extracellular loops reveals an additional acidic residue in the rat receptor: the residues at the homologous position to Gln^{208} in the human are Gln^{207} in the mouse and Glu^{207} in the rat (Table 4). A human GnRH receptor with the Gln^{208}Glu point mutation was used to investigate the effect of this residue on the binding of GnRH II and [D-Lys^6]-GnRH II. The IC_{50} for GnRH II binding to the human wild-type GnRH receptor was 135.6 ± 15.8 nM, compared with 323.0 ± 33.1 nM for binding to the rat wild-type GnRH receptor ($P < 0.05$). The Gln^{208}Glu human receptor mutant had an IC_{50} for GnRH II of 350 ± 15.5 nM, which is significantly different from the human wild-type receptor ($P < 0.001$) but not significantly different from the rat wild-type receptor (Fig. 1).

DISCUSSION

There is now considerable accumulated evidence that mGnRH interacts with its receptor in a β-II′ turn conformation involving residues 5–8 (7–13). This conformation appears to be conferred by interactions between Ser^4 and Arg^8, as well as between pGlu^1 and Gly^{10}-NH_2 (13). Others have presented evidence for interactions of Arg^8 with His^2 and Tyr^5 (9, 10, 13) contributing to the β-II′ turn conformation. An interaction of Arg^8 with an acidic residue in extracellular loop 3 of the receptor is also believed to contribute to the configuration of the ligand in the folded conformation (21, 23).

D-aa^6 Substitution and 6,7 γ-lactam insertion further stabilize this conformation and enhance binding affinity (15–17, 24, 25). Indeed, the D-aa^6 constraint can

Table 3. Summary of Ligand Binding to Rat, Human, *Xenopus* II, and Bullfrog III GnRH Receptors

Ligand	Rat Receptor		Human Receptor		*Xenopus* II Receptor		Bullfrog III Receptor	
	IC_{50}[a] (nM)	Relative Affinity[b]	IC_{50}[a] (nM)	Relative Affinity[b]	IC_{50}[a] (nM)	Relative Affinity[b]	IC_{50}[a] (nM)	Relative Affinity[b]
GnRH II	323.0 ± 33.1		135.6 ± 15.8		3.7 ± 1.3		0.91 ± 0.48	
[D-Trp^6]-GnRH II	111.0 ± 15.3	2.9[c]	106.6 ± 25.4	1.3[d]	1.4 ± 0.23	2.7[d]	0.45 ± 0.08	2.0[d]
[D-Arg^6]-GnRH II	35.2 ± 16.0	9.2[c]	115.0 ± 19.4	1.2[d]	2.6 ± 1.5	1.4[d]	0.32 ± 0.10	2.8[d]
[D]-Lys^6]-GnRH II	9.6 ± 1.1	33.8[c]	19.0 ± 4.8	7.2[e]	1.0 ± 0.21	3.6[d]	0.26 ± 0.01	3.4[d]

Radioligand binding assays were performed on homogenized membranes of COS-7 cells transiently transfected with GnRH receptor cDNA. IC_{50} values were calculated as described in *Materials and Methods.*

[a] Mean ± SEM of between three and six experiments carried out in triplicate.

[b] Fold increase in binding affinity relative to the wild-type ligand.

[c] Significantly different from wild type, $P < 0.05$.

[d] Not significantly different from wild type, $P > 0.05$.

[e] Significantly different from wild type, $P < 0.01$.

Table 4. Comparison of Human, Mouse, and Rat GnRH Receptor Extracellular Loop (ECL) Amino Acid Sequences[a]

Receptor	ECL 1	ECL 2	ECL 3
Human	PLDGMWNITQWYAGE**L**	MI**H**L**A**D**S**SG**QTK**VFSQCVTHCSF**S**QWWH**Q**[b]A	WFDPEMLNR**LS**D**P**V
Mouse	PLDGMWNITQWYAGE**F**	MI**Y**L**A**D**G**SG**PT**—VFSQCVTHCSF**P**QWWH**Q**[b]A	WFDPEMLNR**VSE**P**V**
Rat	PLDGMWNITQWYAGE**F**	MI**Y**L**V**D**G**SG**PA**—VFSQCVTHCSF**P**QWWH**E**[b]A	WFDPEMLNR**VSE**P**V**

[a] Nonconserved residues are shown as *bold letters.*

[b] Residue Q^{208} in human, Q^{207} in mouse, and E^{207} in rat GnRH receptor.

