RECEPTOR ACTIVATION IN GNRH RECEPTORS

by

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ABSTRACT

The cloning of a number of mammalian Gonadotropin-Releasing-Hormone receptors (GnRH receptors) revealed a high homology amongst this group of receptors (>85% sequence identity) and very similar pharmacology [1]. However, the effects of GnRH analogues on hormone release from primary cultures of pituitary cells indicated that non-mammalian GnRH receptors have distinctively different pharmacologies [2]. The recent cloning of non-mammalian GnRH receptors from catfish [3], goldfish [4, 5], frog [6, 7] and chicken [8] confirmed this finding. Notable differences between the mammalian and some non-mammalian GnRH receptors were that certain agonists have different affinities and efficacies. Another difference was that certain antagonists of the mammalian receptors behaved as partial or full agonists in the chicken GnRH receptor [2, 8]. This is also true of the *Xenopus laevis* GnRH receptor (unpublished). It has been demonstrated that GnRH binds to the extracellular loops and the superficial regions of the transmembrane domains (review in ref. [1]). It was therefore considered likely that extracellular loop domains (ELs) of the different receptors would play a role in the recognition of certain ligands and consequently in determining agonist and antagonist activity of ligands.

In this study the possible role of extracellular domains in conferring agonist activity to one of these ligands (Antagonist 135-18) was investigated. Silent mutations close to the extracellular end of the transmembrane domains were introduced into the human GnRH receptor to create restriction endonuclease cutting sites that could be used to exchange ELs. ELs of the *X. laevis* GnRH receptor were amplified by PCR using primers containing the engineered restriction endonuclease recognition sequences. The PCR products were then used to replace ELs of the human GnRH receptor. The resultant chimeric receptors were transiently expressed in COS-1 cells and their pharmacologies were determined. Antagonist 135-18 was found to have agonist activity in the chimeric receptor where EL-2 had been replaced. Human receptors possessing either the *X. laevis* EL-1 or EL-3 still recognised Antagonist 135-18 as an antagonist. These finding implicate the *X. laevis* EL-2 as the arbiter of agonistic activity of Antagonist 135-18 at the chimeric receptor. Chimeric receptors where only parts of EL-2 were replaced

indicated that both the N- and C-terminal sections of EL-2 conferred partial but not full agonist activity to Antagonist 135-18.

In a previous study it has been shown that a positively charged D-Lys or D-Lys(iPr) is needed in position 6 of the antagonist to be recognised as an agonist in the chicken GnRH receptor [8]. It was therefore possible that there would be a counter-ion in EL-2. From comparisons of non-mammalian GnRH receptor sequences a conserved Glu was identified at the C-terminal end of EL-2, which was not present in the mammalian receptors. When Glu in the equivalent position of the human GnRH receptor was mutated, however, Antagonist 135-18 was still recognised as an antagonist. Mutations of each of the other residues in the C-terminal section of EL-2 gave similar results. A series of human GnRH receptors containing multiple mutations showed that the minimal and essential structural determinant in the C-terminal domain of EL-2 for conferring agonist activity to Antagonist 135-18 is a His^{205} combined with Ala^{197}. It is proposed that D-Lys(iPr) in position 6 of the ligand forms a charge supported hydrogen bond with His^{205} which cannot occur between the human wild-type receptor and Antagonist 135-18. A small Ala side chain might be needed in position 197 to overcome steric problems compromising the interaction when a larger side chain is present. This additional interaction presumably allows the receptor to be stabilised in the active conformation.

Results presented here, combined with computer modelling and results from future experiments which are based on this work can ultimately be used to refine the model of receptor activation. Understanding mechanisms involved in the recognition of ligands as agonists or antagonists are of importance for the design of novel pharmaceutical agents, which could be used in the treatment of diseases.

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CHAPTER 1: INTRODUCTION

Background

Gonadotropin-Releasing-Hormone (GnRH[1]) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) released from the hypothalamus to target its cognate receptor in the anterior pituitary. By activating its receptor it regulates the production of gonadotropins, follicle stimulating hormone (FSH) and luteinising hormone (LH), therefore having a pivotal role in the control of the reproductive system [1, 10, 11]. Because of this central role in reproduction, GnRH analogues have been used in the treatment of a wide spectrum of hormone dependent diseases including poly-cystic ovarian syndrome, hirsutism, porphyria, uterine fibrosis, precocious puberty, infertility, endometriosis, endometrial-, breast-, ovarian- and prostate cancer [12]. These analogues may also show promise in the development of a new generation of contraceptives. However, there are a number of disadvantages with using these peptide GnRH analogues. Since these analogues are peptides they have to be administered by injection. Depots are a means to overcome multiple injections and to ensure compliance but patients are not able to stop the treatment when desired [12]. Most of the analogues currently in use are agonists that operate through desensitisation but they initially stimulate the reproductive system for several weeks before the onset of desensitisation. GnRH antagonists are not widely used at present as much higher doses than agonists are required (several mg per day, as opposed to µg per day for agonists) [12]. An alternative to peptide analogues, which would solve these problems, would be to use non-peptide orally active small molecules. The delineation of the ligand binding pocket and mechanisms involved in the stabilisation of the active and inactive conformation of the GnRH receptor may contribute in this quest.

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1 for abbreviations, see page 10

G protein-coupled receptors

GnRH receptors belong to the superfamily of G protein-coupled receptors (GPCRs) [13, 14]. GPCRs form a large functionally diverse family of receptors. GPCRs can be grouped into three major families: the rhodopsin/ β-adrenergic- (family A), glucagon receptor related- (family B) and metabotropic glutamate- (mGlu-) (family C) families. In addition there are another two minor subfamilies of yeast pheromone receptors (families D and E) and a subfamily comprising of four cAMP receptors from *Dictyostelium discoideum* (family F) [15]. Knowledge of mechanisms underlying ligand binding and receptor activation in the family of GPCRs is essential to understand molecular mechanisms underlying GnRH receptor functioning. This introduction mainly focuses on ligand binding and receptor activation in the general context of the largest of the six GPCR families: the rhodopsin/β-adrenergic like GPCRs or family A, of which the GnRH receptor is a member. The GnRH receptor is also reviewed and mechanisms of ligand binding and receptor activation will be compared to what is known from other GPCRs.

Structural features of GPCRs

General structural features of GPCRs and models of the transmembrane domains

The GPCR family of receptors is characterised by comprising seven hydrophobic helices that are proposed to form a helix bundle crossing the cellular lipid bilayer. The N-terminal domain is extracellular, the C-terminal domain intracellular, and the seven helices, or transmembrane domains (TMDs), are connected by alternating intracellular loop domains (ILs) and extracellular loop domains (ELs) (Figure 1). The loops connecting TMDs are as short as 5-12 amino acids in certain receptors. It is therefore understood that consecutive helices are neighbouring in the three-dimensional configuration arranged in a counter-clockwise fashion as seen from the extracellular side of the cell (reviewed in ref. [1, 16, 17]). A hydrophilic pocket is found at the core of the transmembrane helix bundle that is maintained by a number of structural features of the individual helices. The different helices may vary in length: TMD-III, -V and -VI have been proposed to be longer than the other TMDs and therefore extend beyond the

lipid bilayer into the cytosol [15, 18]. It has also been suggested that some TMDs, notably TMDs I-III, are not perpendicular but tilted within the membrane [15, 18-20] (Figure 2), while Pro residues within TMDs are able to kink helices up to 30° [15, 18, 20]. It has been furthermore shown by low resolution density maps that the helix bundle is tightly packed at the intracellular side of the membrane. Near the extracellular side the helix bundle is opened up more forming a hydrophilic cavity mainly formed by TMDs III-VI [15, 20].

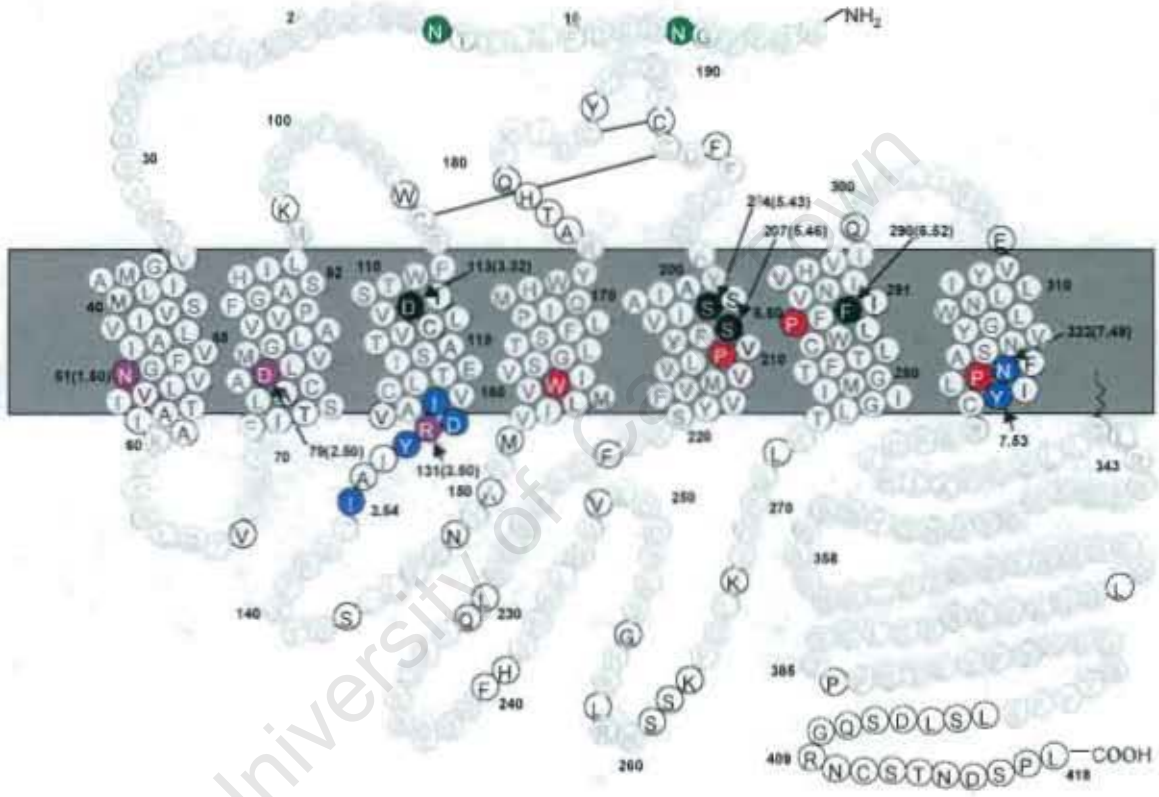

Figure 1: Primary structure of the β-adrenergic receptor, which is a representative member of the rhodopsin family of GPCRs. TMDs are connected by alternating intra- and extracellular loops. The N-terminus is on the extracellular- and the C-terminus is on the intracellular side of the membrane. Residues involved in ligand binding are shown in black. The most conserved residues of GPCRs within each TMD are shown in red. Blue residues are also very conserved and will be discussed in the "Receptor activation" section. Green residues are glycosylation sites. The palmitoylation site in the C-terminal tail is also indicated. The anchoring in the membrane is proposed to form a fourth intracellular loop.

As it has been impossible up to a now to obtain a three-dimensional crystal structure of GPCRs, a number of theoretical approaches suggesting a three-dimensional model have been brought forward [9, 21-24]. These models are mainly based on a projection density map of rhodopsin with a resolution of 7.5-9.0Å ([19, 20, 25], Figure 2). Baldwin

assumed that hydrophobic side chains should be facing the lipids of the membrane while polar side chains of amino acids in the TMDs should either be facing inwards and/or towards other TMDs forming a hydrophilic pocket at the centre of the helix bundle. Moreover, residues that are not conserved between closely related GPCRs should be fairly unimportant and therefore facing the lipid bilayer [23]. Comparing 204 different GPCRs, Baldwin identified clear hydrophobic and hydrophilic faces of each helix [23]. Changes amongst closely related GPCRs occur mainly on the hydrophobic faces. Looking at the size of the hydrophobic and hydrophilic faces it was found that TMD-I, TMD-IV and TMD-V should be most exposed to the lipid while TMD-III should be least exposed. These findings together with the projection density map of rhodopsin and information obtained from mutagenesis studies on ligand binding and the structural integrity of GPCRs provides us with a relatively crude model of the arrangement of the transmembrane helix bundle [23]. The other models were drawn up with more emphasis on computer based modelling [21, 22, 24]. Information from experiments that identified ligand-receptor- and intra-molecular interactions were analysed by computer to draw up three-dimensional models. These models will be progressively refined as new experimental data becomes accessible. On the contrary it has been much more difficult to model the extra- and intracellular domains of GPCRs. One of the major problems is the lack of conservation of these domains amongst all GPCRs. While TMDs are 20-25% homologous amongst GPCRs and 80-85% amongst GPCRs of different species, hardly any homology can be identified for intra- or extracellular domains even amongst closely related receptors [17]. The lack of conservation, both of the amino acid sequence and the length of the loops, is somewhat surprising for the intracellular loop domains as they couple to the same heteromeric G proteins although a consensus site for the coupling of G_s has been identified [26]. Since there is little homology in the primary structure, it has been difficult to model these loops with any reliability. It has also been suggested that the cytoskeleton [27] or RGS proteins (regulators of G protein signals) may play a major role in G protein

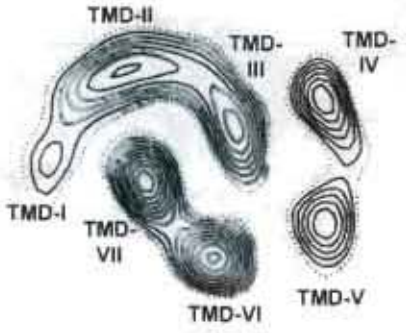

Figure 2: Projection density map of bovine rhodopsin at 9Å. This figure was taken from Schertler and Hargrave [25] and illustrates the arrangement of the helices in the transmembrane bundle as seen from the inside of the cell.

coupling. Interactions of G proteins with the receptor are beyond the scope of this thesis and are therefore only covered where necessary.

Some of the most conserved amino acids amongst GPCRs are two Cys residues at the end of EL-1 and in EL-2 respectively [17]. These two residues are proposed to form a disulphide bond important for the structural integrity of GPCRs. Mutant receptors lacking this disulphide bridge are generally poorly expressed [28-30], but the function other than structural is not properly defined. These findings are in contrast to the AT_1-angiotensin II receptors where mutations of extracellular cysteines do not affect expression and affect agonist but not antagonist binding [31]. It is possible that this bridge between EL-1 and EL-2 might be involved in receptor activation as it connects TMD-II/III with TMD-IV/V and might act like a hinge that is important for the proper movements of all TMDs during receptor activation. Cys residues have also been identified in TMDs but no specific pairs that might stabilise the conformation of the helix bundle has yet been identified [18].

Other conserved residues amongst GPCRs are prolines in TMD-IV, -V, -VI and -VII [17]. Because of steric clashes and a lack of hydrogen bonding capabilities due to its configuration, prolines are expected to break or at least kink α-helices. Since prolines are conserved within helices throughout all GPCRs they are believed to be important in forming the hydrophilic ligand binding pocket and/or being involved in receptor activation by maintaining the correct overall structure and by maintaining the proper positioning of helices [32, 33]. It is also feasible that by bending the helices prolines are important in amplifying conformational changes associated with receptor activation. Pro in TMD-VII on the other hand is proposed to act as a flexible hinge that is needed for receptor activation ([32], discussed below).

Amino acids conserved throughout GPCRs, other than proline, are Asn in TMD-I, Asp in TMD-II, Arg in TMD-III, Trp in TMD-IV and Asn in TMD-VII [17]. Most of these residues are also proposed to form part of the polar pocket [34] and seem to be important in receptor activation [15, 34-36] or in maintaining the structural integrity of GPCRs [34, 37] and will be discussed in greater detail under "Activation of GPCRs". In this chapter the focus will be on the activation of the receptors themselves. Residues and molecular processes involved in making contact and converting the inactive G protein

to its active conformation are very complex and beyond the scope of this thesis. They are the subject of excellent review articles [16, 38-40] and will therefore only be dealt with where necessary.

Post-translational modifications of GPCRs

Consensus N-linked glycosylation sites (Asn-X-Ser/Thr) can be found in the N-terminus of most GPCRs [17]. It has been shown recently that some GPCRs also contain O-linked oligosaccharides. However, no consensus sequence has yet been identified for this O-glycosylation [41, 42]. It has been suggested that there are chaperone proteins, like calnexin and calreticulin, in the endoplasmatic reticulum which anchor proteins, by recognising their carbohydrate moieties, until they are folded correctly [43, 44]. The function of glycosylation varies amongst GPCRs. It has been shown that glycosylation affects expression but not ligand binding in the GnRH- [45], β-adrenergic- [46], V_{1a}-vasopressin- [47] and FSH [48] receptors. For other GPCRs, like rhodopsin [49], the thrombin- [50], $EP_{3\alpha}$-prostaglandin E_2- [51], μ-opioid [52], TSH- [53] and Gastrin-Releasing-Peptide (GRP) [54] receptors, however, glycosylation has been implied for being involved in ligand binding, selectivity and/or G protein coupling. Furthermore glycosylation has no known function in the NK_1-Neurokinin- [55], M_2-muscarinic-acetylcholine- [56], histamine H_2- [57], oxytocin- [58], AT_1- [31] and AT_2-Angiotensin II [59] receptors. Although glycosylation of the N-terminal domain is found in almost every GPCR, its function varies considerably between different receptors. Despite being a conserved feature of GPCRs, its function has therefore not been conserved throughout evolution.

A number of phosphorylation consensus sequences can be found in the C-terminal tail of most GPCRs and to a lessor extent in IL-3. Upon receptor activation these sites are phosphorylated by members of the G protein-coupled receptor kinase (GRKs) family and second-messenger-activated protein kinases such as protein kinase A (PKA) and protein kinase C (PKC) [60]. Phosphorylation of the receptor diminishes its ability to activate G proteins and enables β-arrestin to bind to the receptor [61]. The receptor-β-arrestin complexes are sequestered and internalised through clathrin coated pits. Internalised receptors may ultimately be degraded or dephosphorylated and recycled to the cell surface [62]. It has been suggested that β-arrestin is not only responsible for the

sequestration of GPCRs but also for triggering a second wave of signalling similar to that of receptor tyrosine kinases [63]. It is believed that β-arrestin recruits members of the Src family of tyrosine kinases, which are involved in the activation of mitogen-activated protein (MAP) kinase [63]. These pathways, however, are not the scope of this thesis, and are therefore not dealt with in detail.

Another post-translational modification is palmitoylation. There are a number of semi-conserved Cys residues in the C-terminal tail [17]. These residues seem to be important in maintaining the tertiary structure of GPCRs, not by forming disulphide bridges but by being palmitoylated. Palmitoylated cysteines are believed to anchor the C-terminal tail in the plasma membrane thereby producing extra intracellular loops (e. g. in the adrenoceptors [17] and rhodopsin [64]) (Figure 1). The additional loops seem to have major function in the mobility of GPCRs in the membrane and in coupling of GPCRs to their cognate G protein. This, however, is not a consistent model as, e. g. the mammalian GnRH receptors do not contain C-terminal tails in the cytoplasm.

Dimerisation of GPCRs

Dimerisation is essential for signalling of many classes of cell surface receptors. Early indications that GPCRs may also form dimers (from cross-linking studies [65, 66] and receptor affinity labelling [67, 68]) reaches back as far as 20 years. These findings, however, attracted much controversy as they challenged classical models of GPCR functioning, in which one ligand interacts with only one GPCR.

More recently a more direct approach has been taken to show dimerisation. In one instance the domains from TMD-VI onwards of the α_2-adrenergic receptor were replaced by the equivalent domains of the M_3-muscarinic-acetylcholine receptor. This receptor was unable to bind adrenergic ligands and signalling was abolished [69]. Similarly, when the domains from TMD-VI onwards of the M_3-muscarinic-acetylcholine receptor were replaced by the equivalent domains of the α_2-adrenergic receptor no binding or signalling could be detected for this receptor either. Co-transfection of these two defunct chimeric receptors, however, restored binding for both adrenergic and muscarinic ligands as well as signalling [69]. In a similar experiment two point mutations of the AT_1-angoitensin II receptor exhibited impaired binding.

Upon co-expression of these two mutant receptors binding was restored [70]. These experiments imply that intermolecular contacts between two defunct receptors may restore the binding sites.

Further evidence for dimerisation of GPCRs was derived from co-immunoprecipitation experiments. Western blots often showed bands that might correspond to complexes of two or more equivalents. This, however, is no proof for dimers to occur, as receptors could bind to other proteins as well. For the dimers to be detected under conditions containing SDS also poses questions about whether these complexes can be found under physiological conditions. To show that the obtained high molecular weight bands were indeed from GPCR dimers, Hebert et al co-transfected β_2-adrenergic receptors that contained either HA or Myc tags [71]. Upon immunoprecipitation of receptors containing the Myc tag, HA tagged receptors could also be detected in the high molecular weight bands [71]. HA tagged M_2-muscarinic-acetylcholine receptors could not be detected in high molecular weight bands when co-transfected with Myc tagged α_2-adrenergic receptors indicating that α_2-adrenergic receptors are able to form homodimers but not heterodimers with M_2-muscarinic-acetylcholine receptors [71].

The most recent demonstration of GPCR dimer formation comes from bioluminescence resonance energy transfer (BRET) experiments in living cells [72]. Upon agonist exposure of cells co-transfected with β_2-adrenergic receptors which were fused to either green fluorescent protein (GFP) or *Renilla* luciferase (*R*Luc), fluorescence at 520 nm increased indicating that the agonist either promotes the formation of dimers or changed the conformation in existing dimers [72].

It has also been shown that the $GABA_B$ receptor requires two types (R1 and R2) for proper expression and functioning. $GABA_BR1$ expressed by itself is retained intracellularly as an immature glycoprotein. Co-expression with $GABA_BR2$, which by itself is also inactive, leads to a functional receptor that is expressed on the cell surface [73-77]. $GABA_BR2$ therefore acts as a chaperon for the maturation and/or cell surface expression of the $GABA_BR1$ receptor, but it is unclear whether the dimer is required for signalling.

The formation of heterodimers has also been proposed for κ- and δ-opioid receptors. The resulting heterodimer showed characteristics different from any of the wild type receptors [78]. Heterodimerisation could therefore contribute to pharmacological diversity. Further strength has been added to this line of argument by the observation that the δ-opioid receptor was unable to undergo agonist dependent internalisation upon co-expression with a dimerisation defective mutant δ-opioid receptor [79]. Additionally it has been shown that wild type receptors may lose their function when co-transfected with receptors that contain inactivating mutants [80-82].

In summary, it can be seen that GPCRs are likely to form dimers. Proposed roles for this dimer formation are in receptor maturation, -trafficking, -internalisation and signalling. How dimerisation may affect these pathways, the 3D arrangement of dimer complexes and whether the process of dimerisation is dynamic, however, remains largely unknown, as there is little support to validate the above models.

Nomenclature

Since GPCRs are different in size a common numbering system is needed to be able to compare residues across different receptors. For the remainder of this thesis the numbering system proposed by Ballesteros and Weinstein [9] will be used. The most conserved residue of a TMD is assigned the number 50. For example the most conserved residue in TMD-III is therefore assigned the number 3.50 (see Figure 1 for the most conserved residues in each TMD). Amino acids in the vicinity of this residue will be numbered in relation to this locus (i. e. the preceding residue would be 3.49 while the following residue is assigned to be 3.51). The original number specific for each receptor will follow the general number in brackets (see Figure 3). This combined numbering system simplifies the comparison of amino acid residues between related and completely unrelated GPCRs.

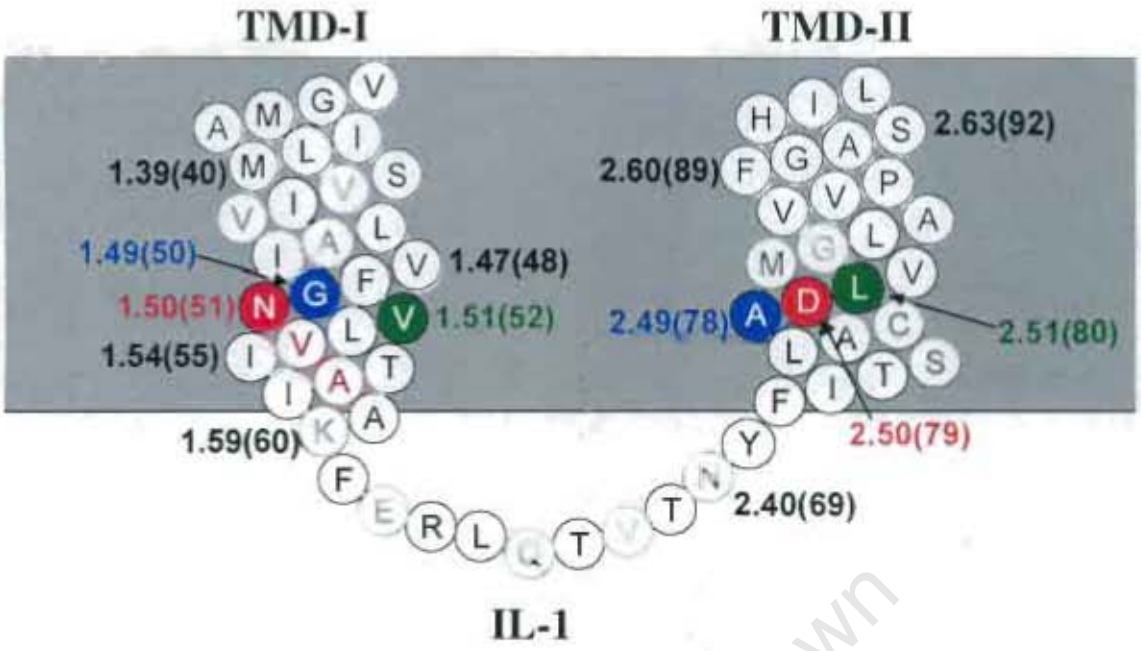

Figure 3: TMD-I and -II of the β-adrenergic receptor to depict the numbering according to Ballesteros and Weinstein [9]. Red residues are the most conserved residues within the TMD and are therefore given the number X.50 where X stands for the number of the TMD. Blue residues precede the most conserved residue of the TMD and are therefore given the number X.49. Green residues follow the most conserved residue in the TMD and are therefore given the number X.51.

Ligand binding in GPCRs

GPCRs respond to a variety of stimuli varying from photons, small cations and catecholamines, nucleotides and neurotransmitters through peptides and proteases to large glycoproteins and phospholipids (reviewed in ref. [15, 18, 39]). In recent years a great deal has been learnt about ligand binding and receptor activation by mutagenesis studies.

