Histidine$^{7.36(305)}$ in the Conserved Peptide Receptor Activation Domain of the Gonadotropin Releasing Hormone Receptor Couples Peptide Binding and Receptor Activation

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Abstract

Transmembrane helix seven residues of G protein-coupled receptors (GPCRs) couple agonist binding to a conserved receptor activation mechanism. Amino-terminal residues of the GnRH peptide determine agonist activity. We investigated GnRH interactions with the His$^{7.36(305)}$

residue of the GnRH receptor, using functional and computational analysis of modified GnRH receptors and peptides. Non-polar His$^{7.36(305)}$ substitutions decreased receptor affinity for GnRH four- to forty-fold, whereas GnRH signaling potency was more decreased (~150-fold). Uncharged polar His$^{7.36(305)}$ substitutions decreased GnRH potency, but not affinity. [2-Nal^{3}]-GnRH retained high affinity at receptors with non-polar His$^{7.36(305)}$ substitutions, supporting a role for His$^{7.36(305)}$ in recognizing Trp^{3} of GnRH. Compared with GnRH, [2-Nal^{3}]-GnRH potency was lower at the wild type GnRH receptor, but unchanged or higher at mutant receptors. Results suggest that His$^{7.36(305)}$ of the GnRH receptor forms two distinct interactions that determine binding to Trp^{3} and couple agonist binding to the conserved transmembrane domain network that activates GPCRs.

Keywords: G protein-coupled receptor (GPCR), peptide hormone, hormone receptor, receptor structure-function, peptide interaction, GnRH

Abbreviations: 2-Nal, 2-naphthylalanine; B_0, radio-ligand bound in the absence of competing unlabeled ligand; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; GnRH, gonadotropin releasing hormone; GPCR, G protein-coupled receptor; IP, inositol phosphate; EC_{50}, half maximal effective concentration; E_{max}, maximal response; IC_{50} half maximal inhibitory concentration; pEC_{50}, negative log value of EC_{50}; PEI, polyethylenimine; pIC_{50}, negative log value of IC_{50}; rNTR1, rat neurotensin receptor type 1; TM, transmembrane helix.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is the central regulator of reproductive function. It is a decapeptide (pGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}-NH$_{2}$) that binds to receptors in the pituitary and stimulates synthesis and secretion of luteinizing hormone and follicle stimulating hormone. These gonadotropic hormones, in turn, regulate gametogenesis and gonadal sex hormone production. The GnRH receptor is a rhodopsin-like, class A, G protein-coupled receptor (GPCR) that transduces the GnRH binding signal across the cell membrane via changes in receptor protein conformation that activate intracellular G proteins and inositol phosphate (IP) signaling (Millar et al., 2004; Naor and Huhtaniemi, 2013; Pincas et al., 2014; Sefideh et al., 2014; Thompson and Kaiser, 2014).

GnRH analogs have been used for treatment of a range of reproductive hormone-dependent disorders, including various forms of infertility as well as hypertrophy and cancers of reproductive tissues (Betz et al., 2008; Kim et al., 2009; Labrie et al., 2005; Millar et al., 2004; Samant et al., 2005; Schally et al., 1990). Many peptide ligands, including chemokines and endogenous opioids, interact with their receptors via two sites, one that determines binding affinity and a second site that induces receptor activation (Choi et al., 2012; Filizola and Devi, 2013; Flanagan, 2014; Granier et al., 2012; Pease and Horuk, 2012; Portoghese, 1992). GnRH structure-activity studies have shown that amino acids at both the amino- and carboxy-termini of the peptide are required for high-affinity binding to the GnRH receptor, whereas the amino-terminal residues determine agonist activity and receptor activation (Karten and Rivier, 1986; Millar et al., 2004; Sealfon et al., 1997).

In the absence of crystal structures of the GnRH receptor, computational models have been used to infer ligand binding interactions (Chauvin et al., 2000; Hovelmann et al., 2002; Li et al., 2005; Soderhall et al., 2005). However, only a few of the proposed contacts have been validated with appropriate ligand modifications (Millar et al., 2004; Sealfon et al., 1997). The $\mathrm{Arg}^{1.35(38)}$ and $\mathrm{Asn}^{2.65(102)}$ residues (Ballesteros and Weinstein receptor residue numbering system, see under “Materials and Methods” for explanation) at the extracellular ends of the first and second transmembrane helices (TM) of the GnRH receptor contribute to recognition of the carboxy-terminal $\mathrm{Gly}^{10}\mathrm{NH}_2$ moiety of GnRH (Davidson et al., 1996; Stewart et al., 2008), an acidic residue at the extracellular end of TM7 recognizes the basic

Arg^8 residue, which is important for high affinity binding of GnRH (Flanagan et al., 1994; Fromme et al., 2001) and the $Tyr^{6.58(290)}$ side chain determines recognition of Tyr^5 of GnRH (Coetsee et al., 2008). Receptor interactions of the amino-terminal residues of GnRH that are important for agonist activity are less well-defined. The His^2 side chain forms a hydrogen bond with $Asp^{2.61(98)}$, which is thought to also form an intramolecular salt bridge with $Lys^{3.32(121)}$ that is important for receptor transition between inactive and activated receptor conformations (Flanagan et al., 2000; Zhou et al., 1995). Other amino-terminal functional groups of GnRH may also induce changes in intramolecular receptor bonds that result in receptor activation. The Trp^3 residue of GnRH has been proposed to interact with receptor residues in the TM6 and second extracellular loop, but some of these are controversial (Chauvin et al., 2000; Coetsee et al., 2006; Forfar and Lu, 2011).

The presence and orientation of an acidic residue ($Glu^{7.32(301)}$ in rodents or $Asp^{7.32(302)}$ in other mammals) at the extracellular end of TM7 of the GnRH receptor is important for binding both GnRH analogs and non-peptide antagonists (Betz et al., 2006b; Betz et al., 2008; Flanagan et al., 1994; Fromme et al., 2004; Fromme et al., 2001; Wang et al., 2004). Mutation of the $His^{7.36(306)}$ residue, one helical turn further along TM7, to Ala, Glu or Lys decreased receptor affinity for GnRH and antagonist and it was suggested that the $His^{7.36(306)}$ side chain might have a function similar to that of $Asp^{7.32(302)}$ (Betz et al., 2006b). It was subsequently shown that the $Asp^{7.32(302)}$ and $His^{7.36(306)}$ side chains form hydrogen bonds with a small molecule antagonist (Betz et al., 2006a), but the roles of $His^{7.36(306)}$ in GnRH binding and receptor signaling were not explored. Although the ligand binding pocket of each GPCR is specific for its cognate ligand, receptor functional groups that interact with agonist ligands are structurally coupled to a network of highly conserved amino acids in the transmembrane domain that constitute a conserved structural mechanism that converts the receptor to the active GPCR conformation (Deupi and Standfuss, 2011). The few published GPCR crystal structures that include peptide ligands show direct (Egloff et al., 2014; White et al., 2012) or water-mediated (Wu et al., 2010) peptide interactions with residues in TM7, including the residue in position 7.36 (Venkatakrishnan et al., 2013). The equivalent residues, Asp$^{7.39(288)}$ of the CXCR4 chemokine receptor and Glu$^{7.39(283)}$ of the CCR5 chemokine receptor, constitute part of the “site two” agonist interaction site that activates these peptide-binding GPCRs (Tan et al., 2013; Wu et al., 2010). More broadly, the position 7.39 residue is considered to be a “consensus” residue that interacts with ligands in many GPCRs and connects to the conserved transmembrane domain network (Venkatakrishnan et al., 2013). Thus, agonist-induced perturbation of the extracellular end of TM7 is part of the GPCR activation process that results in the large rearrangements of the cytosolic receptor surface that activate intracellular signaling molecules (Venkatakrishnan et al., 2013).

His residues are important in the active sites of many enzymes (Meurisse et al., 2003; Vila et al., 2011). Because the imidazole side chain is reversibly protonated and deprotonated at physiological pH and the un-protonated form occurs as two different tautomeric structures (Heyda et al., 2010; Meurisse et al., 2003; Mikulski et al., 2011; Vila et al., 2011; Walters and Allerhand, 1980; Williams et al., 2003) it can simultaneously form aromatic, hydrogen bonding and salt bridge interactions. Thus, His residues in the binding pockets of GPCRs may contribute to coupling agonist binding interactions to changes in receptor conformation that activate cytosolic signaling proteins.

We have investigated the role of the His$^{7.36(305)}$ side chain of the mouse GnRH receptor in GnRH binding and agonist-stimulated cellular signaling, using site-directed mutagenesis and modified GnRH peptides. We show that mutating His$^{7.36(305)}$ to Ala or Phe decreases GnRH-stimulated IP production and decreases receptor binding affinity for GnRH. GnRH analogs have similar decreased affinity for the mutant receptors, except for a position three-substituted analog, [2-Nal^{3}]-GnRH, which has similar affinities for wild type and mutant

GnRH receptors. The [2-Nal^3]-GnRH peptide has lower potency than GnRH in stimulating IP production at the wild type GnRH receptor, but unchanged or higher potency than GnRH at mutant receptors. We provide evidence that distinct polar interactions of His$^{7.36(305)}$ regulate agonist binding affinity and activation of the GnRH receptor.

