BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 517–522 (1997)
ARTICLE NO. RC977331

Differences in Structure–Function Relations between Nonmammalian and Mammalian Gonadotropin-Releasing Hormone Receptors

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Received August 9, 1997

Mammalian gonadotropin-releasing hormone receptors (GnRH-Rs) differ from other G protein-coupled receptors in lacking the intracellular C-terminus and in showing an exchange of two otherwise highly conserved Asp (D) and Asn (N) residues in transmembrane domains (TMD) 2 and 7, respectively. However, the first GnRH-R characterized from a nonmammalian vertebrate, the African catfish, does contain an intracellular C-terminus and has D residues in TMD 2 and 7. The functional relevance of these structural features was analysed with $D^{90}N^{321}$, $N^{90}D^{321}$, $N^{90}N^{321}$ and C-terminally truncated mutant catfish GnRH-Rs. An antiserum raised against the recombinant extracellular domain of the wild-type catfish GnRH-R detected all mutant receptors at the cell surface of transiently transfected 293T cells. However, only the $D^{90}N^{321}$ mutant specifically bound GnRHs and activated signal transduction in response to GnRHs; all other mutants were inactive in both respects. We conclude that the catfish GnRH-R differs from the mammalian GnRH-Rs in that both the C-terminal domain and D^{90} in TMD 2 are important for receptor functioning.

GnRH-R cDNAs have been isolated from six mammalian species. They all show a high degree of sequence similarity and display characteristics of the G protein-coupled receptor (GPCR) family (1). Yet, unlike other GPCRs, mammalian GnRH-Rs lack the intracellular C-terminal tail (Fig. 1). Another notable feature of mammalian GnRH-Rs is the presence of N^{87} in TMD 2. At this position, most other GPCRs show a highly conserved D (Fig. 1). Moreover, mammalian GnRH-Rs show a D^{318} in TMD 7 where other GPCRs usually have a N. The functional significance of this interchange of conserved residues was studied in mouse and rat mutant GnRH-Rs (2-4). A close proximity between N^{87} in TMD 2 and D^{318} in TMD 7 was considered to be necessary for maintaining the functional integrity of the GnRH-R, since the $D^{87}D^{318}$ mutant lost GnRH-binding capacity while the reciprocal $D^{87}N^{318}$ mutant regained wild-type receptor properties (1).

Cloning of the African catfish GnRH-R (5) revealed some interesting features of this nonmammalian receptor. Unlike mammalian GnRH-Rs but in accordance with other GPCRs, the catfish GnRH-R contains an intracellular C-terminal tail of 51 amino acids (Fig. 1). Furthermore, in both TMD 2 and 7, D residues (D^{90} and D^{321}) are located at positions homologous to N^{87} and D^{318} of mammalian GnRH-Rs and homologous to the highly conserved D and N residues in TMD 2 and 7, respectively, in other GPCRs (Fig. 1). In order to investigate the functional significance of these features, the ligand binding and signal transduction properties of several mutants were compared with those of the wild-type catfish GnRH-R. Moreover, an antiserum was raised against the extracellular N-terminus of the receptor to investigate wild-type and mutant receptor expression at the cell surface of transiently transfected human embryonic kidney 293T cells (293T cells).

EXPERIMENTAL PROCEDURES

Generation of GnRH-R mutant constructs. The catfish GnRH-R cDNA insert (5) was excised from pcDNA3 (Invitrogen, San Diego,

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This work has been supported by the European Union (Contract No. AIR2-CT93-0817 to R.W.S.).

Abbreviations used: GnRH, gonadotropin-releasing hormone; GnRH-R, GnRH receptor; TMD, transmembrane domain; human embryonic kidney 293T cells, 293T cells; GPCR, G protein-coupled receptor; cfGnRH, catfish GnRH; cGnRH-II, chicken GnRH-II; sGnRHa, salmon GnRH analogue; Bus, Buserelin; Fab, fraction antigen binding.

0006-291X/97 $25.00

Receptor	TM2	TM7	tail
rat TRH-R	VSLAVA **D** LMVLVA	NSAI **N** PVIYN	+
dopamine D1-R	ISLAVS **D** LLVAVL	NSSL **N** PIIYA	+
human β-adrenergic-R	TSLACA **D** LVMGLA	NSGF **N** PLIYC	+
endothelin B-R	ASLALG **D** LLHIII	NSCI **N** PIAL-	+
catfish GnRH-R	ASLASA **D** LVMTFV	NTCC **D** PVIYG	+
rat GnRH-R	KHLTLA **N** LLETLI	NPCF **D** PLIYG	-
human GnRH-R	KHLTLA **N** LLETLI	NPCF **D** PLIYG	-

FIG. 1. Alignment of the amino acid sequences in TMD 2 and 7 regions of selected members of the GPCR family. N^{87} and D^{318} of mammalian GnRH-Rs, D^{90} and D^{321} of the catfish GnRH-R and conserved D and N residues of other GPCRs are in bold type.