To show a direct interaction between a residue of the receptor and a residue of the ligand, two-dimensional studies are very helpful where residues are mutated both on the receptor and on the ligand. If the two residues are not in direct contact mutation of each residue may have an effect, with the double mutation having an additive effect [16, 39]. If the two residues are in direct contact, single mutations should have a similar effect. The double mutation should have the same effect as a single mutation, or, in some cases, reverse the initial effect. This, however, may be simplistic as residues may

interact with multiple loci. A decreased binding affinity due to a mutation can also be caused by a change in configuration of either the ligand or the receptor.

Comparative studies of related GPCRs and computer modelling programs have been a great help in modelling GPCRs and identifying the ligand-binding pocket [9, 23]. By comparing related receptors, similar residues can be identified which help in the assembly of computer models. Fitting the ligand into the simulated receptor model, putative points of interaction can be identified. Adding new experimental data, these models may then be refined. This might help to make more accurate predictions about the nature and size of the ligand binding pocket. Even in the absence of a model at the molecular level a fairly accurate model may be drawn up. These models will improve as more experimental data are added although precise judgements of distances and orientations of side chains will only be possible when the crystal structure of a GPCR is obtained. This however will still not be enough as the crystal structure of a GPCR will freeze the receptor in a certain conformation. To make an accurate description of the movements of domains during receptor activation, the crystal structure of a number of different conformations of a GPCR needs to be obtained. Until then computer based models will be the closest estimate of the 3D structure of GPCRs. To construct models, however, experimental data obtained from a big range of receptors will be taken. This might have the limitation that each receptor might have its own defined micro-environments, which may vary from receptor to receptor, thereby distorting the overall model.

Non-peptide and small peptide ligands are thought to bind entirely within the transmembrane spanning domain, while many larger peptide ligands appear to bind partly to extracellular domains and to extracellular boundaries of TMDs (reviewed in ref. [16, 18, 39]). Large glycoprotein hormones bind predominantly to the extracellular domains [16, 18, 39]. There seems to be a correlation between the size of the ligand and the size of the N-terminus with the exception of neurotransmitter receptors, such as the Ca^{2+}-, glutamate- and thrombin receptors [18]. Although these family C type receptors bind small ligands they were found to possess very long N-terminal domains which are believed to bind the ligand and then interact with the TMDs to activate the receptor [18]. While receptors binding large glycoprotein hormones have N-termini of up to ~400 residues, adrenoceptors have N-termini 7 to 30 amino acids in length [17].

Although this suggests an involvement of the N-terminus in ligand binding for large ligands, there is little evidence for its involvement in binding smaller peptides. To highlight similarities and differences in ligand binding between different receptors that bind differently sized ligands, representative receptors are discussed below.

Ligand binding in small non-peptide ligand receptors

Receptors binding biogenic amines are some of the most studied GPCRs. Since biogenic amines contain a positive charge it was assumed that there would be a negatively charged counter-ion within the ligand-binding site [16, 83]. $Asp^{3.32(113)}$ was identified in the β-adrenergic receptor to form a salt bridge with the amine group [83]. This residue was found to be conserved in a homologous position in the α_{2A}-adrenergic- [84], M_1-muscarinic-acetylcholine- [85] and histamine H_2 receptors [86]. Glu in position 3.28(113) was identified in a similar position in rhodopsin. This residue is thought to serve as a counter-ion for the Schiff's base that covalently binds retinal in TMD-VII. Lys in the ETB-endothelin receptor [87] and in the equivalent position of the GnRH receptor [88] is also believed to be involved in ligand binding. Although the residue in position 3.32 has an important function in ligand binding, it was shown to have no effect on receptor activation in the GnRH receptor, as mutants in this position can still be activated properly [88]. This, however, is in contrast to observations in the α_{1B}-adrenergic receptor, where $Asp^{3.32(113)}$ has been proposed to form a salt bridge with $Lys^{7.36(331)}$ that stabilises the receptor in the inactive conformation [89, 90]. Interestingly $Gly^{3.36(121)}$, which is found one helical turn further down compared with residue 3.32 and two helical turns away from $Glu^{3.28(113)}$ in rhodopsin, is believed to interact with the C^9-methyl group of retinal [91].

Adrenoceptors bind catecholamines that contain hydroxyl groups in the *para*- and *meta* position of the phenol ring. Two Ser residues in position 5.43(204) and 5.46(207) are believed to hydrogen bond the *meta*- and *para* hydroxyl groups respectively [92]. These residues are conserved in all GPCRs that bind catecholamines, i. e. dopamine, α- and β-adrenergic receptors (reviewed in ref. [16, 17]). On the other hand, receptors that do not bind catecholamines, e. g. the $5\text{-}HT_{2A}$-receptor, which binds mono-hydroxy-tryptamine, the muscarinic-acetylcholine- and histamine receptors, all contain different residues in these positions [15, 16]. Asp in position 5.42 and Thr in position 5.46 of the

histamine receptor are also implicated in binding the immidazol ring of its ligand [86]. In the muscarinic-acetylcholine receptor two Thr residues are found in position 5.38 and 5.42 and this pair is only offset by one helical turn compared with the Asp/Thr pair found in the histamine receptors. These two Thr residues are also believed to be involved in ligand binding by hydrogen bonding the ester moieties of the ligand [93]. It can be seen that this part of TMD-V is very important for binding small non-peptide ligands, even if receptors bind ligands that are not similar in structure.

Another residue important in stabilising the catechol ring in adrenoceptors is $Phe^{6.52(290)}$ [94]. This residue is conserved in all GPCRs that bind aromatic biogenic amines [16]. M_3-muscarinic-acetylcholine receptors, which bind aliphatic amines, contain a Tyr in position 6.51. This residue is believed to form a hydrogen bond with the ligand [16]. In Rhodopsin, $Trp^{6.48(265)}$ and $Tyr^{6.51(268)}$ associate with the β-ionone ring of retinal [95]. This again shows the importance of similar regions in ligand binding for all GPCRs that bind small non-peptide ligands.

Furthermore, computer models suggested an involvement of $Asn^{6.55(293)}$ in binding the β-OH group of agonists of the β_2-adernergic receptor. Mutation of this residue to Leu resulted in a receptor with reduced stereo-specificity for agonists indicating a possible involvement of this residue in forming a hydrogen bond with the β-OH group of the ligand [96, 97].

To date, all residues identified to be involved in ligand binding in the adrenoceptors are conserved amongst all adrenergic receptor subtypes [16, 17]. To explain the differences in pharmacology, one has to look for non-conserved residues. Chimeric exchange of the β_1- and β_2 type adrenergic receptors identified the region from TMD-IV to TMD-V to confer different affinities for certain ligands [98]. No single residue could be identified for being responsible for this phenomenon. It is likely that there are a number of residues involved, and/or the tertiary structure also contributing to the differences in pharmacology. This is a problem often encountered, as most interactions are not straight one on one, but rather involve more than one residue. Not much progress has been made in fine-tuning the ligand binding site, which is made even more difficult by the absence of structural information at the atomic level.

Ligand binding in peptide receptors

In two-dimensional studies a loss in binding affinity might be caused by two events. Firstly a receptor-ligand interaction might be affected by a mutation, and secondly an intra-molecular interaction might be disrupted by changing the configuration of the ligand binding pocket thereby preventing the ligand from entering. Peptide ligands are variable in size ranging from three residues to more than 50. As the size of the ligand increases, the possibilities for interaction with the receptor also increase. Not only are there a greater number of potential points of interaction, but the conformation of the ligand may also affect binding affinity as seen in the GnRH receptor [99]. By mutating a residue of the ligand, its overall structure might be altered rendering the ligand incapable of interacting with receptor binding sites. Analysis of interactions between a receptor and a large ligand may therefore be more difficult than the analysis of interactions between a receptor and a small ligand. Interpreting whether ligand binding was influenced due to a direct interaction being lost or due to an indirect effect (e. g. by a change in the ligand binding pocket or a change in structure of the ligand) is therefore a challenging task, especially for receptors binding larger ligands.

As ligand size increases exclusive binding within the transmembrane bundle is no longer feasible and interactions with the extracellular domains become paramount. The structure of extracellular domains is uncertain, as it is assumed that these domains could be in a number of different conformations (e. g. α-helix, β-sheet or random coil). Due to a vast difference in length and amino acid composition of extracellular domains, it is challenging to compare results from experiments obtained in different receptors. Thus, few advances have been made in attempts to model the extracellular domains, which further complicates identifying ligand binding mechanisms of larger ligands. Like small non-peptide ligands, small peptide ligands, however, are believed to bind within the transmembrane domains, while larger peptide ligands are believed to bind to both, the superficial parts of the TMDs, and to the extracellular domains [15, 16, 18]. Below, two representative examples are discussed: the Thyrotropin-Releasing-Hormone (TRH) receptor, which binds a tripeptide, and the AT_1-angiotensin II receptor, which binds an octapeptide.

Four contact points of TRH (pGlu-His-ProNH_2) have been identified within the transmembrane bundle of the TRH receptor. $Arg^{7.39(306)}$ in TMD-VII is thought to directly interact with the terminal carboxamide [100], while $Tyr^{3.33(106)}$ and $Asn^{3.37(110)}$ in TMD-III are believed to interact with $pyroGlu^1$ by hydrogen bonding [101, 102]. $Tyr^{6.51(282)}$ in TMD-VI was suggested to interact with His^2 [100].

In the AT_1-angiotensin II receptor, $Lys^{5.42(199)}$ in TMD-V has been indicated to interact with the α-carboxy group of Phe^8 [31, 103, 104]. This interaction is made possible by the interaction of $His^{6.51(256)}$ in TMD-VI with Phe^8, which is likely to be responsible for the proper positioning of the α-carboxy group [104]. Arg^2 is believed to form a second salt bridge with $Asp^{7.32(281)}$ in EL-3 [105] while $Asn^{3.35(111)}$ in TMD-III is thought to interact with Tyr^4 [106]. The latter interaction presents is thought to present the first step in receptor activation [107] and will be discussed in more detail under *Activation of GPCRs*. Weak interactions were also shown between $His^{5.26(183)}$ in EL-2 and Asp^1 [105].

As mentioned earlier, an important parameter amongst larger peptide ligands is their conformation. Angiotensin II is proposed to form a horseshoe-like shape, stabilised by a salt bridge between the terminal amino- and carboxy-groups. This structure is also predicted for other peptide ligands such as. GnRH, which is a decapeptide (discussed below).

The above findings indicate that the binding pocket of peptide ligands is very similar to that of small non-peptide ligands (Table 1). Especially residues 6.51 (for the TRH-, muscarinic-acetylcholine- and AT_1-angiotensin II receptor) and 6.52 (for the adrenergic-, 5-HT_{2A}- and histamine receptors) seem to be involved in ligand binding for these classes of GPCRs. It is not clear in many receptors whether residues interact directly with the ligand or whether these residues only influence the overall structure of the ligand-binding pocket. Furthermore, both TRH and angiotensin II seem to bind to their receptors in a similar region as $Asp^{3.32}$ of the biogenic amine receptors. Although no contact of TRH was identified in TMD-V, the 5.42 interaction, similar to the ones observed in the adrenergic-, histamine-, and muscarinic-acetylcholine receptors, has been identified in the AT_1-angiotensin II receptor [103, 104]. Contrary to the small non-peptide ligand receptors, TMD-VII, or the boundary of TMD-VII and EL-3, has also been shown to be involved in ligand binding in both the TRH- (discussed below)

and AT_1-angiotensin II receptor [105]. Although there are small changes to the ligand binding pocket in each receptor, a general theme for ligand binding is observed which, is independent of the nature of the ligand (Table 1).

	Rhod.-R	Musc.-R	Hist.-R	Adren.-R	TRH-R	Ang. II-R	GnRH-R
TMD-II							$Asp^{2.61}$ $Asn^{2.65}$
TMD-III	$Glu^{3.28}$ $Gly^{3.36}$	$Asp^{3.32}$	$Asp^{3.32}$	$Asp^{3.32}$	$Tyr^{3.33}$ $Asn^{3.37}$	$Asn^{3.35}$	$Lys^{3.32}$
EL-2					$Tyr^{5.28}$*	$His^{5.26}$	
TMD-V		$Thr^{5.38}$ $Thr^{5.42}$	$Asp^{5.42}$ $Thr^{5.46}$	$Ser^{5.43}$ $Ser^{5.46}$		$Lys^{5.42}$	
TMD-VI	$Trp^{6.48}$ $Tyr^{6.51}$	$Tyr^{6.51}$	$Phe^{6.52}$	$Phe^{6.52}$	$Tyr^{6.51}$	$His^{6.51}$	
EL-3					$Asn^{6.58}$*	$Asp^{7.32}$	$Asp^{7.32}$
TMD-VII	$Lys^{7.43}$				$Arg^{7.39}$		

Table 1: Comparison of binding sites of rhodopsin and the muscarinic-, histidine, adrenergic, TRH, angiotensin II- and GnRH receptor. Residues are numbered according to Ballesteros and Weinstein (see text and [9]). Contact sites between the ligand and the receptor are mainly conserved in TMD-III, TMD-V and TMD-VI even across receptors with different ligands. It is interesting to note that ELs have only been implicated in binding of peptide- or glycoprotein receptors so far. There is a dispute about whether TRH interacts with residues in TMD-II or TMD-III (*). These interactions, however, indicate that TRH might interact with the extracellular loop domains prior to its insertion into its ligand binding pocket. A similar mechanism where the loops guide the small ligand into its binding pocket might also be employed by biogenic amine receptors.

TRH therefore seems to be embedded in the transmembrane bundle without making any contact with the extracellular domains. This might be due to its small size. Larger peptide ligands often make contact with at least one residue in the extracellular domains, as seen with the $Asp^{7.32(281)}$-Arg^2 interaction of the AT_1-angiotensin II receptor with its ligand. This may position the ligand properly for binding to take place (also see discussion on ligand binding of the GnRH receptor). More recent experiments have shown an interaction between $pyroGlu^1$ of TRH and $Tyr^{5.28(181)}$ in EL-2 [108]. EL-3 has also been implicated in binding TRH [109, 110]. The extracellular domains are proposed to form a channel that facilitates the entry of the ligand into the

transmembrane bundle by low frequency fluctuations [108]. This interaction is believed to happen prior to the insertion of the ligand into the binding pocket as it is sterically not possible for pyroGlu[1] to simultaneously interact with $Tyr^{3.33(106)}$, $Asn^{3.37(110)}$ and $Tyr^{5.28(181)}$ [108]. It has been proposed that this might be a model for all receptors that bind small ligands [108]. More evidence has to be obtained to establish whether the ELs are part of the binding pocket or whether they guide the ligand into it. It remains to be seen whether this mechanism is applied by other receptors than the TRH receptor.

Ligand binding in glycoprotein receptors

Glycoprotein receptors are different from other members of the rhodopsin-like family of GPCRs in having a long N-terminus roughly the same size as the rest of the receptor. The long N-terminus most probably evolved to bind these large heterodimeric ligands. These receptors consist of two distinct domains that can be expressed individually (reviewed in ref. [111, 112]). The N-terminus, or exodomain, containing the high affinity hormone-binding domain, is glycosylated with sialic acid but cannot generate a signal. The rest of the receptor, the endodomain, is very similar in size and nature to all other GPCRs and is responsible for relaying the signal to the heteromeric G proteins. This part of the receptor cannot bind the ligand with high affinity [111, 112]. The ligand generally consists of two subunits: an α-subunit, common for all ligands, and a β-subunit, which is specific for each receptor. Although the α-subunit is conserved, it may function mainly in ligand binding in some, and mainly in receptor activation in other receptors [111]. Despite its size, very few residues of the N-terminus are proposed to interact with the ligand. For the LH/hCG receptor the N-terminus is proposed to be in a horseshoe-like shape that lies flat on the surface of the endodomain, contacting several residues. The ligand is guided into the horseshoe, maybe by some low affinity interactions with the endodomain, where residues of the receptor insert into a groove between the two subunits of the heterodimer ligand [111]. The remainder of the ligand is found in the space between the two arms of the horseshoe, making few, if any, specific interactions. The insertion of residues into the groove triggers a structural change in the ligand that is potentiated by the oligosaccharides of the ligand pushing against the arms of the horseshoe, forcing a structural change of the exodomain/ligand complex [111]. A secondary low affinity contact is made between the ligand and the endodomain, which in turn forces a conformational change in the latter part, eventually

relaying the signal to the G proteins [111]. Although the ligands consist of two subunits and are considerably larger in size, it is believed that the general mechanism underlying receptor activation is similar to the mechanism in other GPCRs [111]. The large exodomain might function as an adapter to facilitate binding of a large ligand to a small receptor, either acting more as a ligand itself or by guiding the large ligand into the correct position. Further experiments are needed to identify residues involved in ligand binding and receptor activation, so that this group of GPCRs can be compared better with other members of the GPCR family.

Activation of GPCRs

Background

Although considerable information has been obtained on the ligand binding pocket and ligand interaction sites of GPCRs, there is less knowledge on the molecular mechanisms mediating activation of GPCRs. Ligand binding and receptor activation are often difficult to separate. Receptor activation, however, is likely to be independent from the ligand binding [15], as seen in rhodopsin where the ligand (11-cis retinal) is covalently bound to the receptor as an inverse agonist. Furthermore there is a number of receptors which have been shown to be constitutively active [113-115] or produce elevated levels of secondary messengers upon overexpression [116-119]. Upon isomerisation to all-trans retinal by a photon, the ligand is converted from an inverse agonist to an agonist. As both forms of the ligand are covalently bound to the receptor, this shows that ligand binding might not be crucial for receptor activation.

Conformational changes of the transmembrane helix bundle are proposed to be involved in the activation and inactivation of the receptor through a network of interactions between residue side chains. It is believed that there is an equilibrium between the inactive (R) and active (R*) conformation of GPCRs [117, 118]. The level of basal receptor activity is dependent on the equilibrium between R and R*. In the absence of ligand the equilibrium is generally shifted towards R and secondary response is at low basal levels [18, 118]. Agonists are defined as ligands that bind to and stabilise the receptor in the R* conformation. Furthermore certain mutations of the receptor may destabilise R, or stabilise R* leading to constitutive activity in the absence of agonist

[113-115]. These mutations have been found in almost every domain of GPCRs (references can be found in [15]). Inverse agonists bind to and stabilise the receptor in the R conformation, while antagonists bind equally well to the active and inactive form of the receptor [117, 118].

The above model, however, has been challenged by Noda et al who propose that the AT_{1A}-angiotensin II receptor must pass through a partially active (R') state to become activated [106]. A similar model has also been proposed by Gether and Kobilka [120]. Noda and co-workers argue that two residues (residues 4 and 8) of the octapeptide angiotensin II are responsible for the conversion of R to R'. In support of this model the authors identified a constitutively active receptor mutant which in their model represents R'. $Asn^{3.35(111)}$ was suggested to form an interaction with $Tyr^{7.43(292)}$ [107] and/or $Asn^{7.46(295)}$ [121] constraining the receptor in an inactive state. This interaction has to be broken for the receptor to become activated. Residues 4 and 8 of angiotensin II are involved in the initial conversion of the receptor from R to R' [106] by breaking the bond between $Asn^{3.35(111)}$ and $Tyr^{7.43(292)}$ or $Asn^{7.46(295)}$. $Tyr^{7.43(292)}$ is now free to bind to $Asp^{2.50(74)}$ [122]. If Tyr in position 4 and Phe in position 8 of angiotensin II are substituted, the receptor cannot assume R' and thus cannot be activated [106, 123]. As the constitutively activated receptor mutant is already in the R' conformation, Tyr and Phe in position 4 and 8 of angiotensin II are not required for receptor activation of the mutant receptor [106]. A model for receptor activation involving more than two receptor states has also been proposed by other groups [15, 124-128].

The breaking of a salt bridge, which stabilises the inactive state of a receptor has also been identified in being the initial step in receptor activation in rhodopsin [95] and in the α_{1B}-adrenergic receptors [89, 90]. In rhodopsin, $Glu^{3.28(113)}$ and $Lys^{7.43(296)}$ are proposed to form a salt bridge that is broken during the initial stage of receptor activation [95]. A similar function has been assigned to the ion pair $Asp^{3.32(125)}$ and $Lys^{7.36(331)}$ of the α_{1B} -adrenergic receptor [89, 90]. It seems to be a common theme having a salt bridge between TMD-III and TMD-VII that stabilises the inactive state of the receptor. All residues identified to participate in these salt bridges are on the same face of the helix. GPCRs might therefore have a common mechanism of receptor activation, at least in the initial stages. Residues participating in these salt bridges are found closer to the cell surface compared with the rest of the receptor activation

machinery (discussed below) and might be in direct contact with the ligand, thereby initiating receptor activation. Whether receptor activation involving an intermediate R' state is a general phenomenon for all GPCRs remains to be shown. It also remains unclear whether receptor activation is ligand induced (as in Noda's model and rhodopsin) or whether a secondary response is triggered by conformational selection of R* by the agonist.

The events linking ligand binding and receptor activation are unclear at present. The possibility exists that residues might have multiple tasks in receptor functioning. Whether residues serve only one or multiple functions, e. g. ligand binding, receptor activation, G protein coupling and G protein activation cannot be dissected easily. It is often difficult to distinguish from experimental data whether a residue mutated is involved in receptor activation and receptor coupling, or whether the mutation only affect one of these properties. Mutations of multitask residues would therefore change more than one function. If these functions involved different residues, mutations would only affect one, while the other functions might still remain intact. It would be advantageous though if different events were guided by residues that are at least in close proximity. Close proximity would also ensure that even subtle changes could trigger the necessary response. The further away these residues are the more complex the mechanism that links the two events becomes. It would therefore be of benefit for the receptor if some residues had multiple tasks, or if residues involved in the different mechanisms were at least in close proximity. For GPCRs with small ligands the agonist binding site can be in close proximity to residues involved in receptor activation. GPCRs with peptide and glycoprotein ligands, however, have evolved away from this model. The process linking ligand binding and receptor activation becomes more complicated when effector residues are far away from each other, and requires a network of interactions to trigger a response distal to the stimulus. It can be seen, however, that there might be important axis connecting residues involved in different functions of the receptors. This axis, however, would provide a mechanism to further fine-tune the action of different ligands. The possibility exists that in some receptors the ligand binding pocket is forced to be in a low affinity state for agonists by a network of interactions that lock the receptor in its inactive conformation. By mutating the axis that connects the ligand binding pocket and the residues for receptor activation, the binding pocket can become more independent of the receptor activation machinery. Likewise,

G proteins cannot be activated when this axis is broken, even when an agonist occupies the binding pocket. With the binding pocket more independent of the receptor activation machinery, it could assume a conformation thermodynamically more stable for this microenvironment, which explains that uncoupling may be associated with increased agonist affinity as reported by Parent et al [129]. On the other hand a constitutively active mutant does not necessarily have to show increased binding affinities for agonists as observed by Cho et al [130]. Although the receptor is in a partially activated state, it might be thermodynamically favourable for the binding pocket to remain in a low affinity state for agonists. Upon agonist binding the receptor could then become fully active and the G proteins would be activated through a mechanism which involved other residues than the mutated one as seen by Noda et al [106]. Because of the huge network of interactions it is unlikely that any process involves only one residue. Mutating a residue involved in such an axis would thus often result in an intermediate between two conformational states.

The precise molecular mechanisms involved in the conversion of receptors between the different states are largely unknown. It is believed that the activation of GPCRs is caused by a conformational change in the arrangement of the TMDs through rotation and/or by changing the network of interactions of TMDs involved, which in turn changes the three-dimensional arrangement of the intracellular loop domains [34, 131]. This process allows a cognate G protein to bind to portions of the intracellular loop, which previously had not been accessible.

As TMD-III, TMD-V and TMD-VI are important for agonist binding in most GPCRs of the rhodopsin family, they are likely to play a role in receptor activation. Indeed TMD-III, containing the counter-ion for the biogenic amines, seems to be the major contributor in receptor activation (discussed below). IL-3, which connects TMD-V and TMD-VI, has also been implicated in coupling to G proteins in a number of receptors (reviewed in ref. [17, 40]). The involvement of TMD-III and TMD-VI in the rearrangement of the transmembrane bundle during receptor activation was also highlighted by spin labelling studies of rhodopsin [132, 133]. Further support of this theory was derived from experiments where free cysteines were labelled with a fluorescent dye in the β_2-adrenergic receptor [15, 33, 134, 135] or exposed to sulfhydryl reactive agents [114, 136]. UV absorbance of Trp and Tyr residues was used as another

tool to show the involvement of TMD-III and TMD-VI in receptor activation [137]. Finally the creation of Zn^{2+} binding sites also hinted towards a movement at the TMD-III/TMD-VI interface during receptor activation. When residues in TMD-III and TMD-VI were mutated in rhodopsin, a Zn^{2+} binding site was created. Upon Zn^{2+} binding the receptor could not be activated which was attributed to the incapability of the TMDs to change the orientation relative to each other due to the binding of Zn^{2+} [138]. Movement of other TMDs is also possible but has yet to be shown [15, 120]. As the above findings were carried out in different GPCRs, these results provide further evidence that mechanisms underlying ligand binding and receptor activation might be conserved amongst the GPCR family.

Due to the difficulty of obtaining crystal structures of GPCRs alternative approaches have been employed to delineate the different conformations of GPCRs. Experiments where receptors have been mutated to constitutively active receptors and/or convert antagonists to partial or full agonists, have shed some light on the process of receptor activation (e. g. [106, 124]). Other experiments have identified microdomains that examine residues that are likely to be in close three-dimensional proximity and involved in receptor activation, i. e. stabilisation of the active conformation [34, 37, 139].

The DR motif at the cytosolic end of TMD-III

As GPCRs are assumed to have a very similar overall structure, it is believed that the mechanisms underlying receptor activation are conserved. Residues involved in receptor activation should therefore be conserved throughout the GPCR family. The most highly conserved amino acid amongst rhodopsin-family GPCRs is an Arg at the intracellular end of TMD-III, which is found within a highly conserved (I/L)xxDRxxx(I/V) motif. It has been shown in rhodopsin [140, 141], adrenergic- [36, 94, 124], muscarinic-acetylcholine- [142, 143], 5-HT_7- [144], V_2-vasopressin- [145], histamine H_2- [146] and GnRH receptors [34, 147] that this $Arg^{3.50}$ is essential for receptor coupling. In all these receptors mutation of this residue severely impairs or completely abolishes signalling in response to agonists. However, when this residue was mutated in the α_{1B}-adrenergic receptor only mutations to Glu, Ile, Asn and Ala completely abolished a secondary messenger response [36]. Although IP production of the $Arg^{3.50(143)}$His and $Arg^{3.50(143)}$Asp mutants were impaired, they had an increased

basal IP production while the $Arg^{3.50(143)}$Lys mutant was even more constitutively active and supercoupled [36]. Similarly, in the oxytocin receptor the $Arg^{3.50(137)}$Ala mutation also gave rise to a constitutively active mutant receptor [148]. Although there was a 50-fold loss in potency, the mutant receptor was still able to produce a maximal IP response [148]. These two receptors clearly do not require Arg in position 3.50 to produce a secondary messenger response, which indicates that $Arg^{3.50}$ is not directly involved in G protein activation. $Arg^{3.50}$ is therefore more likely to act by changing the overall conformation of the receptor from the inactive to the active state. Since $Arg^{3.50}$ was only mutated to very few different residues in most studies, a more thorough investigation of the requirements of this side-chain will be important to elucidate the mechanisms underlying receptor activation in more detail.