2. Materials and Methods

2.1 GnRH Analogs

Mammalian GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$) and GnRH II [His^5,Trp^7,Tyr^8]-GnRH were purchased from Bachem AG (Bubendorf, Switzerland), [His^5,D-Tyr^6]-GnRH, [Ala^4]-GnRH, [AcGly^1]-GnRH and [2-Nal^3]-GnRH were provided by Dr. R. W. Roeske (Indiana University School of Medicine). [D-Trp^6,Pro^9-NHEt]-GnRH and Antagonist 27 ([Ac-D-Nal(2)^1,D-Me-4-Cl-Phe^2,D-Trp^3,Ipr-Lys^5,D-Tyr^6,D-Ala^{10}]-GnRH) were synthesized by Dr. R. Milton. [Trp^2]-GnRH and [Hyp^9]-GnRH were provided by Dr. J. E. Rivier (Salk Institute).

2.2 Amino acid residue numbering system

The Ballesteros and Weinstein consensus numbering system (Ballesteros and Weinstein, 1995) is used to identify receptor amino acids and to facilitate comparison with other rhodopsin-type GPCRs. The most conserved residue of each TM is designated .50 and residues are identified by the TM number and a number that indicates its position relative to the most conserved residue, followed by the receptor sequence number in parenthesis. Thus, the His^{305} residue of the mouse GnRH receptor is designated His$^{7.36(305)}$ because it precedes the most conserved residue of TM7, Pro$^{7.50(319)}$, by 14 residues. The equivalent residue of the human GnRH receptor is designated His$^{7.36(306)}$, because of an additional residue in extracellular loop two. The mouse GnRH receptor was used in this study, because it is better expressed than the human receptor (Arora et al., 1999). The better expression facilitates analysis of mutations that decrease receptor expression or function.

2.3 Site-directed mutagenesis

Polymerase chain reaction (PCR) based site-directed mutagenesis was used to substitute the His$^{7.36(305)}$ residue of the mouse GnRH receptor with Ala, Phe, Asn, Gln, Arg or Trp. The wild type mouse GnRH receptor cDNA in the pcDNAI/Amp vector (Invitrogen, San Diego, USA) was amplified using primers containing the desired mutations and a silent restriction enzyme sequence. PCR products were treated with Dpn I restriction enzyme (40 U, New England Biolabs, Inc, Beverly, USA) and then used to transform DH10B *E. Coli*, which were cultured overnight on ampicillin agar plates. DNA extracted from colonies was screened for the presence of mutations by digestion with silent mutation-specific restriction enzymes. Mutant receptor genes were sequenced to confirm the mutation and ensure absence of PCR-generated errors and subcloned into the Eco RI and Xho I sites of the pcDNAI/Amp vector.

2.4 Cell culture and transfection

COS-1 cells (American Type Culture Collection) were maintained in antibiotic-free Dulbecco's modified eagle's medium (DMEM) (Gibco, Paisley, Scotland) containing 10 % fetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) at 37°C in a 10 % CO_2 humidified incubator. COS-1 cells were transiently transfected using the DEAE-Dextran method, as described previously (Millar et al., 1995). Cells (3 x10^6 per 10cm dish) were incubated (4 hrs, 37° C) with plasmid DNA (15μg) and DEAE-Dextran (0.3 mg/ml) in serum-free DMEM (4 ml), incubated in chloroquine (200 μM in DMEM, 50 min) then treated with dimethylsulfoxide (10 % in DMEM, 2 min) and cultured overnight in DMEM with 10 % FCS and antibiotics (streptomycin sulphate, 2mg/ml and sodium benzylpenicilin, 4000U/ml). Cells were plated in 12-well plates the day after transfection for IP production and whole-cell binding assays.

2.5 IP production assays

Transfected COS-1 cells in 12-well plates were incubated overnight with *myo*-[2-^{3}H] inositol (1μCi/well, Amersham, Arlington Heights, England) in inositol-free Medium 199 (0.5 ml, Gibco, Paisley, Scotland) containing 2 % FCS. Radio-labeled cells were washed twice with buffer I (140mM NaCl; 4mM KCl; 20mM HEPES; 8.6mM glucose; 1mM $CaCl_2$; 1mM $MgCl_2$; 0.1 % BSA, fatty acid free; pH 7.4) containing LiCl (10 mM, 1ml/well) and pre-incubated in buffer I-LiCl (15 min, 37° C) before incubation with varying concentrations of ligand (45 min). The reaction was terminated by removal of the medium and addition of formic acid (10 mM, 1ml/well) which was left on the cells for 30 minutes at 4° C. IP was extracted from the resulting cell lysate on 1X8-200 Dowex-1 ion exchange columns (Sigma-Aldrich, South Africa) as previously described (Millar et al., 1995) and radioactivity was counted in a beta scintillation counter.

2.6 Ligand Binding Assays

[His^5,D-Tyr^6]-GnRH was radio-iodinated as previously described (Flanagan et al., 1998), purified on a QAE-Sephadex column as previously described (Millar et al., 1995), aliquoted and stored (-70° C). Specific activity of ^{125}I-[His^5,D-Tyr^6]-GnRH ranged between 900 and 1800 µCi/µg. For whole-cell binding assays, transfected COS-1 cells in 12-well plates were washed with cold buffer I (1ml/well, 140mM NaCl; 4mM KCl; 20mM HEPES; 8.6mM glucose; 1mM $CaCl_2$; 1mM $MgCl_2$; 0.1 % BSA, fatty acid free; pH 7.4) and incubated (4-5 hours, 4° C) with ^{125}I-[His^5,D-Tyr^6]-GnRH (100,000 cpm) and varying concentrations of unlabeled GnRH agonists (final volume, 0.5 ml). Cells were washed with phosphate-buffered saline (3 x 1ml) before addition of NaOH (0.1 M, 1 ml/well). Radioactivity in the NaOH solution was counted in a gamma counter.

Membrane binding assays were performed as previously described (Millar et al., 1995). Forty eight hrs after transfection COS-1 cells were harvested in detaching buffer (1mM

EDTA, 10mM HEPES, pH 7.4), homogenized with a Dounce homogenizer (Kontes, Vineland, USA) and centrifuged (15,000 x g, 20 min, 4° C). The crude membrane pellet was resuspended in binding buffer (1mM EDTA, 10mM HEPES, 0.1 % BSA, pH 7.4) and diluted to give 1/100 of 10 cm dish (~30 000 cells) per tube for the wild type and $\mathrm{His}^{7.36(305)}$Arg GnRH receptors, 1/10 dish for the $\mathrm{His}^{7.36(305)}$Ala, $\mathrm{His}^{7.36(305)}$Phe and $\mathrm{His}^{7.36(305)}$Trp mutant receptors and 1/50 dish per reaction tube for $\mathrm{His}^{7.36(305)}$Asn and $\mathrm{His}^{7.36(305)}$Gln mutant receptors. Membrane suspensions were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH (50,000 CPM, ~50 pM) and varying concentrations of unlabeled GnRH analogs in a final volume of 0.5 ml of binding buffer (16 h, 4° C). Under these conditions K_d can be estimated directly from IC_{50} values (Flanagan et al., 1998; Hulme and Birdsall, 1992). The incubation was terminated by dilution with polyethylenimine (PEI, 0.01 %, 3ml) and filtration (Brandel Cell Harvester) through glass fiber filters (GF/C, Whatman) pre-soaked for 30 minutes in PEI (1 %). Filters were washed twice with PEI (0.01 %, 3 ml) and retained radioactivity was counted. Antagonist 27 (1 μM) was used to estimate non-specific binding.

2.7 Data Analysis

IP production and whole-cell binding assays were performed at least four times in duplicate and membrane binding assays were performed in triplicate. A non-linear regression curve fitting program, GraphPad Prism (GraphPad Software Inc., La Jolla, CA) was used to calculate the half maximal effective concentration (EC_{50}), maximal response (E_{max}) (sigmoidal dose-response curve) and half maximal inhibitory concentration (IC_{50}) (one site competition curve) values. Homologous membrane binding assays were used to estimate B_{max} and K_d values for [His^5,D-Tyr^6]-GnRH (one site homologous competitive binding, GraphPad Prism). A coupling coefficient was calculated using the previously described formula, $Q = 0.5[(K_d + EC_{50})/EC_{50}] \times (E_{max}/B_{max})$, using the summarized data in Tables 1 and 3, to facilitate comparison of mutant receptor signaling efficiency (Ballesteros et al., 1998). IC_{50}

values for GnRH were used to estimate K_d. P values were calculated using unpaired two tailed T-tests performed on negative log values of IC_{50} (pIC_{50}) and EC_{50} (pEC_{50}) (GraphPad Prism), because the log values have a closer to Gaussian distribution (Motulsky, 1999).

2.8 Molecular Modeling

A homology model for GnRH and mouse GnRH receptor interaction was built with the homology modeling program MODELLER 9v12 (Sali and Blundell, 1993) and was based on the high resolution crystal structures of agonist-bound rat neurotensin receptor type 1 (rNTR1, Protein Data Bank code: 4GRV) (White et al., 2012), which is also a peptide receptor. The sequence of the mouse GnRH receptor was manually aligned with the neurotensin receptor of the crystal structures based on TMHs predicted by TMHMM Server v.2.0 (Krogh et al., 2001) and evolutionarily conserved residues. During homology modeling, distance restraints between the following residue pairs were introduced: His^2 of GnRH ~ $Asp^{2.61(98)}$ of the GnRH receptor (Flanagan et al., 2000); Gly^{10} of GnRH ~ $Asn^{2.65(102)}$ of the GnRH receptor (Davidson et al., 1996); Tyr^5 of GnRH ~ $Tyr^{6.58(289)}$ of the GnRH receptor (Coetsee et al., 2008); Arg^8 of GnRH ~ $Glu^{7.32(301)}$ of the GnRH receptor (Flanagan et al., 1994; Fromme et al., 2001); Ser^4 of GnRH ~ Arg^8 of the GnRH; Gly^{10} of GnRH ~ $pGlu^1$ of the GnRH. The variable target function method with the slow option and the molecular dynamics optimization method with the slow refine option were applied during the model building. All structural analyses and figure preparation were performed with ICM version 3.8-0 (Molsoft, San Diego, CA) and Ligplot+ version 1.4.5 (Laskowski and Swindells, 2011). Structures for the mutants were generated by homology modeling using the model structure of the wild type receptor and GnRH complex as the template.

