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# 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.


## 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).

*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.

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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 $Arg^{1.35(38)}$ and $Asn^{2.65(102)}$ residues (Ballesteros and Weinstein receptor residue numbering system, see Section 2 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 $Gly^{10}NH_2$ 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, 2008; Flanagan et al., 1994; Fromme et al., 2001, 2004; 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