CA) and subcloned into pALTER-1 (Promega, Madison, WI). Mutations were introduced using the pALTER-1 *in vitro* mutagenesis system (Promega) according to the manufacturer's instructions. $Asn^{90}Asp^{321}$ mutant (designated ND), $Asp^{90}Asn^{321}$ mutant (DN), a double mutant $Asn^{90}Asn^{321}$ (NN) and a mutant, lacking the intracellular C-terminal tail (Ser^{331} → stop codon; -tail), were confirmed by sequencing. For expression studies, wild-type and mutant inserts were again subcloned into pcDNA3.

Cell culture and transfection. Human embryonic kidney 293T cells (293T cells) (6) were cultured and transfected as previously described for 293 cells (5).

Peptides. Catfish GnRH (cfGnRH; [His^5,Leu^7,Asn^8]GnRH) and chicken GnRH-II (cGnRH-II; [His^5,Trp^7,Tyr^8]GnRH), the two GnRHs native to catfish (7), and salmon GnRH analogue (sGnRHa; [D-Arg^6,Trp^7, Leu^8, Pro^9-NEt]GnRH) were purchased from American Peptide Company (Sunnyvale, CA), Peninsula Laboratories (Merseyside, England) and Syndel Laboratories (Vancouver, BC), respectively. The mammalian GnRH analogue Buserelin (Bus; [D-Ser^6,Pro^9,NEt]GnRH) was a gift from Hoechst AG (Frankfurt am Main, Germany).

Receptor binding. Salmon GnRHa and cGnRH-II were iodinated using chloramine-T and purified by QAE-Sephadex column chromatography (8) or by C18 column chromatography (9), respectively. Twenty hours after transfection, intact 293T cells were harvested and incubated in binding buffer (DMEM supplemented with 25 mM HEPES, 1 mM EDTA, 0.1% BSA, pH 7.4) containing 50,000 cpm [^{125}I]-cGnRH-II (5×10^5cells/tube, 0.5 ml final volume) at 20°C for 2 h to achieve equilibrium. In binding studies using 50,000 cpm [^{125}I]-sGnRHa (12,500 cells/tube, 0.3 ml final volume) displacement curves were generated using various concentrations of unlabelled sGnRHa, cGnRH-II, cfGnRH or Bus. After separation of bound and free hormone by centrifugation of the cells at 200 g (4°C, 5 min) the radioactivity of cell pellets was determined in a γ-counter. Specific binding was calculated by substracting nonspecific binding (binding in presence of 10^{-5} M unlabelled cGnRH-II or 10^{-6} M sGnRHa) from total binding at each peptide concentration. The peptide concentrations inhibiting 50% of the specific binding of [^{125}I]-sGnRHa (IC_{50}) were calculated from the displacement curves using GraphPad PRISM2 (San Diego, CA) software package.

Northern blot analysis. RNA was isolated 50 h after transfection from approximately 7×10^6 transiently transfected 293T cells using RNAzol B (Campro Scientific, Friendswood, TX), treated with glyoxal-dimethyl sulfoxide, subjected to agarose-gel electrophoresis, Northern blotted and hybridized with antisense ^{32}P-UTP-labelled catfish GnRH-R cRNA probe, according to standard procedures (10). A BaFBr:Eu^{2+}-based phosphor screen was exposed to the membranes for 2 h. After exposure, the phosphor screen was scanned on a Phosphor Imager SI (Molecular Dynamics, Boston, MA) and the phosphor image was analysed with ImageQuaNT version 4.2 software (Molecular Dynamics).

