Identification of a Novel Ligand Binding Residue Arg$^{38(1.35)}$ in the Human GnRH Receptor

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Molecular Pharmacology Fast Forward. Published on October 17, 2007 as DOI: 10.1124/mol.107.040816
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Running Title: $Arg^{38(1.35)}$ in GnRH receptor binding and activation

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Number of Text Pages: 26

Number of Tables: 2

Number of Figures: 6

Number of References: 40

Number of Words in the Abstract: 250

Number of Words in the Introduction: 656

Number of Words in the Discussion: 1446

ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; IP, inositol phosphates; GPCR, G protein-coupled receptor; TM, transmembrane domain; E_{max}, maximal agonist-elicited inositol phosphate response; IN3, (2*S*)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]- 2-(3,5-dimethylphenyl)-1*H*-indol-3-yl]-*N*-(2-pyridin-4-ylethyl) propan-1-amine; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium.

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ABSTRACT

Delineation of peptide ligand binding sites is of fundamental importance in rational drug design and in understanding ligand-induced receptor activation. Molecular modeling and ligand docking to previously experimentally identified binding sites revealed a putative novel interaction between the C-terminus of gonadotropin-releasing hormone (GnRH) and $Arg^{38(1.35)}$, located at the extracellular end of transmembrane domain (TM) 1 of the human GnRH receptor. Mutation of $Arg^{38(1.35)}$ to Ala resulted in 989- and 1268-fold reduction in affinity for GnRH I and GnRH II, the two endogenous ligands. Conservative mutation of $Arg^{38(1.35)}$ to Lys had less effect, giving reduced affinities of GnRH I and GnRH II by 24- and 54-fold. To test whether $Arg^{38(1.35)}$ interacts with the C-terminal Gly^{10}-NH_2 of GnRH, binding of GnRH analogs with substitution of the C-terminal glycinamide with ethylamide ([Pro^9-NHEt]GnRH) was studied with wild-type and $Arg^{38(1.35)}$ mutant receptors. Mutation of $Arg^{38(1.35)}$ to Lys or Ala had much smaller effect on receptor affinity for [Pro^9-NHEt]GnRH analogs and no effect on binding affinity of peptide antagonist cetrorelix. In parallel with the decreased affinity, the mutants also gave a decreased potency to GnRH-elicited inositol phosphate (IP) responses. The mutant receptors had similar effects on [Pro^9-NHEt]GnRH-elicited IP responses as that of the parent GnRHs. These findings indicate that $Arg^{38(1.35)}$ of the GnRH receptor is essential for high-affinity binding of GnRH agonists and stabilizing the receptor active conformation. The mutagenesis results support the prediction of molecular modeling that $Arg^{38(1.35)}$ interacts with the C-terminal glycinamide and likely forms hydrogen-bonds with the backbone carbonyl of Pro^9 and Gly^{10}-NH_2.

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Introduction

The gonadotropin-releasing hormone (GnRH) receptor is a member of the rhodopsin-like family of 7-transmembrane domain (7-TM) G-protein-coupled receptors (GPCRs). It mediates regulation of GnRH to the reproductive hormonal cascades. In humans there is a single functional type of the receptor (type I receptor) and two types of endogenous ligand, GnRH I and GnRH II, although, some vertebrate species are known to contain as many as three types of functional receptors (Millar et al., 2004). GnRH analogs are extensively used clinically in the treatment of disorders such as reproductive cancers, precocious puberty and endometriosis (Casper, 1991). For well over a decade, $G_{q/11}$ has been known to be the predominant G protein coupled to the mammalian GnRH receptors in various cellular environments (Kaiser et al., 1997; Ruf et al., 2003). Binding of agonist to the GnRH receptor triggers gonadotropin secretion from the pituitary following its coupling to $G_{q/11}$ protein, which activates phospholipase C-β to stimulate turnover of inositol phosphates (IP), leading to the release of Ca^{2+} from intracellular stores and activation of protein kinase C by diacylglycerol. There are also reports that the human GnRH receptor is capable of activating other G protein species such as G_s (Ulloa-Aguirre et al., 1998; Liu et al., 2002) and $G_{i/o}$ (Gründker et al., 2001; Krsmanovic et al., 2003) mediating differential physiological and pharmacological effects of GnRH analogs, such as antiproliferative effects of GnRH analogs in cancer cells (Gründker et al., 2001; Maudsley et al., 2004).

These findings give rise to the potential for development of signal-selective GnRH analogs which preferentially activate one signaling pathway, bypassing others, via

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ligand-induced selective receptor active conformations (Lu et al., 2005, 2007; Millar et al., 2007). The binding of various agonists to GnRH receptor may break intramolecular constraint networks that stabilize the receptor in inactive conformations, creating new sets of inter- and intra-molecular contacts that stabilize the receptor in particular active conformations that affect the downstream signaling selectivity. This concept is supported by our recent finding that GnRH I is more potent than GnRH II in stimulating IP responses, but the reverse is true in stimulating antiproliferative effects (Millar et al., 2007). Consistent with this, mutations of GnRH receptor at loci remote from the ligand binding sites specifically increase binding affinity for GnRH II and GnRHs from other species which possess Arg^{8} substitution, but not GnRH I (Lu et al., 2005, 2007), indicating that GnRH I and GnRH II stabilize different receptor active conformations. In order to fully understand this phenomenon and to assist in development of novel signal-selective GnRH analogs directed at different therapeutic end-points, structural characterization of the ligand binding pocket of signal-selective GnRH analogs is essential.

Using these previously identified contact points between GnRH and the receptor we have performed ligand docking experiments with our previously constructed model of the GnRH receptor (Lu et al., 2007; Millar et al., 2007). The resultant model suggested that $Arg^{38(1.35)}$ of the human GnRH receptor (receptor residues are identified by sequence number of amino acids of the receptor followed by nomenclature of Ballesteros and Weinstein in which the position of the most conserved amino acids in the TM domain N is designated as N.50 in parentheses. This distinguishes receptor residues from GnRH peptide residues labeled with sequence number only) is positioned near to the C-terminus of GnRH and therefore may interact directly with

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the peptide. This residue is located at the extracellular end of TM 1 and is completely conserved amongst all known GnRH receptors (Fig. 1). Using site-directed mutagenesis studies we demonstrate that $Arg^{38(1.35)}$ of the GnRH receptor is important for the binding of both endogenous ligands GnRH I and GnRH II, but less so for [Pro^{9}-NHEt]GnRH derivatives of GnRHs and is not important for the binding of the peptide antagonist, cetrorelix which possesses D-Ala^{10}-NH_2. These data suggest that $Arg^{38(1.35)}$ of the GnRH receptor interacts directly with the C-termini of GnRH I and GnRH II which is important for high affinity binding and consequent receptor activation.

Materials and Methods

Materials. GnRH I (pGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}-NH_2, GnRH II ([His^{5},Trp^{7},Tyr^{8}]GnRH) were purchased from Sigma and Bachem. Cetrorelix (Ac-D-Nap-Ala^{1}-D-ClPh-Ala^{2}-D-Pyr-Ala^{3}-Ser^{4}-Tyr^{5}-D-Cit^{6}-Leu^{7}-Arg^{8}-Pro^{9}-D-Ala^{10}-NH_2), [Pro^{9}-NHEt]GnRH I and [Pro^{9}-NHEt]GnRH II were synthesized as described previously (Mamputha et al., 2007). DeepVent polymerase was from Bio-Lab. EcoRI, BsrGI and XhoI restriction endonucleases and T4 ligase were from Promega. *myo*-D-[^{3}H]inositol was from Amersham Biosciences. IN3, (2S)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl]-N-(2-pyridin-4-ylethyl)propan-1-amine (Janovick et al., 2002) was obtained from Merck.