The Asp residue immediately preceding $Arg^{3.50}$ also seems to have an important function in receptor activation. When this residue is mutated the resultant receptor is often constitutively active [114, 131, 146, 149-151] or supercoupled [34, 124, 147, 152]. It was also shown that mutation of $Asp^{3.49}$ in the β_2-adrenergic receptor changed the orientation of TMD-VI, adding further support to the involvement of this residue in the process of receptor activation [114]. In rhodopsin, the protonation of Glu in the homologous position has been shown to be a major step in the initial stages of receptor activation [153]. Despite this finding, it is unlikely that the charge of $Asp^{3.49}$ is the only driving force for keeping the receptor in the inactive state. Substitution of this residue in the α_{1B}-adrenergic receptor by any of the naturally occurring amino acids renders the receptor constitutively active [131]. Basal IP levels increased by 18 to almost 700% compared with basal IP levels of the wild-type receptor. These mutants also retain their response to agonists, showing that these mutant receptors are supercoupled rather than just constitutively active. Mutations of $Asp^{3.49}$ to Asn, a neutral side chain, and to Arg, a positively charged side chain, in the α_{1B}-adrenergic receptor result in much lower constitutive activity than replacement of this residue by an Ala or Thr [124, 131]. There is, however, a trend with the degree of activation decreasing as constitutive activity increases [131]. Other groups, however, found that mutations in this locus decrease or completely abolish signalling in other GPCRs, but this observation was sometimes made in combination with almost undetectable receptor expression [85, 154]. Because of different microenvironments in the various receptors, the effects of mutating this residue might differ amongst the different receptors. As the majority of reports of

mutations of $Asp^{3.49}$ result in supercoupled or constitutively active receptors, it is likely that $Asp^{3.49}$ stabilises the inactive conformation of GPCRs [34].

Ile or Val in position 3.54 of the (I/L)xxDRxxx(I/V) motif has been proposed to form part of a hydrophobic cage as a large hydrophobic side chain is needed in this position for signalling to occur [34]. It has been proposed that $Arg^{3.50}$ interacts with $Asp^{3.49}$ in the inactive conformation [34]. Upon protonation of the latter residue, $Arg^{3.50}$ is released. The bulky side-chain of residue 3.54 is positioned in a manner which restricts the movement of the Arg side chain, preventing it from dipping into the cytosol. The $Arg^{3.50}$ side chain is therefore restricted to move within the transmembrane bundle, ultimately turning TMD-III starting the process of receptor activation [34]. Data from experiments mutating Ile/Leu in position 3.46 indicate that although this residue is very conserved amongst GPCRs, it is not involved in receptor activation [34].

Conserved TMD-II/TMD-VII loci

Other highly conserved motifs in GPCRs are an Asp residue in TMD-II ($Asp^{2.50}$) and an Asn in TMD-VII ($Asn^{7.49}$). The discovery that these two residues are interchanged in mammalian GnRH receptors [139] has sparked experiments where either one or both of these residues have been mutated. A summary of single and double 2.50/7.49 mutations (Asp to Asn and Asp to Asn) can be seen in Table 2. In most cases if $Asp^{2.50}$ is mutated (to Asn or Ala), the secondary messenger response is markedly impaired [84, 85, 148, 155-166] and a change in agonist affinity is observed [84, 148, 157, 158, 160, 163, 164, 166, 167]. Signalling, or in some cases binding, can be rescued, however, by simultaneously mutating $Asn^{7.49}$ to an Asp, resulting in a reciprocal mutant receptor mimicking the motif found in mammalian GnRH receptors [155, 158, 160, 162]. This shows that these two residues might be in close spatial proximity and indicates the requirement for at least one Asp residue in either of the locations for signalling to take place. It is assumed that these two residues are in the immediate vicinity and form a network of hydrogen bonds in this region essential for proper functioning of GPCRs [34]. Asn residues in both loci are generally tolerated but result in receptors which bind ligands with the secondary messenger response severely impaired [37, 84, 155, 156, 158-160, 162, 168]. Two Asp residues in these positions gave inconsistent results with some of the receptors not being expressed [37, 139, 160] while others showed normal

ligand binding properties [155, 158, 162, 165, 166] and even coupling [158, 162, 165, 167]. Some $Asn^{7.49}Asp$ mutants, however, are supercoupled [155, 166]. It is assumed that one of the two Asp residues has to be protonated for hydrogen bonding to occur in order to prevent repulsion of the two negative charges and to obtain a hydrogen bond donor as ionic Asp side chains can only function as hydrogen bond acceptors [34]. The above findings show that a very specific conformation of these two side chains is needed for receptor activation, with one negative charge and a network of hydrogen bonding being essential. This is further highlighted by the finding that, as opposed to $Asn^{7.49}Asp$ mutations, $Asn^{7.49}Ala$ mutations in the 5-HT_{2A}- [155], β_2-adrenergic- [166], AT_1-angiotensin II- [169], TRH- [158], Tachykinin-NK_2- [162], CCK_B- [170] and Platelet-Activating-Factor (PAF) [129] receptors result in uncoupling, while the receptors maintain high expression levels. This indicates that the hydrogen bonding between the 2.50 and 7.49 loci is only important for receptor activation but not for the structural integrity of GPCRs. The exact configuration of these microdomains, however, is dictated by the overall structure of each individual GPCR, which often has its own specific requirements.

The other two residues, which are found in the polar pocket in close proximity to the 2.50/7.49 locus, are the highly conserved $Asn^{1.50}$ and $Tyr^{7.53}$. The role of $Asn^{1.50}$ in G protein coupling seems unclear as mutations of this locus generally result in receptors that are poorly expressed [37, 162]. Perlman et al report that mutation of $Asn^{1.50}Asp$ in the TRH receptor causes low expression with normal binding affinity being retained while G protein coupling is impaired [158]. Similar results were obtained for the $Asn^{1.50}Ala$ mutant [158]. The above observations lead the authors to conclude that residue 1.50 is involved in maintaining the structural integrity of the receptor rather than being involved in receptor activation [37, 158]. Scheer et al, however, argue that this residue constrains the receptor in its inactive configuration in the α_{1B}-adrenergic receptor as mutation of this residue to an Asp or Glu result in the receptor becoming constitutively active [131]. These observations indicate that the role of $Asn^{1.50}$ might be primarily in maintaining the structural integrity of the receptor by hydrogen bonding to residues 2.50 and 7.49 rather than being directly involved in G protein coupling [37].

$Tyr^{7.53}$ also seems to be important in receptor activation but not in receptor expression as receptor mutation of this residue to Phe in the β_2-adrenergic receptor gave rise to

uncoupling [166]. It was shown in the AT_1-angiotensin II- [122, 169], β_2-adrenergic- [166, 171] and GnRH receptors [168] that mutating $Tyr^{7.53}$ to Ala uncouples the receptor as well. From computer modelling of the α_{1A}-adrenergic receptor, $Tyr^{7.53}$ has been proposed to form hydrogen bonds with $Thr^{6.36(295)}$ and $Ser^{7.56(352)}$ at the intracellular end of TMD-VI and at the beginning of the C-terminal tail [124]. More recently, by modelling the 5-HT_{2A}-receptor Konvicka et al have implicated the conserved NP motif in TMD-VII as an important, highly flexible hinge that allows receptor activation [32]. In this model, $Tyr^{7.53}$ hydrogen bonds to the carbonyl group of residue 7.47 on the backbone of the helix, thereby stabilising the regions above and below the hinge [32]. Both the above models have been built on computer modelling data rather than experimental data. However, there is an agreement that $Tyr^{7.53}$ is important for maintaining the receptor in a certain configuration rather than having a direct function in G protein coupling. Arora et al showed that in the GnRH receptors a substitution of $Tyr^{7.53}$ by Phe is tolerated [168], indicating that the aromatic nature of the side chain and not the hydroxyl group of $Tyr^{7.53}$ is important for receptor activation. The GnRH receptors, however, form an exception by having an Asp in position 7.49. This indicates that the overall configuration of the hinge might be slightly different to the majority of GPCRs that have an Asn in this position. It was furthermore shown that Phe substitution of $Tyr^{7.53}$ in the β_2-adrenergic receptor partially uncouples the receptor [166]. Slice et al reported that the $Tyr^{7.54}$Ala mutation neither influenced coupling, nor did it influence agonist binding, internalisation or sequestration in the GRP receptor [172]. This receptor, however, has three residues between $Pro^{7.50}$ and the Tyr in the conserved $NPX_{2-3}Y$ motif. It may therefore behave differently compared with other receptors containing two rather than three residues between $Pro^{7.50}$ and the Tyr, as it is likely that the Tyr side chain points into a different direction. Further experiments are necessary to strengthen any of the above models. A direct involvement of $Tyr^{7.53}$ with the 2.50/7.49 and/or 3.49/3.50 loci, however, has yet to be shown although they are in close proximity.

	wt		DD				NN				ND				DN			
receptor	2.50	7.49	Expr	Ag	Res_{max}	EC_{50}	Expr	Ag	Res_{max}	EC_{50}	Expr	Ag	Res_{max}	EC_{50}	Expr	Ag	Res_{max}	EC_{50}
5-HT_{2A}	D	N	19%	=	95%	↓	177%	=	0	n/a	43%	=	48%	↓	wt (ref. [155])			
TRH	D	N	29%	↓	115%	↓	235%	↓	52%	↓	50%	↓	115%	↓	wt (ref. [158])			
μ-opioid	D	N	0	n/a	n/a	n/a	113%	↓	2%	n/a	90%	=	57%	↓	wt (ref. [160])			
NK_2	D	N	62%	=	93%	n/a	78%	=	7%	n/a	"="	=	57%	n/a	wt (ref. [162])			
CCK_B	D	N	77%	=	100%	n/a	113%	=	50%	n/a	*	*	*	*	wt (ref. [165])			
Ang II	D	N	*	*	*	*	50%	=	0	n/a	*	*	*	*	wt (ref. [156])			
BK	D	N	"="	=	53%	=	*	*	*	*	*	*	*	*	wt (ref. [159])			
M_1-musc	D	N	*	*	*	*	26%	=	9%	n/a	*	*	*	*	wt (ref. [85])			
LH	D	N	*	*	*	*	65%	↓	65%	↓	*	*	*	*	wt (ref. [164])			
PAF	D	D	wt (ref. [129, 157])				*	*	*	*	81%	↓	0	n/a	114%	=	100	=
cf GnRH	D	D	wt (ref. [173])				6%	n/a	n/a	n/a	6%	n/a	n/a	n/a	150%	=	50%	=
h GnRH	N	D	0	n/a	n/a	n/a	67%	=	39%	↓	wt (ref. [37, 139])				29%	=	12%	↓

Table 2: Summary of effect of mutations in postions 2.50 and 7.49 in different GPCRs on expression (Expr), agonist binding affinity (Ag), maximal secondary messenger response ($Resp_{max}$) and EC_{50}. * indicates where no references have been found, n/a depicts no data available, ↓ indicates a decrease in affinity or potency, = means unchanged affinity while "=" was indicated to be similar to wild-type although no data was shown in the reference.

Mechanisms of receptor activation

Two hypotheses of receptor activation have been brought forward which involve residues $Asn^{1.50}$, $Asp^{2.50}$, $Arg^{3.50}$, $Asn^{7.49}$ and $Tyr^{7.53}$ as key players in the mechanism of activation. Olivera et al proposed that in the inactive state $Asp^{2.50}$ acts as a counter-ion for $Arg^{3.50}$, which moves out of the hydrophilic pocket towards the cytosol upon receptor activation [174]. This model has been refined by Scheer et al suggesting that $Asp^{3.49}$ is important in maintaining the receptor in its inactive state. Its protonation causes the side chain to move from the outside to the inside of the hydrophilic pocket, thereby rotating TMD-III. During this process the side chain of $Arg^{3.50}$ is moved out of the hydrophilic pocket away from $Asp^{2.50}$ to rest between TMD-VI and TMD-VII, ultimately exposing previously buried regions in IL-2 and IL-3 important in coupling [35, 36, 124, 131].

Ballesteros et al, however, argue that $Arg^{3.50}$ interacts with the unprotonated $Asp^{3.49}$ in the inactive receptor [34]. This model is mainly based on the observation that mutant receptors at $Asp^{3.49}$ that are expressed properly generally are constitutively active [114, 131, 149-151] or supercoupled [34, 124, 147]. Upon protonation of this residue, the side chain of $Arg^{3.50}$ moves away from this locus to form an ionic interaction with $Asp^{2.50}$ (or $Asp^{7.49}$ in mammalian GnRH receptors). This process is facilitated by a bulky side chain in position 3.54, which prevents the $Arg^{3.50}$ side chain from moving into the cytosol [34]. In order to interact with the 2.50 or 7.49 the $Arg^{3.50}$ side chain has to move from its original interaction with $Asp^{3.49}$ thereby rotating TMD-III. This initial movement, which has been identified mainly along the TMD-III/TMD-VI interface [114, 132-136], represents the initial stage of receptor activation.

In view of the latest studies of the $Arg^{3.50}$ residue, where mutant receptors in this locus can give rise to constitutively active receptors [36, 148], both the above models have to be refined. It is essential to gain more information about the special requirements of the side-chain of residue 3.50 to understand the mechanism of receptor activation better. In most cases $Arg^{3.50}$ plays the pivotal role in receptor activation, but under certain conditions $Arg^{3.50}$ can be replaced by Lys, His and even Glu and Ala [36, 148].

In summary it can be seen that there is a considerable amount of data which implicates $Arg^{3.50}$ in being the heart of the "engine room" of receptor activation. The process of receptor activation is started by the protonation of $Asp^{3.49}$. Joseph et al proposed a model of how this proton is relayed from the ligand via different residues down the transmembrane helices to $Asp^{3.49}$ in the AT_1-angiotensin II receptor [107]. Other GPCRs could have a similar mechanism or share some of the residues involved in this relay of the proton. There are a great number of additional residues involved in binding and/or activation of G proteins, either through direct interactions or through stabilising a certain conformation of the receptor and especially the intracellular domains. Residues important for G protein coupling are mainly found in IL-2 and IL-3. Although very little homology even amongst closely related GPCRs is found in the regions responsible for coupling G proteins, the mechanism of receptor activation seems to be at least partially conserved. It has been shown that other residues involved in this process are $Asp^{2.50}$, $Asp^{3.49}$, $Ile/Val^{3.53}$ and $Asn^{7.49}$. In the absence of structural information of GPCRs in the active or inactive conformation, it is difficult to propose mechanisms involved in the conversion between the two states. More data are needed to shed further light on the mechanisms involved and to settle disputes about the orientation and movement of side chains during this process. Some of the residues involved in G protein coupling and/or activation of the GnRH receptor are reviewed in the GnRH "Receptor activation" section.

The GnRH receptor

This section of the introduction mainly focuses on receptor structure, ligand binding and receptor activation of the GnRH receptor. Direct interactions of GnRH receptors and G proteins, internalisation, desensitisation and downregulation are not the focus of this thesis and will therefore only be dealt with where necessary. The intracellular signalling pathways will also only be mentioned briefly as these are not pertinent to this thesis and are the subject of excellent review articles [10, 11].

General Structure

The first GnRH receptor was cloned from a mouse gonadotrope cell line [13]. The structure and sequence of this receptor was confirmed [175] and five other GnRH receptors were cloned from mammalian species in quick succession from rat [14, 176-178], human [179-181], sheep [182-184], cow [185] and pig [186]. More recently non-mammalian GnRH receptors, from catfish [3], goldfish [4, 5], frog [6, 7], chicken [8] and fruit flies [187], and additional mammalian GnRH receptors from marmoset [188], possum [189], bonnet monkey [190] and dog [191] were cloned. Other GnRH receptor sequences are available on the Genbank which include receptors cloned from three more forms of fish and horse. The GnRH receptor is a member of the rhodopsin like superfamily of GPCRs which includes the families of glycoprotein receptors (including FSH, LH/CG and thyroid stimulating hormone (TSH) receptors), neurotransmitter receptors (e.g. adrenergic-, serotonergic-, dopaminergic- and muscarinic-acetylcholine receptors), opsins and a variety of peptide receptors. The GnRH receptor contains most of the characteristics found in this group of receptors (Figure 4), mainly conserved sequences in the TMDs, which also facilitated its cloning [1, 10, 192]. Mammalian GnRH receptors, however, are unique in lacking a cytoplasmic C-terminal tail, having a relatively large IL-1, an apparent interchange of residues 2.50 and 7.49 ($Asp^{2.50}$ and $Asn^{7.49}$ for most GPCRs, and $Asn^{2.50}$ and $Asp^{7.49}$ for mammalian GnRH receptors), and a Ser or Phe substitution for $Tyr^{3.51}$ in the conserved DR motive in TMD-III, which is involved in receptor activation (discussed below) (Figure 4, reviewed in ref. [1, 10, 11]).

Like most other GPCRs the GnRH receptor contains two Cys residues in EL-1 and EL-2 respectively. These two residues are proposed to form a disulphide bond required for proper folding of the receptor [193, 194]. It was furthermore proposed that this disulphide bridge is important in stabilising the receptor in the active state [193]. Another two Cys residues have been identified in the N-terminus and the C-terminal section of EL-2 of mammalian GnRH receptors, which are also proposed to form a disulphide bond [193]. This finding, however, is somewhat controversial [194].

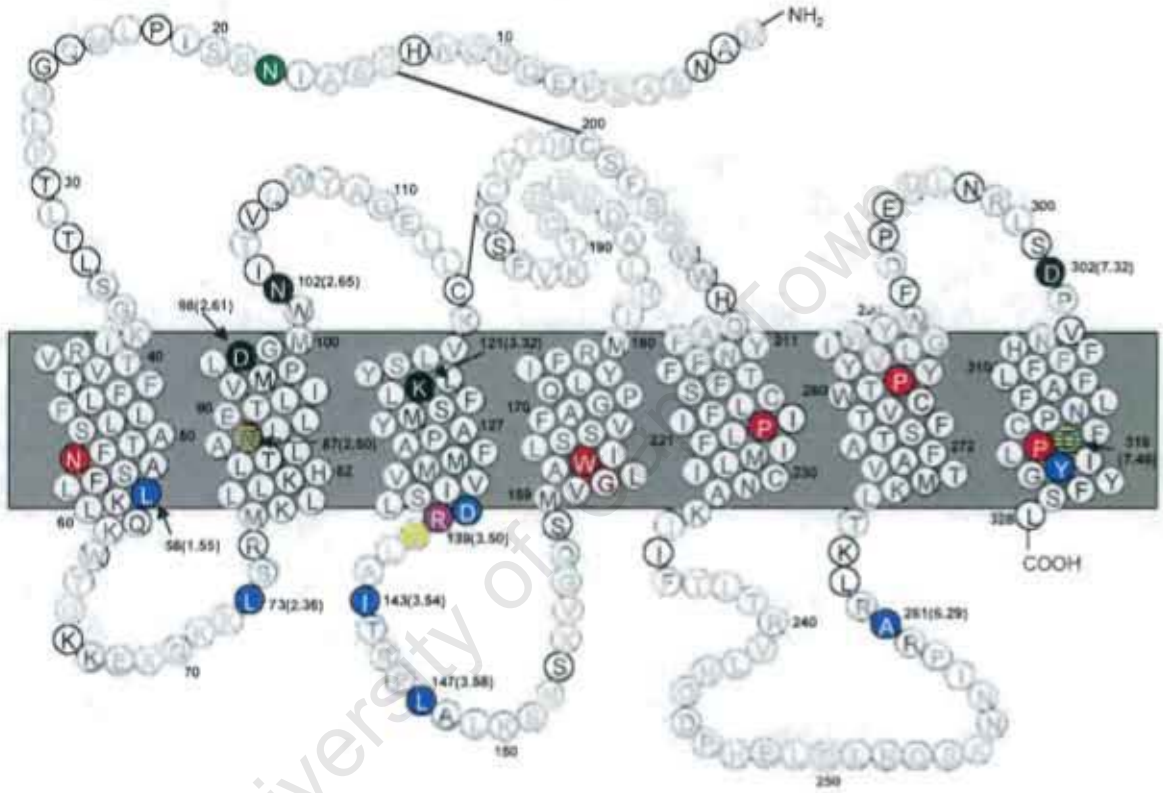

Figure 4: Primary structure of the human GnRH receptor. Red residues indicate the most conserved residues in each domain, yellow residues are different compared with other GPCRs, blue residues are involved in receptor activation and/or G protein coupling, black residues in ligand binding and the green residue is the glycosylation site.

Two potential N-glycosylation sites (Asn-X-Ser/Thr) have been identified in the N-terminus and EL-1 of the cow, pig, sheep and human GnRH receptors, while rodent GnRH receptors have an additional glycosylation consensus sequence in the N-terminus. It was shown that only the sites in the N-terminus are glycosylated in human and mouse GnRH receptors, and that glycosylation contributes to the level of receptor expression. This finding was not associated with the transport of the receptor to

the cell surface, as the mutant receptor did not display abnormal intracellular sequestration [45].

Ligand binding

Structure and function of GnRH

To understand the processes involved in ligand binding it is important to have structure-activity knowledge of the ligand itself. The first molecules of GnRH were isolated from pig and sheep hypothalami [195, 196]. Two or more (usually three) forms of GnRH have been identified in most vertebrate and portochordate species [197]. Table 3 shows all known forms of GnRH to date. GnRH is predominantly expressed in the hypothalamus and regulates the release of gonadotropins from the pituitary. This form of GnRH is generally variable between different species [197]. The most ubiquitous form of GnRH is GnRH-II, also known as chicken GnRH-II or [His^5, Trp^7, Tyr^8]GnRH. GnRH-II has been identified in the extra-hypothalamic areas of the brain of almost every vertebrate species [197]. This form of GnRH was identified to act as a co-neurotransmitter in bullfrog [198]. GnRH-II has also been shown to inhibit K^+ channels in sympathetic ganglia [199, 200]. The question about its function, however, remains intriguing, especially in view of its complete conservation over a time-span of 500 million years.

Although GnRH has been isolated from species spanning over 500 million years of evolution, the length of the molecule (a decapeptide) and the N- and C-terminal residues are conserved (Table 3). The most variable position is the residue in position 8, followed by position 5 and 7. Residue 6 is Gly in all higher vertebrates [1, 197] (Table 3). The same form of GnRH has been found in all mammals except the guinea pig [201]. In mammals, mGnRH was found to be much more active than any other naturally occurring form of GnRH [197]. Since Arg^8 is the only residue that distinguishes mGnRH from any other form of GnRH, this residue was identified for being critical in high affinity binding of mGnRH to the mammalian GnRH receptors [197]. In view of the difference between Guinea Pig GnRH and GnRH found in all other mammalian species, it would be interesting to clone the receptor from this animal as significant changes can be expected in its pharmacology.

The following two subsections summarise the structure-activity requirements of each amino acid residue of GnRH. Thousands of analogues were designed before the first GnRH receptor was cloned. These analogues were therefore only tested in vivo or by radio-immunoassays. Two excellent review articles by Karten and Rivier [202] and by Sealfon et al [1] summarise these findings. The information used for the two subsections below was therefore mainly drawn from these two articles, unless more up to date in vitro information was available, which is indicated in the appropriate places.

	Residue									
Species	1	2	3	4	5	6	7	8	9	10
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH2
Guinea Pig	*	Tyr	*	*	*	*	Val	*	*	*
Chicken I	*	*	*	*	*	*	*	Gln	*	*
Rana	*	*	*	*	*	*	*	Trp	*	*
Seabream	*	*	*	*	*	*	*	Ser	*	*
Catfish	*	*	*	*	His	*	*	Asn	*	*
Salmon	*	*	*	*	*	*	Trp	Leu	*	*
Herring	*	*	*	*	His	*	Leu	Ser	*	*
Dogfish	*	*	*	*	His	*	Trp	Leu	*	*
GnRH-II	*	*	*	*	His	*	Trp	Tyr	*	*
Lamprey III	*	*	*	*	His	Asp	Trp	Lys	*	*
Lamprey I	*	*	Tyr	*	Leu	Glu	Trp	Lys	*	*
Tunicate I	*	*	*	*	Asp	Tyr	Phe	Lys	*	*
Tunicate II	*	*	*	*	Leu	Cys	His	Ala	*	*

Table 3: Primary structure of GnRHs isolated from protochordates and vertebrates. Residues conserved with mammalian GnRH are indicated by *. Different forms of GnRH are named after the species from which they were first isolated, except for GnRH-II, which was first identified in chicken. All GnRH forms are decapeptides with a blocked NH_2- and C-terminus. Variable residues are indicated by blue boxes.

a) Structure and function of the variable region of GnRH

As GnRH-II is more active than chicken GnRH-I (or [Gln^8]GnRH) in mammalian GnRH receptors, it can be seen that the loss of Arg^8 can be somewhat compensated by co-ordinated substitution of residues 5 and 7 as can be seen by the mammalian GnRH receptors having a higher affinity for GnRH-II compared with [Gln^8]GnRH [1]. It is believed that a neutral amino acid in position 8 reduces binding affinity of GnRH for the mammalian receptors. Once the GnRH with a neutral amino acid in position 8 is bound, however, it displays improved efficacy as it shows relatively higher LH-releasing

potency than [Arg^8]GnRH [203]. Contrary to this, His in position 5 seems to enhance binding affinity while reducing efficacy [203].

Position 5 requires an aromatic side chain but not the hydroxyl group of Tyr for high activity in mammalian GnRH receptors. This residue might have an indirect role in receptor activation. The third most variable residue of GnRH is position 7. Amino acids in this position can be variable with large uncharged amino acids being favoured for proper functioning. A basic side chain in position 8, however, is likely to affect the ligand structure by stabilising the active form (for mammalian GnRH receptors) by hydrogen bonding to the side chains of His^2 and Tyr^5. It has been proposed that mGnRH forms a β-II-type bend around residue 6 which is needed for high affinity binding to mammalian GnRH receptors but not to non-mammalian GnRH receptors.