3. Results

3.1 IP production

To identify which mutations affected GnRH receptor function, mutant receptors were screened for their ability to mediate GnRH-stimulated cellular signaling. GnRH stimulated IP production with high potency (EC_{50}, 0.16 ± 0.04 nM) in COS-1 cells transfected with the wild type GnRH receptor. Substituting Ala for the $His^{7.36(305)}$ residue of the GnRH receptor decreased the potency of GnRH-stimulated IP production 150-fold (EC_{50}, 24.6 ± 3.7 nM) and decreased the maximal IP response (Fig. 1, Table 1). This result indicated that the $His^{7.36(305)}$ side chain has a role in GnRH receptor function. The $His^{7.36(305)}$Phe mutant receptor showed a similar decrease in GnRH-stimulated IP production (161-fold) compared with the wild type receptor (Fig. 1, Table 1), showing that the aromatic ring side chain of Phe did not mimic the function of the $His^{7.36(305)}$ side chain. GnRH showed a smaller loss of potency at the $His^{7.36(305)}$Asn (6.6-fold) and $His^{7.36(305)}$Gln (5.7-fold) mutant receptors, compared with the $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe mutants (Fig. 1, Table 1). The Asn and Gln side chains have polar functional groups in the δ- and ε-positions respectively, which potentially mimic the nitrogen atoms at the δ- and ε-positions of the imidazolium side chain of His. GnRH potency at the $His^{7.36(305)}$Arg mutant was similar to that of the wild type receptor (Fig. 1, Table 1). The $His^{7.36(305)}$Trp mutant exhibited low GnRH potency and a low E_{max} (Fig. 1, Table 1).

3.2 Ligand binding

Whole-cell ligand binding assays were used initially to investigate whether decreased IP production mediated by mutant GnRH receptors resulted from decreased cell surface receptor expression, ligand affinity or coupling efficiency. Homologous competition assays were used to assess receptor expression levels, whereas heterologous GnRH competition binding was used to assess whether decreased ligand binding affinity might account for decreased potencies in IP assays. The wild type GnRH receptor bound [His^5,D-Tyr^6]-GnRH with high affinity (IC_{50}, 8.18 ± 4.8 nM) and had lower affinity for native GnRH (IC_{50} 27.0 ± 4.6 nM). Compared with the wild type receptor, all mutant receptors showed decreased total binding of the ^{125}I-[His^5,D-Tyr^6]-GnRH tracer in the absence of competing unlabeled ligand (B_0, Fig. 2, Table 2). Under the non-saturating conditions of competition binding assays, decreased B_0 may reflect decreased receptor expression, decreased affinity for the tracer ligand or both. Cells expressing the His$^{7.36(305)}$Ala mutant GnRH receptor showed decreased affinity for [His^5,D-Tyr^6]-GnRH (IC_{50}, 54.7 ± 22 nM) compared with wild type receptor, as did the His$^{7.36(305)}$Phe mutant (Fig. 2, Table 2). No significant changes in IC_{50} values were observed for the His$^{7.36(305)}$Asn, His$^{7.36(305)}$Gln and His$^{7.36(305)}$Arg mutant GnRH receptors, whereas cells transfected with the His$^{7.36(305)}$Trp mutant GnRH receptor showed no measurable ligand binding (Fig. 2, Table 2). Only the His$^{7.36(305)}$Phe mutant GnRH receptor showed a statistically significant decrease in affinity for GnRH in the whole-cell binding assay (Table 2).

As the whole-cell binding assays showed evidence of ligand depletion for some receptors ($B_0 > 10$ % of total radioactivity) (Hulme and Birdsall, 1992) or insufficient binding of tracer by other receptors to allow accurate estimation of IC_{50} values, we used membrane binding assays, which allow manipulation of GnRH receptor concentration as described under “Materials and Methods”, for subsequent experiments. Similar to the whole-cell binding assay, the wild type GnRH receptor exhibited high affinity for the [His^5,D-Tyr^6]-GnRH (IC_{50} 3.15 ± 0.40 nM) and lower affinity for GnRH (IC_{50} 54.8 ± 3.7 nM, Fig. 3, Table 3) and the His$^{7.36(305)}$Trp mutant receptor showed no specific binding of the tracer ligand. The His$^{7.36(305)}$Asn, His$^{7.36(305)}$Gln and His$^{7.36(305)}$Arg mutant receptors showed no changes in affinity for GnRH or [His^5,D-Tyr^6]-GnRH compared with wild type receptor (Fig. 3, Table 3). The His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutant receptors showed significantly decreased affinity for both GnRH and [His^5,D-Tyr^6]-GnRH. The decreased affinities of mutants with

Ala or Phe substituted for $His^{7.36(305)}$ show that the His side chain may have a role in ligand binding and decreased GnRH binding affinity may partially account for the decreased potency of GnRH-stimulated IP production at these receptors. On the other hand, the unchanged affinities of mutants with Asn, Gln or Arg in position 7.36(305) suggest that these side chains can substitute for His in stabilizing ligand binding interactions and show that decreased binding affinity cannot account for the decreased potency of GnRH-stimulated IP production at the $His^{7.36(305)}$Asn and $His^{7.36(305)}$Gln mutant receptors. Calculation of coupling coefficients from the aggregated membrane binding and IP signaling results showed that all mutant receptors, except for the $His^{7.36(305)}$Arg receptor, have decreased signaling efficiency (Table 1).

3.3 Binding affinities of GnRH analogs at wild type and mutant GnRH receptors

The decreased GnRH binding affinities of the $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe mutant receptors indicate that the mutations may disrupt the receptor-ligand binding interface. We used a series of GnRH analogs with substitutions of each of the 10 amino acids to test whether the $His^{7.36(305)}$Ala and $His^{7.36(305)}$Phe mutations disrupt recognition of a specific functional group of the GnRH ligand. If the receptor mutations disrupt an interaction with a specific functional group of the ligand, then binding of a ligand that lacks the interacting group should not be disrupted by the mutation and should have the same affinity at wild type and mutant receptors.

GnRH analogs with substitutions in positions 1 ([Ac-Gly^1]-GnRH), 4 ([Ala^4]-GnRH) and 9 ([Hyp^9]-GnRH) exhibited affinities for the wild type GnRH receptor that were too low to allow reliable determination of IC_{50} values and it was not possible to determine whether mutant receptors had similar or lower affinities for these peptides (Table 4). [Trp^2]-GnRH bound the wild type receptor with high affinity (IC_{50}, 18.5 ± 4.66 nM) and had lower affinity at the $His^{7.36(305)}$Ala (IC_{50}, 136 ± 40 nM) and $His^{7.36(305)}$Phe (IC_{50}, 362 ± 148 nM) mutant receptors. This result indicates that the $\text{His}^{7.36(305)}$ side chain does not determine receptor recognition of the His^2 side chain of native GnRH. GnRH II, which has substitutions at positions 5, 7 and 8, bound the wild type GnRH receptor with an IC_{50} value of 120 ± 11.9 nM and had lower affinity for the mutant receptors (Table 4). The high-affinity analog, [D-Trp^6,Pro^9-NHEt]-GnRH, also showed lower affinity for the mutant receptors compared with the wild type GnRH receptor (Table 4). In contrast, [2-Nal^3]-GnRH, which has a synthetic amino acid, 2-naphthylalanine (2-Nal), substituted for Trp^3 of GnRH, had higher affinity (IC_{50}, 1.54 ± 0.32 nM) than GnRH (IC_{50}, 54.8 ± 3.7 nM) at the wild type receptor. The higher affinity suggests that the 2-Nal^3 side-chain forms a strong interaction with the receptor or that the substitution changes the peptide conformation and therefore introduces additional peptide-receptor interactions, not directly related to the position 3 side chain. [2-Nal^3]-GnRH also exhibited high affinities at the $\text{His}^{7.36(305)}$Ala (IC_{50}, 2.97 ± 1.62 nM) and $\text{His}^{7.36(305)}$Phe ($\text{IC}_{50,}$ 2.99 ± 0.43 nM) mutant receptors that were not significantly different from its affinity at the wild type receptor (Table 4). The similar affinities of [2-Nal^3]-GnRH for the wild type and mutant receptors show that removing the $\text{His}^{7.36(305)}$ side chain does not disrupt binding of [2-Nal^3]-GnRH and show that $\text{His}^{7.36(305)}$ is not important for binding the [2-Nal^3]-GnRH peptide. Since removing the $\text{His}^{7.36(305)}$ side chain disrupts binding of all GnRH peptides that have the native Trp^3 residue, these results show that the $\text{His}^{7.36(305)}$ side chain is important for binding peptides with Trp in position three. This suggested that the $\text{His}^{7.36(305)}$ side chain may interact with the Trp^3 side chain of native GnRH, whereas it may interact only weakly or not at all with the 2-Nal side chain of [2-Nal^3]-GnRH. Alternatively, the $\text{His}^{7.36(305)}$ side chain may form intramolecular bonds that position another chemical group of the receptor to interact with Trp^3 of GnRH.