GnRH Docking and Molecular Dynamics (MD) Simulations. A model of the human GnRH receptor was built by comparative modeling through MODELLER

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within DS Modeling (version 1.6, Accelrys, San Diego) as described previously (Lu et al., 2007; Millar et al., 2007) using the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (PDB code:2I37; Salom et al., 2006) as a template. A βII'-type turn conformation of GnRH I (derived from an NMR structure, PDB code: 1YY1) and of GnRH II was docked into the model according to the previously experimentally identified contact points between GnRH and receptor ($pGlu^{1}$ with $Asn^{212(5.39)}$, His^{2} with $Asp^{98(2.61)}$/$Lys^{121(3.32)}$, and Tyr^{5}/His^{5} with $Tyr^{290(6.58)}$, and $Gly^{10}NH_2$ with $Asn^{102(2.65)}$; Millar et al., 2004, 2007; Coetsee et al., 2007; Mamputha et al., 2007; Fig. 2). The GnRH-receptor complex was then optimized by energy-minimization and MD simulations of 150 ps by the means of the CHARMM program (Brooks et al., 1983) using a similar setup as described for the oxytoxin receptor (Favre et al., 2005) with harmonic restraints on the receptor backbone atoms except for extracellular loop 2 and its covalently linked N-terminal domain (Millar et al., 2007).

Site-Directed Mutagenesis and Receptor Expression. The GnRH receptor was cloned into the pcDNA1 expression vector. Mutant sequences were constructed using a PCR method (Lu et al., 1997). Wild-type and mutant receptors were transiently expressed in COS-7 cells by transfection using a Bio-Rad Gene Pulsar at 230 V, 960 μF with 15 μg of DNA/0.4 cm cuvette (1.5×10^7 cells; 0.7 ml). After transfection, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics and 2 mM glutamine (complete DMEM) in the absence or presence of 1 μM IN3 (a membrane-permeant, non-peptide GnRH receptor antagonist) (Lu et al., 2005, 2007). Cells were washed four times, each wash lasted for 30 min, with 2% Me_2SO, 0.1% BSA/HEPES/DMEM at 37 ºC after a 48-h of

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incubation. The cells were then continued to be incubated with complete DMEM overnight (~18 h) and were washed again as above prior to assays.

Ligand Binding Assays. Radioligand binding assays were performed on intact cells 72 h after transfection (Lu et al., 2005, 2007). Transfected cells in 12-well culture plates were washed as above and then incubated with ^{125}I-cetrorelix (Hoffmann et al., 2000) at 1×10^5 cpm/well and various concentrations of unlabelled GnRH ligands in 0.1% BSA/HEPES/DMEM for 4 h at 4 ºC. After incubation cells were washed twice with ice-cold phosphate-buffered saline (pH 7.4) and solubilized in 0.5 ml of 0.1 M NaOH. Radioactivity was counted by γ-spectrometry. All experiments were performed in triplicate and repeated at least three times.

IP Accumulation Assays. Assays for ligand stimulation of IP production were carried out as described previously (Lu et al., 2005, 2007). Transfected cells were seeded onto 12-well plates in the absence or presence of 1 μM concentration of IN3. After 48 h, cells were washed as above and labeled overnight with 1 μCi/ml *myo*-D-[^{3}H]inositol in inositol-free DMEM containing 1% dialyzed fetal calf serum. Before conducting IP assay, the medium was removed, and cells were washed again as above. Cells, were then preincubated with 0.5 ml of buffer A (140 mM NaCl, 20 mM Hepes, 8 mM glucose, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml BSA) containing 10 mM LiCl at 37 ºC for 30 min, followed by addition of GnRH peptides for an additional 30 min. This was shown to be within the linear period of the assay. The stimulation was terminated by the removal of the medium and addition of 10 mM formic acid. The ^{3}H-labelled IPs were isolated from the formic acid extracts using

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Dowex AG 1-X8 ion exchange resin, collected with 1 M ammonium formate/0.1 M formic acid, and quantified by liquid scintillation counting.

Data Analysis. Binding curves were fitted to the Hill equation or to the one-site model of the binding using Sigmaplot 9.0 (SPSS) or GraphPad Prism 4.0, yielding an IC_{50} value. The maximum receptor binding sites (B_{max}) were expressed relative to a wild-type control included in each transfection. IP dose response curves were fitted to a sigmoidal dose response model, yielding a basal activity, a maximum response (E_{max}), an EC_{50} value.

RESULTS

GnRH Docking and Molecular Dynamics (MD) Simulations. We performed GnRH docking experiments using our previously constructed model of the human GnRH receptor (Lu et al., 2007; Millar et al., 2007) which was built upon the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (Salom et al., 2006). A βII' conformation of GnRH I and GnRH II was satisfactorily docked into the model according to the previously experimentally identified intermolecular interactions between GnRH and receptor followed by energy-minimization and MD simulations (Favre et al., 2005; Millar et al., 2007) (Fig. 2). In our model, $Arg^{38(1.35)}$ of the GnRH receptor is located in close proximity (ca. 2-3 Å) to Pro^{9} and Gly^{10}-NH_2 of GnRH and may therefore bind directly to the ligand. We have validated the proposed interactions by examining whether mutations of $Arg^{38(1.35)}$ of the receptor affects binding of native GnRHs and their analogs possessing C-terminal modification.

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Expression of Human GnRH Receptors in COS-7 Cells. Wild-type and mutant GnRH receptor constructs were transiently transfected into COS-7 cells and the binding affinity of cetrorelix to each was measured by homologous competition assay. Ala and Lys mutations of $Arg^{38(1.35)}$ of the receptor decreased B_{max} to 25% and 30% of wild type, respectively (Fig. 3A). Expression levels of mutant receptors were moderately increased by preincubation of the transfected COS-7 cells with 1 μM of the membrane-permeant, non-peptide GnRH antagonist, IN3, measured after extensive washes of the cells with 2% Me_2SO, which allowed the removal of IN3 from the cells (Lu et al., 2005, 2007). The receptor expression of the poorly expressed mutant receptors were rescued typically between 15-30% by IN3 pretreatment. No changes in affinity for cetrorelix between IN3 pretreated and untreated cells were observed for wild-type or mutant receptors. Also, neither mutation had any significant effect upon the affinity of the receptor for cetrorelix (Fig. 3B).

Differential Effects of Mutations of $Arg^{38(1.35)}$ of the Receptor on Binding Affinities for GnRH I, GnRH II and their Pro^9-NHEt Analogs. To examine roles of the side chain of $Arg^{38(1.35)}$ of the GnRH receptor in ligand binding, we examined effects of mutation of $Arg^{38(1.35)}$ to Ala and Lys on receptor binding affinity for GnRH I and GnRH II and their Pro^9-NHEt analogs (Fig. 4). Binding curves for each analog are shown in Fig. 5 and the IC_{50} values are summarized in Table 1. The Hill coefficients were unaltered for all mutants. The mutation R38A had a much greater effect upon ligand binding affinity than that of the R38K mutant. GnRH I and GnRH II exhibited IC_{50} values of 3.97 nM and 13.4 nM, respectively to wild-type human GnRH receptor. GnRH I exhibited a 989-fold reduction in affinity toward the R38A

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mutant and a 24-fold reduction in affinity toward the R38K mutant relative to wild-type receptor. Similar to GnRH I, the R38A and R38K mutants gave 1268- and 54-fold reductions in affinity toward GnRH II, respectively. Both mutations of the receptor, however, had much less of an effect upon binding affinity of the Pro^{9}-NHEt analogs. The R38A and R38K mutants only had 35- and 5-fold reductions in affinity for GnRH I analog with substitution of the C-terminal glycinamide by an ethylamine group. The mutations R38A and R38K had similar effect on GnRH II analog with the same substitution of GnRH I, giving reduced affinities by 40- and 11-fold.