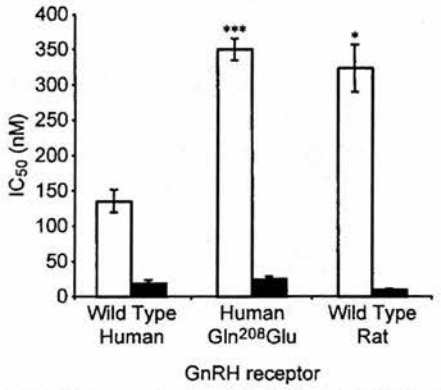

Fig. 1. GnRH II (*open bars*) and [D-Lys^6]-GnRH II (*filled bars*) Binding to the Human Gln^{208} Glu Mutant GnRH Receptor Compared with Binding to the Wild-Type Human and Rat GnRH Receptors

Data are presented as mean ± SEM. IC_{50} values of between three and five experiments carried out in triplicate. IC_{50} values were calculated as described in *Materials and Methods*. *, Significantly different from wild-type human, $P < 0.05$. ***, Significantly different from wild-type human, $P < 0.001$.

abrogate the need for the interaction between Arg^8 of mGnRH and the acidic residue in extracellular loop 3 of the receptor (23). The present studies confirmed these concepts as D-Trp^6, D-Ala^6, and D-Lys^6 substitution and 6,7 γ-lactam insertion all significantly increased the binding affinity of mGnRH at the mouse GnRH receptor.

The potency of mGnRH increases with increasing hydrophobicity of the D-aa^6 (24), and this is exemplified in the current study in which binding affinity successively increased in the series D-Trp^6 > D-Ala^6 > 6,7 γ-lactam > D-Lys^6. Because [D-Trp^6]-GnRH has a much higher affinity than the 6,7 γ-lactam analog, it appears that the substitution with D-Trp^6 makes additional contributions to enhancement of affinity. Explanations for this include: possible hydrophobic interactions with the receptor; a stabilization of the ligand conformation by intramolecular hydrophobic interactions, or a reduction in flexibility due the size of these side chains; and/or a reduction in the desolvation penalty upon binding to the receptor due to the more hydrophobic nature of the ligand (12). [D-Lys^6]-GnRH binds to the mouse and catfish receptors with significantly lower affinity than [6,7 γ-lactam]-GnRH, implying that the basic lysine side chain is detrimental to the binding of the GnRH ligand. The hydrophilic nature of this residue may disrupt one or more of the effects described above. Additionally, the positive charge of lysine may repel the positively charged Arg^8 and so affect ligand conformation. D-Arg^6/D-Lys^6 substitution into sGnRH, which does not contain positively charged amino acids, resulted in a much greater increase in binding affinity in the mouse, chicken, and catfish receptors. This concurs with the recorded effects of D-Arg^6 substitution in sGnRH at the goldfish receptor (26, 27).

Previous studies have shown that some non-mGnRH receptors, unlike mGnRH type I receptors, are

not selective for Arg^8 containing ligands (20, 28). In view of the role of Arg^8 in configuring the ligand, it was suggested that these receptors do not require GnRH to be configured in the β-II′ turn conformation (29). This interpretation was further supported by the observation that D-aa^6 substitution did not enhance binding at the cloned chicken receptor (20) and that NMR showed the N and C termini of cGnRH I were not closely apposed (13).

However, in the present study, D-aa^6 substitution clearly increased the binding affinity of mGnRH, cGnRH I, and sGnRH at mouse, chicken, and catfish receptors.