Immunocytochemical detection of GnRH-R expression. Transfected 293T cells were cultured on chamber slides, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, blocked with 0.5% non-fat dried milk in Coons buffer (0.01 M 5,5′-diethylbarbituric acid sodium salt, 0.85% NaCl, pH 7.4) for 30 min, and incubated with an antiserum raised against the N-terminus of the receptor. To this end, the coding region of the extracellular, N-terminal domain was PCR amplified, using the primers NRN(S), 5′-CAGCGGATCCGGAAACACAACACTTCTACTTTC-3′, and NRN(AS), 5′-CGCGAATTCACGTGGTGAAGGTCGGGGTC-3′, containing *Bam*HI and *Eco*RI sites, respectively (underlined). Using these sites, the amplified fragment was cloned into pGEX-2T. After sequence confirmation of the cloned insert, and induction of the GST-N-terminal catfish GnRH-R fusion-protein expression, the fusion protein was purified and used for immunization. Rats were injected for 3 times in intervals of 4 weeks with 50μg fusion protein in combination with Freuds adjuvant. 10 days after the last injection the antiserum was purified from blood samples. The antiserum was diluted 1:1000 with Coons buffer containing 0.01% BSA-c (Aurion, Wageningen, The Netherlands) and incubated with intact 293T cells overnight at room temperature. After exposure to peroxidase-conjugated goat anti-rat Fab fragments (1:500; Sigma, St. Louis, MO) at room temperature for 90 min, peroxidase was visualized with 4-chloro-1-naphtol (Sigma). The slides were mounted in glycerin/gelatin and cover slipped. For control purposes, wild-type or mutant catfish GnRH-R transfected 239T cells were incubated with rat serum sampled before immunization, or mock-transfected cells were incubated with the antiserum.

Colorimetric detection of GnRH-induced cAMP production. GnRH-induced cAMP production in 293T cells was quantified colorimetrically as described previously (5). Concentration of cAMP are represented as optical densities which are related to the optical density of forskoline stimulation. The GnRH concentrations inducing half-maximal stimulation (EC_{50}) were calculated using GraphPad PRISM2 software package. When two EC_{50} concentrations are given, data sets were best fitted using an equation assuming two binding sites.

RESULTS

Radioligand binding of wild-type and mutant receptors. Apart from the wild-type receptor, only the DN mutant receptor specifically bound the endogenous ligand [^{125}I]-cGnRH-II (data not shown). Under the con-

TABLE 1

Binding Parameters of Wild-Type and Mutant Catfish GnRH-Rs Expressed in 293T Cells

Construct	Specific binding (%)	IC_{50} (nM)			
		sGnRHa	cGnRH-II	Bus	cfGnRH
Wild-type	100.0 ± 2.6	1.0 ± 0.2	4.0 ± 0.9	340 ± 55	7327 ± 196
DN mutant	150.0 ± 4.6	1.2 ± 0.3	9.5 ± 1.4*	379 ± 66	10350 ± 490*
ND mutant	6.0 ± 0.9[a]	—	—	—	—
NN mutant	6.2 ± 1.9[a]	—	—	—	—
-Tail mutant	6.9 ± 1.4[a]	—	—	—	—

Note. Data shown are means ± SEM of three independent experiments. *Indicates a significant difference ($P < 0.05$) compared to the wild-type receptor.

[a] This percentage of specific binding did not allow displacement.

ditions tested, even [^{125}I]-sGnRHa, a high-affinity ligand for the wild-type catfish GnRH-R (11), was hardly bound by the ND or NN mutant receptors, or by the truncated receptors (Tab. 1), whereas [^{125}I]-sGnRHa was clearly bound by wild-type and DN mutant receptors. Salmon GnRHa, cGnRH-II, Bus and cfGnRH inhibited [^{125}I]-sGnRHa binding in a dose-dependent way to intact 293T cells transiently transfected with the wild-type or DN mutant catfish GnRH-R (Fig. 2). IC_{50} values of the DN mutant for sGnRHa and Bus (Tab. 1) were not significantly different from those of the wild-type receptor, while small (1.4- or 2.4-fold) increases in IC_{50} values were recorded for cfGnRH and cGnRH-II, respectively. For NN and ND mutant and C-terminally truncated receptors displacement studies could not be performed.

Expression of wild-type and mutant receptors. Northern blot analysis revealed, that wild-type and mutant GnRH-R steady-state mRNA levels in 293T cells were similar (data not shown). Thus, all mutant cDNAs were veritably transfected into 293T cells. To study if mutant receptor proteins were actually inserted into the plasma membrane of transiently transfected cells, transfected cells were probed with an antiserum raised against the N-terminal extracellular domain of the wild-type GnRH-R. The antiserum recognized the wild-type receptor (Fig. 3A) as well as all mutant receptors (Fig. 3B, data not shown). Considering that the cells were not permeabilized and that the antiserum was raised against an extracellular domain of the receptor, it is not surprising that the staining is localized predominantly to the cell surface. Incubation with rat preimmune serum did not lead to a staining of 293T cells expressing wild-type or mutant receptors; mock-transfected cells were not stained by the antiserum (Fig. 3C).