Effect of Mutations on GnRH Ligand-Induced IP Turnover. GnRH I, GnRH II, [Pro^{9}-NHEt]GnRH I and [Pro^{9}-NHEt]GnRH II elicited robust IP responses from COS-7 cells transfected with the wild-type human GnRH receptor. The E_{max} for all experiments were typically >5 times the basal activity. The effects of mutation of $Arg^{38(1.35)}$ of the receptor to Ala and Lys on the IP responses are shown in Fig. 6. The EC_{50} and E_{max} values are summarized in Table 2. The mutation R38A had a much greater effect upon IP response than that of the R38K mutant. GnRH I and GnRH II exhibited EC_{50} values of 0.87 nM and 26 nM, respectively in COS-7 cells transfected with wild-type GnRH receptor. Mutation of $Arg^{38(1.35)}$ of the receptor to Ala and Lys led to 6520- and 146-fold reductions respectively in potency of GnRH I (EC_{50}) in stimulating IP responses. There was also a reduction in potency of GnRH II in the mutations of $Arg^{38(1.35)}$ to Ala (656-fold reduction) and Lys (76-fold reduction). Interestingly, mutations of $Arg^{38(1.35)}$ of the receptor to Ala and Lys had similar reductions (< 3-fold differences) on potency of [Pro^{9}-NHEt]GnRH I (2405-fold for R38A and 77-fold for R38K) and [Pro^{9}NHEt]GnRH II (374-fold for R38A and 65-fold for R38K) as to GnRH I and GnRH II, although the mutants have significantly

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differential effects on binding affinity for GnRHs and their Pro^{9}-NHEt analogs, the latter exhibited a much smaller decrease toward the mutations. The E_{max} values for all peptide agonists were reduced by ~50% relative to wild-type with the R38A mutant and were little affected by the mutation R38K (Table 2). No increase in basal activity was observed in both mutants R38A and R38K.

DISCUSSION

GPCRs recognize and bind a variety of structurally diverse ligands and modulate majority of physiological processes, and thus are major drug targets. Molecular modeling and site-directed mutagenesis studies have been extensively applied to delineate ligand binding sites in the GnRH receptors (Sealfon et al., 1997; Millar et al., 2004, 2007) and other GPCRs (Lu et al., 2002; Ballesteros et al., 2001). Previous studies have shown that $pGlu^{1}$ of GnRH I interacts with $Asn^{212(5.39)}$ (Hoffmann et al., 2000, Hövelmann et al., 2002, Söderhäll et al., 2005); His^{2} interacts with $Asp^{98(2.61)}$ and $Lys^{121(3.32)}$ (Flanagan et al., 2000; Zhou et al., 1995); Tyr^{5} interacts with $Tyr^{290(6.58)}$ (Coetsee et al., 2007); Arg^{8} of GnRH I interacts with $Asp^{302(7.32)}$ (Fromme et al., 2001) and $Gly^{10}NH_2$ with $Asn^{102(2.65)}$ (Davidson et al., 1996; Hoffmann et al., 2000).

We have successfully docked a βII'-type turn conformation of GnRH derived from a three-dimensional structure of GnRH based on a recent NMR report into the experimentally identified ligand binding sites of the receptor model (Fig. 2). This reveals that $Arg^{38(1.35)}$ of the GnRH receptor may act as a potential binding site for GnRH. $Arg^{38(1.35)}$ of the GnRH receptor is completely conserved in all vertebrate type

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I, II and III GnRH receptors (Fig. 1), implying its functional importance in receptor folding, ligand binding or activation. Mutation of $Arg^{38(1.35)}$ of the GnRH receptor to Ala or Lys markedly reduced receptor binding affinities for GnRH I and GnRH II. The mutation R38A led to 989- and 1268-fold reduction in affinity for both GnRH I and GnRH II as compared with wild-type receptor (Table 1 and Fig. 5). Conservative mutation of $Arg^{38(1.35)}$ to Lys had a lesser effect on the receptor binding affinities for both GnRH I and GnRH II, giving 24- and 54-fold reductions, respectively (Table 1 and Fig. 5). The much smaller effect of mutation to Lys than Ala which deletes the side chain beyond β–carbon, suggests that the side chain of $Arg^{38(1.35)}$ of the GnRH receptor makes multiple contacts with GnRH by forming hydrogen-bond networks and Van der Waals contacts (Fig. 2). Substitution of $Arg^{38(1.35)}$ of the receptor with Lys appears to maintain part of the GnRH receptor-ligand interactions. Mutations of $Arg^{38(1.35)}$ of the receptor to Ala and Lys had much less effect on receptor binding affinity for Pro^{9}-NHEt analogs, with affinity reductions for [Pro^{9}-NHEt]GnRH I of 35- and 5-fold, and for [Pro^{9}-NHEt]GnRH II of 40- and 11-fold (Table 1 and Fig. 5). These data suggest that the side chain of $Arg^{38(1.35)}$ of the receptor is crucial for high affinity binding of GnRH I and GnRH II which contain a C-terminal glycinamide moiety, but is relatively less important for binding of GnRH analogs with glycinamide substitution by an ethylamide group. The much smaller effect of the mutations on ligand binding affinity for [Pro^{9}-NHEt]GnRH analogs than the parent GnRHs indicates that the ethylamide moiety of [Pro^{9}-NHEt]GnRHs may make hydrophobic contacts with some other sites in the receptor which compensate for the loss of interactions between glycinamide of GnRHs and $Arg^{38(1.35)}$ of the receptor. Similarly there was no reduction of binding affinity of the mutants for the peptide antagonist cetrorelix which posseses D-Ala^{10}-NH_2. Consistent with the molecular modeling,

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these results suggest that the side-chain of $Arg^{38(1.35)}$ in the GnRH receptors make direct contacts with the glycinamide moiety of GnRH I and GnRH II. Our molecular docking shows that $Arg^{38(1.35)}$ of the human GnRH receptor, whose side chain is positioned above the C-terminal end of GnRH with potential Van der Waal's contacts along the side chain, may form an additional H-bond with the backbone carbonyl oxygen of Pro^{9}. This may explain why mutations of $Arg^{38(1.35)}$ of the receptor had small effects on receptor binding affinity for [Pro^{9}-NHEt]GnRH analogs. Ligand docking experiments performed with GnRH II on the human GnRH receptor model also suggested that GnRH II is likely to interact with $Arg^{38(1.35)}$ of the receptor in a similar manner as GnRH I which is supported by the mutagenesis studies.

