Chimaeric gonadotropin-releasing hormone (GnRH) peptides with improved affinity for the catfish (Clarias gariepinus) GnRH receptor

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The gonadotropin-releasing hormone (GnRH) receptor in catfish differs from its mammalian counterparts in showing a very low affinity for the hypothalamic GnRH form [i.e. catfish GnRH (cfGnRH)] and a very high affinity for the highly conserved mesencephalic GnRH, chicken GnRH-II (cGnRH-II). In the present study we investigated the molecular interactions between ligand and receptor involved in determining the ligand selectivity of the catfish GnRH receptor. Studies on the binding characteristics of the catfish GnRH receptor for cfGnRH and cGnRH-II as well as for mammalian GnRH (mGnRH) and synthetic chimaeric GnRHs, differing at positions 5, 7 and 8, revealed that the low affinity of the catfish receptor for cfGnRH can be improved by replacing Leu7 by a tryptophan residue and/or Asn6 by either a tyrosine or an arginine residue. Testing cfGnRH and cGnRH-II as well as mGnRH and the chimaeric GnRHs on Asp204→Ala, Asp204→Glu and Asp204→Asn mutant catfish GnRH receptors revealed that Asp204 of the catfish receptor mediates the recognition of Arg6 in mGnRH, as well as in the chimaeric peptides [Arg6]cGnRH and [Arg6]cGnRH-II, but seems to be less important for the recognition of Tyr6 in cGnRH-II. On the basis of these results, a three-dimensional model for the binding of [Arg6]cGnRH-II to the catfish GnRH receptor is proposed.

Key words: computational modelling, G-protein-coupled receptor, ligand selectivity, site-directed mutagenesis.

INTRODUCTION
Gonadotropin-releasing hormone (GnRH) is a decapeptide that is essential in the control of reproduction in all vertebrates [1]. Many agonistic and antagonistic analogues have been synthesized and are used clinically for the treatment of GnRH deficiency states, infertility, hormone-dependent cancers, precocious puberty and different gynaecological disorders [2]. So far, 13 different native GnRHs have been characterized from various species [3]. In addition to a hypothalamic GnRH of variable sequence, many vertebrate species, including humans, have been shown to express a second, invariant GnRH form designated chicken GnRH-II (cGnRH-II; [His6,Trp7,Tyr9]GnRH) in the midbrain [4]. The African catfish (Clarias gariepinus) has two endogenous ligands: the hypothalamic catfish GnRH (cfGnRH; [His6,Leu7,Asn6]GnRH) and the mesencephalic cGnRH-II [5].

GnRH receptors belong to the family of rhodopsin-like G-protein-coupled receptors (GPCRs). Previous studies have shown that mammalian GnRH receptors are selective for mammalian GnRH (mGnRH), but can also be substantially activated by GnRHs, differing at positions 5, 7 and 8, revealed that the low affinity of the catfish GnRH receptor mediates the recognition of Arg6 in mGnRH, as well as in the chimaeric GnRHs [6–8]. Non-mammalian GnRH receptors are promiscuous in interacting well with most of the vertebrate GnRHs but seem to have a preference for cGnRH-II [8,9]. In accordance with this, we have shown that the catfish GnRH receptor has a 1000-fold lower affinity for cfGnRH than for cGnRH-II [10,11] and that the lower biological activity of cfGnRH is compensated for by its higher concentration in the catfish pituitary [10].

It has been shown that the negatively charged Glu93 residue of the mouse GnRH receptor has a major role in the receptor recognition of the Arg6 residue in mGnRH [6]. In this context it is intriguing that the catfish GnRH receptor contains a negatively charged Asp93 residue at the position similar to Glu93 in the mouse receptor, but nevertheless has a low affinity for mGnRH [12]. Such differences in agonist selectivity between vertebrate GnRH receptors might form the basis for elucidating specific receptor–ligand interactions. This knowledge, in turn, might facilitate the rational design of novel peptide and/or non-peptide GnRH analogues.

In the present study we tried to shed more light on the molecular mechanism involved in the ligand selectivity of the catfish GnRH receptor, with the intention of answering two main questions. First, which residue(s) in the ligand enable the catfish GnRH receptor to bind cGnRH-II with high selectivity? Secondly, what is the function of Asp93 in the catfish GnRH receptor, because the African catfish has no endogenous ligand containing a positively charged amino acid residue at position 8? To answer these questions we performed functional studies with the two endogenous ligands (namely cfGnRH and cGnRH-II) and with mGnRH, as well as various chimaeric GnRH analogues in combination with the site-directed mutagenesis of Asp93 in the catfish GnRH receptor. In line with our experimental findings, we describe molecular models of the catfish GnRH receptor in which endogenous cGnRH-II as well as the potent [Arg6]cGnRH-II peptide were docked into the presumed GnRH-binding pocket of the catfish GnRH receptor.

This comparative study exemplifies how structural and functional similarities and differences between GPCRs of evolutionarily distant animal species might help in identifying residues involved in mediating ligand selectivity.

EXPERIMENTAL
Peptides
mGnRH (pGlu1-His2-Trp3-Ser4-Tyr5-Gly6-Leu7-Arg8-Pro9-Gly10-NH2) was purchased from Sigma (St Louis, MO, U.S.A.).
cGnRH-II ([His]³,Trp³,Tyr [GnRH] and cGnRH ([His]³,Leu³, Asn [GnRH]), as well as [His]³,Leu³,Tyr [GnRH], [His]³,Trp³, Asn [GnRH], [His]³,Trp³,Arg [GnRH], [His]³,d-Trp³,Trp³,Arg³] GnRH and [His]³,Leu³,Arg³ [GnRH], were synthesized at the Institute of Molecular Pharmacology (Berlin, Germany). In brief, peptides were synthesized automatically (MilliGen 9050 Peptide Synthesizer) by solid-phase methods with standard fluoroen-9-ylmethoxycarbonyl chemistry in continuous-flow mode as described previously for the synthesis of corticotropin-releasing factor analogues [13]. Purification was performed by preparative reverse-phase HPLC to give final products of more than 95 % purity. Characterization of accurate peptide synthesis was achieved by MS, yielding the expected [M + H]⁺ mass peaks, and by determining the amino acid composition.