3.4 [2-Nal^3]-GnRH stimulation of IP production at wild type and mutant GnRH receptors

The amino-terminal residues of GnRH, including Trp^3, are important for agonist activity of the peptide at the GnRH receptor, in contrast to the carboxy terminal residues, which are important primarily for high binding affinity (Millar et al., 2004). The decreased potency of GnRH at receptor mutants lacking His$^{7.36(305)}$, suggests that, in addition to its contribution to ligand binding, a Trp^3-His$^{7.36(305)}$ interaction may have an additional role in transducing the agonist binding signal, i.e. receptor activation. We therefore investigated [2-Nal^3]-GnRH stimulation of IP production at wild type and mutant receptors. [2-Nal^3]-GnRH was less potent than native GnRH (3.1-fold) in stimulating IP production at the wild type GnRH receptor (Fig. 4, Table 5), even though it had higher binding affinity (35.6-fold) than GnRH. Although the coupling coefficient is usually used to assess the signaling efficiency of mutant receptors, calculation of a coupling coefficient using [2-Nal^3]-GnRH IC$_{50}$ and EC$_{50}$ values, yielded a coefficient of 1.07 units, which is 0.29 % of the coupling coefficient for GnRH (368 units). This indicates that the wild type GnRH receptor couples binding of [2-Nal^3]-GnRH to IP signaling less efficiently than it does binding of GnRH. The lower potency and coupling coefficient of [2-Nal^3]-GnRH suggest that Trp^3 is required for full agonist activity of the GnRH peptide. In contrast, [2-Nal^3]-GnRH was more potent than GnRH at the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutant receptors (Fig. 4, Table 5). Since the low potency of GnRH compared with [2-Nal^3]-GnRH at the mutant receptors could be a result of the decreased GnRH binding affinity (150-fold), we also tested [2-Nal^3]-GnRH stimulation of IP production at the His$^{7.36(305)}$Asn mutant, which had unchanged affinity for GnRH. The potencies of GnRH (EC$_{50}$, 1.07 ± 0.36) and [2-Nal^3]-GnRH (EC$_{50}$, 0.81 ± 0.14) were similar at the His$^{7.36(305)}$Asn mutant receptor (Fig. 4, Table 5). The [2-Nal^3]-GnRH peptide also showed a minimal decrease in potency at the His$^{7.36(305)}$Asn mutant receptor compared with the wild type receptor (Table 5). These results suggest that, although Trp^3 is necessary for full agonist potency at the wild type GnRH receptor, substituting Trp^3 with 2-Nal does not decrease agonist potency when the His$^{7.36(305)}$ side chain is not present in the receptor. Like native GnRH, the high affinity agonist, [His^5,D-Tyr^6]-GnRH, which has Trp^3, had decreased potency at all mutant receptors lacking His$^{7.36(305)}$ (Table 5).

3.5 Molecular model of interactions of Trp^3 of GnRH and His$^{7.36(305)}$ of the GnRH receptor

A three-dimensional homology model of the GnRH-GnRH receptor interaction was built, based on the rNTR1 neurotensin receptor (White et al., 2012) and constrained to include ligand-receptor interactions previously identified by site-directed mutagenesis studies, but not constrained to include an interaction between Trp^3 of GnRH and His$^{7.36(305)}$ of the receptor. After molecular dynamics optimization, the model had no intramolecular salt bridge between Asp$^{2.61(98)}$ and Lys$^{3.32(121)}$ because pGlu^1 and His^2 of GnRH were located between the two receptor residues. The model showed that Trp^3 of GnRH is not near His$^{7.36(305)}$ of the GnRH receptor, suggesting no direct interaction between them (Figure 5A). Instead, Trp^3 of GnRH is located near Asp$^{2.61(98)}$ and Phe$^{7.39(308)}$, which is 0.83 helical turn towards the intracellular side of the His$^{7.36(305)}$ residue in TM7 of the GnRH receptor. Trp^3 of GnRH may form a π-π interaction with Phe$^{7.39(308)}$, which can be quite strong. The side chain of His$^{7.36(305)}$ points to the extracellular end of TM1. Further analysis revealed that the δ1-nitrogen and ε2-nitrogen of His$^{7.36(305)}$ in the receptor are close to the carbonyl oxygen atoms of the amino acids Val$^{1.30(33)}$ and Ser$^{1.31(34)}$ of the receptor (Figure 5B). This arrangement suggests that positively charged side chains at the position of His$^{7.36(305)}$ would interact with the carbonyl oxygen atoms by ion-dipole force, whereas polar side chains at the position would interact with the carbonyl oxygen atoms by dipole-dipole force, which is weaker than ion-dipole force. Even though His$^{7.36(305)}$ is not directly involved in the GnRH binding, it seems still to participate in the formation and maintenance of the network of interactions that configure the GnRH binding pocket and allow the amino-terminal residues of the peptide, including Trp^3, to enter the transmembrane domain of the receptor and interact with residues, including Phe$^{7.39(308)}$ and Asp$^{2.61(98)}$, that initiate receptor activation. Therefore, substitution of His$^{7.36(305)}$ with positively-charged Arg would fully preserve the architecture of the ligand binding pocket (Figure 5C), whereas substitutions with non-polar Ala (Figure 5D), Phe or large Trp residues disrupts the relative orientation of TM1 and TM7, decreasing both binding affinity and signaling potency of GnRH. Substituting His$^{7.36(305)}$ with uncharged, polar residues, Asn or Gln (Figure 5E), appears to partially disrupt the intramolecular network, such that GnRH binding affinity is not affected, but the intramolecular interactions that initiate receptor activation are disrupted. The naphthalene ring of [2-Nal^{3}]-GnRH potentially makes a strong π-π interaction with the benzene ring of the Phe$^{7.39(308)}$, which could compensate or overcome the weakened or disrupted ligand binding pocket network.

In summary, mutating the His$^{7.36(305)}$ side chain of the GnRH receptor to Ala or Phe severely decreased the potency of GnRH-stimulated IP production, whereas mutation to Asn, Gln or Arg had lesser effects. The His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutant GnRH receptors had decreased affinity for GnRH, but mutants with polar amino acid substitutions for His$^{7.36(305)}$ retained high affinity for GnRH. The His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutant receptors also had decreased affinity for a series of GnRH analogs, but retained high affinity for [2-Nal^{3}]-GnRH, suggesting that the His$^{7.36(305)}$ side chain regulates receptor recognition of the Trp^{3} residue of native GnRH. A three dimensional molecular model showed that Trp^{3} of GnRH may form a π-π interaction with Phe$^{7.39(308)}$ of the receptor and that His$^{7.36(305)}$ forms intramolecular interactions. Compared with GnRH, [2-Nal^{3}]-GnRH had decreased potency at the wild type GnRH receptor, showing that Trp^{3} is required for full agonist activity of GnRH. In contrast, [2-Nal^{3}]-GnRH did not show decreased potency, compared with GnRH, at mutant receptors with substitutions of the His$^{7.36(305)}$ side chain. Taken together, these results show that a dipole-dipole interaction of the His$^{7.36(305)}$ side chain is required for high affinity binding of GnRH, whereas an ion-dipole interaction of His$^{7.36(305)}$, which can be mimicked by Arg, but not other substitutions tested, is required for full agonist potency of GnRH.

4. Discussion and Conclusions

We have investigated the role of the His$^{7.36(305)}$ residue of the GnRH receptor in ligand binding and receptor activation. We show that substituting the His$^{7.36(305)}$ residue with small (Ala) or non-polar aromatic (Phe) amino acids decreases receptor affinity for GnRH four- to forty-fold, whereas these mutations decrease the potency of GnRH-stimulated IP signaling to a greater extent (~150-fold). Uncharged polar amino acid substitutions (Asn and Gln) that can partially mimic the polar groups in the imidazole side chain of His$^{7.36(305)}$ did not affect GnRH binding affinity, but decreased the potency of GnRH-stimulated signaling five- to six-fold. The His$^{7.36(305)}$Arg mutation had a minimal effect on affinity and potency of GnRH, suggesting that the positive charge and the geometry of the amine groups of the guanidinium side chain of Arg can substitute for His$^{7.36(305)}$. These results suggested that the two nitrogen atoms of the imidazole side chain of His$^{7.36(305)}$ may have distinct, but interdependent functions in agonist binding and receptor activation. To characterize the ligand binding interaction, we investigated the binding of modified GnRH peptides to mutant receptors. We found that the affinity of the Trp^3-substituted analog, [2-Nal^3]-GnRH, was not affected by the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutations. Since [2-Nal^3]-GnRH differs from native GnRH in having a six-carbon ring substituted for the nitrogen-containing pyrrole ring of the indole side chain of Trp^3, it is possible that the His$^{7.36(305)}$ side chain determines receptor recognition of the amino group in the side chain of Trp^3 of GnRH. However, our molecular model shows no direct interaction between the Trp^3 and His$^{7.36(305)}$ side chains, but shows that His$^{7.36(305)}$ may configure the ligand binding pocket via interactions with TM1. In spite of its higher binding affinity, [2-Nal^3]-GnRH had lower potency (3.1-fold) than GnRH in stimulating IP signaling at the wild type GnRH receptor, showing that the pyrrole ring of Trp^3 of GnRH contributes to activation of the receptor. [2-Nal^3]-GnRH had further decreased potency (6 to 20-fold) at mutant receptors with Ala or Phe substitutions, in spite of unchanged binding affinity, showing that a polar functional group of His$^{7.36(305)}$ contributes to receptor activation. Consistent with this, [2-Nal^3]-GnRH showed only a minimal decrease in potency (1.7-fold) at the mutant receptor with a polar, Asn, substitution for His$^{7.36(305)}$. These results suggest that the His$^{7.36(305)}$ side chain of the GnRH receptor forms two distinct interactions, one that indirectly determines binding of Trp^3 in the ligand and one that mediates agonist-induced receptor activation.