Position 6 is invariably Gly for GnRH molecules of higher vertebrates (Table 3). A small side-chain is needed in this position to allow for the β-II-type bend. Not even an Ala is tolerated in this position. D-amino acids in this position, however, were often found to compensate for the loss of Arg in position 8. When residue 6 is substituted by a D-amino acid, the binding affinity of [Gln^8]GnRH is increased by about 1000 fold in the mammalian GnRH receptors [99, 203]. There is not much change in the affinity of non-mammalian GnRH receptors for these analogues, indicating that these receptors are less sensitive for the conformation of GnRH [203]. Gly and D-amino acids share the ability to have positive phi dihedral angles. They therefore more readily allow a β-II-type bend at position 6, which is considered to be the active conformation of GnRH at the mammalian GnRH receptors. Because of its configuration the side chain of the D-amino acid in position 6 faces away from, instead towards, the receptor. Bulky side-chains are therefore tolerated when residue 6 is substituted by a D-amino acid. The observation that side chains of D-amino acids are facing away from the receptor has been exploited for the design of a radioligand with a D-Tyr in position 6. Besides containing the β-II-type bend, this ligand has the further advantage of being iodinated in position 6 where the large iodine group cannot interfere with the binding of the molecule [204].

b) Structure and function of the conserved terminal regions of GnRH

Both the N- and C-terminal residues of GnRH are extremely conserved amongst all forms of GnRH. The N-terminus of GnRH was shown to be involved in both ligand binding and receptor activation. This is highlighted by the fact that pyroGlu in position 1 is substituted in almost every antagonist synthesised. A requirement in this position is that this residue is uncharged. Position 2 requires an aromatic and possibly an immidazol moiety, but not an ε-position hydrogen bond for activity. His in this position is an ideal residue to interact with the receptor. Bulky hydrophobic side-chains are generally found in antagonists in this position. The aromatic nature of residue 3 is essential for activity of GnRH. This residue seems to be crucial for high potency of GnRH in mammalian GnRH receptors. Although position 4 is 100% conserved amongst all naturally occurring forms of GnRH isolated to date, this position is relatively tolerant to substitutions. Spatial constraint, however, seems paramount, as no large side chains are allowed in this position.

Contrary to the N-terminus, the C-terminal residues of GnRH are not involved in receptor activation but only in binding. Pro in position 9 is believed to constrain the conformation of GnRH that is essential for binding. Removal of the amide of Gly-NH_2 in position 10 results in low activity most probably due to decreased binding affinity, as alkyl amines maintain or enhance activity. Small, uncharged moieties are generally tolerated while large side chains most probably pose a steric problem during binding.

It can be seen that no single residue alone is crucial for activity. Figure 5 shows a summary of the functional properties of each residue. It should be borne in mind that changing a residue might alter either an interaction with the receptor or the conformation of the ligand itself, which makes interpretations of two dimensional mutagenesis, where amino acid residues in both the receptor and the ligand have been mutated, more difficult. This is a general problem encountered by receptors that bind large ligands.

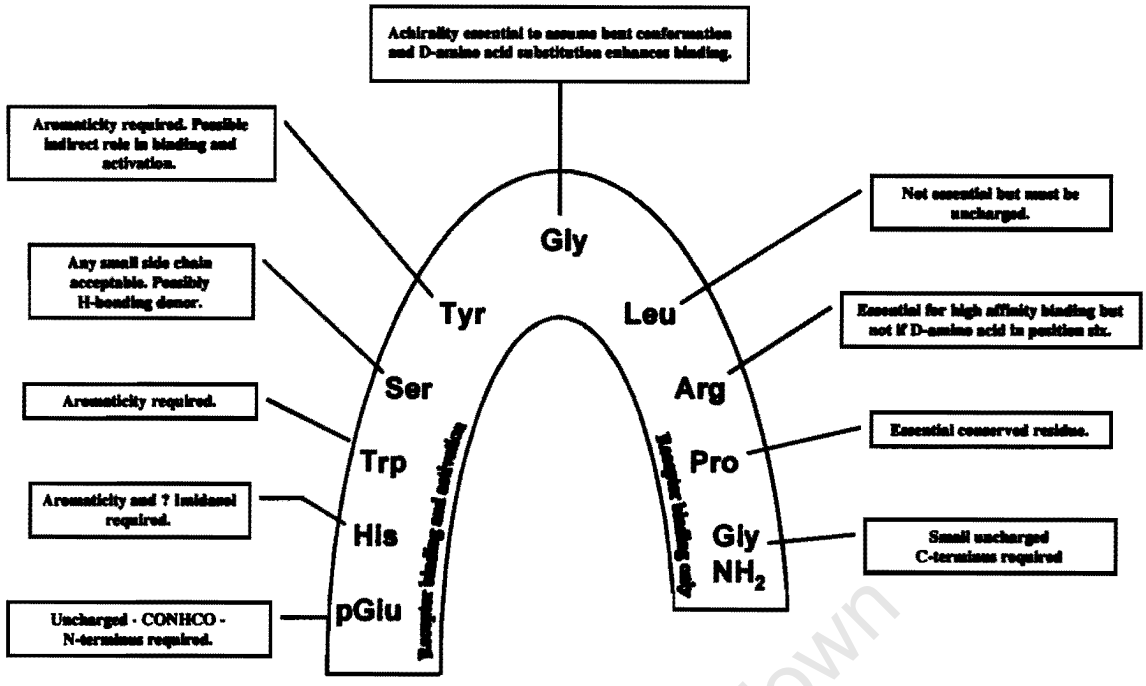

Figure 5: Summary of properties of individual residues of GnRH (as in Sealfon et al [1])

Agonist binding

Binding sites for GnRH on its cognate receptor, $Asp^{2.61(98)}$ [205], $Asn^{2.65(102)}$ [206], $Lys^{3.32(121)}$ [88] and $Asp^{7.32(302)}$ [99], have been identified in the extracellular domains and close to the extracellular boundaries of the transmembrane domains and are conserved in all vertebrate- and non-mammalian GnRH receptors isolated to date [1]. $Asp^{2.61(98)}$ has recently been proposed to hydrogen bond to the δ-NH group of His^2 [205]. Furthermore, it has been suggested that $Asp^{2.61(98)}$ hydrogen bonds with the backbone NH group of Trp^3 as well as forming an interaction with $Lys^{3.32(121)}$ [205]. It was furthermore shown that $Lys^{3.32(121)}$ requires a charge-strengthened hydrogen-bonding residue for high affinity agonist binding, suggesting an interaction of this locus with His^2 or $pyroGlu^1$ [88].

$Asn^{2.65(102)}$ at the beginning of EL-1 has been implicated in an interaction with the C-terminal glycinamide [206]. Mutation of $Asn^{2.65(102)}$ to Ala had a much greater effect on GnRH analogues containing the natural glycinamide moiety compared with the effect seen for analogues having an ethylamide at the C-terminus. It was suggested that $Asn^{2.65(102)}$ hydrogen bonds with the C-terminal Gly-amide moiety [206].

In the mouse GnRH receptor, $Glu^{7.32(301)}$ at the end of EL-3 was identified to be involved in agonist binding in a study aimed at identifying an interacting residue for Arg^8 of mGnRH [99]. Mutations of all acidic residues in the extracellular domains and at the superficial regions of the transmembrane domains revealed the importance of $Glu^{7.32(301)}$ in the mouse GnRH receptor for selectivity for Arg^8 [99]. The $Glu^{7.32(301)}$Gln mutant had a much lesser effect on [Gln^8]GnRH binding than it had on [Arg^8]GnRH, while the affinity for [Glu^8]GnRH was increased more than 10 fold, adding further support to the above hypothesis [99]. $Glu^{7.32(301)}$ is not conserved amongst mammalian GnRH receptors. In the human GnRH receptor this residue is conservatively replaced by $Asp^{7.32(302)}$. Unpublished results from our laboratory from similar experiments on $Asp^{7.32(302)}$ in the human GnRH receptor indicate that this residue has the same function as $Glu^{7.32(301)}$ in the mouse receptor. It is believed that this residue is important in the initial contact of GnRH with its receptor.

The above points of interaction have been conserved amongst all cloned GnRH receptors, including the non-mammalian ones. As these receptors have very different pharmacologies it can be assumed that there are more points of interactions between the receptor and the ligands which account for the different specificities unless they are entirely due to conformational constraints.

It can be seen that the binding pocket of the GnRH receptor is at least partially conserved with other GPCRs (Table 1). Lys in position 3.32(121) is found in the homologous position to the Asp residue which acts as the counter-ion of the amine group of small biogenic amines in the adrenergic-, muscarinic-acetylcholine-, histamine-, dopamine- and 5-HT_{2A}- receptors. $Asp^{7.32(302)}$ is conserved with the AT_1-angiotensin II receptor. In both, the AT_1-angiotensin II- and GnRH receptors this residue at the end of EL-3 is important for high affinity peptide-agonist binding. It is possible that this part of EL-3 evolved to facilitate binding of medium sized peptides (about 10 amino acids in size), as this region does not seem important for high affinity binding of biogenic amines. The small tripeptide TRH also seems to bind entirely in the TMDs, although it has been suggested that EL-3 might provide points of interaction [109, 110] or at least guides TRH into its binding pockets [108].

The GnRH ligand-binding site seems to be different compared with receptors binding small non-peptide ligands in having two contact sites in TMD-II instead of having contact sites in TMD-V and/or TMD-VI (Table 1). Neither has TMD-II been implicated in ligand binding in the TRH- or AT_1-angiotensin II receptor. These receptors, however, were shown to have contact sites in TMD-V and/or TMD-VI. TMD-II, however, is in close proximity to TMD-III, TMD-VII, and to a certain degree TMD-VI (Figure 2), all of which are implicated in ligand binding, indicating that the same might be true for TMD-II. Besides in the GnRH receptor, it has been suggested that the Melanocortin-1-(MCR-1) receptor [207] also contains ligand binding sites in TMD-II. TMD-II contains a major constituent of the receptor activation machinery, further highlighting the importance of this region for proper receptor functioning.

Antagonist binding

Agonists and antagonists are believed to bind to different conformations of the receptor (R* and R respectively). It is therefore feasible that the ligand-binding site for agonists and antagonists should be at least partly different. Competitive antagonists should have an overlapping binding site with the agonist, and therefore compete on the principle of volume exclusion. Antagonists may also have a different binding site from that of agonists and compete via an allosteric mechanism or conformational selection, binding to and stabilising the inactive form of the receptor. An example of this is the tachykinin NK_1 receptor [208]. The majority of GnRH antagonists are peptides, with active non-peptide antagonists only being discovered recently [209-211]. The GnRH peptide antagonists are generally known to be competitive inhibitors on the basis of volume exclusion mechanisms. It was shown that the pre-treatment of pituitary membranes with trypsin, chymotrypsin or sulfhydryl blocking agents decreased subsequent binding of labelled antagonists. The binding of labelled agonist was decreased to a lesser extent indicating that the agonist-binding pocket is less exposed and more buried than the antagonist binding pocket [212]. It was also observed that mono- and divalent cations had more influence on agonist than on antagonist binding. This adds further support to the proposal that the agonist and antagonist binding pocket are not necessarily the same [212].

Janovick et al covalently bound photoactive radiolabelled agonists and antagonists to GnRH receptors [213]. The antagonists and agonists bound to different parts of the receptor as shown by different ligand/receptor complexes isolated after trypsin treatment. This shows that the photoactive group of the antagonists and agonists are exposed to different environments [213].

Another indication for a difference in contact sites of the receptor with agonists and antagonists came from the study that identified $Lys^{3.32(121)}$ as a point of interaction for agonists. Mutation of this residue affected agonist but not antagonist binding affinities [88].

Most antagonists are decapeptides like the agonist. In a number of antagonists mainly the N-terminal residues are substituted. It is therefore feasible that the binding site of those antagonists and agonists is at least overlapping. Antagonists containing Arg^{8} or the glycinamide can still bind to $Asp^{7.32(302)}$ and $Asn^{2.65(102)}$, while the N-terminus would be able to bind to different loci than the TMD-II contact sites. The recently published PAnt-1 antagonist ([Ac-D-Lys^{1}, D-4-Cl-Phe^{2}, D-Trp^{3}, D-Arg^{6}, D-Ala^{10}]GnRH), was shown to have its N-terminus close to residues 11 and 19 of the N-terminus of the receptor [214]. Further studies are required to define the antagonist binding pocket more specifically. This antagonist binding pocket may, however, vary between different antagonists, as points of interactions with the receptor might be different when residues are substituted. The knowledge of the exact dimensions of the binding pocket for selected antagonists may ultimately lead to the design of orally active non-peptide antagonists which could be used in the treatment of a number of hormone dependent diseases or as a novel method of contraception for men and women.

Receptor activation

As mentioned previously, residues 2.50 and 7.49 are interchanged in the mammalian GnRH receptors. Although Asn is found in position 2.50 and Asp in position 7.49, GnRH receptors still conform to the model of receptor activation as proposed for other GPCRs. Asp in position 7.49 is essential for coupling as a mutation to Asn [37, 139, 215] or Ala [37] severely impairs secondary messenger response to agonists. When $Asp^{7.49(318)}$ was substituted by Glu, receptor expression was decreased considerably but

coupling efficiency for this mutant still remained at high levels [37]. This indicated the necessity of a carboxyl group for efficient coupling to occur. An introduction of an Asp or other amino acids in position 2.50 resulted in the receptor not being expressed and makes it difficult to identify the role of this residue in receptor activation [37, 139, 215]. This observation is consistent with results from the μ-opioid receptors [160] but is opposed to findings in other GPCRs that allow Asp residues in both the 2.50 and 7.49 loci [155, 158, 162, 167]. This finding is unexpected as the non-mammalian GnRH receptors cloned to date (from catfish [3], goldfish [4, 5], frog [6, 7] and chicken [8]) all contain two Asp residues in these positions. This highlights the special evolution of mammalian GnRH receptors and shows the importance of other residues in this microdomain which are responsible for maintaining the structural integrity of the receptor. A reciprocal $Asp^{2.50(87)}$ $Asn^{7.49(318)}$ mutation, however, which mimics the motif found in most other GPCRs and the *Drosophila* GnRH receptor, restores ligand binding [37, 139, 215]. This finding presents further evidence that an acidic and a hydrogen bond accepting residue is needed in this microdomain of the receptor for activation to occur. Non-mammalian GnRH receptors have two Asp residues in positions 2.50 and 7.49 which is common to some GPCRs [17]. It is believed, however, that one of these residues is protonated in order to form an uncharged hydrogen bond donating side chain [34]. Another GPCR where this motif is found is the PAF receptor. This receptor retains the coupling function of Asp in position 2.50 as the mutation of this residue to Asn results in an uncoupled receptor, while $Asp^{7.49}$Asn almost behaves like the wild-type receptor [157]. In the non-mammalian GnRH receptor, however, the function of locus 2.50 in receptor activation is unclear as mutation of this residue in the catfish GnRH receptor results in poor expression of the receptor at the cell surface, similar to the findings of mutations in the mammalian GnRH receptors [173]. There is, however, a decrease in signalling in response to agonist when $Asp^{7.49}$ is mutated to Asn in the catfish GnRH receptor, indicating that activation of the receptor depends on an Asp in this position [173]. The non-mammalian GnRH receptors therefore represent intermediates in evolution between the $Asp^{2.50}$ $Asn^{7.49}$ configuration found in most GPCRs, and what looks like the ancient form of GnRH receptors isolated from *Drosophila*, and $Asn^{2.50}$ $Asp^{7.49}$ found in mammalian GnRH receptors. In non-mammals GnRH receptors seems to have already switched the activating function from TMD-II to TMD-VII.

GnRH receptors couple to the PLC pathway. Interestingly, short-term activation of PLD via small G proteins (ADP-ribosylation factor (ARF) and RhoA) requires an Asn in position 7.49 [216]. Since GnRH receptors contain an Asp in this position, they are unable to directly couple to the PLD pathway. The reciprocal mutant ($Asp^{2.50(87)}$ $Asn^{7.49(318)}$), however, is able to mediate a PLD response [37], indicating that GnRH receptors might have been selected during evolution to prevent coupling to PLD via ARF/RhoA rather than PLD.

Another residue that is not conserved between mammalian GnRH receptors and other GPCRs is residue 3.51 of the highly conserved DR motif in TMD-III involved in receptor activation. $Tyr^{3.51}$ found in most GPCRs is substituted by Ser in all mammalian GnRH receptors except for the marmoset GnRH receptor where Tyr is replaced by Phe. Mutations of $Ser^{3.51(140)}$ in the human GnRH receptor to Ala [147] or Tyr [217] did not alter expression levels or the ability to produce a secondary messenger response consistent with mutations in this locus in other receptors. The $Ser^{3.51(140)}$Tyr mutation, however, changed the rate of internalisation and increased the binding affinity of the mutant receptor for GnRH agonist [217]. In the rat GnRH receptor substitution of $Ser^{3.51(140)}$ by Phe resulted in a receptor where ligand affinity and potency was similar to those of the wild-type receptor [188]. The B_{max} and the maximal IP response were slightly reduced [188]. This indicates that substitution of Ser with Phe reduces receptor expression. The rate of internalisation was increased marginally [188]. Although residue 3.51 is very conserved amongst GPCRs, this residue is not very conserved as Tyr within the GnRH receptor family. The above findings suggest that this amino acid is not directly involved in any of the major pharmacological pathways.

All other residues in the (I/L)xxDRxxx(I/V) motif are conserved in the human GnRH receptor. Mutations of $Asp^{3.49(138)}$ decrease receptor expression but increase the coupling efficiency [34, 147]. This locus is thought to interact with $Arg^{3.50(139)}$ in the inactive state of the receptor [34]. As with most other GPCRs, $Arg^{3.50(139)}$ is important for receptor activation and mutation to Gln [34, 147] leads to uncoupling while mutations to His or Lys result in the receptor not being expressed [34]. If mutated, the receptor becomes uncoupled. During receptor activation $Asp^{3.49(138)}$ becomes protonated and the predicted salt-bridge with $Arg^{3.50(139)}$ is broken. $Arg^{3.50(139)}$ might then be guided by the highly conserved $Ile^{3.54(142)}$ to form an ionic interaction with $Asp^{7.49(318)}$, which

constitutes the activated receptor and facilitates G protein coupling [34]. Mutations of $Ile^{3.46(134)}$ showed that this residue is not important in receptor activation or expression [34].

Other residues involved in G protein coupling are $Leu^{3.58(147)}$ [218, 219], $Ala^{6.29(261)}$ [220], $Leu^{1.55(58)}$ [221], $Leu^{2.36(73)}$ [221], and $Tyr^{7.53(322)}$ [168]. $Leu^{3.58(147)}$ is found in IL-2 and is conserved in several GPCRs. In the muscarinic-acetylcholine receptors this residue has been shown to be involved in G protein coupling and receptor sequestration [218, 219]. $Leu^{3.58(147)}$Ala and $Leu^{3.58(147)}$Asp have been shown to have no effect on receptor expression levels and affinity for GnRH agonist but severely impaired G protein coupling and decreased the rate of internalisation [217]. It is unclear whether this locus is directly involved in G protein coupling, or indirectly through stabilising a specific conformation of IL-2 needed for coupling or activation of the G protein [217].

$Ala^{6.29(261)}$ is found in the C-terminal domain of IL-3. Gly and Ser substitutions of this locus result in mutant receptors with normal coupling abilities, while Pro and Val substitutions give rise to partially uncoupled receptor mutants [220]. Leu, Ile, Phe, Glu and Lys substitutions give rise to completely uncoupled receptors that retain normal ligand binding affinities [220]. Thus the amino acid side chain in this position has to be smaller than 40 Da for normal coupling to occur, while side chains of 40-50 Da result in partially uncoupled mutant receptors. Receptors containing side chains larger than 50 Da are completely uncoupled independent of the charge of the side chain. Ala in position 6.29 is conserved amongst a great number of GPCRs [17]. Mutation of this locus in the α_{1B}-adrenergic- [222], TSH- [223] and LH receptors [224] result in constitutively active receptor mutants. Given the nature of the side chain of this residue and the opposite effects of mutations of this locus, it is unlikely that this residue interacts directly with G proteins. It therefore appears that this locus is important in allowing the assumption of a tertiary structure of IL-3 in the active conformation that is necessary to bind and/or activate G proteins [220, 222]. Bulky residues prevent this in the mammalian GnRH receptor but favour it in other GPCRs resulting in constitutive activity.

In another study Arora et al identified $Leu^{1.55(58)}$, $Leu^{2.36(73)}$ at the border of and in IL-1 as important for coupling to G_s and the generation of cAMP. The ability of the mutant

receptors to couple to $G_{q/11}$, however, was not altered [26]. This study therefore supports a number of studies showing that GnRH receptors are also able to couple to the G_s pathway [221, 225-227].

$Tyr^{7.53(322)}$ is found in TMD-VII as part of a highly conserved $NPX_{2-3}Y$ motif in GPCRs. Through the interchange of residues 2.50 and 7.49 this motif becomes DPLIY in the mammalian GnRH receptors. Although $Tyr^{7.53(322)}$Ala mutants were better expressed with the receptor having normal ligand binding characteristics, this mutation completely abolishes G protein coupling [168]. $Tyr^{7.53(322)}$Phe substitutions resulted in a receptor mutant with low expression, but it seemed to be supercoupled [168]. These findings highlight the importance of this residue in receptor activation. Contrary to findings in β_2-adrenergic receptor the aromatic nature of this side chain seems sufficient for G protein coupling to take place [166]. This might be due to the conserved $NPX_{2-3}Y$ sequence being DPLIY in GnRH receptors but further experiments are needed to clarify this point further. As for other GPCRs, this microdomain seems to be important in creating a flexible hinge that is involved in allowing the receptor to switch its three-dimensional arrangement between the inactive and active conformations [32].

Coupling and downstream signalling

GnRH receptors interact with trimeric G proteins that target phospholipase C. As this process is pertussis toxin insensitive it is assumed that the G proteins involved are G_q and/or G_{11} [10, 11]. When the receptor is in the active configuration, GDP is displaced by GTP. This results in activation of the α-subunit of $G_{q/11}$ and dissociates from the $\beta\gamma$-subunit. The free α-subunit then activates PLC-β, which in turn cleaves phosphatidyl inositol bisphosphate into IP_3 and diacyl glycerol. IP_3 is a small water-soluble molecule, which diffuses from the membrane into the cytosol. Here it binds to IP_3 receptors that activate Ca^{2+} channels on the endoplasmatic reticulum [10, 11]. IP_3 is then dephosphorylated and dissociates from the receptors resulting in channel closure. Diacyl glycerol, however, remains in the plasma membrane where it activates Ca^{2+} dependent PKC. PKC is involved in the phosphorylation of proteins and in increasing the transcription of several genes. Measuring the accumulation of IP_3 in response to ligands in combination with binding studies can therefore be used as a tool in establishing agonist or antagonist activity of ligands [10, 11]. Despite not being activated by small

G-proteins ARF and RhoA, PLD is activated in gonadotrope cell lines a few minutes after PLC activation [228]. PLD in turn is responsible for a late generation of DAG which might activate Ca^{2+} independent PKCs [229]. Under certain circumstances, the GnRH receptor is also capable to activate G_s which ultimately leads to the formation of cAMP [26, 221]. However, the role of this pathway under normal physiological conditions is not well defined. The signalling pathways of the GnRH receptor are very complex and not the scope of this thesis. They are however comprehensively covered in a review article [229].

A number of potential phosphorylation sites are found in the intracellular domains of the GnRH receptor. They could be involved in modulating receptor responsiveness, intracellular trafficking and desensitisation [1] as well as the activation of MAP kinases [63]. It has been noted, however, that desensitisation in mammalian GnRH receptors is not rapid but rather slow which is attributed to the absence of the C-terminal tail [230, 231]. As non-mammalian GnRH receptors contain a C-terminal tail and are desensitised rapidly it can be assumed that the mammalian GnRH receptors evolved to be desensitised slowly in order to facilitate the prolonged LH surge [173, 230]. Phosphorylation, desensitisation and internalisation are complex mechanisms and beyond the scope of this thesis and are therefore only mentioned where necessary.

Concluding remarks

It can be seen that there are similarities in ligand binding and receptor activation between all members of the GPCR family. Each receptor, however, deviates somewhat from the general mechanisms of GPCR functioning as each receptor evolved to accommodate specific requirements. One example is that glycoprotein receptors evolved to have long N-terminal regions in order to bind their large ligands. There seems to be a relationship between the size of the ligand and the size of the N-terminus except for in the family C of GPCRs. Another example might be that mammalian GnRH receptors have lost the C-terminal tail. This happened so that these receptors do not get desensitised rapidly. Prolonged exposure to GnRH eventually results in a prolonged LH surge, which is necessary for mammalian but not non-mammalian reproduction. Major thrusts of future experiments will not only be focused on mechanisms conserved amongst all GPCRs but will aim at understanding the special requirements and mechanisms needed for the proper functioning of each individual receptor.

The cloning of a number of mammalian GnRH receptors revealed high homology amongst this group of receptors (>85% sequence identity) and very similar ligand selectivity and pharmacology [1]. However, the effects of GnRH analogues on hormone release from primary cultures of pituitary cells indicated that non-mammalian GnRH receptors have distinctively different pharmacologies [2]. Pharmacological studies on non-mammalian GnRH receptors recently cloned from catfish [3], goldfish [4, 5], frog [6, 7] and chicken [8] confirmed this finding. Notable differences in pharmacology between the mammalian and non-mammalian GnRH receptor are that (a) agonists have different potencies in the mammalian and non-mammalian receptors, and that (b) certain antagonists of the mammalian receptors behaved as partial or full agonists in the non-mammalian GnRH receptors such as the *X. laevis* GnRH receptor [2, 8]. GnRH binds to the extracellular loops and the superficial regions of the transmembrane domains. It was therefore likely that the extracellular loops would play a role in high affinity binding of agonists and in conveying agonist and antagonist activity to ligands in the different receptors. Extracellular loops of the human GnRH receptor were therefore replaced with the equivalent domains of the *X. laevis* GnRH receptor to identify which domains are important for these phenomena.

This thesis focuses on the differences in pharmacology between mammalian- and non-mammalian GnRH receptors and tries to elucidate which regions and residues are conveying some of these differences observed. One aspect investigated was to identify regions that convey different agonist affinities to the *X. laevis* GnRH receptor compared with the human GnRH receptor. The main focus is to try to identify residues that confer agonist activity to certain antagonists in the human GnRH receptor. The findings will help to acquire more knowledge about the ligand binding pocket and mechanisms underlying receptor activation in different subtypes of GnRH receptors.

CHAPTER 2: MATERIAL AND METHODS

This Chapter outlines materials and methodology used in this study. The construction and characterisation of a human GnRH receptor containing silent mutations to obtain restriction enzyme cutting sites, as well as the iodination of GnRH ligands was deemed as basic groundwork which would not merit a separate chapter. Results of these experiments are therefore covered in this section.