Studies on other peptide GPCRs have also shown that the extracellular end of TM 1 is important for high affinity binding of peptide agonists (Hawtin et al., 2005; Wesley et al., 2002; Silvente-Poirot et al., 1998; Anders et al., 1999; Marco et al., 2007). Mutation of the residue $Glu^{1.35}$ of the V_{1a} vasopressin receptor (which is positionally equivalent to $Arg^{38(1.35)}$ and is totally conserved among vasopressin and oxytocin receptors), to Ala leads to a 1700-fold decrease in affinity for peptide agonist vasopressin, but has no effect on peptide antagonist binding affinity (Hawtin et al., 2005). The equivalent residue $Arg^{1.35}$ in the cholecystokinin-2 receptor has also been shown to be important for peptide ligand binding (Silvente-Poirot et al., 1998; Marco et al., 2007). Direct evidence on the role of the extracellular end of TM 1 in peptide agonist binding was obtained via the covalent linking experiment in which a photoreactive tritiated analog of sulfated cholecystokinin octapeptide was covalently attached to the exofacial sequences of TM 1 (Anders et al., 1999). Together with our

studies, we propose that the extracellular end of TM 1 of peptide GPCRs may play a common role for peptide agonist binding.

In parallel with the reduced receptor binding affinity and expression levels, the receptor mutants R38A and R38K also gave markedly decreased potencies in mediating IP responses with increased EC_{50} values for GnRH I by 6520- and 146-fold, for GnRH II by 656- and 76-fold (Table 2 and Fig. 6). The mutations had similar effect on IP responses elicited by Pro^{9}-NHEt analogs as that of the parent GnRHs (less than 3-fold differences, Table 2 and Fig. 6), although they had much less effect on the binding affinity of [Pro^{9}-NHEt]GnRHs than GnRHs (Table 1 and Fig. 5). Mutation of $Arg^{38(1.35)}$ of the receptor to Ala also resulted in about 50% reduction in maximum IP responses for both GnRH and [Pro^{9}NHEt]GnRH analogs, whilst the receptor mutation R38K had no significant effect on E_{max} (Table 2 and Fig. 6). These results indicate that $Arg^{38(1.35)}$ of the receptor plays an important role in stabilizing the receptor active conformation through forming a new set of inter- and intra-molecular interactions (Hulme et al., 1999). This is in agreement with previous report suggesting that the N-terminal and C-terminal domains are important in receptor binding and activation (Sealfon et al., 1997). The reduction in receptor expression levels caused by mutations of $Arg^{38(1.35)}$ to Ala and Lys, which were moderately increased by IN3 preincubation, suggests that the side chain of $Arg^{38(1.35)}$ may form intramolecular interactions that stabilize receptor folding (Lu et al., 1997). When these interactions are disrupted, incorrect folding of the receptor protein increases, resulting in increased degradation (Lu and Hulme, 1999). The side chain of $Glu^{1.35}$ in the human V_2 receptor (equivalent to $Arg^{38(1.35)}$ of the GnRH receptor) has been shown to make intramolecular contacts with $Gln^{2.61}$ and $Lys^{2.65}$ in TM 2, but this is not the case in the murine V_2 receptor in

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which $Glu^{1.35}$ is proposed to interact with $Arg^{7.32}$ in TM 7 (Oksche et al., 2002). Apparently the interactions depend on the local environments. The extracellular ends of TM 1 and TM 7 are also shown in proximity in opioid receptors (Xu et al., 2005). In our molecular model, the side chain of $Arg^{38(1.35)}$ of the GnRH receptor is located in a close proximity with the residues $Asp^{98(2.61)}$, $Trp^{101(2.64)}$ and $Asn^{102(2.65)}$ in TM 2, and $Asp^{302(7.32)}$ and $His^{306(7.36)}$ in TM 7 and is able to make a H-bond network. However, no constitutive activation was observed in both receptor mutants R38A and R38K. This is consistent with our previous proposal that the GnRH receptor might be strongly constrained in the inactive state because none of the mutations of the equivalent residues of other GPCRs whose mutation leads to constitutive activity (Lu et al., 2002; Smit et al., 2007) gives rise to constitutive activity in the GnRH receptor (Lu et al., 2005, 2007). We therefore propose that $Arg^{38(1.35)}$ of the GnRH receptor may participate with other residues as a ligand-dependent receptor activation switch.

In summary, we have shown that the side chain of $Arg^{38(1.35)}$ of the GnRH receptor may act as a direct binding site for both endogenous ligands GnRH I and GnRH II. Molecular modeling and site-direct mutagenesis studies in combination with ligand modification suggest that $Arg^{38(1.35)}$ of the GnRH receptor interacts directly with the backbone carbonyl oxygen of Pro^{9} and C-terminal glycinamide in both GnRH I and GnRH II. The reduced receptor expression levels and signalling potency given by mutation of $Arg^{38(1.35)}$ to Ala or Lys suggest that $Arg^{38(1.35)}$ may make intramolecular interactions which stabilise the receptor in the ground state, but are broken by ligand binding, creating a new set of inter- and intra-molecular interactions that stabilize receptor active conformations.

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Acknowledgments

We thank Ryan Gallagher and Nicola Miller for technical support.

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Molecular Pharmacology Fast Forward. Published on October 17, 2007 as DOI: 10.1124/mol.107.040816
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Footnotes

This work was supported by the Medical Research Council, UK and Ardana Biosciences.

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Figure legends

Fig. 1. ClustalW alignment of sequences of TM 1 from vertebrate GnRH type I, II and III receptors. $Arg^{38(1.35)}$ in human GnRH receptor and the corresponding residue in other GnRH receptors from different species is shown by the black box. "*****" indicates identical or conserved residues in all sequences in the alignment, "**:**" indicates conserved substitutions and "**.**" indicates semi-conserved substitutions.

Fig. 2. Molecular model of human receptor-GnRH I complex. A βII′ conformation of GnRH I derived from the NMR structure (1YY1) was docked into the receptor model according to the experimentally identified intermolecular interactions between GnRH I (cyan/black) and the receptor contact sites (yellow/magenta), i.e. $pGlu^{1}$ interacts with $Asn^{212(5.39)}$, His^{2} with $Lys^{121(3.32)}$/$Asp^{98(2.61)}$, Tyr^{5} with $Tyr^{290(6.58)}$ and Arg^{8} with $Asp^{302(7.32)}$ and Pro^{9}-$Gly^{10}NH_2$ with $Trp^{101(2.64)}$/$Asn^{102(2.65)}$. The H-bonds are indicated by dashed lines. The model shows that the side-chain of $Arg^{38(1.35)}$ in TM (green) 1 of the receptor is capable of making H-bonds with the carbonyl oxygen of Pro^{9} and $Gly^{10}NH_2$ of GnRH, in addition to the intramolecular interactions with $Trp^{101(2.64)}$ and $Asn^{102(2.65)}$ in TM 2 and $Asp^{302(7.32)}$ and $His^{306(7.36)}$ in TM 7.

Fig. 3. Homologous inhibition binding of cetrorelix. A, binding curves showing homologous competitive binding of peptide antagonist cetrorelix in wild-type and mutant receptors in IN3 pretreated (dashed line) and non-pretreated (solid line) cells. Details are given in Materials and Methods. Results are representative experiments, which were repeated at least three times with essentially the same results. B, normalized binding curves which show no significant differences in receptor affinity

for cetrorelix between the IN3-pretreated and the untreated cells in wild-type and mutant receptors. ●, wild type; ○, wild type with IN3 pretreatment; ■, R38A; □, R38A with IN3 pretreatment; ▲, R38K; △, R38K with IN3 pretreatment. Points, mean ± standard error of triplicate measurements.