Previous studies have shown that mutations of residues at the extracellular end of TM7 of the GnRH receptor disrupt GnRH binding and the resulting models of receptor-ligand binding have included direct interactions of these residues with specific residues of GnRH or GnRH peptide analogs (Betz et al., 2006b; Chauvin et al., 2001; Coetsee et al., 2008; Flanagan et al., 1994; Fromme et al., 2004; Fromme et al., 2001; Hovelmann et al., 2002; Millar et al., 2004; Wang et al., 2004). Mutations of His$^{7.36(306)}$ of the human GnRH receptor had disruptive effects on binding of GnRH and a non-peptide antagonist that were similar to the effects of mutating Asp$^{7.32(302)}$ (Betz et al., 2006b). Subsequent structure-activity relationship studies concluded that the Asp$^{7.32(302)}$ and His$^{7.36(306)}$ side chains form hydrogen bonds with adjacent functional groups of the antagonist (Betz et al., 2006a), but the roles of the His$^{7.36}$ side chain in binding GnRH peptides and in receptor activation were not reported. The imidazole side chain of His can form π-stacking interactions with other aromatic side chains (Meurisse et al., 2003; Mikulski et al., 2011; Wang et al., 2006; Williams et al., 2003). It is also reversibly protonated at physiological pH (Heyda et al., 2010; Vila et al., 2011) and the neutral form occurs in both of its tautomeric structures, which have a hydrogen atom attached to either the δ1- or the ε2-nitrogen atom. Hence, the His side chain can act as a proton shuttle, a donor and an acceptor of hydrogen bonds and it can rotate (Heyda et al., 2010; Meurisse et al., 2003;

Mikulski et al., 2011; Vila et al., 2011; Walters and Allerhand, 1980; Williams et al., 2003). This structural flexibility makes the His$^{7.36(305)}$ residue of the GnRH receptor an attractive candidate for a role in coupling ligand binding to receptor activation. Indeed, crystal structures have shown that the His$^{7.43(278)}$ residue is important for agonist binding and activation of the A_{2A} adenosine receptor (Lebon et al., 2012).

Recent GPCR crystal structures have allowed identification of a conserved network of non-covalent interhelical interactions that is important for receptor protein structure and function and includes six residues in TM7 (Venkatakrishnan et al., 2013). Small molecule ligands, including retinal, monoamines and opioids, interact with a consensus ligand binding pocket made up of topologically equivalent residues in TM3, TM6 and TM7 (Venkatakrishnan et al., 2013). The residue in position 7.39 forms part of both the consensus ligand binding pocket and the interhelical network and thus potentially couples binding of diverse ligands with the conserved receptor structure (Venkatakrishnan et al., 2013). Crystal structures of GPCRs in both inverse agonist-stabilized inactive conformations and agonist-bound fully activated (rhodopsin, β_2 adrenergic and M2 muscarinic receptors) or partially activated (A_{2A} adenosine and $P2Y_{12}$ purinergic receptors) conformations have provided insight into how agonist binding changes receptor conformation. Compared with inverse agonists, agonist interactions with residues in TM3, TM5, TM6 and TM7 cause small (in the cases of rhodopsin, β adrenergic and adenosine receptors) or large (in the cases of muscarinic and purinergic receptors) changes in the ligand binding pocket that draw the extracellular ends of these helices together and contract the pockets of receptors that bind dissociable ligands, but widen the retinal pocket in rhodopsin (Deupi et al., 2012a; Deupi et al., 2012b; Katritch and Abagyan, 2011; Kruse et al., 2013; Lebon et al., 2012; Rasmussen et al., 2011a; Ring et al., 2013; Venkatakrishnan et al., 2013; Xu et al., 2011; Zhang et al., 2014a; Zhang et al., 2014b). These diverse agonist binding interactions, nevertheless, induce common changes in the transmembrane domain and the cytosolic receptor surface that interacts with G proteins. Movement of the extracellular ends of helices changes the interhelical interactions. A change in the conformation of TM5 around the conserved Pro$^{5.50}$ residue (except in the P2Y$_{12}$ receptor, which has Asn$^{5.50}$) is transmitted to the cytoplasmic end of the helix, which moves outwards. This is associated with a change in the contacts between the conserved hydrophobic residues (Ile/Leu$^{3.40}$, Leu$^{5.51}$, Phe$^{6.44}$ and Trp$^{6.48}$) that constitute the "transmission switch" and rotation of TM6, which moves the cytosolic end of the helix outwards, exposing the G protein binding surface. Changes in the intramolecular hydrogen bond network, which includes water molecules, change the conformation of the TM7 helix and move the side chain of Tyr$^{7.53}$ (of the NPxxY motif) to the interior if the transmembrane bundle, where it interacts with residues in the cytosolic ends of TM3, TM5 and TM6 to stabilize the active receptor conformation (Deupi et al., 2012a; Deupi and Standfuss, 2011; Deupi et al., 2012b; Kruse et al., 2013; Standfuss et al., 2011; Trzaskowski et al., 2012; Venkatakrishnan et al., 2013). It is notable that the key receptor residues that mediate agonist activity differ. In the β_2 adrenergic receptor the TM5 Ser$^{5.42}$ and Ser$^{5.46}$ residues are key for agonist activity, whereas the TM7 residues, Ser$^{7.42}$ and His$^{7.43}$, are key for activation of the A$_{2A}$ adenosine receptor (Katritch and Abagyan, 2011; Lebon et al., 2012; Xu et al., 2011). Similarly, agonist ligands interact directly with Trp$^{6.48}$ and Tyr$^{6.51}$ (in the CWxPY motif) of rhodopsin and the A$_{2A}$ adenosine receptor, but not the β_2 adrenergic receptor (Deupi and Standfuss, 2011; Katritch et al., 2013). These results show that agonists interact with different combinations of residues to trigger receptor activation. Although small ligands bind in the transmembrane domain and interact with some of the highly conserved residues that regulate receptor activation, much biochemical evidence suggests that larger, peptide ligands bind to the extracellular GPCR surface (Ji et al., 1998). Although crystal structures of a number of peptide-binding GPCRs have now been reported, only a few include peptide ligands. Structures of the CXCR4 chemokine receptor in complex with an antagonist peptide, CVX15, and the rNTR1 neurotensin receptor complexed with an agonist peptide have confirmed that peptide binding pockets are predominantly extracellular and that agonists may not penetrate into the transmembrane domain (Egloff et al., 2014; Trzaskowski et al., 2012; White et al., 2012; Wu et al., 2010). This suggests that intramolecular interactions may couple extracellular agonist peptide binding pockets to the conserved transmembrane interaction network that mediates receptor activation.

Agonist peptides form hydrogen bond interactions with Tyr$^{7.30(347)}$ of the NTR1 neurotensin receptor (Egloff et al., 2014; White et al., 2012) and van der Waals interactions with other residues in TM7 (White et al., 2012). The CVX15 antagonist peptide makes a water-mediated contact with Asp$^{7.39(288)}$, which is part of the activation site of the CXCR4 chemokine receptor (Wu et al., 2010). Small molecule antagonists that block orthosteric peptide binding sites make contact with Asp$^{7.39(288)}$ or Glu$^{7.39(283)}$ in chemokine receptors and Tyr$^{7.43}$ in opioid receptors (Filizola and Devi, 2013; Granier et al., 2012). Since these TM7 residues are also important for agonist activity, they likely participate in coupling agonist binding to the conserved transmembrane domain scaffold (Venkatakrishnan et al., 2013) and the molecular pathways that mediate receptor activation (Deupi et al., 2012b).

4.1 A polar functional group of the His$^{7.36(305)}$ side chain of the GnRH receptor is required for GnRH binding

Our mutations of the GnRH receptor were designed to address how the His$^{7.36(305)}$ side chain affects receptor interactions with GnRH. The decreased GnRH affinity and potency resulting from the His$^{7.36(305)}$Ala mutation confirms a role for the His$^{7.36(305)}$ side chain in ligand binding and signaling. The similar phenotype of the His$^{7.36(305)}$Phe mutant receptor shows that non-polar aromatic interactions of His$^{7.36(305)}$ do not contribute significantly to GnRH binding or receptor activation. The substitutions with Asn and Gln, which have hydrogen bond donor and acceptor groups at the δ and ε positions respectively, were designed to test the contribution of the δ1- and ε2-nitrogen atoms of the imidazole ring to binding GnRH. Surprisingly, both the His$^{7.36(305)}$Asn and His$^{7.36(305)}$Gln mutations had no effect on receptor affinity for GnRH. This flexibility suggests that the His$^{7.36(305)}$ interaction that determines GnRH affinity may be indirect or water-mediated. The decreased coupling efficiencies of the His$^{7.36(305)}$Asn and His$^{7.36(305)}$Gln mutant receptors show that neither amide group can fully substitute for His$^{7.36(305)}$ in coupling ligand binding to receptor activation. Our molecular models show interaction of the His$^{7.36(305)}$ side chain with two carbonyl groups of TM1, whereas Asn and Gln can form only one of these interactions. An Arg substitution was chosen to mimic the protonated form of His$^{7.36(305)}$, because the delocalized charge of the protonated guanidinium side chain more closely resembles the protonated His side chain than does the amino side chain of Lys. The minimal effects of the His$^{7.36(305)}$Arg mutation on GnRH affinity and signaling potency suggest that either protonation or a second polar group is required to fully couple ligand binding to receptor activation. It was previously reported that a His$^{7.36}$Lys mutation of the human GnRH receptor decreased receptor affinity for GnRH (Betz et al., 2006b), suggesting that a positive charge is not sufficient to substitute for His$^{7.36(305)}$. Combined with our results, this suggests that the His$^{7.36(305)}$ side chain of the wild type GnRH receptor forms a hydrogen bond that is important for ligand binding, whereas protonation contributes to receptor activation.