**Mutant catfish GnRH receptor constructs**

Mutations in the catfish GnRH-R cDNA insert [12] were introduced by using the pALTER-1 in vitro mutagenesis system (Promega, Madison, WI, U.S.A.) in accordance with the manufacturer’s instructions. Asp³⁰⁴ was mutated to an alanine residue, a glutamate residue or an asparagine residue, generating Asp³⁰⁴ → Ala, Asp³⁰⁴ → Glu and Asp³⁰⁴ → Asn mutant catfish GnRH receptor constructs. The mutations introduced in these constructs were confirmed by sequence analysis. The mutant cDNA inserts were then subcloned into pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) for expression studies.

**Cell culture and transfection**

HEK-293T cells were cultured as described previously [11] and transiently transfected with wild-type or mutant catfish GnRH-R cDNA constructs (5 μg of DNA per 100 mm² dish, or 2.5 μg of DNA per 60 mm² dish) with the SuperFect transfection method (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. To prevent cells from detaching, 24-well and 48-well plates were coated with poly-D-(lysine) (Sigma) and 48-well plates (5 μg of DNA per 100 mm² dish) with the SuperFect transfection method (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. After 24 h, extracts were transferred to columns containing Dowex 

**ELISA detection of GnRH receptor expression**

HEK-293T cells in 60 mm² plates were transfected with 2.5 μg of wild-type receptor cDNA-pcDNA3 construct (positive control), mutant receptor cDNA-pcDNA3 construct, or pcDNA3 only (negative control). After 24 h, cells were transfected to 24-well plates (5 x 10⁵ cells per well) and, after an additional 24 h, were fixed with 4 % (w/v) paraformaldehyde in PBS at 20 °C for 30 min. Samples were then blocked with 1 % (w/v) non-fat dried milk in 0.1 M NaHCO₃ at 20 °C for 4 h, then incubated overnight at 4 °C with an antiserum raised against the N-terminus of the catfish GnRH receptor [11] (diluted 1:100 in 10 mM Tris/HCl (pH 7.5)/150 mM NaCl containing 0.1 %, BSA). After exposure to peroxidase-conjugated goat anti-rat IgG (diluted 1:1000 in 0.1 M NaHCO₃/1 %, non-fat dried milk (Sigma)) at 20 °C for 2 h, peroxidase was detected with TMB liquid substrate system (Sigma) for 30 min. The A₅₇₀ of the negative control was subtracted and the values were expressed as a percentage of the positive control. All constructs were tested in triplicate in three separate experiments.

**Receptor binding assay**

cGnRH-II (2.5 μg) was iodinated with the chloramine-T method and subsequently purified by C₁₄ column chromatography [11]. The specific radioactivity of the radioligand was 111 μCi/mmol. Ligand-binding assays were performed on cell membranes from receptor-expressing HEK-293T cells as described previously [14].

In brief, purified membranes were incubated in 0.5 ml of assay buffer [40 mM Tris/HCl (pH 7.4)/2 mM MgCl₂/0.1 %, BSA] at 4 °C for 2 h with increasing concentrations of ¹²⁵I-labelled cGnRH-II (up to 133000 d.p.m.) in the presence or absence of 1 μM unlabelled cGnRH-II. For displacement studies, purified membranes were incubated at 4 °C for 2 h with approx. 1 nM ¹²⁵I-labelled cGnRH-II (133000 d.p.m.) in the presence of various concentrations of unlabelled native and chimaeric GnRH analogues. The concentration of ¹²⁵I-labelled cGnRH-II approximated its Kᵦ at the wild-type catfish GnRH receptor (2 nM; see the Results section). The membranes were then filtered and the radioactivity was counted. All binding studies were performed in triplelicate in three independent experiments. Binding parameters were determined from saturation and displacement curves with the PRISM2 (GraphPad, San Diego, CA, U.S.A.) software package.

**Total inositol phosphates**

Total inositol phosphates were extracted and separated as described previously [15]. In brief, 24 h after transfection, cells were transferred to 48-well plates (2.5 x 10⁷ cells per well in 0.5 ml of inositol-free Dulbecco’s modified Eagle’s medium (DMEM) containing 10 % (v/v) dialysed fetal calf serum) and incubated for 4 h with [³²P]inositol (1 μCi/ml; Amersham, Little Chalfont, Bucks., U.K.). Medium was removed and cells were washed and preincubated for 10 min with assay medium (Hepes-modified DMEM containing 10 mM LiCl). Various concentrations of different native and chimaeric GnRH analogues were added at 37 °C for 45 min; the assay medium was then aspirated. After extraction with 10 mM formic acid at 4 °C for at least 90 min, extracts were transferred to columns containing Dowex (AG 1X8-200) anion-exchange resin (Sigma). Total inositol phosphates were then eluted and the amount of radioactivity was counted. Assays were performed in duplicate in three separate experiments. EC₅₀ values were determined from concentration–response curves by using PRISM2.

**Statistical analysis**

All results are presented as means ± S.E.M. for three independent experiments. Statistical analysis was performed with a one-way analysis of variance and, where P < 0.05, was followed by the Bonferroni test. P < 0.05 was considered to be significant.