Although these observations appear to be contradictory, closer inspection reveals that the primary data concur. The interpretation that D-aa^6 substitution does not enhance binding affinity at the cloned chicken receptor was based on [D-Arg^6]-GnRH II having the same binding affinity as GnRH II and a GnRH analog incorporating D-Ala^6 having the same affinity as mGnRH (20). The present study found a lack of enhancement for D-Arg^6 substitution into GnRH II, and that D-Ala^6 substitution into mGnRH produced a relatively small enhancement. Our data showing an increase in affinity resulting from D-Trp^6 substitution are supported by a study testing LH releasing activity using dispersed chicken anterior pituitary cells, in which D-Trp^6 analogs of cGnRH I and mGnRH were approximately 20-fold more potent than cGnRH I and mGnRH, respectively (30). Furthermore, our data showing increased binding affinity of sGnRH at non-mGnRH receptors after D-aa^6 substitution are supported by two studies that showed D-Arg^6 substitution in sGnRH enhanced binding affinity at the goldfish receptor (26, 27).

The NMR data show that the N and C termini of cGnRH I are not closely apposed; however, a turn conformation around Gly^6 was still identified (13). Conformational energy analysis indicates that cGnRH I can adopt the β-II′ turn conformation (16). It is therefore likely that mGnRH and cGnRH I have similar conformations around Gly^6, but different conformations of the termini. This may explain how these ligands have such different affinities for the same receptor.

Studies using fluorescence (10) and NMR (13) have indicated a greater flexibility of the cGnRH I ligand compared with mGnRH. This implies that the nonmammalian receptors are able to stabilize the bioactive ligand conformation, either as a result of additional receptor contact sites, or as a result of a different spatial arrangement of conserved receptor contact sites as previously proposed (20). The latter concept is supported by the observation that non-mGnRH receptors have a different conformation of extracellular loop 3 due to the altered positioning of a proline residue (31).

Most D-aa^6 substitutions had limited effects on the binding affinity of GnRH II in contrast to that observed for mGnRH, cGnRH I, and sGnRH. D-Trp^6 substitution, which gave the greatest increase in binding affinity of mGnRH (8.6- to 74-fold), did not substantially increase the binding affinity of GnRH II to any of the receptors (Tables 1 and 3), and none of the D-aa^6 substitutions substantially increased the binding affinity of GnRH II at the chicken, catfish, *Xenopus* II, or bullfrog III GnRH receptors. Because GnRH II binds with high affinity to these receptors and D-aa^6 substitution does not substantially increase affinity, GnRH II would appear to be preconfigured in a bioactive conformation suitable for binding non-mGnRH receptors, as proposed previously (32). A more recent study also found that D-aa^6 substitution did not improve the binding affinity of GnRH II at the catfish receptor, again concluding that this ligand interacts with the catfish receptor in a constrained β-II′ turn conformation (33).

Only D-Lys^6 substantially increased the binding affinity of GnRH II at the human and mouse GnRH receptors. In view of the failure of other D-aa^6 substitutions to increase binding affinity, we considered that D-Lys^6 might provide an additional ligand-receptor interaction that does not occur with non-mGnRH receptors. A candidate interaction is between the basic D-Lys^6 and an acidic residue. To address this possibility, extracellular domain acidic residues conserved in mGnRH type I receptors were screened with GnRH II and [D-Lys^6]-GnRH II, using point-mutated mouse receptors. These mutations did not alter the binding affinity of either ligand (Table 2). Although there are other amino acid residues that can interact with a Lys side chain, we have not investigated these. Instead, we revisited the possibility that D-Lys^6 substitution can contribute to the configuration of GnRH II.

Although we have evidence that GnRH II is preconfigured, its lower affinity at mGnRH type I receptors (IC_{50} of 128–323 nM compared with 0.75–3.7 nM at non-mGnRH receptors) suggests the interaction is not optimal. We cannot therefore rule out the possibility that D-Lys^6 substitution may alter the conformation of GnRH II to improve its binding to mGnRH type I receptors.