Fig. 4. A, primary structures of GnRH peptides used in this study. B, the chemical structures of the C-termini of GnRH I, GnRH II and their Pro^9-NHEt derivatives.

Fig. 5. Binding of GnRH peptides. A, inhibition curves of GnRH I (solid line) and [Pro^9-NHEt]GnRH I (dashed line) in human GnRH wild-type and R38A and R38K mutant receptors. B, inhibition curves of GnRH II (solid line) and [Pro^9-NHEt]GnRH II (dashed line) in human GnRH wild-type and R38A and R38K mutant receptors. The radiolabeled ligand used in the assays is ^{125}I-cetrorelix. ●, wild-type with GnRH I/GnRH II; ○, wild-type with Pro^9-NHEt analogs; ■, R38A with GnRH I/GnRH II; □, R38A with Pro^9-NHEt analogs; ▲, R38K with GnRH I/GnRH II; △, R38K with Pro^9-NHEt analogs.

Fig. 6. GnRH peptide stimulated increases of total IP in COS-7 cells transiently expressing the wild-type and mutant receptors. A, GnRH I- (solid line) and [Pro^9-NHEt]GnRH I- (dashed line) elicited IP responses in human GnRH wild-type, R38A and R38K mutant receptors. B, GnRH II- (solid line) and [Pro^9-NHEt]GnRH II- (dashed line) elicited IP responses in wild-type, R38A and R38K mutant receptors. ●, wild-type with GnRH I/GnRH II, ○, wild-type with Pro^9-NHEt analogs; ■, R38A with GnRH I/GnRH II; □, R38A with Pro^9-NHEt analogs; ▲, R38K with GnRH I/GnRH II; △, R38K with Pro^9-NHEt analogs.

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TABLES

TABLE 1.

Binding of GnRH peptides to human wild-type and mutant receptors

The IC_{50} values were measured by inhibition of ^{125}I-cetrorelix binding by increasing concentrations of unlabeled GnRH peptides and are expressed as the mean ± standard error of three or more experiments performed in triplicates. Fold-increases in IC_{50} values of R38A and R38K mutants are expressed relative to those measured for the wild-type receptor.

	Wild-type	R38A		R38K	
Peptide	IC_{50}	IC_{50}	Fold-increase	IC_{50}	Fold-increase
	nM	*nM*		*nM*	
GnRH I	3.97 ± 0.25	3530 ± 233	989	95.2 ± 8.0	24
[Pro^9-NHEt]GnRH I	2.34 ± 0.08	82 ± 11	35	11.9 ± 3.0	5
GnRH II	13.4 ± 1.1	17000 ± 1681	1268	721 ± 33.3	54
[Pro^9-NHEt]GnRH II	6.43 ± 0.22	255 ± 35	40	72.4 ± 20.2	11

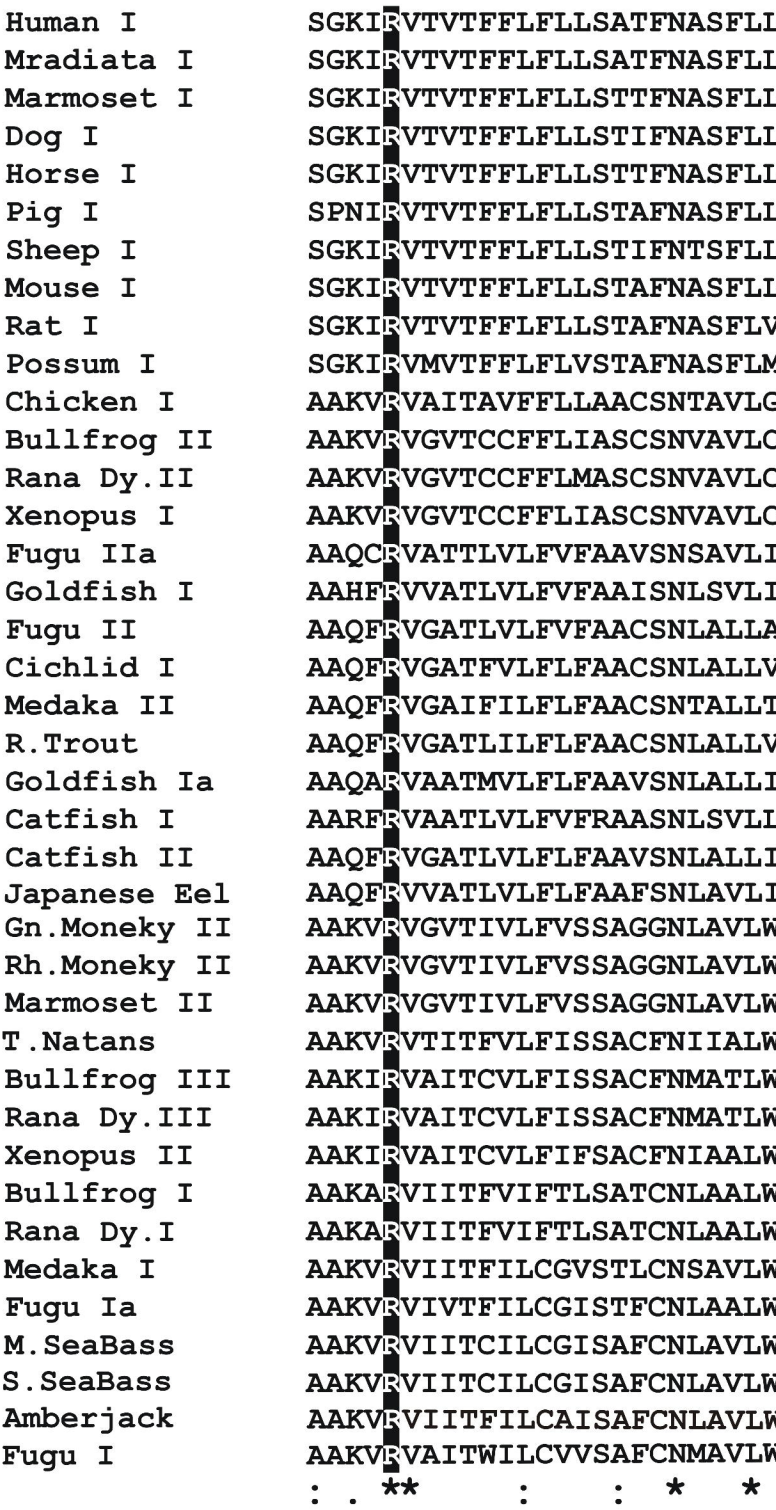
TABLE 2.

Effect of mutations of $Arg^{38(1.35)}$ of the receptor to Ala and Lys on the GnRH peptide-stimulated IP responses

EC_{50} and E_{max} values are presented as mean ± standard errors of three or more independent experiments. The fold-change was calculated as the ratio of the EC_{50} values in the mutant and wild-type receptors. E_{max} is expressed relative to a wild-type control included in each transfection.