GnRH Analogues

Peptide	Amino acid residue 1	2	3	4	5	6	7	8	9	10	MW
Agonists											
mGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2	1411
[Gln^8]GnRH	*	*	*	*	*	*	*	Gln	*	*	1267
GnRH II	*	*	*	*	His	*	Trp	Tyr	*	*	1463
[D-Trp^6]GnRH	*	*	*	*	*	D-Trp	*	*	*	*	1312
GnRH A	*	*	*	*	*	D-Ala	N-Me-Leu	*	Pro-NHEt		1294
Antagonists											
Ant 135-18	Ac-D-Nal(2)	D-4-Cl-Phe	D-Pal(3)	*	Ile	D-Lys(iPr)	*	Lys(iPr)	*	D-Ala-NH_2	1311
Ant 27	Ac-D-Nal(2)	D-α-Me-4-Cl-Phe	D-Trp	*	Lys(iPr)	D-Tyr	*	*	*	D-Ala-NH_2	1614

Table 4: Table of GnRH analogues and their molecular weitghts (MW) used in this study; amino acids in common with mGnRH are indicated by "*"; Nal(2): 3-(2-naphtyl)alanine; Pal: 3-(3-pyridyl)alanine; Lys(iPr): N^{ε}-isopropyl-lysine; 4-Cl-Phe: 3-(4-chlorophenyl)alanine; α-Me-4-Cl-Phe: 2-methyl-4-(chlorophenyl)alanine

Peptides in Table 4 were prepared by solid-phase synthesis in our laboratories and purified by C-18 reverse-phase chromatography to more than 98% purity.

Introduction and Effects of Silent Mutations in the Human GnRH Receptor:

To determine the function of the extracellular loop domains, the three domains were replaced in the human GnRH receptor with the equivalent parts of the *X. laevis* GnRH receptor. As there was only one naturally occurring unique restriction endonuclease cutting site close to the extracellular end of the TMDs (PflMI in TMD-II, Figure 6), it was necessary to introduce suitable cutting sites into the human GnRH receptor gene to allow exchange of extracellular loop domains and to introduce point mutations in EL-2. Silent mutations were introduced into the human GnRH receptor to obtain unique ScaI, BsrGI, StuI, HpaI and SnaBI sites at the extracellular ends of TMDs III-VII (Figure 6) using the Kunkel Method ([232], Figure 21 in Appendix I; for primer sequences see Appendix II). Another two restriction sites were introduced by silent mutations near the intracellular end of TMD-II (NruI) and at the intracellular end of TMD-VII (VspI) for other projects (for primer sequences see Appendix II). Mutations were verified by manual sequencing (T7 sequenase kit, Version 2.0, Amersham, RSA). The resultant receptor showed binding properties and secondary messenger responses identical to those of the wild-type human GnRH receptor (Figure 7, Table 5, Figure 8, Table 6). Expression of the engineered receptor also seems normal as the total counts bound in the absence of cold ligand were equivalent to levels measured in the wild type receptor (Figure 7).

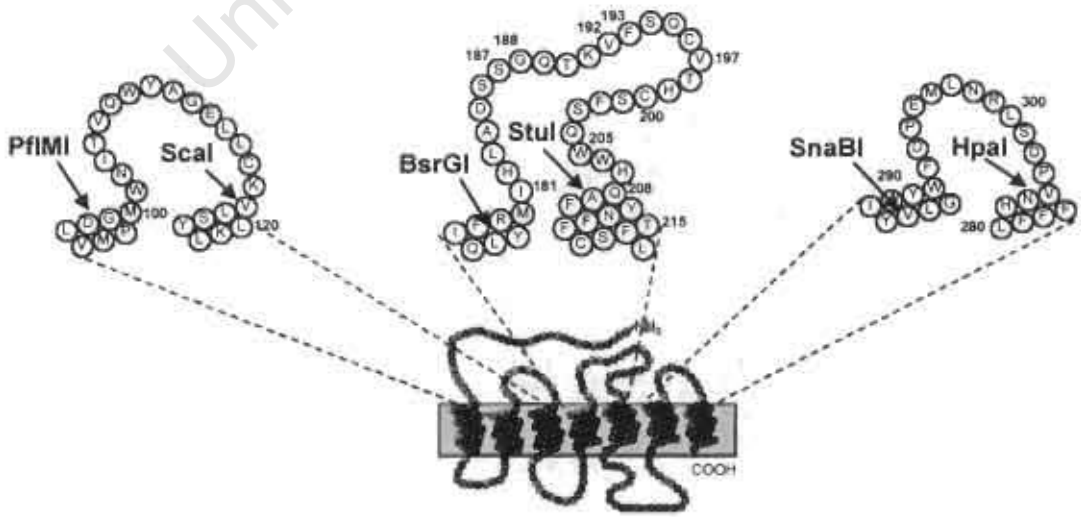

Figure 6: Human GnRH receptor with engineered restriction endonuclease cutting sites near the extracellular end of TMD-III (ScaI), TMD-IV (BsrGI), TMD-V (StuI), TMD-VI (SnaBI) and TMD-VII (HpaI). PflMI near the extracellular end of TMD-II is a naturally occurring cutting site.

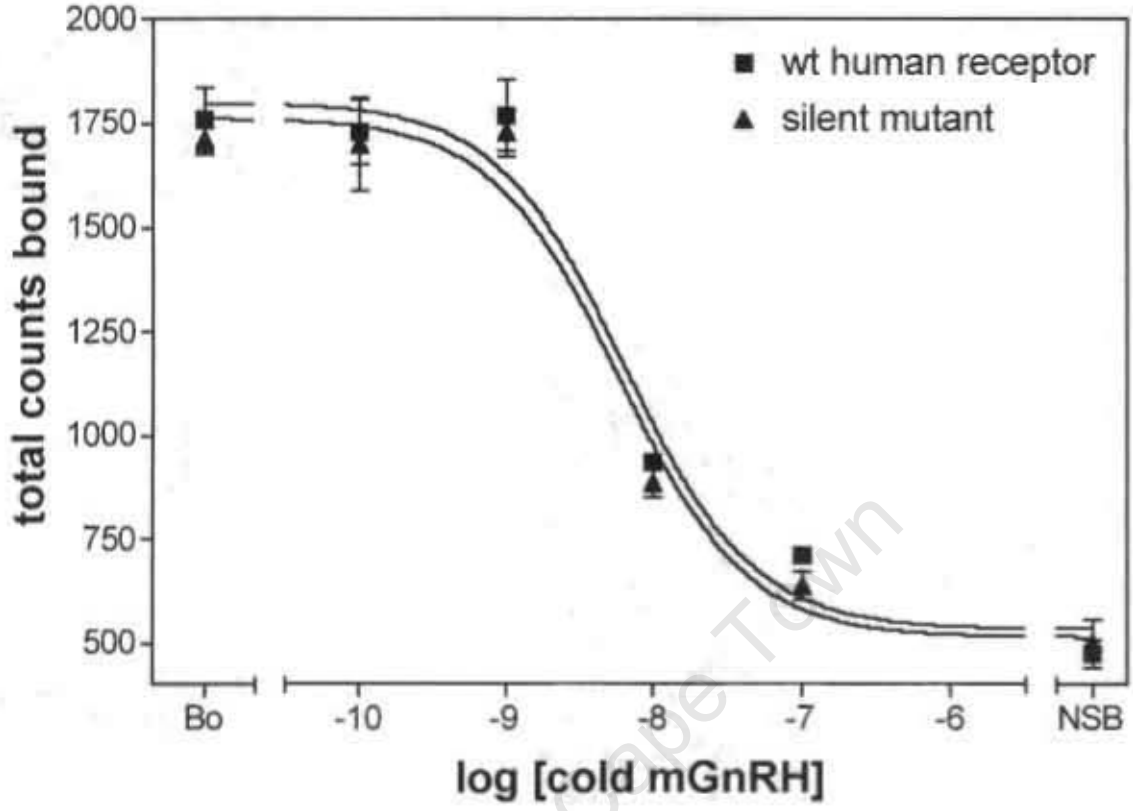

Figure 7: Binding assay to compare affinities of mGnRH in the wild-type human GnRH receptor and the engineered human GnRH receptor with seven silent mutations. Data presented are a representative of at least three independent experiments.

Ligand	IC_{50} of wild-type (nM)	IC_{50} of engineered GnRH-R (nM)
mGnRH	4.8 ± 1.2	4.4 ± 1.5
[Gln^8]GnRH	174 ± 69	164 ± 43
GnRH II	39 ± 9	60 ±10

Table 5: Comparison of IC_{50}s of various ligands for the wild-type human GnRH receptor and the engineered human GnRH receptor containing silent restriction sites. Data presented are a mean of at least three independent experiments.

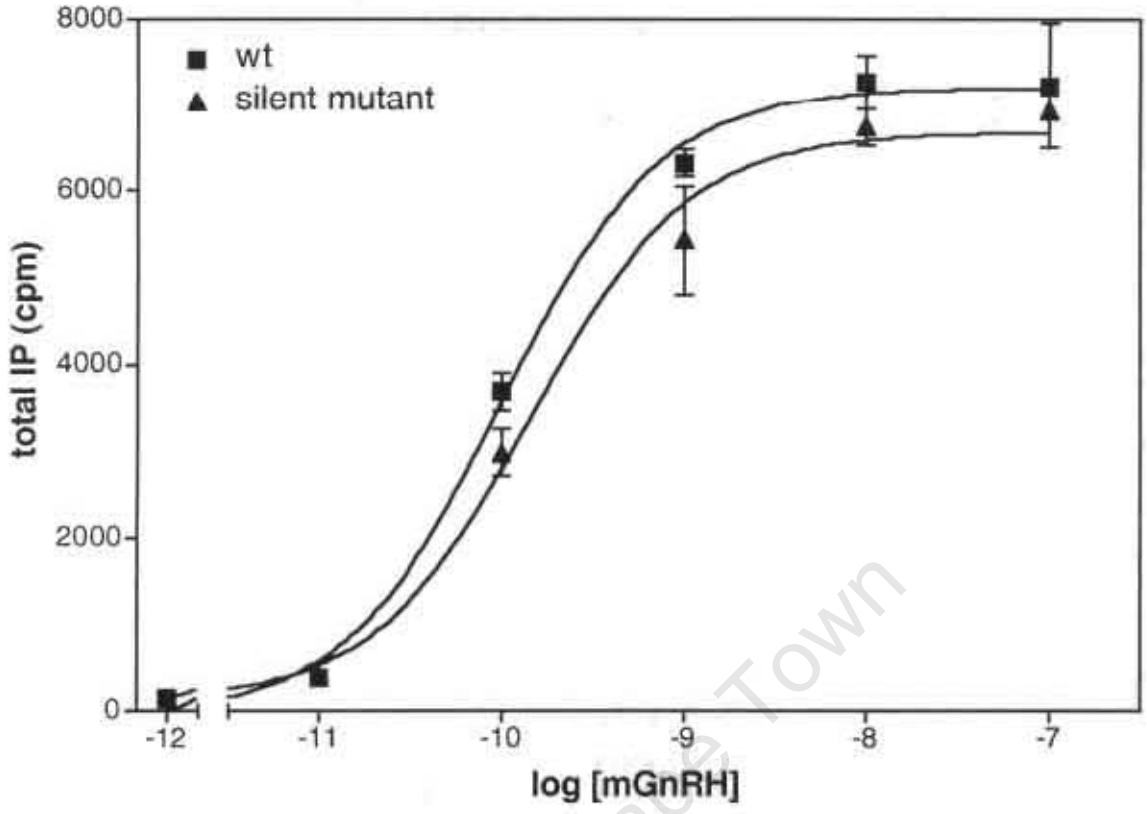

Figure 8: IP assay to compare efficacies of mGnRH in the wild-type human GnRH receptor and the engineered human GnRH receptor with seven silent mutations. Data presented are a representative of at least three independent experiments.

Ligand	EC_{50} of wild-type	EC_{50} of engineered GnRH-R
mGnRH	0.02 ± 0.005	0.06 ± 0.02
[Gln^8]GnRH	0.9 ± 0.1	0.6 ± 0.2
GnRH II	0.5 ± 0.2	1.6 ± 0.3

Table 6: Comparison of EC_{50}s of various ligands in the wild-type human GnRH receptor and the engineered human GnRH receptor containing silent restriction sites. Data presented are a mean of at least three independent experiments.

Construction of Extracellular Loop Chimeras

For a flow diagram of the construction of extracellular loop chimeras see Figure 22 in Appendix I. Chimeric GnRH receptors were constructed by excising the extracellular loops of the human GnRH receptor and replacing them with those of the *X. laevis* GnRH receptor using suitable restriction sites. Extracellular loops 1, 2 and 3 of the *X. laevis* GnRH receptor were amplified by PCR using proof-reading Deep-Vent DNA polymerase (New England Biolabs, RSA) and mutagenic primers flanked by the appropriate restriction endonuclease cutting sites (for primer sequences see Appendix II). The PCR products were precipitated, digested with the appropriate restriction endonucleases and gel purified. Extracellular loops 1, 2 and 3 of the *X. laevis* GnRH receptor were then ligated into the appropriately digested human GnRH receptor in pBluescript SK(-) (Stratagene, RSA) to produce chimeric receptors 1, 2 and 3 (CHR-1, CHR-2 and CHR-3, respectively). Recombinant colonies were screened for correctly sized receptors by colony PCR screening and restriction endonuclease digestions. Positive plasmids were used to subclone the chimeric receptors into pcDNAI/Amp for expression and characterisation of the receptors in COS-1 cells. All sequences were confirmed by manual sequencing. Residues substituted can be seen in Figure 9.

The following chimeras were constructed replacing sub regions of EL-2: (a) residues Ile^{181} to Ser^{187} in the human GnRH receptor were replaced by Arg^{174} to Asn^{180} of the *X. laevis* GnRH receptor (CHR-4, Figure 10), (b) residues Ile^{181} to Val^{192} of the human GnRH receptor were replaced by Arg^{174} to Asn^{180} of the *X. laevis* GnRH receptor (CHR-5, Figure 10) (EL-2 in this construct has the same length as EL-2 in the *X. laevis* GnRH receptor), (c) residues Phe^{193} to Gln^{208} in the human GnRH receptor were replaced by Phe^{181} to Glu^{196} of the *X. laevis* GnRH receptor (CHR-7, Figure 10), and (d) residues Gly^{188} to Gln^{208} in the human GnRH receptor were replaced by Phe^{181} to Glu^{196} of the *X. laevis* GnRH receptor (CHR-6, Figure 10) (EL-2 in this construct has the same length as EL-2 in the *X. laevis* GnRH receptor).

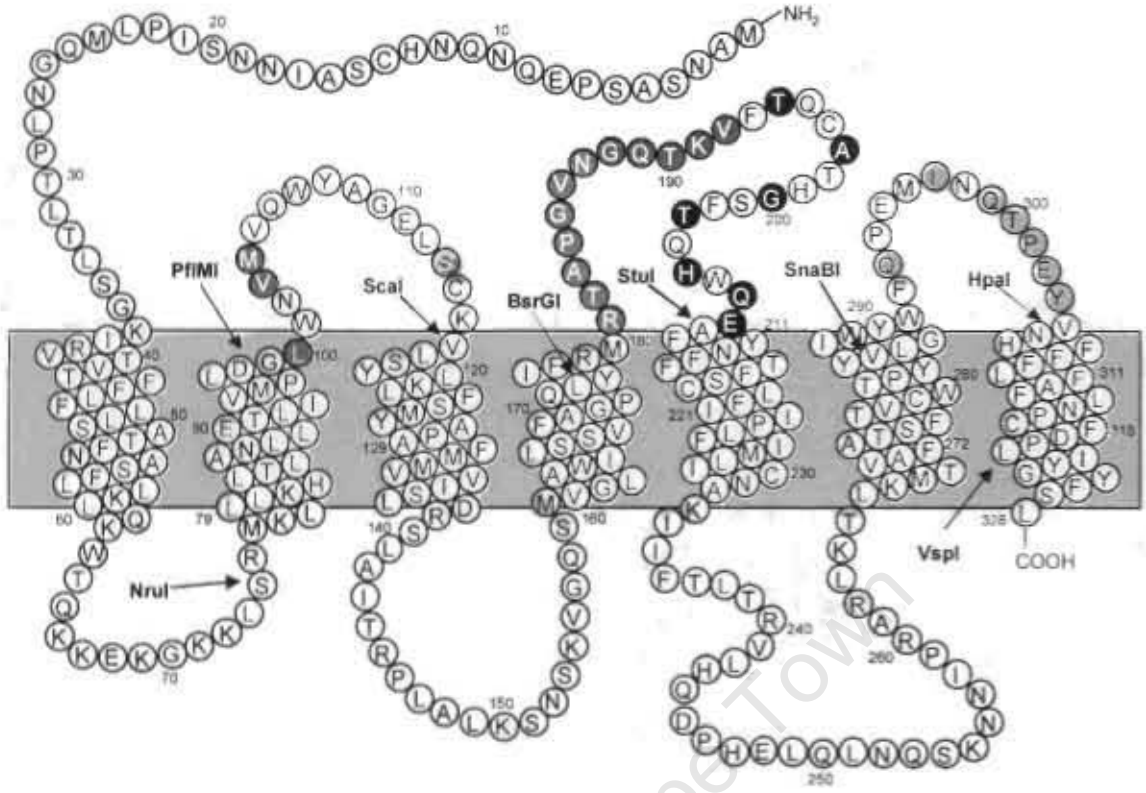

Figure 9: Human GnRH receptor with silent mutations to allow for chimeric exchange of the extracellular loops. Red residues indicate *X. laevis* GnRH receptor residues in EL-1 which are different compared with the human sequence, blue residues indicate *X. laevis* GnRH receptor residues in the N-terminal section of EL-2 which are different compared with the human sequence, green residues are residues not present in the *X. laevis* GnRH receptor, black residues are *X. laevis* GnRH receptor residues in the C-terminal section of EL-2 which are different compared with the human sequence, and yellow residues indicate *X. laevis* GnRH receptor residues in EL-3 which are different compared with the human sequence. In CHR-2 blue and black residues were substituted while the green residues were deleted. In CHR-4 and -5 blue residues were substituted. Additionally green residues were deleted in CHR-5. In CHR-6 and -7 black residues were substituted. Additionally green residues were deleted in CHR-6.

For CHRs 4-7 chimeric antisense primers were designed for both the human and *X. laevis* GnRH receptor containing 18 bases of the other receptor at their 5' end to produce the constructs shown in Figure 10 (for primer sequences see Appendix II). These primers were used in combination with a sense primer, which aligned to the N-terminal portion of EL-2 containing the BsrGI recognition sequence at its 5' end. At the same time the C-terminal section of EL-2 of the other receptor was amplified using a sense primer complementary to the 18 bases on the chimeric primer and an antisense primer which aligned to the C-terminal section of EL-2 containing the StuI recognition sequence 5'. In a second round of PCR the two products of the first round of PCR were used in combination with primers containing the BsrGI and StuI recognition sequences (from earlier mutagenesis experiments) to amplify the full length chimeric EL-2. Since

these chimeric receptors were flanked by BsrGI and StuI sites the resultant chimeric fragments were cloned into the human GnRH receptor identical to the way CHR-2 was cloned. A flow diagram of the construction of these chimeras is shown in Figure 23 in Appendix I.

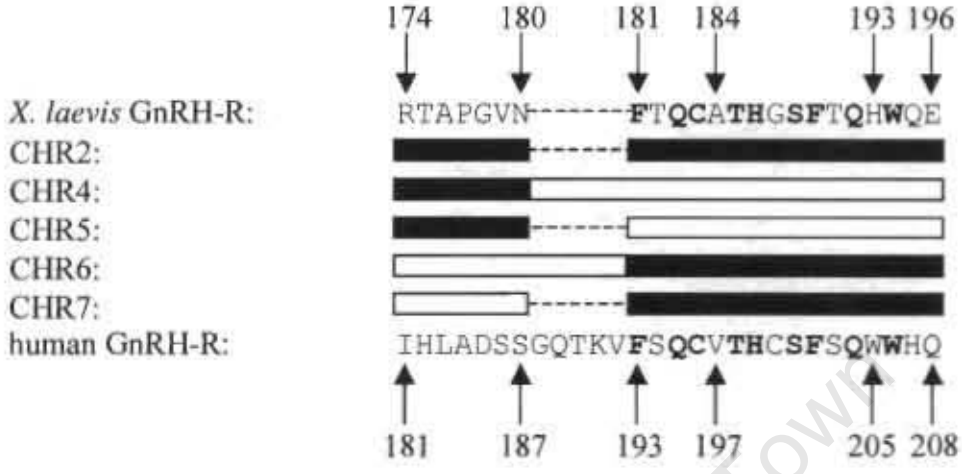

Figure 10: Comparison of the *X. laevis* (top) and human (bottom) EL-2. Bold letters indicate conserved residues. To show the construction of CHR-2 and 4-7, solid boxes indicate *X. laevis* GnRH receptor residues while open boxes represent the human GnRH receptor residues. The dashed line represents a gap of 5 amino acids in the *X. laevis* GnRH receptor meaning that the *X. laevis* EL-2 and EL-2 of CHR-2, CHR-5 and CHR-7 are 5 amino acids shorter than the human GnRH receptor EL-2.

Point Mutations in the Human GnRH Receptor

Point mutations were introduced into the human GnRH receptor using a PCR based method similar to the one used to create chimeric receptors 4-7 (Figure 23). Antisense primers were constructed containing the wanted mutations flanked by at least 12 bases of the wild-type sequence on either side. Sense primers were constructed to align with the 12 bases 5' of the mutations on the antisense primers (for primer sequences see Appendix II). Antisense primers were used together with SP6 and sense primers together with T7 to obtain two PCR products. These products were used together as a template in a second round of PCR in which T7 and SP6 were used to obtain the full length mutant GnRH receptor. All PCR reactions were performed using Deep-Vent DNA polymerase (New England Biolabs, Hotfordshire, UK). The resultant mutant PCR products were precipitated, digested with the appropriate restriction endonucleases and gel purified. The mutant receptors were then cloned into pcDNAI/Amp and sequences were confirmed by automated sequencing (ABI Prism, BigDye™ Terminator Cycle Sequencing, Perkin-Elmer Applied Biosystems, Warrington, UK).

Transfection

DNA for transfection was prepared using Qiagen (West Sussex, UK) Maxipreps. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum. The cells were transfected as previously described [233]. Cells were seeded at a density of 200 000 to 300 000 cells per well in DMEM containing 10% foetal calf serum, penicillin (0.2 U/ml) and streptomycin sulphate (100µg/ml) into 12 well plates (Corning, Bucks, UK) coated with poly-D-Lysine. DNA was transfected using an adapted DEAE-Dextran method [233]. COS-1 cells were incubated for 4h in serum free DMEM containing DEAE-Dextran and 2µg of DNA. This was followed by a 60 min incubation in DMEM containing 2% foetal calf serum and chloroquine (200 µM) and by a 2 minute dimethylsulfoxide shock. Transfected cells were grown for 48h in DMEM containing 10% foetal calf serum and antibiotics prior to IP and binding experiments.

Inositol Phosphate Assay

IP assays were carried out as previously described [233]. Briefly, transfected COS-1 cells were labelled for 16-18h in 0.5ml Special DMEM without inositol (Gibco BRL RSA and Paisley, UK), containing 2% foetal calf serum and 2µCi/ml myo[2-^{3}H]inositol (Amersham, RSA and Buckinghamshire, UK). Cells were stimulated in the presence and/or absence of agonists and antagonists in Buffer I (140mM NaCl/4mM KCl/20mM Hepes/8mM glucose/0.1% fatty-acid-free BSA/1mM $CaCl_2$/1mM $MgCl_2$, pH 7.4) [233] containing 10mM LiCl for 60 minutes. Inositol phosphates were extracted at 4°C with 10mM formic acid for a minimum of 30 minutes [234] and separated using a Dowex ion exchange resin as previously described [233]. Total inositol phosphates were eluted and radioactivity was measured using a scintillation counter. Basal IP production was measured in the absence of ligand. There was no significant difference between basal IP and mock-transfected cells stimulated with 1µM mGnRH.

Receptor Binding Studies

^{125}I-GnRH A used for radioligand assays for agonists was radio-iodinated as previously described [233] using chloramine T. Briefly, 5µg of peptide was labelled in a 0.5M phosphate buffer in the presence of 1.0mCi ^{125}I. The reaction was started by adding chloramine T at a final concentration of 2.5mM. After 10 seconds the reaction was

stopped by adding sodium-*meta*-bisulfite at a final concentration of 3mM. The radioligand was purified on a C18 HPLC column using an acetonitrile gradient. A typical elution profile is shown in Figure 11. The assay was carried out as previously described [220]. Transfected cells were incubated with 100 000 cpm ^{125}I-labelled GnRH A in 500µl Buffer I in the presence/absence of cold ligand for 2 hours. Cells were washed 2x with ice-cold Buffer I containing 0.5% BSA before being lifted with 1.0ml 0.1M NaOH and radioactivity was determined. Non-specific binding was determined in the presence of 1µM unlabelled Antagonist 26.

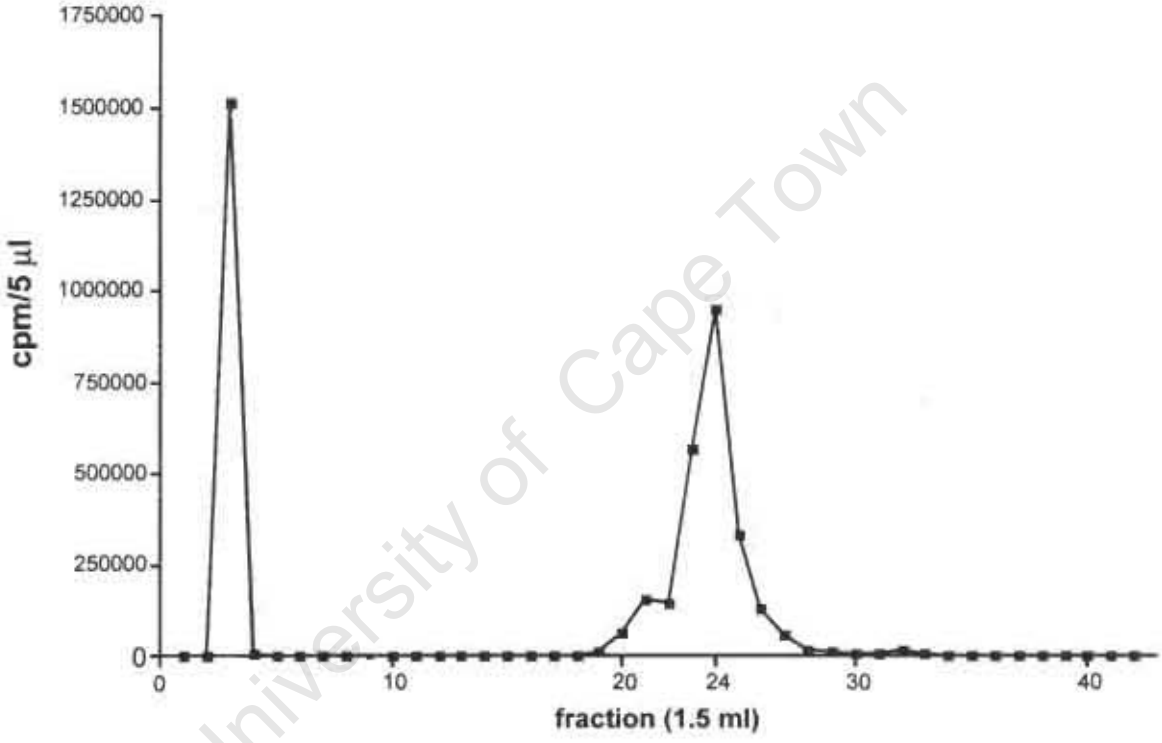

Figure 11: Representative elution profile after iodination of GnRH A on a C18 HPLC column using an acetonitrile gradient for elution; in this iodination samples fractions 23 to 25 were individually tested before use in receptor binding assays.