**Computational methods**

The catfish GnRH receptor model was based on Baldwin’s template of the electron-density structure of bovine rhodopsin [16] and built from an existing human GnRH receptor model [17] by substituting the appropriate amino acids in the transmembrane regions with the program SYBYL 6.4. Intracellular loops 1 and 3, extracellular loop 2 (EL 2) and the intracellular C-terminus were rebuilt with the use of the loop search option in SYBYL 6.4. By analogy with the human GnRH receptor [18], one disulphide bridge (Cys¹⁴¹-Cys¹⁴⁹) was included in the model. Start coordinates of the docked GnRH peptides cGnRH-II and [His]³,Trp³,Arg³]GnRH were also abstracted from the human GnRH receptor model and in accordance with existing experimental site-directed mutagenesis data on that receptor [17]. The catfish GnRH receptor complexes were minimized with AMBER 4.1 FF before running a molecular dynamics (MD) simulation of 500 ps in vacuo [time step = 2 fs, T = 300 K, ε = 1, and using a cut-off for non-bonded interactions of 9 Å (0.9 nm)]. For representation, the frame with the lowest interaction enthalpy (ΔHᵦ = ΔHᵦ(complex) - ΔHᵦ(receptor) - ΔHᵦ(ligand)) was selected out of 500 frames (every 1 ps) generated.
RESULTS

Activities of cGnRH-II analogues with and without Trp7 and/or Tyr8

From previous work we knew that the catfish GnRH receptor expressed in HEK-293T cells has an approx. 1000-fold higher affinity for cGnRH-II than for cGnRH [11]. In addition, cGnRH-II is approx. 1000-fold more potent than cGnRH in stimulating second messenger production in these cells [11]. In the present study we confirmed that the catfish GnRH receptor has high-affinity binding characteristics for cGnRH-II, while the $K_i$ value of the receptor for cGnRH was approx. 1083-fold higher (Figure 1A and Table 1).

The catfish GnRH receptor binds cGnRH-II, from all GnRHs tested, with the highest affinity. To study which amino acid residue(s) in cGnRH-II are important to enable the catfish GnRH receptor to bind cGnRH-II with this high affinity, we created two chimaeric GnRH analogues, namely [His5,Leu7, Tyr8]GnRH and [His5,Trp7,Asn8]GnRH. These analogues represent two intermediate peptides of the two endogenous African catfish GnRH ligands cGnRH-II and cfGnRH [5], which differ from each other at positions 7 and 8 (Table 1). Surprisingly, the affinity of the receptor for these cGnRH-II analogues was decreased significantly compared with that for cGnRH-II (52-fold and 15-fold higher $K_i$ values for [His5,Leu7, Tyr8]GnRH and [His5,Trp7,Asn8]GnRH respectively; $P < 0.001$) (Figure 1A and Table 1).

In addition to the binding studies, we also compared the activity of cGnRH-II with that of the cGnRH-II analogues containing substitutions on position 7 and/or position 8 to stimulate phospholipase C activity in HEK-293T cells expressing the catfish GnRH receptor. After 24 h of labelling with $[\text{H}]$inositol and 45 min of stimulation with various concentrations of the different GnRH peptides, the cellular accumulation of total $[\text{H}]$inositol phosphates was determined. The signal-transduction data reflected the results from the binding studies. Thus the high potency of cGnRH-II was diminished significantly by replacing amino acid residues at either position 7 or position 8 or both (93-fold, 76-fold and 976-fold higher EC50 values for [His5,Leu7, Tyr8]GnRH, [His5,Trp7,Asn8]GnRH and [His5,Leu7, Asn8]GnRH (cfGnRH) respectively; $P < 0.001$) (Figure 1B and Table 1).

Activities of GnRH analogues with and without Arg8

The arginine residue on position 8 of mGnRH is necessary for the mouse GnRH receptor to bind mGnRH with high affinity [6].

The pK values for the receptors for cfGnRH was approx. 1083-fold higher (Figure 1A and Table 1). Asn both (93-fold, 76-fold and 976-fold higher EC50 values for [His5,Leu7, Tyr8]GnRH (cGnRH-II), [His5,Leu7,Asn8]GnRH (cGnRH, [])) and the two intermediate GnRH peptides, [His5,Leu7,Tyr8]GnRH ([]) and [His5,Trp7,Asn8]GnRH (()), of HEK-293T cells transiently expressing the wild-type catfish GnRH receptor were measured as described in the Experimental section. Results are means ± S.E.M. for triplicate observations from a single representative experiment.

![Figure 1](image-url) Activities of cGnRH-II analogues with and without Trp7 and/or Tyr8 Binding of $[\text{H}]$GnRH-II (A) and $[\text{H}]$inositol phosphate ($[\text{H}]$-IP) production (B) in the presence of various concentrations of [His5,Trp7,Tyr8]GnRH (cGnRH-II, ●); [His5,Leu7,Asn8]GnRH (cGnRH, □) and the two intermediate GnRH peptides, [His5,Leu7,Tyr8]GnRH (■) and [His5,Trp7,Asn8]GnRH (▲), of HEK-293T cells transiently expressing the wild-type catfish GnRH receptor were measured as described in the Experimental section. Results are means ± S.E.M. for triplicate observations from a single representative experiment.

Table 1 Summary of ligand binding and $[\text{H}]$inositol phosphate production of HEK-293T cells expressing wild-type catfish GnRH receptor by native and chimaeric forms of GnRH

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pK ($^\dagger$)</th>
<th>pEC50 ($^\ddagger$)</th>
</tr>
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<tbody>
<tr>
<td><strong>Native peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[His5,Leu7,Asn8]GnRH (cGnRH)</td>
<td>6.09 ± 0.13$^\dagger$</td>
<td>5.91 ± 0.16$^\ddagger$</td>
</tr>
<tr>
<td>[Tyr5,Leu7,Arg8]GnRH (mGnRH)</td>
<td>6.18 ± 0.04$^\dagger$</td>
<td>n.d.</td>
</tr>
<tr>
<td>[His5,Trp7,Trp8]GnRH (cGnRH-II)</td>
<td>9.12 ± 0.09$^\dagger$</td>
<td>8.90 ± 0.17$^\ddagger$</td>
</tr>
<tr>
<td><strong>Chimaeric peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[His5,Leu7,Arg8]GnRH</td>
<td>7.01 ± 0.13$^\dagger$</td>
<td>n.d.</td>
</tr>
<tr>
<td>[His5,Leu7,Tyr8]GnRH</td>
<td>7.41 ± 0.06$^\dagger$</td>
<td>6.93 ± 0.03$^\ddagger$</td>
</tr>
<tr>
<td>[His5,Trp7,Asn8]GnRH</td>
<td>7.95 ± 0.07$^\dagger$</td>
<td>7.01 ± 0.07$^\ddagger$</td>
</tr>
<tr>
<td>[His5,Trp7,Arg8]GnRH</td>
<td>9.27 ± 0.02$^\dagger$</td>
<td>n.d.</td>
</tr>
<tr>
<td>[His5,Trp7,Trp8,Arg8]GnRH</td>
<td>9.41 ± 0.09$^\dagger$</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 2 Alignment of the amino acid sequences in EL 3 of selected members of the GnRH receptor family