	Wild-type		R38A			R38K		
	EC_{50}	E_{max}	EC_{50}	EC_{50} fold-increase	E_{max}	EC_{50}	EC_{50} fold-increase	E_{max}
	(nM)	(% Wt)	(nM)		(% Wt)	(nM)		(% Wt)
GnRH I	0.87 ± 0.06	(100)	5672 ± 302	6520	42 ± 4	129 ± 9	146	100 ± 9
[Pro^9,NHEt]GnRH I	0.18 ± 0.01	(100)	433 ± 21	2405	56 ± 3	14 ± 2	77	91 ± 6
GnRH II	26 ± 3	(100)	17070 ± 690	656	41 ± 5	1978 ± 334	76	104 ± 7
[Pro^9,NHEt]GnRH II	1.9 ± 0.15	(100)	711 ± 62	374	58 ± 3	125 ± 12	65	96 ± 4

Fig. 1.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Horse I SGKIRVTVTFFLFLLSTTFNASFLL
Pig I SPNIRVTVTFFLFLLSTAFNASFLL
Sheep I SGKIRVTVTFFLFLLSTIFNTSFLL
Mouse I SGKIRVTVTFFLFLLSTAFNASFLL
Rat I SGKIRVTVTFFLFLLSTAFNASFLV
Possum I SGKIRVMVTFFLFLVSTAFNASFLM
Chicken I AAKVRVAITAVFFLLAACSNTAVLG
Bullfrog II AAKVRVGVTCCFFLIASCSNVAVLC
Rana Dy.II AAKVRVGVTCCFFLMASCSNVAVLC
Xenopus I AAKVRVGVTCCFFLIASCSNVAVLC
Fugu IIa AAQCRVATTLVLFVFAAVSNSAVLI
Goldfish I AAHFRVVATLVLFVFAAISNLSVLI
Fugu II AAQFRVGATLVLFVFAACSNLALLA
Cichlid I AAQFRVGATFVLFLFAACSNLALLV
Medaka II AAQFRVGAIFILFLFAACSNTALLT
R.Trout AAQFRVGATLILFLFAACSNLALLV
Goldfish Ia AAQARVAATMVLFLFAAVSNLALLI
Catfish I AARFRVAATLVLFVFRAASNLSVLL
Catfish II AAQFRVGATLVLFLFAAVSNLALLI
Japanese Eel AAQFRVVATLVLFLFAAFSNLAVLI
Gn.Moneky II AAKVRVGVTIVLFVSSAGGNLAVLW
Rh.Moneky II AAKVRVGVTIVLFVSSAGGNLAVLW
Marmoset II AAKVRVGVTIVLFVSSAGGNLAVLW
T.Natans AAKVRVTITFVLFISSACFNIIALW
Bullfrog III AAKIRVAITCVLFISSACFNMATLW
Rana Dy.III AAKIRVAITCVLFISSACFNMATLW
Xenopus II AAKIRVAITCVLFIFSACFNIAALW
Bullfrog I AAKARVIITFVIFTLSATCNLAALW
Rana Dy.I AAKARVIITFVIFTLSATCNLAALW
Medaka I AAKVRVIITFILCGVSTLCNSAVLW
Fugu Ia AAKVRVIVTFILCGISTFCNLAALW
M.SeaBass AAKVRVIITCILCGISAFCNLAVLW
S.SeaBass AAKVRVIITCILCGISAFCNLAVLW
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Fugu I AAKVRVAITWILCVVSAFCNMAVLW
: . ** : : * *

Fig. 2.

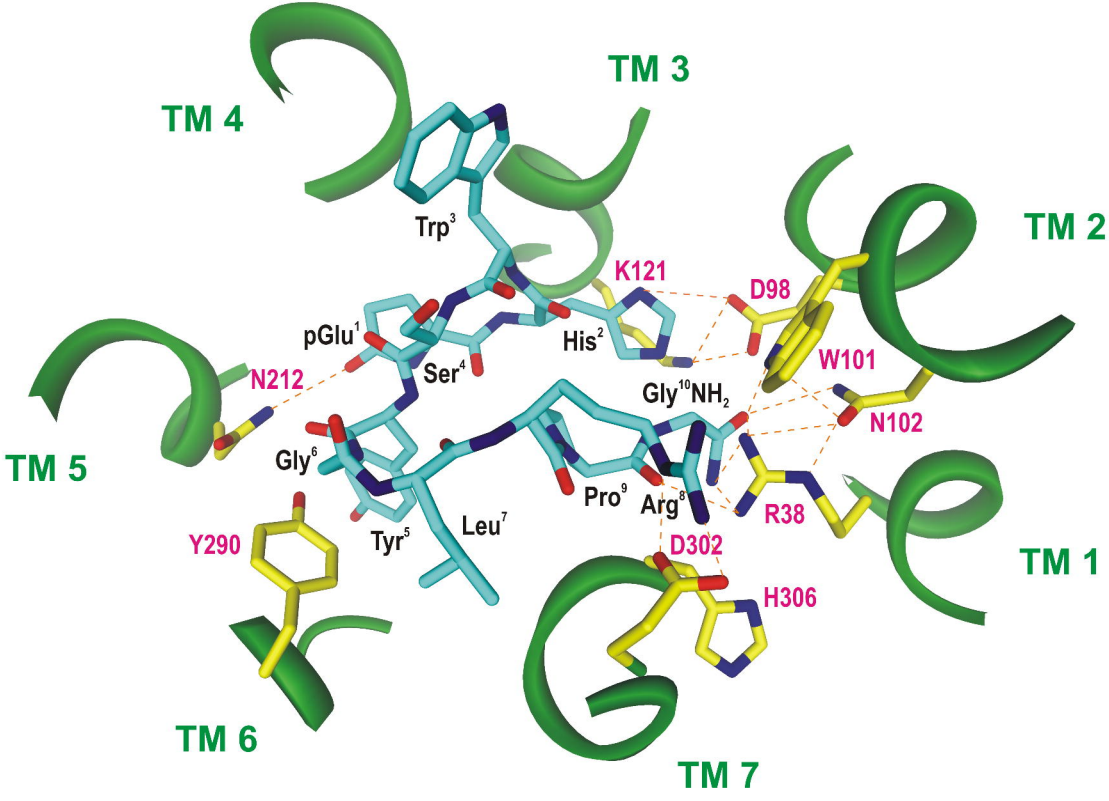
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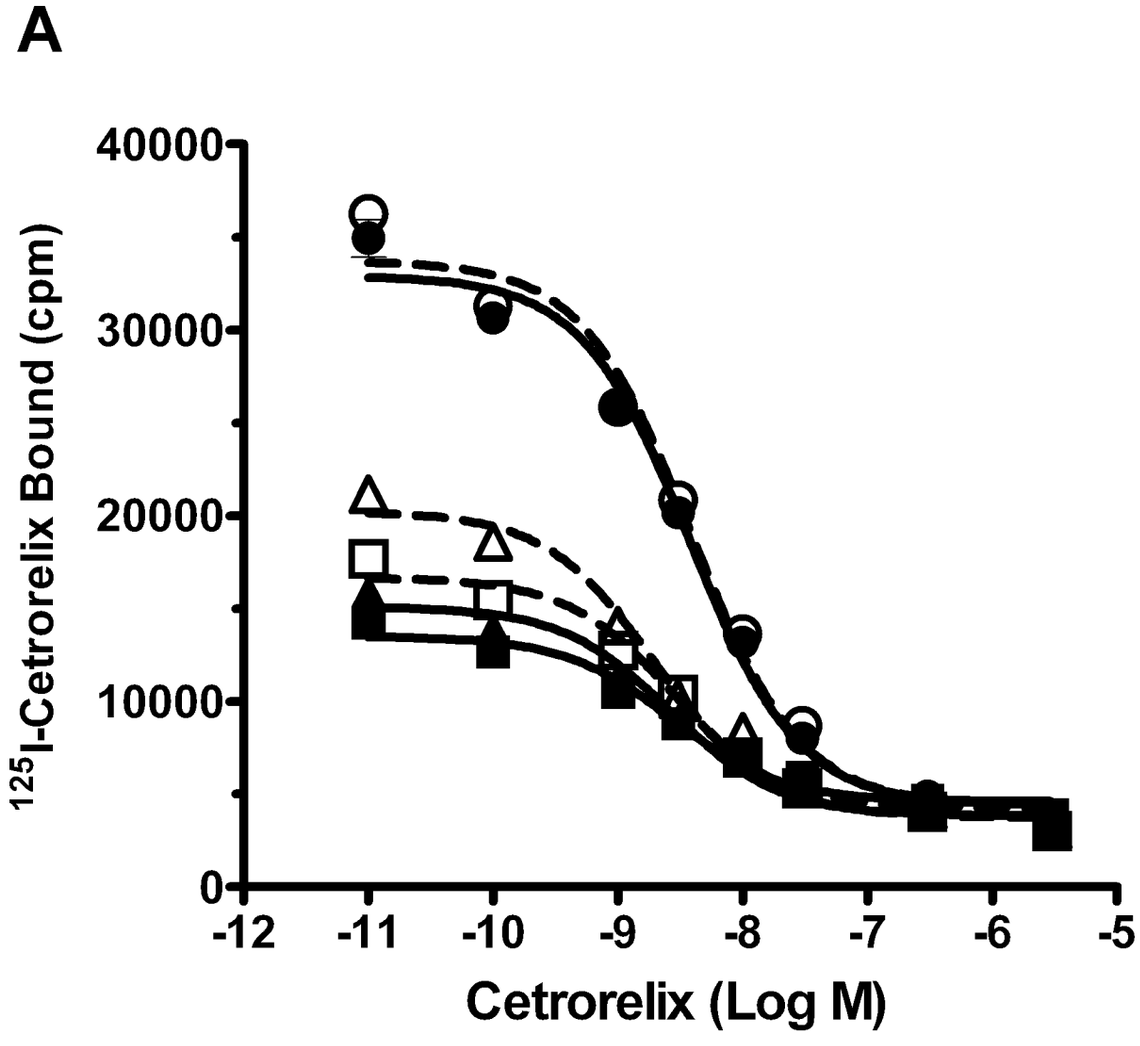
Fig. 3.