^{125}I-[D-Trp^{6}]GnRH used for radioligand assays for Antagonist 135-18 was radio-iodinated using the Iodogen (Pierce and Warriner, Chester, UK) method. Briefly, 5µl of 1mM [D-Trp^{6}]GnRH was iodinated in phosphate buffered saline in the presence of 40µg Iodogen and 1.0mCi ^{125}I^{-}. The reaction was stopped after 90 seconds by adding 500µl elution buffer (0.01M acetic acid/0.1%BSA). The radioligand was purified using a Sephadex G25 column using the elution buffer. A typical elution profile is shown in

Figure 12. Transfected cells were incubated with 200 000 cpm ^{125}I-labelled [D-Trp^{6}]GnRH in 500µl HEPES/DMEM + 0.1% BSA in the presence/absence of cold ligand for 2 hours. Cells were washed 2x with ice-cold phosphate buffered saline before being lifted with 1.0ml 0.1M NaOH and radioactivity was determined. Non-specific binding was determined in the presence of 1 µM unlabelled Antagonist 26.

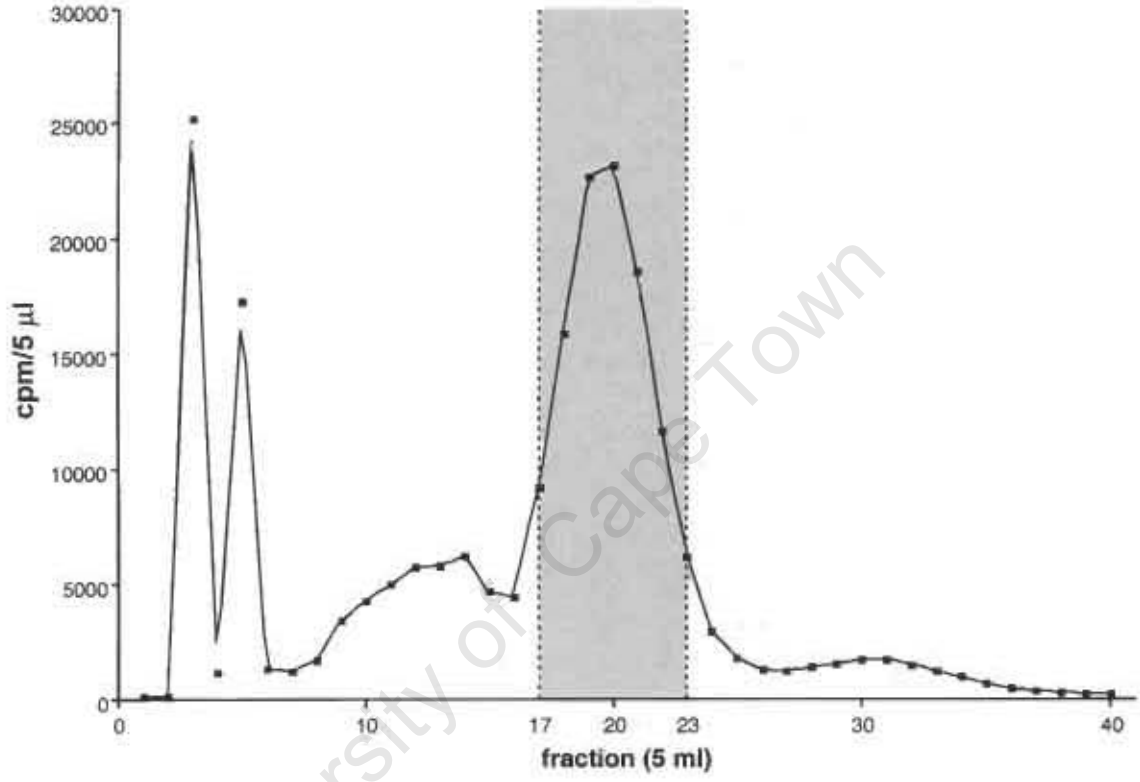

Figure 12: Representative elution profile after iodination of [D-Trp^{6}]GnRH on a Sephadex G25 column using 0.01M acetic acid/0.1%BSA for elution; in this iodination samples between fractions 17 and 23 were pooled, alliquoted and tested before use in receptor binding assays.

Data Analysis

Experiments were carried out in duplicate. Graphs shown are a representative of at least 3 independent experiments or the mean of at least 3 independent experiments. The graphs were plotted and IC_{50} and EC_{50} values were obtained using the "One site competition" formula of Prism® Version 2.0 (GraphPad, San Diego, USA) for the IC_{50} values and "Sigmoidal dose response" formula of Prism® Version 2.0 (GraphPad, San Diego, USA) for the EC_{50} values.

CHAPTER 3: AGONIST ACTIVITY OF ANTAGONISTS IN CHIMERIC AND MUTANT GNRH RECEPTORS

Introduction

GPCRs are involved in a number of important physiological systems. Since antagonists and inverse agonists are able to bind to, but cannot activate GPCRs, receptor activation plays a crucial part in relaying a signal into the cell. The identification of determinants of the receptor and ligand which can stabilise the receptor in an active or inactive state would be fundamental for understanding molecular mechanisms of receptor activation and therefore be crucial determinants in developing novel drugs for the treatment of a number of diseases.

As discussed in the introduction, the determination of the precise molecular mechanisms underlying receptor activation is a major challenge in understanding the functioning of GPCRs. Given that ligands can vary considerably in size and structure even for the same target receptor [15], it is difficult to dissect which residues and domains are involved in activating the receptor by stabilising the receptor in the active state. In the extended ternary complex model it was proposed that GPCRs can exist in two conformations, the inactive or R state and the active or R* state [116-118]. As in the absence of ligands GPCRs are predominantly in the R state, they are believed to be constrained in this conformation. The energy barrier between R and R*, however, is low enough for some receptors to spontaneously switch to the active conformation. In the absence of ligands, the equilibrium is generally very much in favour of the inactive conformation, although some receptors are active at any given point [118]. The present notion is that a ligand has high affinity for a defined conformation of the receptor [117, 118]. Upon binding to the receptor the ligand stabilises the receptor in this particular conformation, thus removing it from the equilibrium between unliganded R and R*. Antagonists are thought to bind equally well to the active and inactive states of the receptor and therefore preserve the equilibrium. Inverse agonists are believed to bind predominantly to the inactive state [117]. In the wild-type GPCR, both antagonists and inverse agonists therefore do not change or even lower basal secondary messenger production. Agonists, however, preferentially bind to activated receptors. As a

consequence the equilibrium shifts towards the active conformation. Agonists are therefore able to produce an elevated secondary messenger response on the basis of conformational selectivity [15]. This theory, however, is disputed by Noda et al [106] who argue that the agonist plays an active role in inducing the active conformation. In their paper the authors argue that an interaction between Tyr^{4} of angiotensin II and $Asn^{3.35(111)}$ on the AT_1-angiotensin II receptor is needed to release $Asn^{3.35(111)}$ from a constraint that stabilises the receptor in the inactive conformation. When this constraint is released, either by agonist binding or by mutating $Asn^{3.35(111)}$, the receptor assumes an intermediate R' conformation before it becomes fully activated by other interactions between the agonist and the receptor. Another group identified the possibility of an interaction between $Tyr^{7.43(292)}$ and $Asn^{3.35(111)}$ [107]. This hydrogen bond might account for the constraint of the receptor in the inactive state. Upon agonist binding, Tyr^{4} of angiotensin II replaces $Tyr^{7.43(292)}$, which is released to bind to $Asp^{2.50(74)}$ [122]. This process represents the first step in a chain of events ultimately leading to full receptor activation. This model therefore proposes an induced activation rather than a conformational selection of the active state by agonists. Furthermore, this work presents some of the first experimental data indicating that GPCRs can exist in more than just the inactive R and active R* conformations as removal of the constraint on $Asn^{3.35(111)}$ does not activate the receptor fully [106]. The possibility of GPCRs existing in more than two states was initially proposed by Schwartz et al [235]. It has since been documented by other groups [15, 124-128]. Whether this mechanism is unique to certain receptors, or whether this is a general mechanism followed by other GPCRs has yet to be demonstrated.

Antagonists and agonists are often structurally similar in nature. It is assumed that in this scenario they share a similar binding pocket. Whether a ligand is an agonist or an antagonist is therefore determined by whether the ligand can or cannot make crucial contact with a specific receptor residue. It is possible that residues interacting with non-peptide and small peptide ligands are directly involved in receptor activation or at least in very close proximity to residues that constitute the receptor activation machinery. Residues involved in receptor activation are generally found deep within the TMDs [15]. Larger peptide ligands and glycoproteins are understood to bind to more superficial residues. As discussed earlier, mechanisms linking ligand binding and receptor activation for these receptors are therefore more complex. Non-peptide and

peptide agonists for the same receptor (e. g. in the somatostatin receptors [236]) are another example indicating that GPCRs can be activated by different mechanisms. This further complicates proposing a common mechanism of receptor activation for all GPCRs.

In GnRH receptors non-peptide antagonists have only been discovered recently [209-211]. To date, not much is known about their method of action and their binding pocket. In order to explain the mechanisms underlying activation of GnRH receptors better, it would be helpful to determine which residues and interactions of GnRH are needed for agonist activity. So far the N-terminal region of GnRH has been identified to be involved in conferring agonist activity to GnRH (as discussed earlier, [1]). At present only $Lys^{3.32(121)}$ has been implicated to influence agonist but not antagonist binding [88]. More information about receptor residues important in binding ligands and recognising them as agonists or antagonists is needed for a further refinement of the receptor activation model as proposed by Ballesteros et al (discussed previously, [34]).

One of the most striking pharmacological differences of mammalian- and non-mammalian GnRH receptors is that some of the antagonists of the mammalian GnRH receptors, such as Antagonist 135-18, act as partial or complete agonists in the non-mammalian GnRH receptors [2, 8]. Studies indicated the necessity of a D-Lys or a D-Lys(iPr) in position 6 of the ligand as crucial for agonist activity of those ligands in the non-mammalian GnRH receptors [8]. This chapter attempts to identify domains and specific residues that are needed to recognise these ligands as agonists. As the ligands are believed to mainly bind to the extracellular domains of the GnRH receptor [1], EL-1, EL-2 and EL-3 of the human GnRH receptor were exchanged individually with the equivalent regions of the *X. laevis* GnRH receptor in which Antagonist 135-18 has agonistic properties. After identifying EL-2 as the major determinant for conferring agonist activity to Antagonist 135-18 point mutations were made in the C-terminal region of EL-2 to determine the individual amino acids responsible.

Results

Agonistic/Antagonistic activities of Antagonist 135-18 in chimeric receptors

Extracellular loops of the human GnRH receptor were individually replaced with equivalent domains of the *X. laevis* GnRH receptor and tested for their ability to produce inositol phosphates in response to Antagonist 135-18. When EL-1 (CHR-1) and EL-3 (CHR-3) of the human GnRH receptor were replaced with *X. laevis* receptor domains, Antagonist 135-18 was still unable to produce a secondary messenger response (Figure 13). When EL-2 was replaced (CHR-2), however, Antagonist 135-18 was converted from an antagonist to an agonist as the ligand did not inhibit IP production in the presence of mGnRH and alone stimulated the production of IPs to levels similar to maximal levels generated by saturating concentrations of mGnRH (Figure 13, Figure 14). To determine whether the exchange of EL-2 had an effect on other antagonists, Antagonist 27 was tested and found to still behave as an antagonist in inhibiting the IP response to mGnRH (Figure 15). This correlates with Antagonist 27 being a full antagonist in the *X. laevis* GnRH receptor (data not shown).

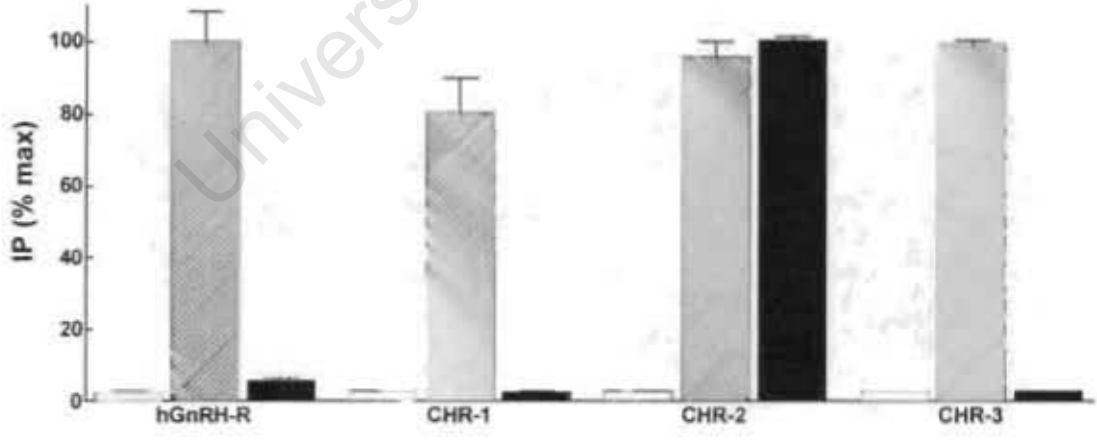

Figure 13: Total IP produced by extracellular loop chimeras in response 10^{-6}M Antagonist 135-18 in comparison to basal IP produced. To establish which extracellular domain is responsible for conferring agonist activity to Antagonist 135-18 COS-1 cells were transfected with pcDNAI/Amp containing the human GnRH receptor and CHRs 1-3 as described in "Materials and Methods". Forty-eight hours after transfection, cells were stimulated with mGnRH (hashed bars) and 10^{-6}M Antagonist 135-18 (solid bars). Basal IP production was measured by measuring the IP production of unstimulated, transfected cells (open bars). IP production was presented as percentage of maximal stimulation of the wild-type human GnRH receptor by mGnRH. Data presented are a representative of at least three independent experiments carried out in duplicate.

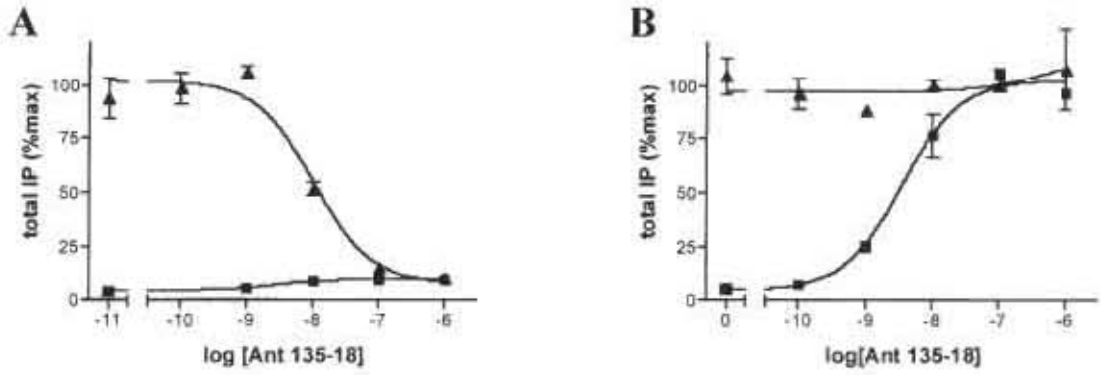

Figure 14: Competition IP assay (mGnRH vs. Antagonist 135-18) and IP response to Antagonist 135-18 of the human GnRH receptor and CHR-2. To show the antagonistic or agonistic effect of Antagonist 135-18, COS-1 cells were transfected with pcDNAI/Amp containing the human wild-type GnRH receptor (A) and CHR-2 (B) as described in "Materials and Methods". Forty-eight hours after transfection, cells were stimulated with increasing concentrations of Antagonist 135-18 in the presence (c=10x higher than the ED_{50}) (▲) and absence (■) of mGnRH. IP production was measured and expressed as a percentage of the maximal stimulation of the receptor with mGnRH. Data presented are a representative of at least three independent experiments carried out in duplicate.

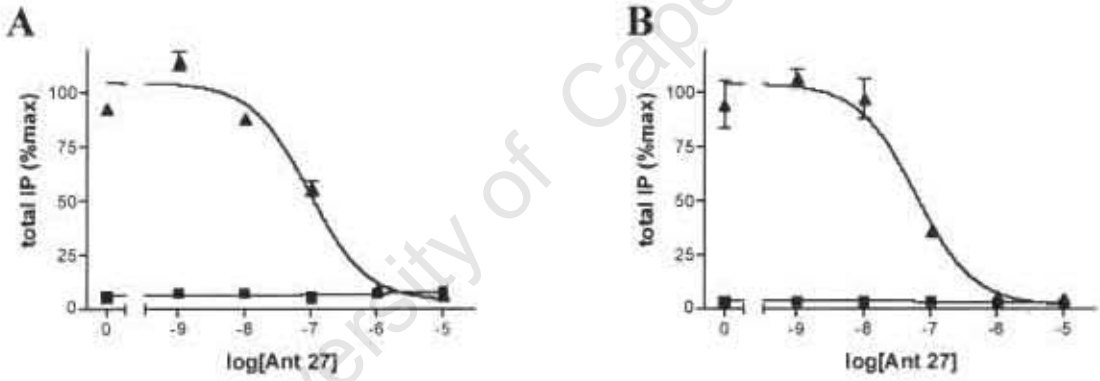

Figure 15: Competition IP assay (mGnRH vs. Antagonist 27) and IP response to Antagonist 27 of the human GnRH receptor and CHR-2. To show the antagonistic effect of Antagonist 27 in both the human GnRH receptor and CHR-2, COS-1 cells were transfected with pcDNAI/Amp containing the human wild-type GnRH receptor (A) and CHR-2 (B) as described in "Materials and Methods". Forty-eight hours after transfection, cells were stimulated with increasing concentrations of Antagonist 27 in the presence (c=10x higher than the ED_{50}) (▲) and absence (■) of mGnRH. IP production was measured and expressed as a percentage of the maximal stimulation of the receptor with mGnRH. Data presented are a representative of at least three independent experiments carried out in duplicate.

EL-2 of the *X. laevis* and other non-mammalian GnRH receptors is 5 amino acids shorter than the human GnRH receptor EL-2. According to alignments the equivalent of residues 188 to 192 of the human GnRH receptor are missing in the non-mammalian GnRH receptors. To investigate the possible role of this gap and to determine which part of EL-2 confers agonist activity to the antagonist a series of other chimeric constructs were tested (Figure 10). It was found that for all four constructs

Antagonist 135-18 was converted to a partial agonist (Figure 16). The antagonist by itself was able to stimulate the receptor but not up to the IP_{max} levels achieved with mGnRH (Figure 16). When the percentage activity of the long over the short constructs were calculated (CHR-4/CHR-5 and CHR-6/CHR-7, see Figure 10 and Figure 16) it was found that they were 107% and 75% indicating that the length of the loop did not have any significant influence on the activity of Antagonist 135-18 (Figure 16).

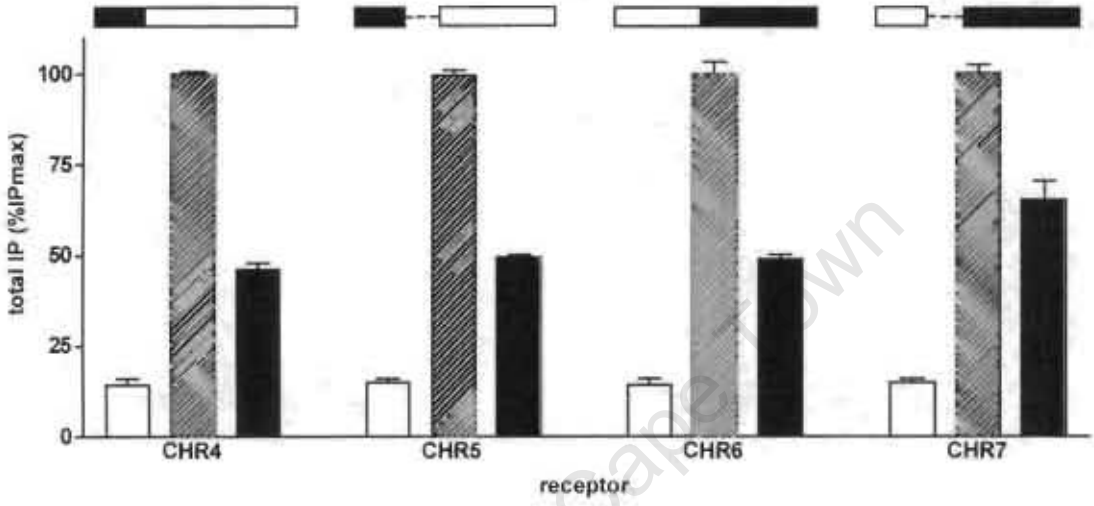

Figure 16: Total IP produced by short EL-2 chimeras (CHRs 4-7) in response to mGnRH, 10^{-6}M Antagonist 135-18 and basal IP production. To determine which part of EL-2 conferred agonist activity to Antagonist 135-18, COS-1 cells were transfected with pcDNAI/Amp containing the human wild-type receptor and CHRs 4-7 as described in "Materials and Methods". Forty-eight hours after transfection, cells were stimulated with 10^{-9}M mGnRH (hashed bars), and 10^{-6}M Antagonist 135-18 (solid bars). Open bars represent basal IP production (measured for mock-transfected cells). IP production was measured and expressed as a percentage of the maximal stimulation of the receptor by mGnRH. Data presented are a representative of at least three independent experiments carried out in duplicate. Above the graph the different EL-2s of the constructs are indicated. Black boxes indicate the *X. laevis* EL-2 residues, open boxes are human residues. The *X. laevis* GnRH receptor EL-2 is five amino acids shorter than the human GnRH receptor EL-2. To investigate the effect of the length of EL-2 five amino acids were deleted in CHRs 5 and 7 (deletion indicated by the dashed line).

Effects of point mutations on the activity of Antagonist 135-18

As earlier studies identified the necessity of a positively charged residue in position 6 of the antagonists to be necessary for agonist activity in the chicken GnRH receptor, it was possible that there would be a negatively charged counter ion in EL-2. Comparison of the sequences in EL-2 revealed that there was a Glu residue at the end of EL-2 which was conserved amongst the non-mammalian but not mammalian GnRH receptors. As a candidate for a possible interaction with the antagonist, the equivalent residue (Gln^{208})

was mutated to Glu. The resultant receptor showed no agonist activity in response to Antagonist 135-18 (Figure 17). As the C-terminal portion of the *X. laevis* EL-2 has only seven differences from the human GnRH receptor (Figure 10), these residues were investigated to elucidate their involvement in conferring agonist activity to Antagonist 135-18. The seven amino acids were individually mutated in the human GnRH receptor and tested for their ability to stimulate inositol phosphate production. In the $Ser^{194}Thr$, $Val^{197}Ala$, $Cys^{200}Ser$, $Ser^{203}Thr$, $Trp^{205}His$, $His^{207}Gln$ and $Gln^{208}Glu$ mutants Antagonist 135-18 did not stimulate inositol phosphate production significantly higher than basal levels (Figure 17). To investigate the requirements for agonist activity of Antagonist 135-18 in the C-terminal section of EL-2, a series of combinations of the mutations were constructed. Ser^{194}, Val^{197}, Ser^{203} and Trp^{205} were mutated in the human GnRH receptor construct to the residues present in the *X. laevis* receptor (viz. Thr, Ala, Thr and His respectively). Of all the possible double amino acid substitution mutants only $Ser^{194}Thr/Trp^{205}His$ displayed partial agonistic activity (Figure 18). This suggested that these residues were important in conferring agonist activity but not sufficient alone to confer full agonist activity to Antagonist 135-18. Triple and quadruple mutations were then studied. $Val^{197}Ala/Ser^{203}Thr/Trp^{205}His$, $Ser^{194}Thr/Val^{197}Ala/Trp^{205}His$ showed that Antagonist 135-18 was able to stimulate IP production to about 40% of the maximal stimulation level. (Figure 19). On the other hand $Ser^{194}Thr/Ser^{203}Thr/Trp^{205}His$ and $Ser^{194}Thr/Val^{197}Ala/Ser^{203}Thr$ tipple mutants had no agonist activity in response to this ligand (Figure 19). This confirmed the essential requirement of His^{205} and Ala^{197} for conferring agonist activity to Antagonist 135-18.

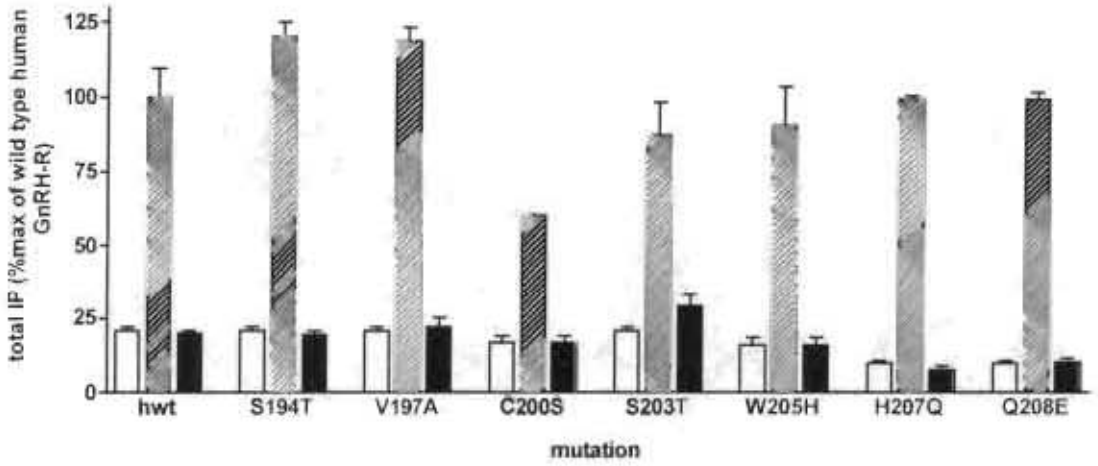

Figure 17: Total IP produced by point mutants of the human GnRH receptor in the C-terminal section of EL-2 in response to 10^{-9}M mGnRH, 10^{-6}M Antagonist 135-18 and basal IP production. To determine whether any single amino acid in the C-terminal section of EL-2 of the human GnRH receptor conferred agonist activity to Antagonist 135-18, COS-1 cells were transfected with pcDNAI/Amp containing the human wild-type GnRH receptor and receptors with mutations $Ser^{194}Thr$, $Val^{197}Ala$, $Cys^{200}Ser$, $Ser^{203}Thr$, $Trp^{205}His$, $His^{207}Gln$ and $Gln^{208}Glu$ as described in "Materials and Methods". Forty-eight hours after transfection, cells were stimulated with 10^{-9}M mGnRH (hashed bars), and 10^{-6}M Antagonist 135-18 (solid bars). Open bars represent basal IP production (measured for unstimulated transfected cells). IP production was measured and expressed as a percentage of the maximal stimulation of the wild-type receptor by mGnRH. Data presented are a representative of at least three independent experiments carried out in duplicate.