D-Arg^6 as well as D-Lys^6 substantially increased the binding affinity of GnRH II at the rat GnRH receptor. This concurs with previous findings using rat pituitary membranes (34). D-Lys^6 increased the binding affinity of GnRH II at the rat receptor much more (33.8-fold) than at the human (7.2-fold) and mouse receptors (8.4-fold) (Tables 1 and 3). A search for amino acids different in the rat from the mouse and human revealed the presence of a glutamate residue in extracellular loop 2 of the rat in the homologous position to a glutamine residue in the human and mouse receptors. The mutation of Gln^{208} to Glu in the human receptor reduced the binding affinity of GnRH II to that of the rat receptor, and D-Lys^6 substitution increased binding affinity by a similar amount to that found in the rat receptor. The deleterious effect of Glu^{208} may be due to charge repulsion between this acidic residue and acidic residues in the N-terminal region of extracellular loop 3, such as Asp^{293} and Glu^{295}, causing distortion of binding site configuration and/or influencing the ease with which the ligand can interact with contact

sites within the transmembrane domains. The binding affinity of [D-Lys^6]-GnRH II is not significantly different between the three receptors, suggesting that D-Lys^6 substitution overcomes the deleterious effect of Glu^{208}.

In conclusion, we have obtained data in support of the concept that, as for the mGnRH type I receptors, the non-mGnRH receptors have a preference for GnRH in the folded conformation involving a β-II′ turn for residues 5–8, which is enhanced in mGnRH, cGnRH I, and sGnRH by D-aa^6 substitution. In contrast, the evolutionarily conserved GnRH II ligand appears to have a preconfigured β-II′ turn that accounts for its relatively high affinity for all GnRH receptors and a failure, in most instances, of any enhancement of binding affinity with D-aa^6 substitution. The surprising total conservation of GnRH II's primary structure from bony fish to man appears to have been a product of the coordinated evolutionary selection of amino acids contributing to binding, activation, and configuration such that its structure cannot be improved by substitution with any natural amino acid at any position.

MATERIALS AND METHODS

GnRH Analogs

mGnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$), [D-Trp^6]-GnRH, [D-Ala^6]-GnRH, [D-Lys^6]-GnRH, cGnRH I ([Gln^8]-GnRH), sGnRH ([Trp^7,Leu^8]-GnRH), and GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) were supplied by Bachem (Saffron Walden, Essex, UK) (Table 5). [D-Ala^6]-cGnRH I, [D-Arg^6]-sGnRH, [D-Lys^6]-sGnRH, [D-Trp^6]-GnRH II, [D-Arg^6]-GnRH II, and [D-Lys^6]-GnRH II were gifts from University of Cape Town (Cape Town, South Africa). [6,7 γ-Lactam]-GnRH and [6,7 γ-lactam]-cGnRH I were gifts from R. Freidinger (Merck & Co., Inc., West Point, PA) and R. Roeske (Indiana University School of Medicine, Indianapolis, IN).

GnRH Receptor cDNA

The human (35), mouse (36), chicken (20), and *Xenopus* II (Troskie, B., N. Illing, and R. Millar, unpublished results) GnRH receptor cDNA constructs were gifts from Cape Town University. The rat GnRH receptor was cloned by this laboratory (37). The catfish GnRH receptor cDNA (38) was a gift from Utrecht University (Utrecht, The Netherlands). The bullfrog III GnRH receptor cDNA was a gift from Chonnam National University (Kwangju, Republic of Korea) (28). The bullfrog III receptor has greatest sequence homology for designated type II receptors (Troskie, B., N. Illing, and R. Millar, unpublished results) and should be regarded as a type II receptor. Its classification as type III was based on tissue distribution.

The mouse GnRH receptors, each having one of the conserved extracellular domain acidic residues mutated to its isosteric amide, were gifts from C. Flanagan (University of Cape Town). They were produced as described previously (21). The human receptor containing the Gln^{208} Glu mutation was also produced previously (39).

Cell Culture and Transfection

COS-7 cells were seeded in 100-mm^2 dishes at a density of 1.2×10^6 cells per dish. Cells were maintained at 37 C, 5% CO_2 in DMEM containing 10% fetal calf serum, 0.3 mg/ml glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich Corp., Poole, Dorset, UK). After 24 h, the cells were transiently transfected with GnRH receptor cDNA from various species (10 μg of DNA per 100-mm^2 dish) using Superfect (QIAGEN, Crawley, West Sussex, UK) according to manufacturer's instructions (30 μl Superfect per 100-mm^2 dish for 8 h). After a further 48-h incubation, cells were scraped in PBS, pelleted, and stored at −70 C.