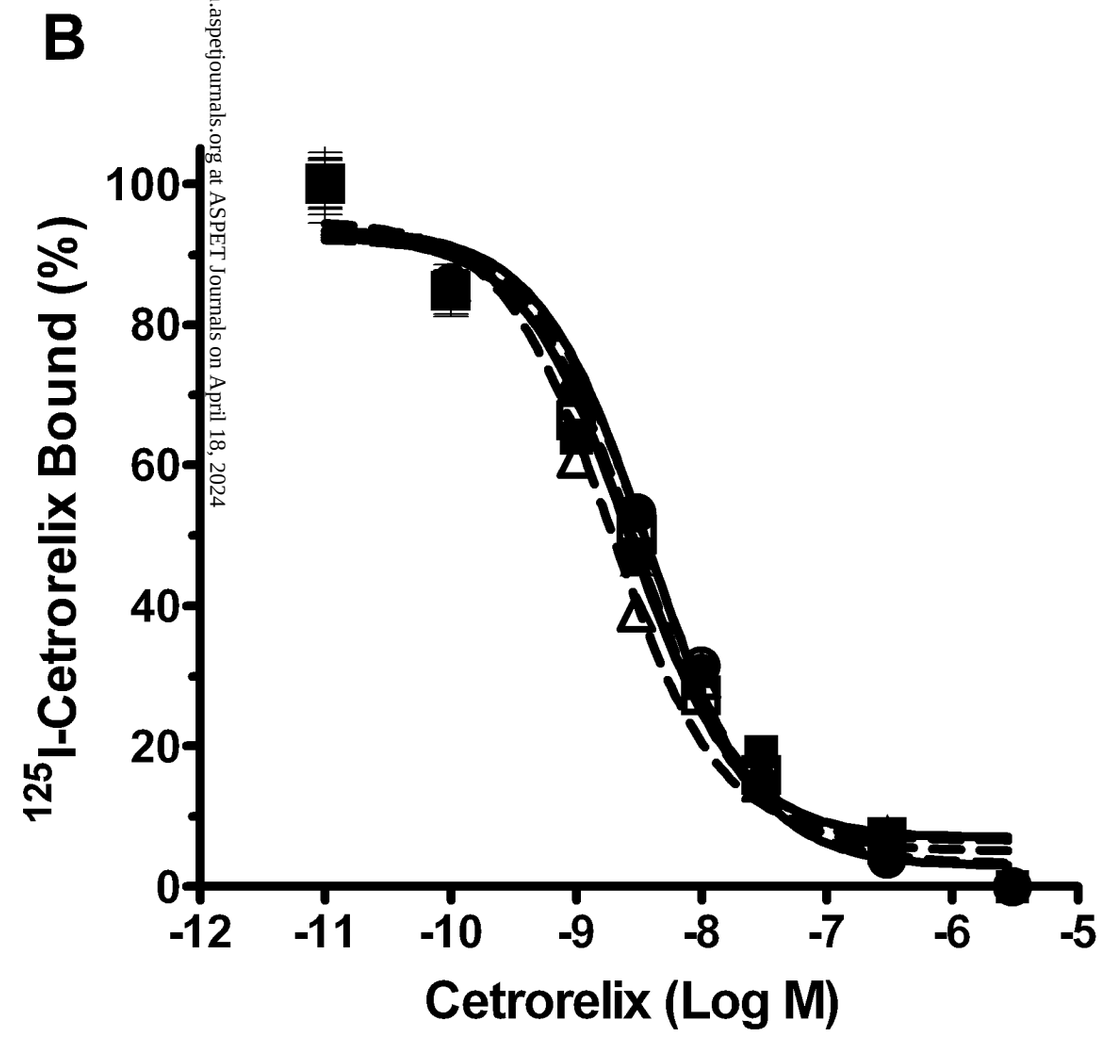


Fig. 4.