4.2 The His$^{7.36(305)}$ side chain of the GnRH receptor discriminates Trp^{3} of GnRH

All GnRH analogs that had measurable affinity for the wild type GnRH receptor, had lower affinity at the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutant receptors, except for [2-Nal^{3}]-GnRH, which had similar affinity at the wild type and mutant receptors. The retention of high affinity [2-Nal^{3}]-GnRH binding at the mutant receptors suggests that the Trp^{3}-substituted peptide lacks a feature of native GnRH that is recognized by the wild type receptor and not by the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutants. [2-Nal^{3}]-GnRH has a six-carbon ring substituted for the nitrogen-containing pyrrole ring of Trp^{3} of native GnRH. The Nal side chain potentially mimics the π-stacking interactions of Trp, but not the hydrogen bonds formed by the pyrrole NH group. The four-fold decrease in affinity of native GnRH at the His$^{7.36(305)}$Ala mutant receptor, compared with the wild type receptor, is consistent with loss of a single hydrogen bond (Fersht et al., 1985). On the other hand, three substitutions of His$^{7.36(305)}$ did not measurably affect GnRH affinity, suggesting that the His$^{7.36(306)}$Ala mutation may disrupt recognition of Trp^{3} via indirect effects, such as a change of receptor conformation. Our molecular model, which includes the previously identified His^{2}-Asp$^{2.61(98)}$, Tyr^{5}-Tyr$^{6.58(289)}$, Arg^{8}-Glu$^{7.32(301)}$ and Gly^{10}NH$_{2}$-Asn$^{2.65(102)}$ interactions, but does not show a direct His$^{7.36(305)}$-Trp^{3} interaction, supports an indirect role for His$^{7.36(305)}$ in binding GnRH. Our molecular models suggest that the Trp^{3} side chain interacts with the consensus binding pocket residue, Phe$^{7.39(308)}$ and with Asp$^{2.61(98)}$. The Asp$^{2.61(98)}$, which potentially forms four non-covalent bonds, also interacts with His^{2} of GnRH in the current model and others (Coetsee et al., 2008; Flanagan et al., 2000; Forfar and Lu, 2011; Soderhall et al., 2005).

Based on a small number of mutations, many GnRH-receptor models have included contacts of Trp^{3} of GnRH agonists with the Trp$^{6.48}$ residue (of the CWxPY motif) (Betz et al., 2006b; Chauvin et al., 2000; Chauvin et al., 2001; Hovelmann et al., 2002; Millar et al., 2004; Soderhall et al., 2005). However, systematic mutagenesis and rigorous determination of mutant receptor affinity showed that all mutations of Trp$^{6.48(280)}$ decreased mutant receptor expression, but had no significant effect on GnRH affinity or signaling, excluding a direct role for the Trp$^{6.48}$ side chain in binding GnRH (Coetsee et al., 2006). More recently, a Phe$^{4.64(178)}$Ala mutation of the human GnRH receptor decreased GnRH affinity and potency by two to three orders of magnitude, but had lesser effects on the affinity and potency of [Phe^{3}]-GnRH. Although a π-stacking interaction was proposed (Forfar and Lu, 2011), loss of a weak aromatic interaction is unlikely to account for the large decrease in GnRH binding affinity at the Phe$^{4.64(178)}$Ala mutant receptor (Fersht et al., 1985). Indeed disruption of a crystallographically-confirmed van der Waals interaction using a similar mutation decreases affinity of the neurotensin receptor, NTSR1, five to ten-fold (White et al., 2012). The results suggest rather, that the Phe$^{4.64(178)}$Ala mutation disrupts the protein structure (Fersht, 1987) and decreases GnRH affinity via indirect effects that distort the ligand binding surface.

4.3 Trp^{3} of GnRH and His$^{7.36(305)}$ of the GnRH receptor are both required for full agonist activity of GnRH

Agonist ligand interactions induce or stabilize receptor conformations that are distinct from the inactive conformations of the unoccupied receptor, via changes in intramolecular bonds (Jacobson and Costanzi, 2012; Katritch et al., 2013; Rasmussen et al., 2011b; Samama et al., 1993; Venkatakrishnan et al., 2013). It is well-established that modifications of amino terminal residues decrease efficacy of GnRH peptides, converting them to antagonists (Sealfon et al., 1997). As we have previously demonstrated significant receptor reserve in the current GnRH receptor expression system (Zhou et al., 1995), the lower potency of [2-Nal^{3}]-GnRH, compared with GnRH, in spite of its higher binding affinity strongly suggests that the position three-substituted peptide is a partial agonist of the wild type GnRH receptor and lacks a chemical functional group that contributes to induction or stabilization of the active receptor conformation. [2-Nal^{3}]-GnRH showed a smaller decrease in potency at the His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutant GnRH receptors (6 to 20-fold) than did GnRH (150-fold, Table 5), suggesting a less than additive effect of substituting both Trp^{3} and His$^{7.36(305)}$, but interpretation is complicated by the lower affinity of GnRH at the mutant receptors. We therefore investigated [2-Nal^{3}]-GnRH signaling in cells expressing the His$^{7.36(305)}$Asn mutant receptor, which had unchanged affinity for GnRH. In contrast to GnRH, which has decreased potency at the His$^{7.36(305)}$Asn mutant receptor, potency of [2-Nal^{3}]-GnRH was minimally decreased (less than two-fold). Thus, in the absence of Trp^3 of the ligand, mutation of $His^{7.36(305)}$ of the receptor did not disrupt ligand-stimulated receptor activation. This result, combined with the larger loss of GnRH potency than loss of GnRH affinity at all mutant receptors (except for the $His^{7.36(305)}$Arg receptor), suggests that the $His^{7.36(305)}$ side chain has a role in coupling agonist binding to receptor activation and this function depends on Trp^3 in the ligand. Our results therefore suggest that an interaction of the $His^{7.36(305)}$ side chain with TM1 stabilizes a binding pocket configuration that allows the pyrrole ring of Trp^3 of GnRH to interact with the $Phe^{7.39(308)}$ residue, which is part of the consensus GPCR ligand binding pocket (Venkatakrishnan et al., 2013) and with $Asp^{2.61(98)}$ in TM2. A second, ion-dipole, interaction of the $His^{7.36(305)}$ side chain is needed to couple ligand binding to the conserved transmembrane domain network that induces the active GPCR conformation (Deupi et al., 2012b; Venkatakrishnan et al., 2013).

In conclusion, we have confirmed the importance of the $His^{7.36(305)}$ side chain of the GnRH receptor in binding GnRH and shown that it may also have a role in receptor activation. A polar functional group of the side chain is necessary for receptor recognition of the Trp^3 residue of GnRH. The Trp^3 side chain of GnRH and two polar functional groups of the $His^{7.36(305)}$ side chain of the receptor are necessary for full agonist potency. Our results suggest that hydrogen bond interactions of the Trp^3 side chain of GnRH and the $His^{7.36(305)}$ side chain and protonation of the $His^{7.36(305)}$ side chain are needed to couple agonist binding to the conserved GPCR transmembrane triggering mechanism that activates the GnRH receptor.

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Figure legends

FIGURE 1. GnRH stimulated IP production in COS-1 cells expressing wild type and mutant GnRH receptors. COS-1 cells transfected with wild type (●) or His$^{7.36(305)}$Ala (○), His$^{7.36(305)}$Phe (■) or His$^{7.36(305)}$Trp (▼) mutant GnRH receptor constructs (top panel) and wild type (●) or His$^{7.36(305)}$Asn (□), His$^{7.36(305)}$Gln (▲) or His$^{7.36(305)}$Arg (△) mutant GnRH receptor constructs (lower panel) were labeled with ^{3}H-*myo*-inositol and stimulated with increasing concentrations of GnRH before extraction of IP. Data are mean ± range from a single experiment representative of at least 4 independent experiments performed in duplicate. B, basal IP production in the absence of added ligand.

FIGURE 2. Whole cell competition binding of GnRH by wild type and mutant GnRH receptors. COS-1 cells transfected with wild type (●) or His$^{7.36(305)}$Ala (○), His$^{7.36(305)}$Phe (■), His$^{7.36(305)}$Asn (□), His$^{7.36(305)}$Gln (▲), His$^{7.36(305)}$Arg (△) or His$^{7.36(305)}$Trp (▼) mutant GnRH receptor constructs were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH and increasing concentrations of GnRH. Data are mean ± range from a single experiment representative of at least 4 independent experiments performed in duplicate. B0, total ^{125}I-[His^5,D-Tyr^6]-GnRH bound in the absence of competing unlabeled ligand.

FIGURE 3. Membrane binding of [His^5,D-Tyr^6]-GnRH by wild type and mutant GnRH receptors. Cell membranes from COS-1 cells expressing wild type (●) or His$^{7.36(305)}$Ala (○), His$^{7.36(305)}$Phe (■), His$^{7.36(305)}$Asn (□), His$^{7.36(305)}$Gln (▲) or His$^{7.36(305)}$Arg (△) mutant GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH and increasing concentrations of [His^5,D-Tyr^6]-GnRH. Data are mean ± SEM from a single experiment representative of at least 4 independent experiments performed in triplicate. B0, total ^{125}I-[His^5,D-Tyr^6]-GnRH bound in the absence of competing unlabeled ligand.

FIGURE 4. [2-Nal^3]-GnRH-stimulated IP production in cells expressing wild type and mutant GnRH receptors. COS-1 cells transfected with wild type (●) or His$^{7.36(305)}$Ala (○), His$^{7.36(305)}$Phe (■) or His$^{7.36(305)}$Asn (□) mutant GnRH receptor constructs were labelled with ^{3}H-*myo*-inositol and stimulated with increasing concentrations of GnRH (top panel) or [2-Nal^3]-GnRH (lower panel) before extraction of IP. Data are mean ± range from a single experiment representative of 4 independent experiments performed in duplicate. To facilitate comparison of EC_{50} values, data were normalized relative to the lowest (0%) and highest (100%) IP production in each curve (GraphPad Prism). B, basal IP production in the absence of added ligand.