Receptor Binding Assays

The cell pellets were homogenized in ice-cold buffer (20 mM Tris, 2 mM $MgCl_2$, pH 7.2) and centrifuged at 15,000 rpm for 10 min at 4 C. The crude membrane pellet was then resuspended in ice-cold assay buffer (40 mM Tris, 2 mM $MgCl_2$, 0.1% BSA, pH 7.4). Competition binding assays were carried out using radiolabeled ^{125}I-[His^5, D-Tyr^6]-GnRH (~120,000 cpm/tube). The high binding affinity of this tracer compared with conventional tracers was established previously (40). The membrane suspension was incubated overnight at 4 C with labeled ligand and varying concentrations of unlabeled GnRH analogs in triplicate. The suspensions were then filtered through a membrane harvester (Brandel, St. Albans, Herts, UK) onto Whatman GF/B filter paper (Merck, Lutterworth, Leics, UK) (presoaked in assay buffer containing 0.01% polyethylenimine) and washed three times with ice-cold assay buffer. Bound radioactivity was counted using a multigamma counter [Perkin-Elmer Corp. (Wallac, Inc.), Cambridge, UK]. Maximum specific binding ranged between approximately 5,000 and 10,000 cpm/tube with nonspecific binding ranging between approximately 2,000 and 4,000 cpm/tube. No specific binding was detected with COS-7 cells transfected with vector only. Membrane concentration was varied to control for expression levels, with cells from two 100-mm^2 dishes being used for each binding curve, with the exception of the human, chicken, and catfish receptors. The human and chicken receptors exhibited particularly low expression levels; therefore, four 100-mm^2 dishes per curve were used. Conversely, the catfish receptor exhibited particularly high expression levels, and so one 100-mm^2 dish per curve was used. Therefore, similar maximal specific radioligand binding was observed at all receptors.

Table 5. Sequences of GnRH Ligands

Ligand	Sequence									
	1	2	3	4	5	6	7	8	9	10
mGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH$_2$
cGnRH I	[a]	[a]	[a]	[a]	[a]	[a]	[a]	Gln	[a]	[a]
sGnRH	[a]	[a]	[a]	[a]	[a]	[a]	Trp	Leu	[a]	[a]
GnRH II	[a]	[a]	[a]	[a]	His	[a]	Trp	Tyr	[a]	[a]

[a] Residue identical to mGnRH.

Data Reduction and Statistical Analysis

Binding curves were generated by Prism graphing software (GraphPad Software, Inc., San Diego, CA) using nonlinear regression, assuming one-site competition. Significant differences in wild-type to mutant IC_{50} values were assessed using a two-tailed, unpaired Student's *t* test with Welch's correction (does not assume equal variances).