<table>
<thead>
<tr>
<th>Receptor</th>
<th>EL 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse GnRH receptor</td>
<td>WYWFDPEMLNVS E PVNH</td>
</tr>
<tr>
<td>Human GnRH receptor</td>
<td>WYWFDPEMLRNS E PVNH</td>
</tr>
<tr>
<td>Rat GnRH receptor</td>
<td>WYWFDPEMLRNS E PVNH</td>
</tr>
<tr>
<td>Catfish GnRH receptor A</td>
<td>WYWFDPEMLQYP E YIHH</td>
</tr>
<tr>
<td>Goldfish GnRH receptor B</td>
<td>WYWFDPEMLQYTP E YIHH</td>
</tr>
</tbody>
</table>

However, the catfish GnRH receptor has only a poor affinity for mGnRH [11], although the receptor contains negatively charged Asp$^{202}$ at a position similar to Glu$^{201}$ in the mouse GnRH receptor (Table 2). To obtain further insight into the specific interaction between GnRH receptor and GnRH ligand, we synthesized [Arg$^8$]cGnRH and [Arg$^8$]cGnRH-II analogues...
Figure 2 Ligand binding of [His\(^5\),Leu\(^7\),Asn\(^8\)]GnRH (cfGnRH, □) and [His\(^5\),Leu\(^7\),Arg\(^8\)]GnRH (■) to membranes prepared from HEK-293T cells transiently expressing wild-type (A), Asp\(^{304}\)→Ala mutant (B), Asp\(^{304}\)→Asn mutant (C) or Asp\(^{304}\)→Glu mutant (D) catfish GnRH receptor.

Displacement of \(^{125}\)I-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing either wild-type catfish GnRH receptor or one of the mutant catfish GnRH receptors by various concentrations of cfGnRH or [His\(^5\),Leu\(^7\),Arg\(^8\)]GnRH were measured as described in the Experimental section. Results are means ± S.E.M. for triplicate observations from a single representative competition binding experiment.

Figure 3 Ligand binding of [His\(^5\),Trp\(^7\),Tyr\(^8\)]GnRH (cGnRH-II, □) and [His\(^5\),Trp\(^7\),Arg\(^8\)]GnRH (■) to membranes prepared from HEK-293T cells transiently expressing wild-type (A) or Asp\(^{304}\)→Ala mutant (B) catfish GnRH receptor.

Displacement of \(^{125}\)I-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing either wild-type catfish GnRH receptor or the Asp\(^{304}\)→Ala mutant catfish GnRH receptor by various concentrations of cGnRH-II or [His\(^5\),Trp\(^7\),Arg\(^8\)]GnRH were measured as described in the Experimental section. Results are means ± S.E.M. for triplicate observations from a single representative competition binding experiment.

(namely [His\(^5\),Leu\(^7\),Arg\(^8\)]GnRH and [His\(^5\),Trp\(^7\),Arg\(^8\)]GnRH respectively). The catfish GnRH receptor had a higher affinity for [His\(^5\),Leu\(^7\),Arg\(^8\)]GnRH than for [His\(^5\),Leu\(^7\),Asn\(^8\)]GnRH (cfGnRH) and [Tyr\(^7\),Leu\(^7\),Arg\(^8\)]GnRH (mGnRH) (\(P < 0.001\); Table 1 and Figure 2A). Furthermore, the catfish GnRH receptor showed a \(K_i\) for the [His\(^5\),Trp\(^7\),Arg\(^8\)]GnRH peptide similar to that for [His\(^5\),Trp\(^7\),Tyr\(^8\)]GnRH (cGnRH-II) (\(P > 0.05\); Table 1 and Figure 3A).

In addition, a conformationally constrained [d-Trp\(^8\),Arg\(^8\)] cGnRH-II analogue ([His\(^5\),d-Trp\(^8\),Trp\(^7\),Arg\(^8\)]GnRH) was used to investigate whether the catfish GnRH receptor binds cGnRH-II in a constrained conformation, analogous to the binding mode of mGnRH to mammalian GnRH receptors [8]. The affinity of the catfish GnRH receptor for this constrained peptide was similar to that for cGnRH-II and [His\(^5\),Trp\(^7\),Arg\(^8\)]GnRH (\(P > 0.05\); Table 1).

Characteristics of Asp\(^{304}\)→Ala, Asp\(^{304}\)→Glu and Asp\(^{304}\)→Asn mutant catfish GnRH receptors

Next we investigated the role of the Asp\(^{304}\) residue in EL 3 of the catfish GnRH receptor. To obtain further insight into the GnRH-selectivity characteristics of the catfish GnRH receptor, mediated by the presumed interaction of Asp\(^{304}\) of the receptor with
Table 3 Characteristics of wild-type and mutant catfish GnRH receptors transiently expressed in HEK-293T cells