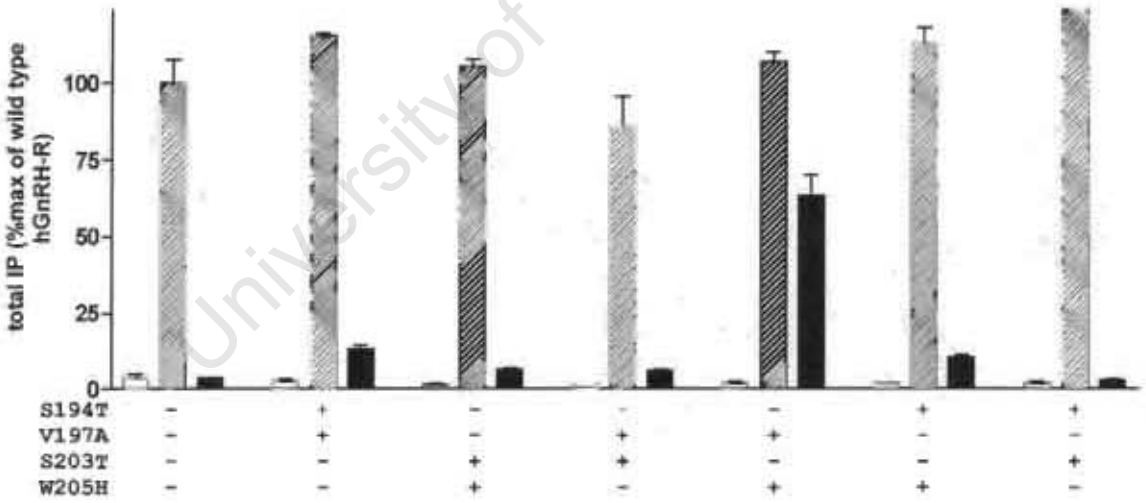

Figure 18: Total IP produced by double mutants of the human GnRH receptor in the C-terminal section of EL-2 in response to 10^{-9}M mGnRH, 10^{-6}M Antagonist 135-18 and basal IP production. To determine whether a double mutation in the C-terminal section of EL-2 of the human GnRH receptor conferred agonist activity to Antagonist 135-18, COS-1 cells were transfected with pcDNAI/Amp containing the human wild-type GnRH receptor and receptors with mutations indicated below the graphs as described in "Materials and Methods". Forty-eight hours after transfection, cells were stimulated with 10^{-9}M mGnRH (hashed bars), and 10^{-6}M Antagonist 135-18 (solid bars). Open bars represent basal IP production (measured for unstimulated transfected cells). IP production was measured and expressed as a percentage of the maximal stimulation of the wild-type receptor by mGnRH. Data presented are a representative of at least three independent experiments carried out in duplicate.

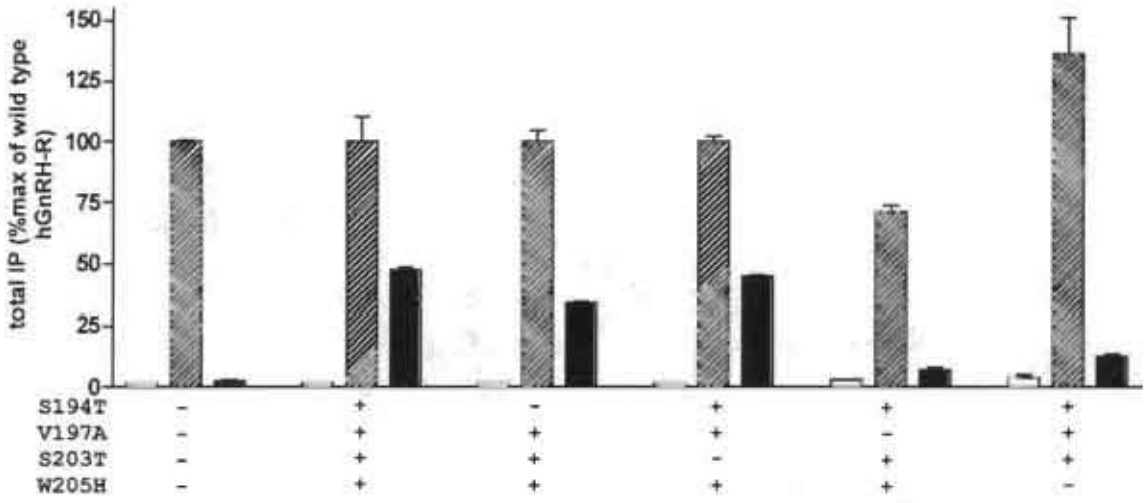

Figure 19: Total IP produced by multiple mutants of the human GnRH receptor in the C-terminal section of EL-2 in response to 10^{-9}M mGnRH, 10^{-6}M Antagonist 135-18 and basal IP production. To determine whether any multiple mutation in the C-terminal section of EL-2 of the human GnRH receptor conferred agonist activity to Antagonist 135-18, COS-1 cells were transfected with pcDNAI/Amp containing the human wild-type GnRH receptor and receptors with mutations indicated below the graphs as described in "Materials and Methods". Forty-eight hours after transfection, cells were stimulated with 10^{-9}M mGnRH (hashed bars), and 10^{-6}M Antagonist 135-18 (solid bars). Open bars represent basal IP production (measured for unstimulated transfected cells). IP production was measured and expressed as a percentage of the maximal stimulation of the wild-type receptor by mGnRH. Data presented are a representative of at least three independent experiments carried out in duplicate.

Binding of Antagonist 135-18 to chimeric receptors and receptor mutants which recognise Antagonist 145-18 as an agonist

To test the possibility that Antagonist 135-18 had gained an extra point of contact with receptors that recognised Antagonist 135-18 as an agonist, receptor-binding assays were performed on the human wild type GnRH receptor, and chimeric and mutant human GnRH receptors. The IC_{50}s of all these receptors were similar at approximately $1x10^{-8}$M (Figure 20, Table 7). The expression levels of all mutant and chimeric GnRH receptors were variable but higher than the wild type receptor expression (Table 7)

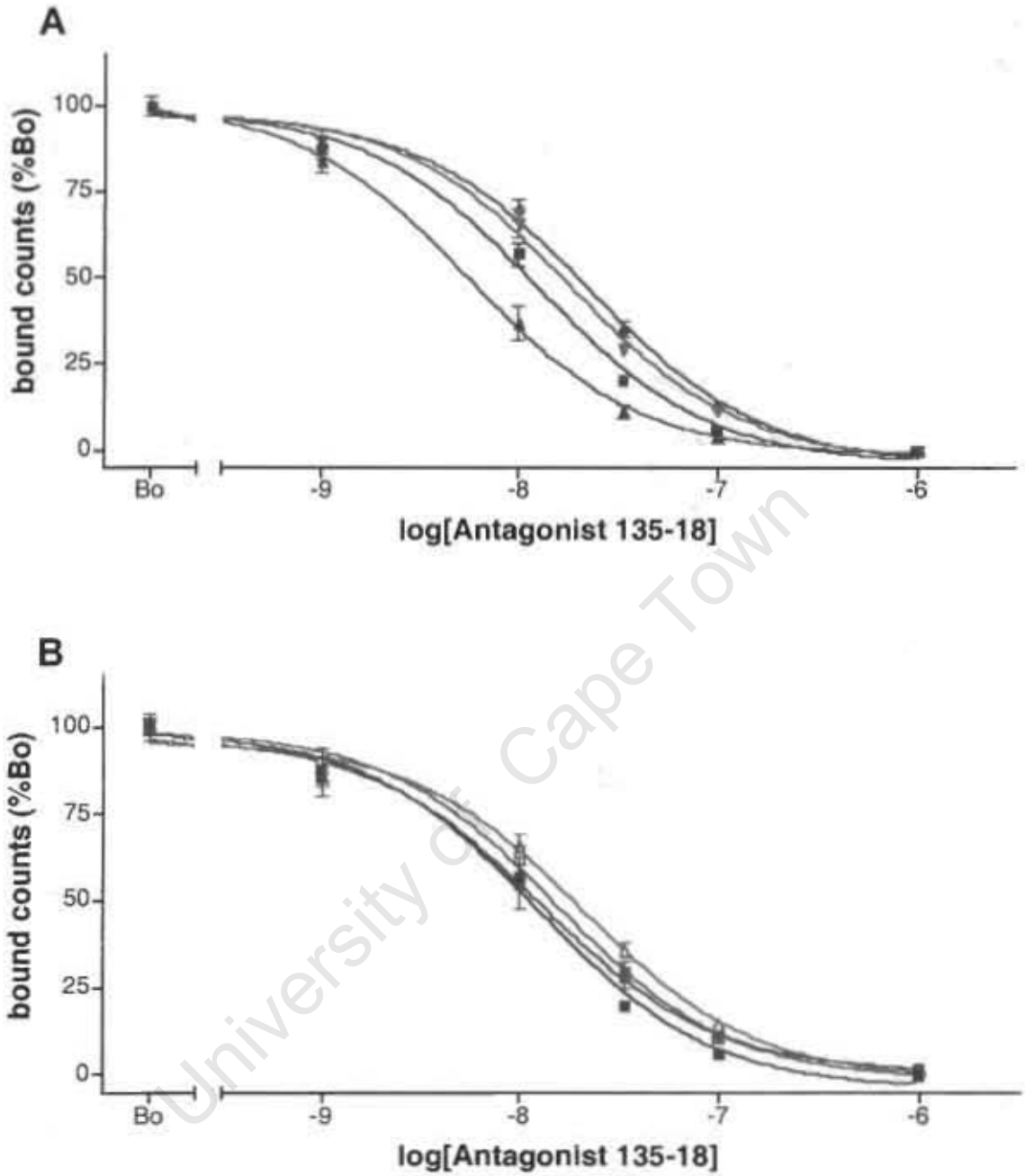

Figure 20: Competition binding assays of the human GnRH receptor and chimeric and mutant receptors, which recognise Antagonist 135-18 as an agonist to determine the binding affinity for Antagonist 135-18. To determine the binding affinity of Antagonist 135-18 for the wild-type human GnRH receptor and the receptors that recognise this ligand as an agonist, COS-1 cells were transfected with **A**: pcDNAI/Amp containing the human wild-type GnRH receptor (■), CHR-2 (▼), CHR-7 (▲) and receptors with the following mutations: $Ser^{194}Thr$, $Val^{197}Ala$, $Ser^{203}Thr$, $Trp^{205}His$ (◆), and **B**: pcDNAI/Amp containing the human wild-type GnRH receptor (■), $Val^{197}Ala$, $Ser^{203}Thr$, $Trp^{205}His$ (□), $Ser^{194}Thr$, $Val^{197}Ala$, $Trp^{205}His$ (△) and $Val^{197}Ala$, $Trp^{205}His$ (●) as described in "Materials and Methods". Forty-eight hours after transfection, cells were incubated with 200 000cpm ^{125}I-[D-Trp^6]GnRH in the presence/absence of cold Antagonist 135-18 for 2 hours. After 2 washes with PBS, cells were solubilised with 0.1M NaOH and total counts bound were measured. Data shown were expressed as percentage of maximal binding (Bo, in the absence of Antagonist 135-18) where the basal level has been subtracted from the total counts obtained (y=(total counts-basal counts)/(Bo-basal counts)).

Receptor	IC_{50} (nM)	Total counts bound (% wt)
wild type	14 ± 3	100 ± 2
CHR-2	19 ± 3	358 ± 45
CHR-7	6.4 ± 2.0	126 ± 7
$Ser^{194}Thr$, $Val^{197}Ala$, $Ser^{203}Thr$, $Trp^{205}His$ mutant	23 ± 3	120 ± 4
$Val^{197}Ala$, $Ser^{203}Thr$, $Trp^{205}His$ mutant	16 ± 3	156 ± 12
$Ser^{194}Thr$, $Val^{197}Ala$, $Trp^{205}His$ mutant	22 ± 4	158 ± 9
$Val^{197}Ala$, $Trp^{205}His$ mutant	15 ± 5	137 ± 8

Table 7: IC_{50} of Antagonist 135-18 and total counts bound for wild type and chimeric and mutant receptors which recognise Antagonist 135-18 as an agonist. Total count values are a percentage of the total counts bound of the wild type GnRH receptor in the absence of any cold ligand.

Discussion

In recent years a number of studies of GPCRs have demonstrated the conversion of antagonists to agonists. Mutations in TMD-III of rhodopsin [237], β-adrenoceptors [238], V_2-vasopressin receptors [151] and AT_{1A}-angiotensin II receptors [106, 239], in TMD-IV of opioid receptors [240], in TMD-VI of the D_1-dopamine receptor [130], and in IL-3 of α_{2A}-adrenergic receptors [241] all showed the conversion of antagonists to partial or full agonists.

In a number of studies some of the ligands classified as antagonists had slight agonist activities in the wild-type receptors. Mutations often render receptors more permissive for activation as the receptors become constitutively active [106, 130, 151, 239, 241]. Since these receptors seem to be activated more easily, an explanation may be that the antagonists had very weak undiscernible agonist activity that is revealed in the constitutively active mutants.

Another explanation how antagonists can be converted to agonists is that they closely resemble the structure of agonists but cannot form the crucial interaction with the receptor, which is needed to stabilise the receptor in its active conformation. A mutation on the receptor could therefore compensate for the shortcoming of the antagonist to form this interaction thereby enabling the receptor ligand complex to be formed in the active conformation [238].

The exchange of EL-2 in the human GnRH receptor by the equivalent domain of the *X. laevis* GnRH receptor converted Antagonist 135-18 from an antagonist to a full agonist (Figure 13). When only portions of EL-2 were replaced Antagonist 135-18 was converted to a partial agonist, indicating that the entire region is an important determinant for conferring agonist activity to Antagonist 135-18 (Figure 16). It appears that agonist activity of Antagonist 135-18 is due to a combination of determinants in both the N- and C-terminal portion of EL-2. This highlights the importance of EL-2 in receptor activation. The length of the EL-2, however, has no influence on whether Antagonist 135-18 is recognised as an agonist or an antagonist in the chimeric receptors where only small sections of EL-2 were replaced (Figure 16).

It has been reported previously that antibodies raised to EL-2 of the α_1- [242], β_1- [243] and β_2-adrenergic- [244], AT_1-angiotensin II- [245], bradykinin B_2- [246], M_1- [247] and M_2-muscarinic-acetylcholine [248] receptor respectively, can activate a secondary messenger response presumably by stabilising the receptor in its active conformation. This further highlights the importance of this domain in receptor activation.

When sub-regions of EL-2 were substituted in the human GnRH receptor, only partial agonist activity of Antagonist 135-18 was observed while the exchange of the entire EL-2 yielded a receptor which conferred full agonist activity to Antagonist 135-18. Previous reports from our laboratory demonstrate that D-Lys(iPr) or D-Lys is needed in position 6 of antagonists in order for the ligand to act as an agonist for the chicken GnRH receptor [8]. If this moiety is also responsible in the *X. laevis* GnRH receptor for conferring agonist activity to Antagonist 135-18, a residue conserved between the chicken and *X. laevis* GnRH receptor, which is not conserved with the mammalian GnRH receptors, would be expected as a possible point of interaction in EL-2. By comparing non-mammalian EL-2 sequences a Glu residue was identified at the end of the loop which was not conserved in the mammalian GnRH receptors. This residue might act as a counter-ion for D-Lys(iPr) in position 6 of Antagonist 135-18. Mutation of the equivalent residue in the human GnRH receptor (Gln^{208}) did not result in agonist activity in response to Antagonist 135-18. Mutating each of the other non-conserved residues in this section of EL-2 resulted in similar results (Figure 17).

A number of multiple mutants were made in order to establish the minimal requirement needed to obtain agonist activity in response to Antagonist 135-18 (Figure 18, Figure 19). For Antagonist 135-18 to be recognised as a partial agonist in the human GnRH receptor, two mutations ($Val^{197}Ala$, $Trp^{205}His$) are sufficient. A candidate interaction is a charge supported hydrogen bond between D-Lys(iPr)^6 of Antagonist 135-18 and His^{205} stabilising the receptor in the active conformation. For agonist activity to be realised, however, a second mutation is needed in position 197 (Val to Ala). This residue is the equivalent of two turns of an alpha helix away from the His. The smaller Ala residue may therefore permit an orientation of the His^{205} side chain to allow the formation of a hydrogen bond with the D-Lys(iPr)^6 side chain. An alternative possibility is that removing the large side-chain of Val enables the ligand to position itself sufficiently close to His^{205} to form the hydrogen bond.

Receptor activation is a complex mechanism in which the TMDs appear to play an important role. It has been proposed that the rotation of TMDs changes the three dimensional arrangement of the intracellular domains which allows interaction with, and activation of, G proteins [34, 114, 132, 133, 135]. $Arg^{3.50}$ might play a central role in the activation of GPCRs as it is proposed to interact with residues in TMD-I, TMD-III and TMD-VII in the active and inactive state [34, 37]. Since antibodies to EL-2 can stabilise receptors in the active conformation [242-248], extracellular domains might play a crucial role in positioning and stabilising different arrangements of the helix bundle. Therefore slight changes in EL-2 have the potential to translate into substantial changes in the pharmacology of a receptor by inducing changes in the tertiary structure of the helix bundle.

The binding data revealed that there is no significant change in the affinity for Antagonist 135-18 in any of the receptors that recognise this ligand as an agonist compared with the affinity of the ligand in the human GnRH receptor where it is a pure antagonist (Figure 20, Table 7). An increase in binding affinity is anticipated if an additional contact site is created (i. e. between His^{205} of the receptor and D-Lys(iPr)^6 of the antagonist). However most studies reporting mutations which convert antagonists into agonists also find no change in binding affinity. Cho et al [130], Claude et al [240] and Morin et al [151] all reported similar binding affinities of the antagonist for the mutant and wild-type receptors, while Wurch et al [241] reported a small decrease in binding affinities (1.8 – 2.9 fold). Strader et al [238] reported a loss of detectable binding for the radioligand and were hence unable to make any conclusion about the affinity of the antagonist. The only incidence where there was a marked increase in binding affinity for the antagonist was reported by Groblewski et al [239]. However, only one non-peptide antagonist was found to have a 29-fold increase in binding affinity while all peptide antagonists retained their binding affinities at the mutant receptor. Since there is no change in binding affinities of the chimeric and mutant receptors which recognise Antagonist 135-18 as an agonist was observed compared with the wild-type human GnRH receptor, it is likely that D-Lys(iPr)^6 of GnRH forms a charge supported hydrogen bond with His^{205} which would only give rise to a 2-3 fold change in binding affinity [249]. This may not be easily detectable. Another explanation for the failure to detect a difference in binding affinities would be that the Antagonist 135-18

forms an alternative bond with the mutant receptor at the expense of another interaction which is formed at the wild-type receptor. Instead of binding the receptor in both the active and inactive states, the ligand can now stabilise the receptor in its active conformation.

There is an intriguing increase in expression of some of the chimeric and mutant receptors, which recognise Antagonist 135-18 as an agonist (Table 7). The observation that a deletion of an amino acid in EL-2 leads to an increased expression of the human GnRH receptor further shows the importance of EL-2 in receptor expression [250]. It is unknown whether this is due to an extended half-life of the receptor, improved trafficking to the cell surface or to an increase in transcription or translation. Further experiments are needed to shed light on this. Over-expression of receptors can lead to accentuating low intrinsic agonist activity [251-253]. The expression levels of the receptors, however, does not appear to be an explanation for agonistic behaviour of Antagonist 135-18 as the level of expression is not proportional to the degree of agonist activity of Antagonist 135-18 (Table 7). This observation is further supported by a mutant CHR-2, which expresses at lower levels than the human wild-type GnRH receptor and has the highest agonistic response to for Antagonist 135-18 (data not shown). The full agonism observed in the EL-2 chimeric receptor compared with all the other chimeric and mutant receptors that recognise Antagonist 135-18 as an agonist might be explained by the fact that this receptor seems to be expressed much higher than the other receptors (Table 7). As seen in the α_2-adrenergic [251], dopamine D_4- [253] and mGlu1α receptors [252], the potency of partial agonists may increase compared with the potency of full agonists as receptor expression increases. This, however, might not be the case here, as it is likely that there is a determinant that confers agonist activity to Antagonist 135-18 in both the N- and C-terminal domain of EL-2 (Figure 16). It is therefore possible that these two effects are additive accounting for the full agonism observed in the EL-2 chimera. In future experiments, CHR-4 might be combined with the $Val^{197}Ala$, $Trp^{205}His$ double mutation to establish whether this is true.

In Summary EL-2 was identified in being an important determinant in conferring activity to Antagonist 135-18. The minimal and essential structural determinant in the C-terminal domain of EL-2 for conferring agonist activity to Antagonist 135-18 in the

human GnRH receptor is a $Trp^{205}His$ mutation combined with a $Val^{197}Ala$ substitution. However, these mutations are not sufficient to confer full agonistic activity to Antagonist 135-18 as seen with the exchange of EL-2 by the equivalent domain of the *X. laevis* GnRH receptor. It is likely that full agonist activity can be conferred to Antagonist 135-18 when additional mutations are made to the $Val^{197}Ala/Trp^{205}His$ mutant in the N-terminal domain of EL-2, as this domain of the *X. laevis* GnRH receptor is also able to confer partial agonist activity to this ligand indicating there might be another contact point in this region of EL-2. The mechanism involved in conferring activity to Antagonist 135-18 is very specific, however, as Antagonist 27 retains its antagonist activity at the chimeric receptors. It is therefore proposed that by mutating Trp^{205} to a His, a charge supported hydrogen bond can be formed with D-Lys(iPr) in position 6 of GnRH which allows Antagonist 135-18 to stabilise the receptor in its active conformation. Experiments are currently carried out where agonist activity of Antagonist 135-18 is tested at different pH levels. Protonation of His^{205} might impede with the hydrogen bond forming between the protonated His^{205} and D-Lys(iPr) which in turn should abolish agonist activity of Antagonist 135-18 thereby strengthening the above proposal. Val in position 197 might be in close proximity of the 205 locus. A bulky side-chain might therefore interfere sterically, so that the hydrogen bond cannot be formed. A small Ala side chain may be necessary in this position to overcome this problem. The exact requirement of residue 197 could be further tested by substituting this amino acid by residues of different chain lengths (e. g. Gly, which is smaller, and Leu of which the side chain is one carbon atom longer). This study represents the first report of mutations in an extracellular domain which confer agonist activity to an antagonist.

CONCLUDING DISCUSSION

The introduction in Chapter 1 focused on the molecular functioning of ligand binding and receptor activation in the broader context of GPCRs. Current models were discussed in the light of experimental data from the literature. The data were taken from representatives of a wide variety of rhodopsin-like GPCRs, which constitute the largest family of this type of receptor. It was shown that there are similarities in ligand binding and the mechanism underlying receptor activation for most of these receptors.

As opposed to large peptides and glycoproteins, however, small ligands are believed to bind entirely within the transmembrane bundle [15, 16, 18]. Potential residues involved in interactions with the ligand might therefore also function in receptor activation or be at least in close proximity to these residues. As discussed in Chapter 1, peptide receptors were shown to involve extracellular domains in ligand binding. Thus the binding pocket of these receptors is removed from residues involved in receptor activation. Residues involved in linking ligand binding and receptor activation therefore may provide a means to fine-tune the efficacy of different ligands. It is possible that receptors in which the receptor activation machinery is spatially close to residues involved in ligand binding have a highly polarised activity status depending on which type of ligand is bound. This scenario would thus minimise the capacity for ligands to exhibit partial agonist activity. Knowledge of the configuration of the TMD bundle and of mechanisms underlying receptor activation might help designing a small ligand for receptors that normally bind larger ligands. This small ligand could therefore activate or inactivate the receptor more efficiently than the natural ligands as it binds more closely or even directly to the receptor activation machinery.

An advantage of peptide ligands is the great number of changes that can be made to the ligand by substituting a residue. Small biogenic amines are much more rigid in structure and allow very few changes of functional groups. It should therefore be possible to analyse the peptide binding pocket in greater detail than the binding pocket of receptors that bind small biogenic amines. Small non-peptide analogues, however, are more practical for applications as they are easily absorbed through the gut without being

broken down. Ultimately advances in the characterisation of the binding pocket might lead to the design of ligands that can act on a number of different GPCRs.

It has been shown in the parathyroid hormone (PTH) receptor that the size of the peptide ligand may influence which type of G-protein the receptor activates [254, 255]. This further highlights the importance of extracellular binding domains in receptor activation. It has been proposed that peptide receptors may exist in a number of partially activated stages [15]. In view of the findings in the PTH receptors it might therefore be possible that different intermediate states couple G-proteins in a different way. Depending on the nature of the ligand, a specific configuration of the receptor would be stabilised resulting in a specific secondary response for this ligand. This mechanism of receptor activation was also proposed for opioid receptors by Selley et al [127]. Further elucidation of these problems would improve our understanding of the requirements needed to target a certain G protein.

The second part of the introduction focused on the molecular functioning of the GnRH receptor, which was the subject of this study. Although there are a number of differences between the GnRH receptor and other members of the rhodopsin-like family of GPCRs, mechanisms underlying ligand binding and receptor activation appear to be conserved. By working on the GnRH receptors it might therefore be possible to draw conclusions for general mechanistics of receptor function for the greater family of GPCRs. The work presented here attempted to shed some light on how ligands are recognised as agonists or antagonists, the knowledge of which is fundamental to GPCR function and could have impact on the design of new pharmaceutical agents.

Introduction of silent mutations into the human GnRH receptor

The first part of the experimental work involved the engineering of the GnRH receptor to produce cassettes that would enable the exchange of EL domains. Seven silent mutations were incorporated in strategic places into the wild-type human GnRH receptor. These silent mutations created new restriction endonuclease cutting sites, which are unique for the GnRH receptor. Care was also taken that the restriction sites chosen were not abundant in vectors such as pBluescript and pcDNA. In fact, most of these restriction sites are unique for the GnRH receptor in pBluescript SK(-) and pcDNAI/Amp, which makes these plasmids excellent tools for chimeric exchange

experiments. Since the mutations introduced were silent, the primary structure of the receptor had not been changed. From IP and binding experiments it became apparent that the engineered receptor behaved identically in comparison with the wild-type receptor. This new construct was not only used for the work carried out for this thesis but also forms an important starting point for a number of other projects pursued in our laboratories. One example is a project which attempted to identify the binding domains for a non-peptide GnRH antagonist using chimeric receptors between sheep and human, where the antagonist binding affinity at the sheep receptor was more than one order of magnitude lower than at the human receptor (paper in preparation).