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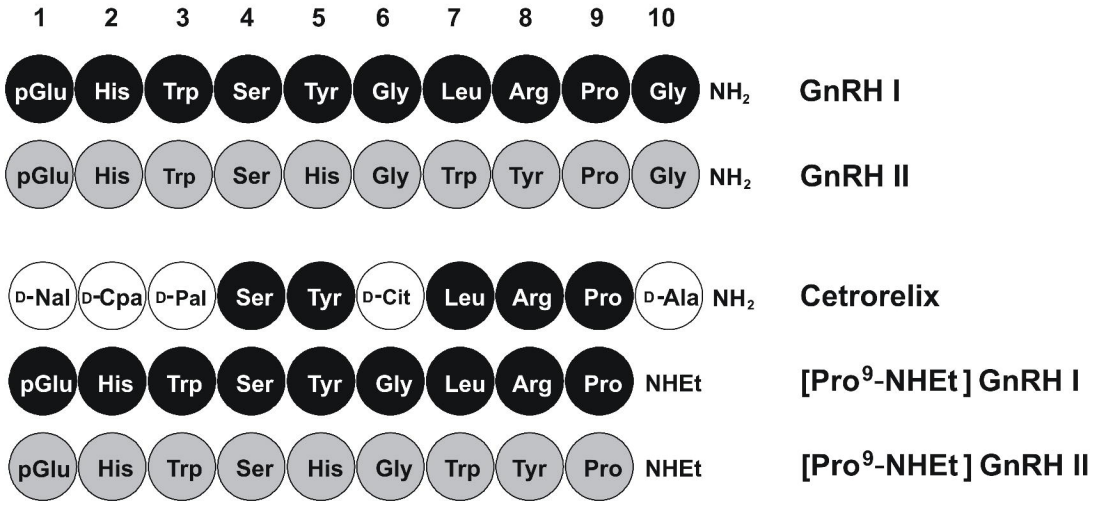
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GnRH I
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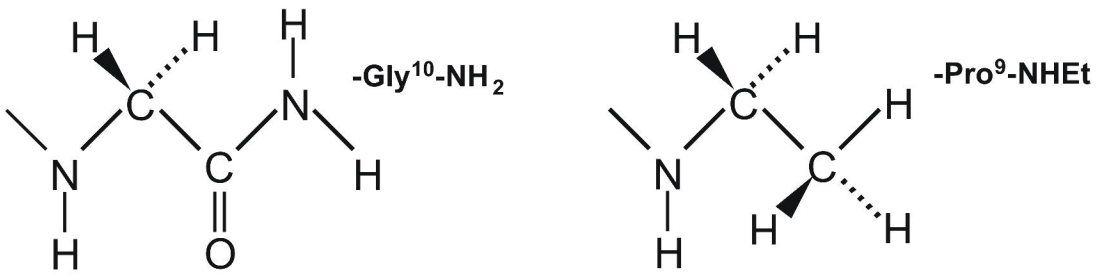
GnRH II
D-Nal D-Cpa D-Pal Ser Tyr D-Cit Leu Arg Pro D-Ala NH2
Cetrorelix
pGlu His Trp Ser Tyr Gly Leu Arg Pro NHEt
[Pro9-NHEt] GnRH I
pGlu His Trp Ser His Gly Trp Tyr Pro NHEt
[Pro9-NHEt] GnRH II

B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-Pro9-NHEt

Fig. 5.

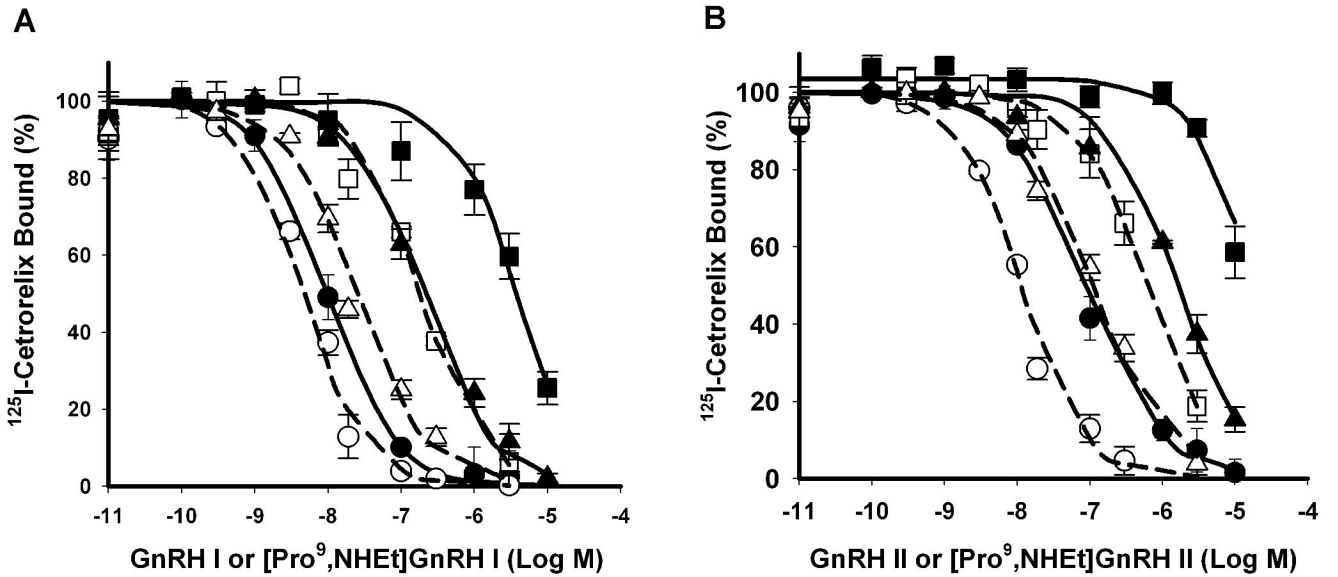
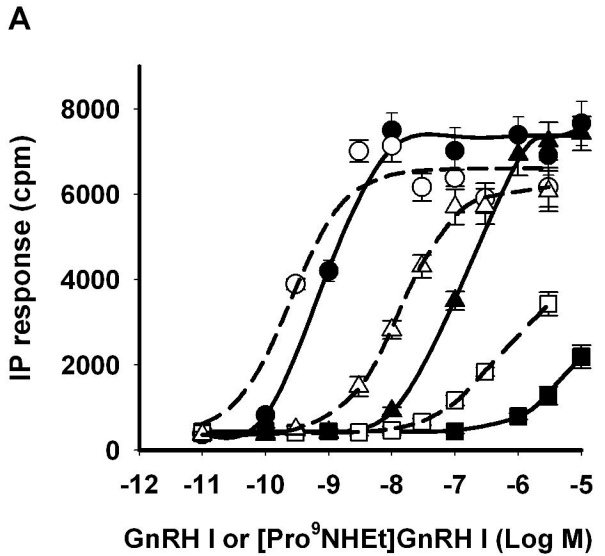

A
B
^{125}I-Cetrorelix Bound (%)
0
20
40
60
80
100
-11
-10
-9
-8
-7
-6
-5
-4
GnRH I or [Pro^{9},NHEt]GnRH I (Log M)
GnRH II or [Pro^{9},NHEt]GnRH II (Log M)

Fig. 6.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IP response (cpm)
8000
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4000
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-11
-10
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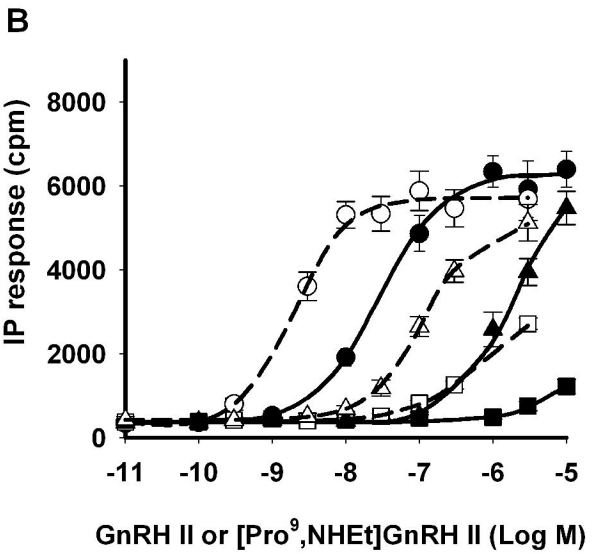
GnRH I or [Pro9NHEt]GnRH I (Log M)


B
IP response (cpm)
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4000
2000
0
-11
-10
-9
-8
-7
-6
-5
GnRH II or [Pro9,NHEt]GnRH II (Log M)