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REFERENCES

1. Conn PM, Crowley Jr WF 1994 Gonadotropin-releasing hormone and its analogs. Annu Rev Med 45:391–405
2. Millar R, Lowe S, Conklin D, Pawson A, Maudsley S, Troskie B, Ott T, Millar M, Lincoln G, Sellar R, Faurholm B, Scobie G, Kuestner R, Terasawa E, Katz A 2001 A novel mammalian receptor for the evolutionarily conserved type II GnRH. Proc Natl Acad Sci USA 98: 9636–9641
3. Millar RP 2002 Gonadotropin-releasing hormones and their receptors. In: Fauser BCJM, ed. Reproductive medicine: molecular cellular and genetic fundamentals. Lancaster, UK: Parthenon Publishing; 199–224
4. Troskie B, Illing N, Rumbak E, Sun YM, Hapgood J, Sealfon S, Conklin D, Millar R 1998 Identification of three putative GnRH receptor subtypes in vertebrates. Gen Comp Endocrinol 112:296–302
5. White RB, Eisen JA, Kasten TL, Fernald RD 1998 Second gene for gonadotropin-releasing hormone in humans. Proc Natl Acad Sci USA 95:305–309
6. King JA, Millar RP 1992 Evolution of gonadotropin-releasing hormones. Trends Endocrinol Metab 3: 339–346
7. Momany FA 1976 Conformational energy analysis of the molecule, luteinizing hormone-releasing hormone. I. Native decapeptide. J Am Chem Soc 98:2990–2996
8. Millar RP, Tobler CJ, King JA, Arimura A 1984 Region-specific antisera in molecular biology of neuropeptides: application in quantitation, structural characterization and metabolism of luteinizing hormone-releasing hormone. In: Soreq H, ed. Molecular biology approach to the neurosciences. New York: Wiley; 221–230
9. Shinitzky M, Fridkin M 1976 Structural features of luliberin (luteinizing hormone-releasing factor) inferred from fluorescence measurements. Biochim Biophys Acta 434: 137–143
10. Milton RCdL, King JA, Badminton MN, Tobler CJ, Lindsey GG, Fridkin M, Millar RP 1983 Comparative structure-activity studies on mammalian [Arg8] LH-RH and chicken [Gln8] LH-RH by fluorimetric titration. Biochem Biophys Res Commun 111:1082–1088
11. Guarnieri F, Weinstein H 1996 Conformational memories and the exploration of biologically relevant peptide conformations: an illustration for the gonadotropin-releasing hormone. J Am Chem Soc 118:5580–5589
12. Mezei M, Guarnieri F 1998 Computer simulation studies of the fully solvated wild-type and mutated GnRH in extended and β-turn conformations. J Biomol Struct Dyn 16:723–732
13. Maliekal JC, Jackson GE, Flanagan CA, Millar RP 1997 Solution conformations of gonadotropin releasing hormone (GnRH) and [Gln(8)]GnRH. S Afr J Chem 50: 217–219
14. Rivier JE, Struthers RS, Porter J, Lahrichi SL, Jiang G, Cervini LA, Ibea M, Kirby DA, Koerber SC, Rivier CL 2000 Design of potent dicyclic (4–10/5–8) gonadotropin releasing hormone (GnRH) antagonists. J Med Chem 43:784–796
15. Monahan MW, Amoss MS, Anderson HA, Vale W 1973 Synthetic analogs of the hypothalamic luteinizing hormone releasing factor with increased agonist or antagonist properties. Biochemistry 12:4616–4620
16. Momany FA 1976 Conformational energy analysis of the molecule, luteinizing hormone-releasing hormone. 2. Tetrapeptide and decapeptide analogues. J Am Chem Soc 98:2996–3000
17. Freidinger RM, Veber DF, Perlow DS, Brooks JR, Saperstein R 1980 Bioactive conformation of luteinizing hormone-releasing hormone: evidence from a conformationally constrained analog. Science 210:656–658
18. Flanagan CA, Zhou W, Chi L, Yuen T, Rodic V, Robertson D, Johnson M, Holland P, Millar RP, Weinstein H, Mitchell R, Sealfon SC 1999 The functional microdomain in transmembrane helices 2 and 7 regulates expression, activation, and coupling pathways of the gonadotropin-releasing hormone receptor. J Biol Chem 274:28880–28886
19. Blomenröhr M, Bogerd J, Leurs R, Schulz RW, Tensen CP, Zandbergen MA, Goos HJ 1997 Differences in structure-function relations between nonmammalian and mammalian gonadotropin-releasing hormone receptors. Biochem Biophys Res Commun 238:517–522
20. Sun YM, Flanagan CA, Illing N, Ott TR, Sellar R, Fromme BJ, Hapgood J, Sharp P, Sealfon SC, 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. J Biol Chem 276: 7754–7761
21. Flanagan CA, Becker II, Davidson JS, Wakefield IK, Zhou W, Sealfon SC, Millar RP 1994 Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone. J Biol Chem 269:22636–22641
22. Flanagan CA, Rodic V, Konvicka K, Yuen T, Chi L, Rivier JE, Millar RP, Weinstein H, Sealfon SC 2000 Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction. Biochemistry 39:8133–8141
23. Fromme BJ, Katz AA, Roeske RW, Millar RP, Flanagan CA 2001 Role of aspartate7.32(302) of the human gonadotropin-releasing hormone receptor in stabilizing a high-affinity ligand conformation. Mol Pharmacol 60:1280–1287
24. Karten MJ, Rivier JE 1986 Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective. Endocr Rev 7:44–66
25. Sealfon SC, Weinstein H, Millar RP 1997 Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. Endocr Rev 18:180–205
26. Murthy CK, Turner RJ, Nestor JJJ, Rivier JE, Peter RE 1994 A new gonadotropin-releasing hormone (GnRH) superagonist in goldfish: influence of dialkyl-D-homoargin-