The cell-surface expression of wild-type and mutant receptor constructs, transiently transfected in HEK-293T cells, was measured in an ELISA with an antibody raised against the N-terminal part of the catfish GnRH receptor. Binding and IP values were obtained from saturation studies of $[^{125}]$I-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing wild-type or mutant GnRH receptors. The agonist-induced accumulation of $[^{3}H]$inositol phosphates was determined in HEK-293T cells transiently expressing wild-type and mutant GnRH receptors after stimulation for 45 min with 10 nM cGnRH-II as described in the Experimental section. Results are means ± S.E.M. for three independent experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Asp$^{304}$ → Ala</th>
<th>Asp$^{304}$ → Glu</th>
<th>Asp$^{304}$ → Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA-detected expression (%) of wild-type</td>
<td>100</td>
<td>68 ± 6*</td>
<td>110 ± 4</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>$B_{max}$ (pmol/mg of protein)</td>
<td>10.4 ± 1.7</td>
<td>6.3 ± 2.0</td>
<td>10.7 ± 1.8</td>
<td>9.0 ± 2.4</td>
</tr>
<tr>
<td>$pK_{i}$</td>
<td>8.75 ± 0.11</td>
<td>8.53 ± 0.19</td>
<td>8.58 ± 0.12</td>
<td>8.56 ± 0.09</td>
</tr>
<tr>
<td>IP response (%) of wild-type</td>
<td>100</td>
<td>81 ± 7</td>
<td>110 ± 7</td>
<td>86 ± 7</td>
</tr>
</tbody>
</table>

Figure 4 Ligand binding of [Tyr$^{5}$,Leu$^{7}$,Arg$^{8}$]GnRH (mGnRH) to membranes prepared from HEK-293T cells transiently expressing wild-type (■), Asp$^{304}$ → Ala mutant (○), Asp$^{304}$ → Glu mutant (△) or Asp$^{304}$ → Asn mutant (●) catfish GnRH receptor.

Displacement of $[^{125}]$I-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing either wild-type catfish GnRH receptor or one of the mutant catfish GnRH receptors by various concentrations of mGnRH was measured as described in the Experimental section. Results are means ± S.E.M. for triplicate observations from a single representative competition binding experiment.

Table 4 Summary of the affinities of the Asp$^{304}$ → Ala, Asp$^{304}$ → Glu and Asp$^{304}$ → Asn mutant catfish GnRH receptors for various native and chimaeric GnRH peptides in comparison with the wild-type GnRH receptor

The $pK_{i}$ values were obtained from displacement studies of $[^{125}]$I-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing wild-type or mutant GnRH receptors. Results are means ± S.E.M. for three independent experiments. *P < 0.05 compared with the wild-type receptor.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Wild-type</th>
<th>Asp$^{304}$ → Ala</th>
<th>Asp$^{304}$ → Glu</th>
<th>Asp$^{304}$ → Asn</th>
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<tr>
<td>native peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[His$^{5}$,Leu$^{7}$,Asn$^{8}$]GnRH (cGnRH)</td>
<td>6.09 ± 0.13</td>
<td>5.95 ± 0.13</td>
<td>5.86 ± 0.10</td>
<td>6.02 ± 0.01</td>
</tr>
<tr>
<td>[Tyr$^{5}$,Leu$^{7}$,Arg$^{8}$]GnRH (mGnRH)</td>
<td>6.18 ± 0.04</td>
<td>4.98 ± 0.28*</td>
<td>5.85 ± 0.17</td>
<td>5.51 ± 0.06*</td>
</tr>
<tr>
<td>[His$^{5}$,Trp$^{7}$,Tyr$^{8}$]GnRH (cGnRH-II)</td>
<td>9.12 ± 0.09</td>
<td>8.72 ± 0.10</td>
<td>9.12 ± 0.11</td>
<td>9.04 ± 0.03</td>
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<tr>
<td>Chimaeric peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[His$^{5}$,Leu$^{7}$,Arg$^{8}$]GnRH</td>
<td>7.01 ± 0.13</td>
<td>5.84 ± 0.08*</td>
<td>6.45 ± 0.06*</td>
<td>5.99 ± 0.07*</td>
</tr>
<tr>
<td>[His$^{5}$,Leu$^{7}$,Tyr$^{8}$]GnRH</td>
<td>7.41 ± 0.06</td>
<td>6.91 ± 0.07*</td>
<td>7.43 ± 0.03</td>
<td>6.95 ± 0.09*</td>
</tr>
<tr>
<td>[His$^{5}$,Trp$^{7}$,Asn$^{8}$]GnRH</td>
<td>7.95 ± 0.07</td>
<td>7.68 ± 0.12</td>
<td>7.83 ± 0.07</td>
<td>7.91 ± 0.03</td>
</tr>
<tr>
<td>[His$^{5}$,Trp$^{7}$,Arg$^{8}$]GnRH</td>
<td>9.27 ± 0.02</td>
<td>7.99 ± 0.10*</td>
<td>8.65 ± 0.01*</td>
<td>8.29 ± 0.06*</td>
</tr>
<tr>
<td>[His$^{5}$,d-Trp$^{7}$,Trp$^{7}$,Arg$^{8}$]GnRH</td>
<td>9.41 ± 0.09</td>
<td>8.35 ± 0.20*</td>
<td>9.01 ± 0.11</td>
<td>8.57 ± 0.08*</td>
</tr>
</tbody>
</table>

Asp$^{304}$ of the catfish GnRH receptor is important in the recognition of [Arg$^{i}$]GnRH peptides

In an initial binding study we tested whether the affinity of the catfish GnRH receptor for [Tyr$^{5}$,Leu$^{7}$,Arg$^{8}$]GnRH (mGnRH) decreased when Asp$^{304}$ was replaced by an alanine, glutamic acid or asparagine residue. Figure 4 shows that the Asp$^{304}$ → Glu mutant receptor, in which Asp was replaced by another negatively charged residue, had an affinity for mGnRH that was not significantly different from that of the wild-type receptor ($P > 0.05$) (Table 4). However, replacement of Asp$^{304}$ by an uncharged residue (Asp$^{304}$ → Ala and Asp$^{304}$ → Asn mutant receptors) resulted in a decreased affinity for mGnRH ($P < 0.001$ and $P < 0.01$ respectively) (Table 4 and Figure 4).