Characterisation of CHRs 1-3

The primary structure of all mammalian GnRH receptors cloned to date is highly conserved [1]. As expected the pharmacologies of these receptors are almost identical [1]. The recent cloning of the first non-mammalian GnRH receptors confirmed the hypothesis that these receptors possess completely different pharmacologies [3-8]. These non-mammalian receptors are therefore ideally suited to investigate the importance of structure-function of certain domains by creating receptor chimeras.

GnRH is a decapeptide which is thought to bind to the extracellular domains and the extracellular surface of the TMDs [1]. EL-1, EL-2 and EL-3 of the human GnRH receptor were therefore replaced with the equivalent domains of the *X. laevis* GnRH receptor in the next part of the project to create CHR-1, CHR-2 and CHR-3. Initial stages of this work focused on the ability of these chimeric receptors to recognise agonists. CHR-1 and CHR-3, however, were expressed very poorly and could therefore not be used to establish the affinity for agonist ligands. These constructs are currently being cloned into an adenovirus vector to increase transfection efficiency in order to assess their pharmacology. Employing these chimeras may allow the identification of domains that are important for the selectivity of the receptor for certain ligands. These experiments could ultimately lead to the identification of further binding sites between GnRH ligands and cognate receptors.

Identification of residues conferring agonist activity to Antagonist 135-18

As previously mentioned the most intriguing difference in pharmacology between the mammalian and non-mammalian GnRH receptors is that the non-mammalian GnRH

receptors recognise certain antagonists as partial or full agonists. The third part of the project, which is presented in Chapter 3, looked into which domains of the GnRH receptor confer agonist activity to these ligands. It was found that the exchange of the human EL-2 by the equivalent domain of *X. laevis*, but not the exchange of EL-1 and EL-3, conferred agonist activity to one of these ligands: Antagonist 135-18.

Antibodies raised to EL-2 of a number of receptors were able to produce a secondary messenger response [242-248]. EL-2 might be important in receptor activation as it is linked to EL-1 by a highly conserved disulphide-bond. Although the extracellular domains might not contain much of a secondary structure, this disulphide-bond brings some rigidity to the extracellular domains. Furthermore it could act as a hinge connecting TMDs II-V which border EL-1 and EL-2. The disulphide bridge might therefore be of importance for mediating a co-ordinated rearrangement of all TMDs. As antibodies to EL-2 can also activate receptors which bind small non-peptide ligands within the transmembrane bundle [242-244, 247], it may be possible to activate these receptors by ligands that recognise the extracellular domains. Identifying residues that are involved in ligand binding and/or receptor activation in the extracellular loop domains of peptide receptors may therefore lead to the discovery of universal ligands which could be used for a variety of receptors.

It was previously identified that a D-Lys or D-Lys(iPr) in position 6 of the antagonist is needed for it to be recognised as an agonist in a non-mammalian GnRH receptor [8]. This suggested that there might be a negatively charged counter-ion in the non-mammalian GnRH receptors which interacts with this residue stabilising the receptor in the active conformation. Indeed there is a conserved negatively charged residue at the C-terminal end of EL-2 of the non-mammalian GnRH receptors which is not present in the mammalian GnRH receptors. Mutation of the equivalent residue in the human GnRH receptor, however, did not confer agonist activity to Antagonist 135-18. Further point mutations in the C-terminal section of the human GnRH receptor EL-2 demonstrated that no single residue confers agonist activity to Antagonist 135-18. A series of multiple mutations in the C-terminal section of EL-2 identified that agonist activity can be conferred to Antagonist 135-18 by mutating Trp^{205} and Val^{197} to His and Ala respectively. It is possible that a charge strengthened hydrogen bond between His^{205} and D-Lys(iPr)^6 of the ligand can stabilise the receptor in the active conformation. The

small Ala side chain might be needed in position 197 to overcome steric problems compromising the interaction when a larger side chain is present.

The scope of future experiments is to identify further points of interaction between Antagonist 135-18 and the N-terminal region of EL-2, as well as by expressing CHR-1 and CHR-3 by means of the adenovirus expression system. These data, together with the aid of computer modelling might then be used to refine the model of the ligand binding site of GnRH receptors. The model of receptor activation proposed for the GnRH receptors might also be improved, which may lead to the design of novel non-peptide contraceptives or drugs which could ultimately be used in the treatment of a number of hormone-dependent diseases.

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APPENDIX I: ADDITIONAL FIGURES

Kunkel's Method of Mutation

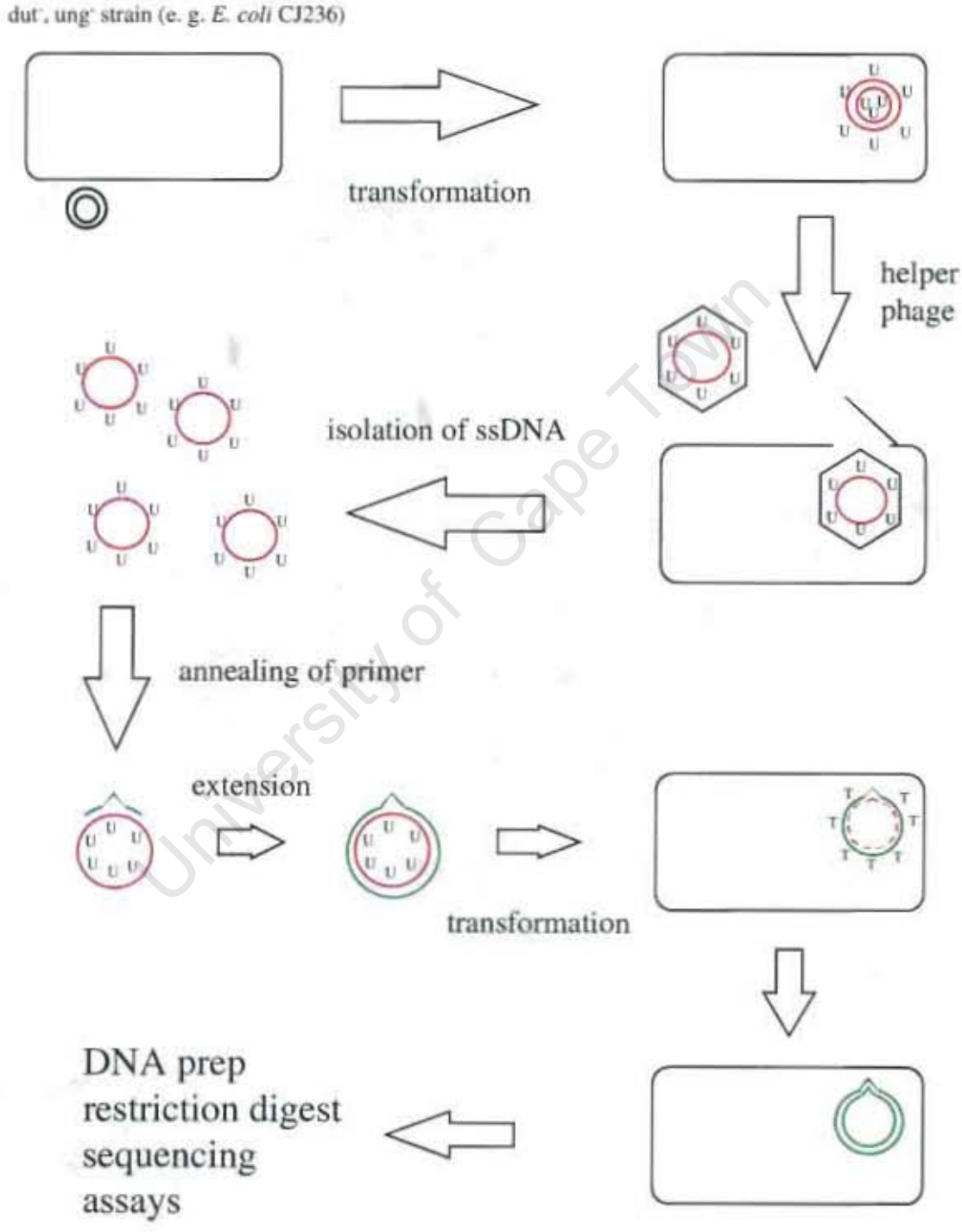

Figure 21: Flow diagram of Kunkels methods

Construction of extracellular loop chimeras

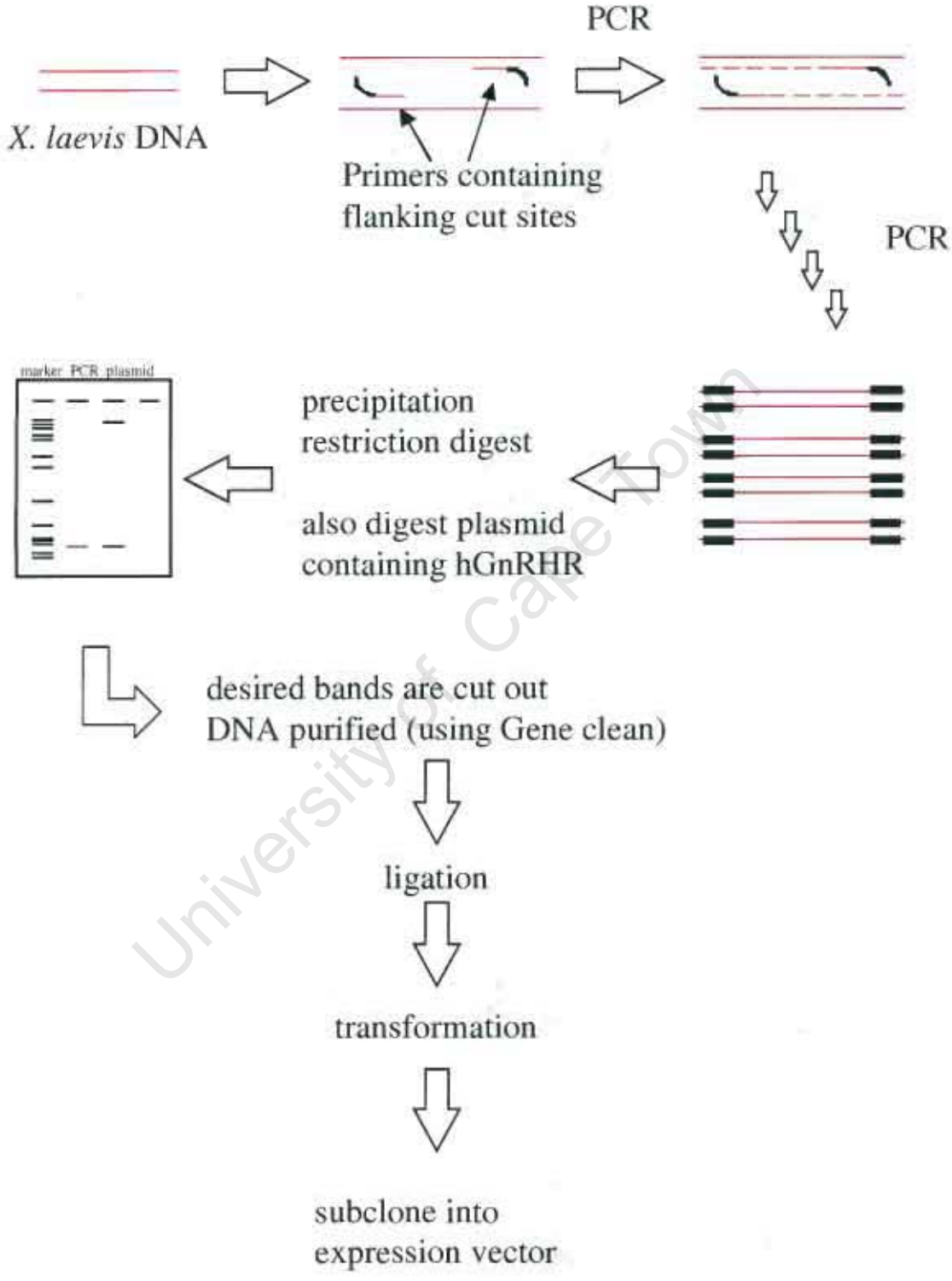

Figure 22: Flow diagram of the construction of extracellular loop chimeras

Bridge PCR to create short EL-2 chimeras (CHRs 4-7):

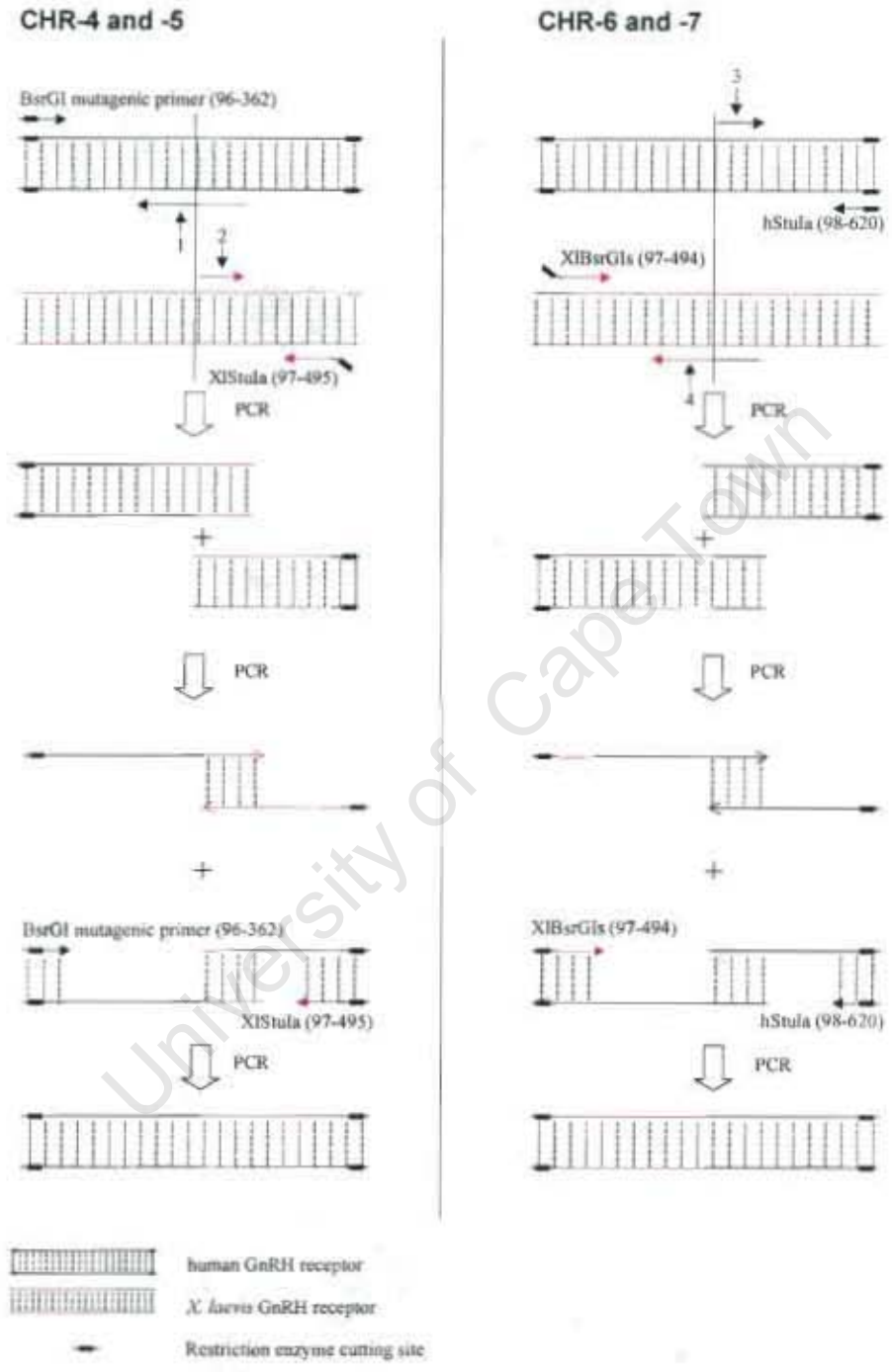

Figure 23: Flow diagram of production of half chimeras. For primer sequences see Appendix I.

APPENDIX II: PRIMER SEQUENCES

Primers used for the introduction of silent restriction sites into the human GnRH receptor

Bold letter indicate restriction endonuclease cutting sites, small letters indicate changes of the wild-type sequence.

TMD-I:

There is a naturally occurring Tth111I (GACN↓NNGTC) site at the extracellular end of TMD-I:

117 **GACC↓TTGTC** 125

TMD-II:

There is a naturally occurring PflMI site (CCANNNN↓NTG) at the extracellular end of TMD-II:

310 **CCACTGG↓ATG** 319

A NruI site (TC↓GCGA) was introduced near the intracellular end of TMD-II:

```
235 AAAAAGCTC**TCAAGA**ATGAAGCTG 258          using primer
      AAAGCTC**TCgcGA**ATGAAGC
    K  K  L  S  R  M  K  L
             ↑
```

TMD-III:

A ScaI site (AGT↓ACT) was introduced at the extracellular end of TMD-III:

```
361 CTCTGCAA**AGTTCT**CAGTTATCTA 384          using primer
     TCTGCAA**AGTaCT**CAGTTATC
    L  C  K  V  L  S  Y  L
             ↑
```

TMD-IV:

A BsrGI site (T↓GTACA) was introduced at the extracellular end of TMD-IV:

```
538 GGACCACAGT**TATACA**TCTTCAGG 561          using primer
     GACCACAGT**TgTACA**TCTTCA
    G  P  Q  L  Y  I  F  R
             ↑
```

TMD-V:

A StuI site (AGG↓CCT) was introduced near the extracellular end of TMD-V:

```
637 TGGTGGCATCAAGCATTTTATAACTTT 653          using primer
     GGTGGCATCAgGCcTTTTATAACT
    W  W  H  Q  A  F  Y  N  F
               ↑
```

TMD-VI:

A SnaBI site (TAC↓GTA) was introduced at the extracellular end of TMD-VI

```
865 ACTCCCTACTATGTCCTAGGAATTTGGTAT 894        using primer:
     CTCCCTACTAcGTaCTAGGcATTTGG
    T  P  Y  Y  V  L  G  I  W  Y
               ↑
```

TMD-VII:

A HpaI site (GTT↓AAC) was introduced on extracellular end of TMD-VII

```
922 TTGTCAGACCCAGTAAATCACTTCTTCTTT 951        using primer:
      GTCAGACCCAGTtAAcCACTTCTTCTT
    L  S  D  P  V  N  H  F  F  F
               ↑
```

A VspI site (AT↓TAAT) was introduced at the intracellular end of TMD-VII using primer 10 665:

```
973 TGCTTTGATCCACTTATCTATGGATATTTT 1002
     GCTTTGATCCAtTAATCTATGGATATT
    C  F  D  P  L  I  Y  G  Y  F
               ↑
```

All the above restriction sites are unique for pBluescript SK(-) containing the human GnRH receptor. VspI is unique for the receptor but there are 3 other VspI cutting sites in pSK Bluescript: 924, 983 + 2218 (plasmid size: 4.0 kb). Mutagenesis and chimeric exchange was therefore carried out in pBluescript SK(-).

Primers used for the exchange of EL-1, EL-2 and EL-3:

Bold letter indicate restriction endonuclease cutting sites, small letters indicate changes of the wild-type sequence; where possible the DNA sequence was kept identical to the human wild-type sequence in the TMD regions. Boxes around amino acids indicate TMDs.

XlPflmIs (97-492):

This primer is designed for the extracellular end of TMD-II of the *X. laevis* GnRH receptor. It is a sense primer.

X. laevis aa sequence	:	V M P L D A L W N V M
X. laevis sequence	: 5′	GTGATGCCTTTGGATGCTTTGTGGAACGTGATG 3′
Primer sequence	: 5′	GATG**CCacTGGATG**ggTTGTGGAACGTGAT 3′
changed amino acids	:	G M

XlScaIa (97-493):

This primer is designed for the extracellular end of TMD-III of the *X. laevis* GnRH receptor. It is an antisense primer.

X. laevis aa sequence	:	G E L S C K V L N F
X. laevis sequence	: 5′	GGGGAGCTCTCCTGTAAGGTCCTCAACTT 3′
primer sequence	: 3′	CTCGAGAGGACATT**tCAtGA**GTTG 5′
new DNA sequence	: 5′	GGGGAGCTCTCCTGTAA**AGTACT**CAACTT 3′
changed amino acids	:	

XlBsrGIs (97-494):

This primer is designed for the extracellular end of TMD-IV of the *X. laevis* GnRH receptor. It is a sense primer.

X. laevis aa sequence	:	Q L F L F R L R T A P G
X. laevis sequence	: 5′	CAGCTATTTCTCTTTCGATTACGTACTGCTCCTGGA 3′
Primer sequence	: 5′	CAGC**TgTaca**TCTTcaGgaTgCGTACTGCTCCTGG 3′
changed amino acids	:	Y I M

Primers to construct short EL-2 chimeras (CHRs 4-7)

```
2a:                  5' TTCACACAGTGTGCCACC 3' (EL2IABs, 98-614)
1a: 3' GATCGTCTGTCGAGAAAGTGTGTCACACGGTGG 5' (EL2IAa , 98-613)

2b:                 5' TTCACACAGTGTGCCACC 3' (EL2IABs, 98-614)
1b: 3' CTGTCTGTTTTCAAAAGTGTGTCACACGGTGG 5' (EL2IBa , 98-615)

3a:                  5' GGACAGACAAAAGTTTTCTC 3' (EL2IIAs, 98-617)
4a: 3' CGAGGACCTCAaTTGCCTGTCTGTTTTCAAAAGAG 5' (EL2IIAa, 98-616)


3b:                  5' TTCTCTCAATGTGTAACACA 3' (EL2IIBs, 98-619)
4b: 3' CGAGGACCTCAaTTGAAGAGAGTTACACATTGTGT 5' (EL2IIBa, 98-618)


hStuIa: 5' TAAAAGGCCTGAT 3' (98-620)
```

Black indicates human GnRH receptor sequence while red indicates the *X. laevis* GnRH receptor sequence (as in Figure 23). Where possible silent mutations (small letters) were introduced to create restriction endonuclease cutting sites to simplify screening for correct chimeric receptors. For a flow diagram of the construction of the short EL-2 chimeric receptors (CHRs 4-7) see Figure 23.

Primers used for mutagenesis in EL-2:

Bold letter indicate restriction endonuclease cutting sites, small letters indicate changes of the wild-type sequence.

$Ser^{194}Thr$:

```
aa sequence     :        Q  T  K  V  F  S  Q  C  V  T  H
wt human seq.   :    5'  CAGACAAAAGTTTTCTCTCAATGTGTAACACAC  3'
primer          :    3'       TTTCAAAAGtGtGTTACACATTG      5'
                     5'       AAAGTTTTCACACAATGTGTAAC      3'
changed aa      :                        T  DraIII
```

$Val^{197}Ala$:

```
aa sequence     :        Q  T  K  V  F  S  Q  C  V  T  H  C  S  F
wt human seq.   :    5'  CAGACAAAAGTTTTCTCTCAATGTGTAACACACTGCAGTTTT  3'
primer          :    3'       TCAAAAGAGtGTTACACggTGTGTGACGTCAAAA  5'
                     5'       AGTTTTCTCACAATGTGCCACACACTGCAGTTTT  3'
changed aa      :                           DraIII A        PstI
```

$Ser^{194}Thr$, $Val^{197}Ala$:

```
aa sequence     :        Q  T  K  V  F  S  Q  C  V  T  H  C  S  F
wt human seq.   :    5'  CAGACAAAAGTTTTCTCTCAATGTGTAACACACTGCAGTTTT  3'
primer          :    3'       TTTCAAAAGtGtGTTACACggTGTGTGACGTCAAAA  5'
                     5'       AAAGTTTTCACACAATGTGCCACACACTGCAGTTTT  3'
new aa:                          T  DraIII A        PstI
```

$Ser^{203}Thr$:

```
aa sequence     :        T  H  C  S  F  S  Q  W  W  H  Q  A  F  Y
wt human seq.   :    5'  ACACACTGCAGTTTTTCACAATGGTGGCATCAGGCCTTTTAT  3'
primer          :    3'       GACGTCAAAAtGgGTTACCACCGTAGTtCGaAAAATA  5'
                     5'       CTGCAGTTTTaCcCAATGGTGGCATCAAGCTTTTTAT  3'
changed aa      :                PstI     T              HindIII
```

$Trp^{205}His$:

```
aa sequence     :        H  C  S  F  S  Q  W  W  H  Q  A  F  Y
wt human seq.   :    5'  CACTGCAGTTTTTCACAATGGTGGCATCAGGCCTTTTAT  3'
primer          :    3'          AAAAGTGTTgtgACCGTAGTtCGaAAAAT  5'
                     5'          TTTTCACAAcacTGGCATCAAGCTTTTTA  3'
changed aa      :                        H          HindIII
```

Ser[203]Thr, Trp[205]His:

```
aa sequence   :        H  C  S  F  S  Q  W  W  H  Q  A  F  Y
wt human seq. :   5' CACTGCAGTTTTTCACAATGGTGGCATCAGGCCTTTTAT 3'
primer        :   3'   GACGTCAAAAtGgGTTgtgACCGTAGTtCGaAAAAT  5'
                  5'   CTGCAGTTTTACCCAACACTGGCATCAAGCTTTTTA  3'
changed aa    :          PstI      T     H          HindIII
```

His[207]Gln:

```
aa sequence   :        S  Q  W  W  H  Q  A  F  Y
wt human seq. :   5' TCACAATGGTGGCATCAGGCCTTTTAT 3'
primer        :   3'   GTTACCACCGTcGTtCGaAAAAT  5'
                  5' TCACAATCCTGGCAGCAAGCTTTTTA  3'
changed aa    :                    Q   HindIII
```

Gln[208]Glu:

```
aa sequence   :        S  Q  W  W  H  Q  A  F  Y
wt human seq. :   5' TCACAATGGTGGCATCAGGCCTTTTAT 3'
primer        :   3'   GTTACCACCGTAcTtCGaAAAAT  5'
                  5' TCACAATGGTGGCATGAAGCTTTTTA  3'
changed aa    :                    E HindIII
```

The introduction of DraIII and HindIII sites were made in order to simplify screening for mutant receptors. Other double, triple and quadruple mutants were constructed by cutting and ligating the appropriate single/double mutants together using the PstI site. The Cys[200]Ser mutant was a gift from Dr. Adam Pawson.

Other primers used

T7: 5' TAATACGACTCACTATAGGG 3'

SP6: 5' ATTTAGGTGACACTATAG 3'

T3: 5' AATTAACCCTCACTAAAGGG 3'