FIGURE 5. Molecular models of wild type and mutant GnRH-GnRH receptor binding complexes. Homology models of the mouse GnRH receptor were built based on the crystal structure of the agonist-bound rNTR1 neurotensin receptor and four restrained interactions with GnRH. (A) Enlarged view of the wild type GnRH receptor bound to native GnRH showing interactions of Trp^3 with Phe$^{7.39(308)}$ and His$^{7.36(305)}$ with carbonyl oxygen atoms of TM1. The carbon, nitrogen and oxygen atoms of the residues are colored gray, blue, and red, respectively. GnRH is colored cyan. The orientation of each transmembrane helix is indicated (TM1 to TM7). The amino-terminus and the first extracellular loop of the receptor are "undisplayed" for clarity. The side chains of His$^{7.36(305)}$ (B), Arg$^{7.36(305)}$ (C), Ala$^{7.36(305)}$ (D), Gln$^{7.36(305)}$ (E) of mutant GnRH receptors are shown in relative to two carbonyl oxygen atoms of the TM1. The 33o and 34o indicate the carbonyl oxygens of Val$^{1.30(33)}$ and Ser$^{1.31(34)}$, respectively.

Table 1. GnRH-stimulated IP production. COS-1 cells transfected with wild type or mutant GnRH receptor constructs were incubated with varying concentrations of GnRH in the presence of LiCl, followed by extraction of IP. Data are means ± SEM of the indicated numbers of experiments performed in duplicate. All experiments included the wild type GnRH receptor and fold change values are the ratio of mutant to wild type receptor EC_{50} values. * Significantly different from the wild type receptor, $p < 0.05$; NMB, no measurable binding

Receptor	**EC_{50}** (nM)	**pEC_{50}**	**Fold Change**	**E_{max}** (% wild type)	**Coupling Coefficient** (%wild type)
Wild type	0.16 ± 0.04 (n = 14)	9.96 ± 0.13		100	100
$His^{7.36(305)}Ala$	24.6 ± 3.7 (n = 8)	7.65 ± 0.07*	152	70.0	1.70
$His^{7.36(305)}Phe$	26.0 ± 3.9 (n = 4)	7.60 ± 0.07*	161	61.7	5.35
$His^{7.36(305)}Asn$	1.07 ± 0.36 (n = 9)	9.19 ± 0.17*	6.6	56.9	11.2
$His^{7.36(305)}Gln$	0.92 ± 0.19 (n = 10)	9.21 ± 0.19*	5.7	58.9	13.4
$His^{7.36(305)}Arg$	0.24 ± 0.04 (n = 9)	9.41 ± 0.27*	1.5	79.3	112
$His^{7.36(305)}Trp$	770 ± 267 (n = 8)	6.59 ± 0.38*	4745	29.4	NMB

Table 2. Whole-cell competition binding of mutant GnRH receptors. COS-1 cells expressing wild type or mutant GnRH receptor receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH and varying concentrations of unlabeled ligands. Data are mean ± SEM for three to eight independent experiments, all of which included the wild type GnRH receptor. * Significantly different from the wild type receptor, $p < 0.05$; B_0, total binding of ^{125}I-[His^5,D-Tyr^6]-GnRH in the absence of unlabeled ligand as % total radioactivity; NMB, no measurable binding.

	GnRH		**[His^5,D-Tyr^6]-GnRH**		**B_0**
Receptor	IC_{50} (nM)	pIC_{50}	IC_{50} (nM)	pIC_{50}	(%)
Wild type	27 ± 4.6 (n = 8)	7. 61 ± 0. 08	8.18 ± 4.8 (n = 7)	8.94 ± 0.31	13.5 ± 5.5
His$^{7.36(305)}$Ala	119 ± 100 (n = 5)	7.30 ± 0.29	54.7 ± 22.0 (n = 3)	7.34 ± 0.18*	3.5 ± 1.6
His$^{7.36(305)}$Phe	1050 ± 24 (n = 3)	6.14 ± 0.26*	146 ± 119 (n = 3)	7.19 ± 0.39*	5 ± 2.5
His$^{7.36(305)}$Asn	46.9 ± 11.7 (n = 5)	7.39 ± 0.13	5.60 ± 2.64 (n = 4)	8.56 ± 0.36	6 ± 2.7
His$^{7.36(305)}$Gln	18 ± 5.7 (n = 4)	7.94 ± 0.31	7.91 ± 3.19 (n = 3)	8.21 ± 0.25	5.5 ± 2.5
His$^{7.36(305)}$Arg	25.7 ± 5.1 (n = 6)	7.67 ± 0.14	5.40 ± 4.10 (n = 3)	8.67 ± 0.47	10.8 ± 4.4
His$^{7.36(305)}$Trp	NMB	NMB	NMB	NMB	NMB

Table 3. Membrane competition binding of mutant GnRH receptors. Cell membranes from COS-1 cells expressing wild type or mutant GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH and varying concentrations of unlabeled ligands. Data are mean ± SEM of the indicated numbers of experiments, all of which included the wild type GnRH receptor and a homologous competition curve, performed in triplicate. * significantly different from the wild type receptor, $p < 0.05$; NMB, no measurable binding.

	GnRH		**[His^5,D-Tyr^6]-GnRH**		**B$_{max}$**
Receptor	IC$_{50}$ (n) (nM)	pIC$_{50}$	IC$_{50}$ (n) (nM)	pIC$_{50}$	(sites/cell x 10^{-5})
wild type	54.8 ± 3.7 (n = 16)	7.29 ± 0.04	3.15 ± 0.40 (n = 29)	8.60 ± 0.06	46.7 ± 6.7
His$^{7.36(305)}$Ala	213 ± 39 (n = 8)	6.73 ± 0.09*	26 ± 7.5 (n = 11)	7.95 ± 0.17*	54.0 ± 13.8
His$^{7.36(305)}$Phe	541±111 (n = 5)	6.30 ± 0.09*	19 ± 9.4 (n = 7)	7.88 ± 0.15*	34.2 ± 8.2
His$^{7.36(305)}$Asn	57 ± 12.3 (n = 5)	7.29 ± 0.10	3.9 ± 1.09 (n = 15)	8.60 ± 0.14	37.5 ± 21.6
His$^{7.36(305)}$Gln	41.8 ± 8.7 (n = 4)	7.41 ± 0.09	2.7 ± 0.59 (n = 8)	8.60 ± 0.08	27.8 ± 9.7
His$^{7.36(305)}$Arg	86.4 ± 31.7 (n = 3)	7.13 ± 0.16	2.2 ± 0.63 (n = 13)	8.80 ± 0.08	34.7 ± 7.6
His$^{7.36(305)}$Trp	NMB		NMB		

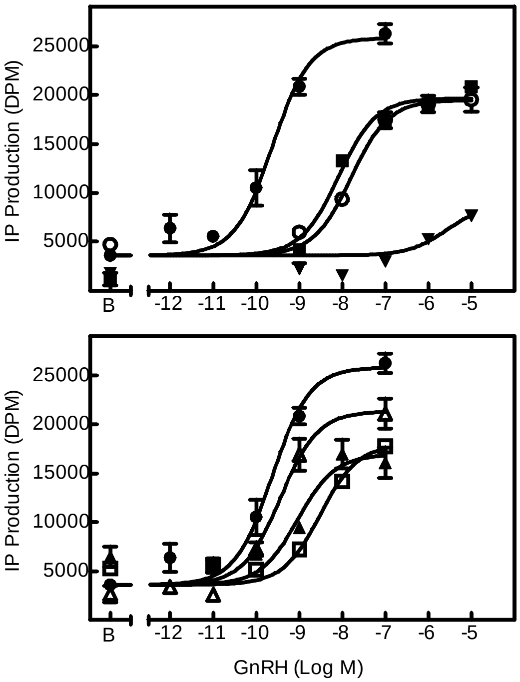
Table 4. Competition binding of GnRH analogs to His$^{7.36(305)}$Ala and His$^{7.36(305)}$Phe mutant GnRH receptors. COS-1 cell membranes expressing wild type or His$^{7.36(305)}$Ala or His$^{7.36(305)}$Phe mutant receptors were incubated with ^{125}I-[His^{5},D-Tyr^{6}]-GnRH in presence of varying concentrations of GnRH analogs. Data are means ± SEM for the indicated numbers of independent experiments (in parenthesis) performed in triplicate. * Significantly different from the wild type receptor, $p< 0.05$

Receptor	**Wild type**		**His$^{7.36(305)}$Ala**		**His$^{7.36(305)}$Phe**	
GnRH peptide	IC$_{50}$ (nM)	pIC$_{50}$	IC$_{50}$ (nM)	pIC$_{50}$	IC$_{50}$ (nM)	pIC$_{50}$
GnRH	54.8 ± 3.7 (16)	7.29 ± 0.04	213 ± 39 (8)	6.73 ± 0.09*	541 ± 111 (5)	6.30 ± 0.09*
[AcGly^{1}]-GnRH	> 1000 (4)	<6	>1000 (4)	<6	<1000 (3)	<6
[Trp^{2}]-GnRH	18.5 ± 4.66 (8)	7.88 ± 0.16	136 ± 39.8 (8)	7.02 ± 0.15*	362 ± 148 (4)	6.57 ± 0.19*
[2-Nal^{3}]-GnRH	1.54 ± 0.32 (4)	8.84 ± 0.10	2.97 ± 1.62 (3)	8.69 ± 0.27	2.99 ± 0.43 (3)	8.5 ± 0.08
[Ala^{4}]-GnRH	>1000 (5)	<6	>1000 (6)	< 6	>1000 (3)	<6
[His^{5},D-Tyr^{6}]-GnRH	3.15 ± 0.40 (29)	8.60 ± 0.06	26 ±7.5 (3)	7.95 ± 0.18*	19 ± 9.4 (7)	7.88± 0.15*
GnRH II	120 ± 11.9 (10)	6.94 ± 0.04	684 ± 143 (7)	6.23 ± 0.11*	1504 ± 638 (5)	6.02 ± 0.21*
[D-Trp^{6},Pro^{9}NHEt]-GnRH	0.186 ± .052 (7)	9.77 ± 0.11	5.04 ± 3.88 (7)	8.60 ± 0.36*	143 ± 102 (4)	7.26 ± 0.51*
[Hyp^{9}]-GnRH	>1000 (7)	< 6	>1000 (4)	< 6	>1000 (4)	< 6

Table 5. GnRH analog-stimulated IP production. COS-1 cells expressing wild type or mutant GnRH receptors were incubated with various concentrations of GnRH analogs in the presence of LiCl before extraction of IP. Data are means ± SEM of four independent experiments performed in duplicate. Fold change is the ratio of mutant and wild type EC_{50} values.