To substantiate these results we investigated whether the Asp$^{304}$ → Glu, Asp$^{304}$ → Ala and Asp$^{304}$ → Asn mutant catfish GnRH receptors were able to discriminate between cGnRH and the [Arg$^{i}$]cGnRH peptide, like the wild-type receptor (see above). The Asp$^{304}$ → Ala and Asp$^{304}$ → Asn mutant receptors had similar $K_{i}$ values for [His$^{5}$,Leu$^{7}$,Asn$^{8}$]GnRH (cGnRH) and [His$^{5}$,Leu$^{7}$,Arg$^{8}$]GnRH ($P > 0.05$) (Figures 2B and 2C, and Table 4). In contrast, the Asp$^{304}$ → Glu mutant receptor showed a higher affinity for [His$^{5}$,Leu$^{7}$,Arg$^{8}$]GnRH than for cGnRH ($P < 0.05$) (Figure 2D and Table 4), although the difference in affinities was less pronounced for the Asp$^{304}$ → Ala mutant receptor than for the wild-type receptor.

In addition, we determined the affinity of the Asp$^{304}$ → Ala, Asp$^{304}$ → Glu and Asp$^{304}$ → Asn mutant catfish GnRH receptors for cGnRH-II and [Arg$^{i}$]cGnRH-II. The Asp$^{304}$ → Glu mutant receptor showed no significant difference in affinity for either [His$^{5}$,Trp$^{7}$,Tyr$^{8}$]GnRH (cGnRH-II) or [His$^{5}$,Trp$^{7}$,Arg$^{8}$]GnRH ($P > 0.05$) (Table 4), in accord with our findings on the wild-type receptor (see above). In contrast, the Asp$^{304}$ → Ala and Asp$^{304}$ → Asn mutant catfish GnRH receptors were comparable with those of the wild-type receptor ($P > 0.05$; Table 3).
Figure 5 Molecular model of the catfish GnRH receptor with [His$^5$,Trp$^7$,Arg$^8$]GnRH docked into the binding pocket

(A) The green tube represents catfish GnRH receptor backbone, capped sticks with orange carbon atoms represent [His$^5$,Trp$^7$,Arg$^8$]GnRH and capped sticks with white carbon atoms represent receptor side chains. (B) Detail of the molecular model of the binding of [His$^5$,Trp$^7$,Arg$^8$]GnRH to the catfish GnRH receptor. Arg$^8$ makes two hydrogen bonds with Arg$^{304}$, Gly$^6$ is near the entrance of the receptor and permits a $\beta$-turn-type conformation of the agonist, the N-terminal pGlu$^1$ interacts with Lys$^{124}$, and the C-terminal Gly$^{10}$-NH$_2$ hydrogen bonds with Asn$^{105}$ in EL1 in agreement with experimental results (see the text for details).
Asn mutant receptor constructs had a lower affinity for [His\(^8\), Trp\(^7\), Arg\(^4\)]GnRH than for cGnRH-II (P < 0.01 and P < 0.001 respectively) (Figure 3B and Table 4). In addition, the conformationally constrained [His\(^8\), d- Trp\(^6\), Trp\(^7\), Arg\(^4\)]GnRH peptide reflected the findings for the [His\(^8\), Trp\(^7\), Arg\(^4\)]GnRH analogue (Table 4).

**Asp\(^{204}\) of the catfish GnRH receptor is not essential for the recognition of [Tyr\(^8\)]GnRH peptides**

To test whether Tyr\(^8\) of cGnRH-II was able to interact with Asp\(^{204}\) of the catfish GnRH receptor, we studied whether the Asp\(^{204}\)→ Ala, Asp\(^{204}\)→ Glu and Asp\(^{204}\)→ Asn mutant catfish GnRH receptors were able to discriminate between [His\(^8\), Trp\(^7\), Tyr\(^4\)]GnRH (cGnRH-II) and [His\(^8\), Trp\(^7\), Asn\(^4\)]GnRH, and between [His\(^8\), Leu\(^7\), Tyr\(^4\)]GnRH and [His\(^8\), Leu\(^7\), Asn\(^4\)]GnRH (cGnRH). Ligand binding studies on wild-type and Asp\(^{204}\)-mutant receptors revealed that these receptors had similar affinities for cGnRH-II (P > 0.05) (Table 4) and that the Asp\(^{204}\)-mutant receptors did discriminate between [His\(^8\), Trp\(^7\), Tyr\(^4\)]GnRH (cGnRH-II) and [His\(^8\), Trp\(^7\), Asn\(^4\)]GnRH, similarly to the wild-type receptor (P < 0.001) (Table 4). In contrast, Asp\(^{204}\)→ Ala and Asp\(^{204}\)→ Asn mutant catfish GnRH receptors had a lower affinity for [His\(^8\), Leu\(^7\), Tyr\(^4\)]GnRH than for the wild-type and the Asp\(^{204}\)→ Glu mutant receptors (P < 0.01) (Table 4). Nevertheless, all Asp\(^{204}\)-mutant receptors could still discriminate between [His\(^8\), Leu\(^7\), Tyr\(^4\)]GnRH and [His\(^8\), Leu\(^7\), Asn\(^4\)]GnRH (P < 0.001) (Table 4).

**Computational modelling**

On the basis of the above findings, we developed models of the catfish GnRH receptor with either [His\(^8\), Trp\(^7\), Arg\(^4\)]GnRH (Figure 5) or [His\(^8\), Trp\(^7\), Tyr\(^4\)]GnRH (cGnRH-II; results not shown) docked into the catfish GnRH receptor-binding pocket. These models were built in agreement with published data from mutagenesis studies on mammalian GnRH receptors [17]. For example, experimental results suggest that the C-terminal Gly\(^18\)-NH\(^2\) of GnRH interacts with Asn\(^192\) of the human GnRH receptor [7]. Photoaffinity labelling studies also identified the side chain of [d-Trp\(^{20}\)]GnRH in the proximity of Cys\(^14\) of the receptor, thus accommodating the ligand in the region between the ELs. Moreover, mutation of Lys\(^121\) to Gln affects GnRH agonist binding but not antagonist binding to the human GnRH receptor [19]. In our previous GnRH receptor model we proposed that the hydrogen-bond donor of Lys\(^121\) is in contact with the N-terminal pGlu\(^1\) residue of GnRH, which is lacking in peptide antagonists [17]. The present molecular models of the catfish GnRH receptor obtained from 500 ps of MD simulations are also in accord with these data.