ine at position 6 on gonadotropin-II and growth hormone release. Regul Pept 53:1–15
27. Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE, Millar RP 1999 Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (*Carassius auratus*). Proc Natl Acad Sci USA 96: 2526–2531
28. Wang L, Bogerd J, Choi HS, Seong JY, Soh JM, Chun SY, Blomenröhr M, Troskie BE, Millar RP, Yu WH, McCann SM, Kwon HB 2001 Three distinct types of GnRH receptor characterized in the bullfrog. Proc Natl Acad Sci USA 98:361–366
29. Millar RP, King JA 1984 Structure-activity relations of LH-RH in birds. J Exp Zool 232:419–423
30. Millar RP, King JA 1983 Synthesis and biological activity of [D-Trp6] chicken luteinizing hormone-releasing hormone. Peptides 4:425–429
31. Millar RP, Troskie B, Sun YM, Ott T, Wakefield I, Myburgh D, Pawson A, Davidson JS, Flanagan C, Katz A, Hapgood J, Illing N, Weinstein H, Sealfon SC, Peter RE, Terasawa E, King JA, Plasticity in the structural and functional evolution of GnRH: a peptide for all seasons. Advances in comparative endocrinology. Proc 13th International Congress of Comparative Endocrinology, Yokohama, Japan, 1997, pp 17–27 (Scharrer-Bargmann lecture—transcript of lecture published by Monduzzi Editore S.p.A., Bologna, Italy, 1997)
32. Pfleger KDG, Millar RP, D-Amino acid incorporation in position 6 of GnRH II does not enhance binding affinity: evidence for conformational constraint. Program of the 83rd Annual Meeting of The Endocrine Society, Denver, CO, 2001, p 550 (Abstract P3-476)
33. Blomenröhr M, ter Laak T, Kuhne R, Beyermann M, Hund E, Bogerd J, Leurs R 2002 Chimaeric gonadotropin-releasing hormone (GnRH) peptides with improved affinity for the catfish (*Clarias gariepinus*) GnRH receptor. Biochem J 361:515–523
34. Millar RP, Milton RC, Follett BK, King JA 1986 Receptor binding and gonadotropin-releasing activity of a novel chicken gonadotropin-releasing hormone ([His5, Trp7, Tyr8]GnRH) and a D-Arg6 analog. Endocrinology 119: 224–231
35. Chi L, Zhou W, Prikhozhan A, Flanagan C, Davidson JS, Golembo M, Illing N, Millar RP, Sealfon SC 1993 Cloning and characterization of the human GnRH receptor. Mol Cell Endocrinol 91:R1–R6
36. Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC 1992 Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol Endocrinol 6:1163–1169
37. Eidne KA, Sellar RE, Couper G, Anderson L, Taylor PL 1992 Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. Mol Cell Endocrinol 90:R5–R9
38. Tensen C, Okuzawa K, Blomenröhr M, Rebers F, Leurs R, Bogerd J, Schulz R, Goos H 1997 Distinct efficacies for two endogenous ligands on a single cognate gonadoliberin receptor. Eur J Biochem 243:134–140
39. Ott TR, Troskie BE, Roeske RW, Illing N, Flanagan CA, Millar RP 2002 Two mutations in extracellular loop 2 of the human GnRH receptor convert an antagonist to an agonist. Mol Endocrinol 16:1079–1088
40. Flanagan CA, Fromme BJ, Davidson JS, 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