	GnRH		**[His^5,D-Tyr^6]-GnRH**		**[2-Nal^3]-GnRH**	
Receptor	EC_{50} (nM)	fold change	EC_{50} (nM)	fold change	EC_{50} (nM)	fold change
Wild type	0.162 ± 0.04		0.24 ± 0.14		0.49 ± 0.07	
His$^{7.36(305)}$Ala	24.6 ± 3.7	152	35 ± 17.6	145	9.73 ± 3	20
His$^{7.36(305)}$Phe	26 ± 3.9	160	21.7 ± 6.2	90	2.98 ± 0.4	6.0
His$^{7.36(305)}$Asn	1.07 ± 0.36	6.6	1.5 ± 0.27	6.3	0.81 ± 0.14	1.7

Figure 1.



IP Production (DPM)
25000
20000
15000
10000
5000
B
-12
-11
-10
-9
-8
-7
-6
-5
IP Production (DPM)
25000
20000
15000
10000
5000
B
-12
-11
-10
-9
-8
-7
-6
-5
GnRH (Log M)

Figure 2.

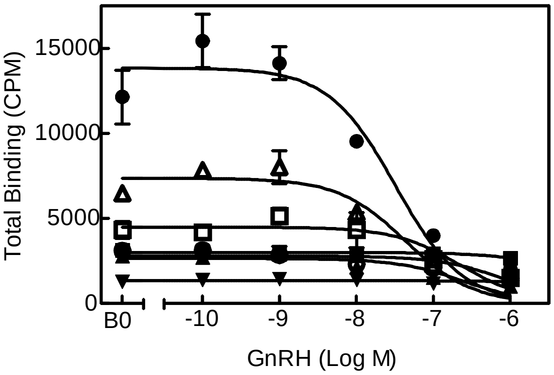


Figure 3.

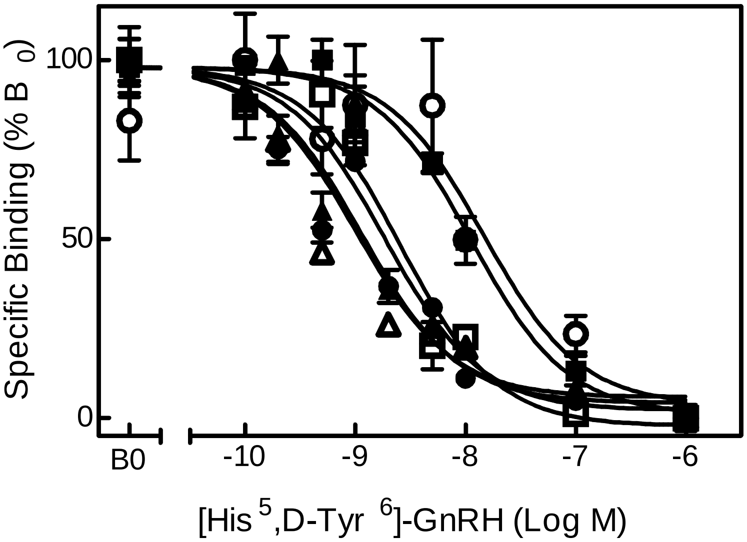


Figure 4.

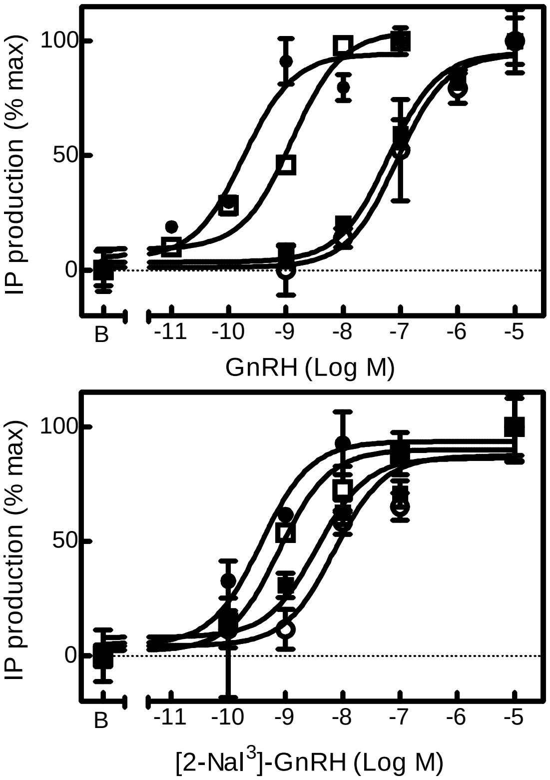
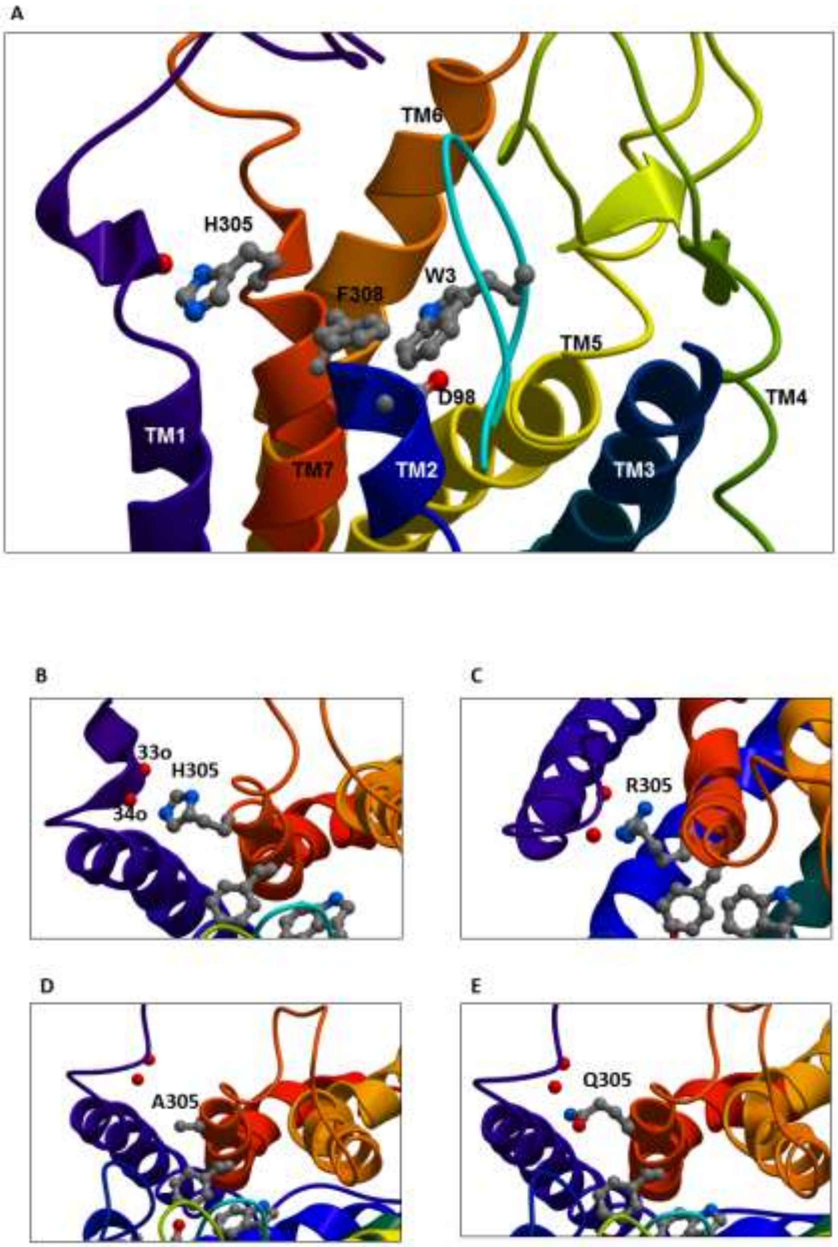

IP production (% max)
100
50
0
B
-11
-10
-9
-8
-7
-6
-5
GnRH (Log M)
IP production (% max)
100
50
0
B
-11
-10
-9
-8
-7
-6
-5
[2-Nal3]-GnRH (Log M)

Figure 5


A
TM6
H305
F308
W3
TM5
D98
TM4
TM1
TM7
TM2
TM3
B
33o
H305
34o
C
R305
D
A305
E
Q305