According to our model, in which [His\(^8\), Trp\(^7\), Arg\(^4\)]GnRH was docked into the catfish GnRH receptor-binding pocket, the positively charged Arg\(^4\) of [His\(^8\), Trp\(^7\), Arg\(^4\)]GnRH makes strong ionic hydrogen bonds with the negatively charged Asp\(^{204}\) (Figure 5B). In addition, MD simulations, docking [His\(^8\), Trp\(^7\), Tyr\(^4\)]GnRH (cGnRH-II) into the catfish GnRH receptor-binding pocket, revealed that Tyr\(^4\) is able to form a hydrogen bond with Asp\(^{204}\) in the wild-type catfish receptor (results not shown). Additional MD simulations of cGnRH-II, now docked into the binding pocket of the Asp\(^{204}\)→ Ala mutant catfish receptor, showed that, in the absence of the negatively charged side chain of the Asp\(^{204}\) residue in the receptor, Tyr\(^4\) of cGnRH-II can also make a hydrogen bond with the backbone carbonyl group of Ala\(^193\) in this receptor (results not shown). The aromatic ring of Tyr\(^4\) seems to be embedded in a hydrophobic pocket formed by the aromatic rings of His\(^{307}\) and Trp\(^{104}\) of the receptor.

**DISCUSSION**

In the present study we used the catfish GnRH receptor as a model to investigate which amino acid residues in GnRHs are important to enable the receptor to bind cGnRH-II with the highest affinity. In agreement with previous studies, we demonstrated that the catfish GnRH receptor has a higher affinity for cGnRH-II than for cGnRH and mGnRH [11,12], and that cGnRH-II has a higher potency than cGnRH for second messenger production in HEK-293T cells expressing the catfish GnRH receptor. In the African catfish pituitary, the lower affinity of the catfish GnRH receptor for cGnRH than for cGnRH-II is compensated for by an approx. 700-fold higher concentration of cGnRH [10]. It has also been reported that cGnRH-II is the most potent endogenous ligand for the chicken GnRH receptor and the two goldfish GnRH receptors [9,20]. Moreover, apart from the highly potent mGnRH, cGnRH-II is also able to stimulate the mouse and human GnRH receptors to accumulate inositol phosphates with an EC\(^{50}\) value in the nanomolar range, comparable with the EC\(^{50}\) value of cGnRH-II for the catfish GnRH receptor [6,7].

**His\(^8\), Trp\(^7\) and Tyr/Arg\(^4\) are important residues in the GnRH ligand, allowing the catfish GnRH receptor to bind GnRH with high affinity**

The fact that the catfish GnRH receptor has a lower affinity for [Tyr\(^8\), Leu\(^7\), Arg\(^4\)]GnRH (mGnRH) than for [His\(^8\), Leu\(^7\), Arg\(^4\)]GnRH, which differs from mGnRH only at position 5, demonstrates that the presence of His\(^8\) in the ligand is needed for the high-affinity interaction of the catfish GnRH receptor with GnRH ligands. Chicken, *Xenopus laevis* and goldfish (*Carassius auratus*) GnRH receptors differ in this respect, because [His\(^8\)]mGnRH resulted in a marked decline in their activity, whereas mammalian GnRH receptors showed similar or higher affinities for [His\(^8\)]mGnRH compared with mGnRH [9,21].

In addition, we identified the Trp\(^7\) and Tyr\(^4\) residues as important residues in the GnRH ligand that contribute to the high affinity of the catfish GnRH receptor for cGnRH-II and thus confer on the cGnRH-II ligand its high potency towards the catfish receptor. The fact that the catfish GnRH receptor prefers a GnRH ligand with Trp\(^7\) rather than Leu\(^7\) is supported by the finding that this receptor showed a higher affinity for [His\(^8\), Trp\(^7\), Tyr\(^4\)]GnRH and [His\(^8\), Trp\(^7\), Arg\(^4\)]GnRH than for [His\(^8\), Leu\(^7\), Tyr\(^4\)]GnRH and [His\(^8\), Leu\(^7\), Arg\(^4\)]GnRH. In this respect, the catfish GnRH receptor is similar to the chicken GnRH receptor but different from mammalian GnRH receptors, which tolerate rather than favour a tryptophan residue at position 7 in the GnRH ligand [9].

Surprisingly, we found that the catfish GnRH receptor has a similar affinity for [His\(^8\), Trp\(^7\), Arg\(^4\)]GnRH to that for [His\(^8\), Trp\(^7\), Tyr\(^4\)]GnRH (cGnRH-II) but the receptor has a lower K\(_f\) for [His\(^8\), Leu\(^7\), Arg\(^4\)]GnRH than for [His\(^8\), Leu\(^7\), Asn\(^4\)]GnRH (cGnRH). From these findings we conclude that the catfish GnRH receptor recognizes GnRHs with an arginine residue at position 8 equally as well as GnRHs with a tyrosine residue at position 8, and that GnRHs having an arginine or tyrosine residue at position 8 interact better with the catfish GnRH receptor than GnRHs with an asparagine residue at position 8.

Mammalian GnRH receptors have been reported to prefer a constrained \(\beta\)-II-type turn conformation of mGnRH, which is favoured by amino acid substitutions in the \(\beta\)-configuration at position 6 in the ligand [8]. Amino acids at this position can be replaced by large polar \(\delta\)-amino acids, suggesting that these side chains point to the extracellular surroundings of the receptor.
Asp^{304} of the catfish GnRH receptor mediates the recognition of [Arg^{a}]GnRHs but is not essential for the recognition of [Tyr^{b}]GnRHs

Non-mammalian GnRHs differ from mGnRH by the substitution of a neutral amino acid for the positively charged arginine residue at position 8. For that reason, non-mammalian GnRH receptors were not expected to contain a negatively charged residue at the position similar to Glu^{520} of the mouse GnRH receptor that confers the high affinity of the mouse receptor for [Arg^{a}]GnRH ligands [6]. However, the catfish GnRH receptor contains an aspartic acid residue at position 304, similar to Glu^{520} of the mouse GnRH receptor [12]. We therefore wished to investigate the function of Asp^{544} in the catfish receptor. Replacement of the negatively charged Asp^{544} residue with an uncharged (alanine or asparagine) residues abolished the preference of this receptor for ligands with Arg^{a}, demonstrating that Asp^{544} of the catfish GnRH receptor is able to mediate the recognition of [Arg^{a}]GnRH peptides. Because the conserved Asp/Glu residue in EL 3 is preceded by a proline residue in the catfish receptor compared with a serine residue in mammalian GnRH receptors, such an interaction in non-mammalian GnRH receptors had previously been doubted [21].

Our three-dimensional model with [His^{a},Trp^{b},Arg^{a}]GnRH docked into the ligand-binding pocket of the catfish GnRH receptor confirms that the orientation of the acidic side chain of Asp^{544} in the catfish receptor is such that it can make strong ionic hydrogen bonds with Arg^{a} residues in GnRH ligands. This is in accordance with the experimental results in this study, namely that the mutation of Asp^{544} in the catfish GnRH receptor to a neutral alanine residue resulted in a more-than-10-fold decrease in affinity for all four agonists (namely mGnRH, [His^{a},Leu^{b},Arg^{a}]GnRH, [His^{a},Trp^{b},Arg^{a}]GnRH and [His^{a},D-Trp^{b},Trp^{b},Arg^{a}]GnRH), which contain a positively charged arginine residue on position 8. In contrast, mutation of Asp^{544} in the catfish GnRH receptor to the negatively charged Glu residue had a less marked effect on the affinity of the receptor for these [Arg^{a}]GnRH peptides. Moreover, the observed interaction of Arg^{a} in [His^{a},Trp^{b},Arg^{a}]GnRH with Asp^{304} of the catfish GnRH receptor is analogous to the binding of Arg^{a} in mGnRH to the aspartic or glutamic residues present at the similar position in human and mouse GnRH receptors respectively [6, 17].

Thus the poor affinity of the catfish GnRH receptor for mGnRH is not due to a lack of specific recognition of Arg^{a} in the ligand. Instead, the side chains of the amino acid residues on position 5 (tyrosine) and/or position 7 (leucine) of mGnRH are likely to fit better in mammalian GnRH receptors than in the catfish GnRH receptor.

We reported previously that a model of [D-Trp^{b}]mGnRH binding to the human GnRH receptor favours a hydrogen-bond interaction between the hydroxy group of Tyr^{b} in the ligand and Asp^{304} in the receptor [17]. The residue in a similar position in the catfish GnRH receptor is Glu^{393}. The model of cGnRH-II binding to the catfish GnRH receptor supports a close proximity between His^{a} and Glu^{393} but also between His^{a} and His^{397} (Asn^{305} in the human receptor). Future studies will be aimed at investigating these predicted interactions. According to our models, the position of residue 7 of cGnRH-II is predicted to be located in a hydrophobic pocket formed by the residues of transmembrane regions 3, 4 and 7 together with residues of EL 2. This is in accordance with the localization of residue 7 of [D-Trp^{b}]mGnRH in the human GnRH receptor model [17].

Because the African catfish does not contain endogenous GnRH ligands with an arginine residue at position 8, we hypothesized that Asp^{304} of the catfish GnRH receptor might be important for the recognition of Tyr^{b} in the endogenous cGnRH-II ligand. Indeed, the molecular model with cGnRH-II docked into the ligand-binding pocket of the catfish GnRH receptor predicts that Tyr^{b} is able to hydrogen bond with Asp^{304} of the receptor. However, our experimental results suggest only a small contribution of Asp^{304} to the binding of Tyr^{b}-containing agonists.

The Glu^{520} → Gln mutant mouse GnRH receptor shares characteristics with the Asp^{304} → Asn mutant catfish GnRH receptor in that the high affinity of the mouse GnRH receptor for cGnRH-II was not influenced by this mutation [6]. The molecular model for the binding of cGnRH-II to the Asp^{304} → Ala mutant catfish GnRH receptor demonstrates that, in the absence of the negatively charged side chain of Asp^{304}, Tyr^{b} of cGnRH-II makes a hydrogen bond with Ala^{395} in EL 2 of the catfish receptor. Moreover, the aromatic residues His^{397} and Trp^{398} (Asn^{305} and Trp^{398} in the human GnRH receptor) also might contribute to the hydrophobic pocket contacting the aromatic ring of Tyr^{b}. Future mutagenesis studies will test the role of these residues of the catfish GnRH receptor on the binding of cGnRH-II.

Our experimental findings have demonstrated that Asp^{304} of the catfish GnRH receptor is unlikely to be the sole residue that determines the high-affinity binding of [Tyr^{b}]GnRH peptides. However, Asp^{304} of the catfish GnRH receptor does clearly contribute to the recognition of [Arg^{a}]GnRH peptides.

In conclusion, the differences in agonist selectivity between non-mammalian and mammalian GnRH receptors in combination with computational three-dimensional molecular models of the receptor–ligand complexes might help us to unravel the molecular mechanisms involved in ligand selectivity. The GnRH receptor might therefore serve as a model for GPCRs in general, demonstrating the usefulness of comparative studies between receptors of evolutionarily distant animal species for the understanding of the mechanism underlying ligand recognition.

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