Agonist-stimulated cAMP production. Mock-transfected cells, ND and NN mutant receptors as well as the receptor missing the C-terminal tail, did not transduce the GnRH signal to an elevated intracellular cAMP response (data not shown). Apart from the wild-type receptor, only the DN mutant responded to the

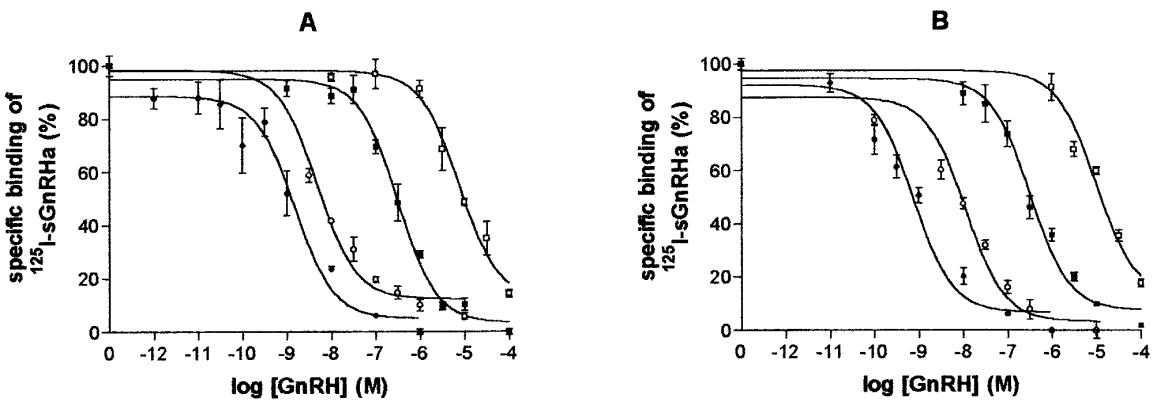

FIG. 2. Displacement of [^{125}I]-sGnRHa binding to 293T cells transiently expressing wild-type (A) or DN mutant (B) catfish GnRH-Rs by sGnRHa (●), cGnRH-II (○), Bus (■) and cfGnRH (□). Data are given as means ± SEM of triplicate determinations from one out of three independent experiments giving similar results. The 100% specific binding corresponds to 4519 dpm and 7038 dpm or 9 % and 14 % of the total counts added for the wild-type and the DN mutant receptor, respectively.

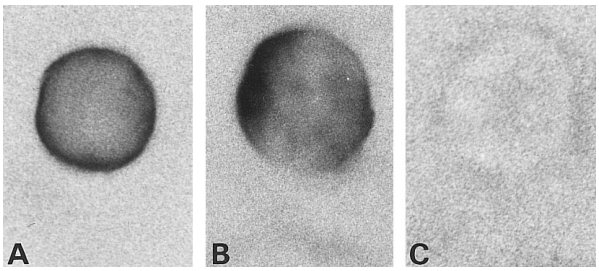

FIG. 3. Immunocytochemical staining of 293T cells transiently transfected with the wild-type receptor (A) or the C-terminally truncated receptor (B) using an antiserum raised against the N-terminal extracellular domain of the catfish GnRH-R. Mock-transfected cells were not stained (C).

various GnRHs with an elevation in cAMP in a dose-dependent manner (Fig. 4 A-C). Stimulation with cGnRH-II resulted in biphasic dose-response curves with the wild-type receptor and the DN mutant. The concentrations of cGnRH-II and Bus inducing half-maximal stimulation (EC_{50}, Tab. 2) did not differ significantly between wild-type and DN mutant receptors while a small (1.6-fold) increase was recorded for cfGnRH. However, the DN mutant receptor showed a significantly impaired maximal stimulation in response to all three agonists compared to the wild-type receptor (E_{max}, Tab. 2).

DISCUSSION

Four mutant catfish GnRH-Rs were constructed; a receptor missing the intracellular C-terminal tail, and mutants in which D^{90} and/or D^{321} in TMDs 2 and 7, respectively, were mutated to N residues. Only one of the four mutants, the DN mutant, harboring the arrangement found in most other GPCRs, showed ligand-binding activity and generated elevated intracellular cAMP levels upon agonist stimulation. In the other mutants, failure to couple to cAMP production was associated with the lack of ligand binding while the mutant receptor protein apparently was inserted into the plasma membrane.

Importance of the C-terminal tail. The C-terminally truncated receptor lost both the ability for agonist binding and GnRH-stimulated cAMP-production, suggesting that the intracellular C-terminal tail of the catfish GnRH-R is essential for receptor functioning. At first, it may appear odd that the intracellular C-terminal domain is involved in ligand binding, also in view of the fact that the known mammalian GnRH-Rs lack this domain. However, in the catfish GnRH-R the C-terminal tail might be necessary for proper receptor folding, in such a way that loss of the tail disturbs the conformation of the ligand binding site. For the histamine H_2 receptor, for example, it has also been reported that C-terminally truncation results in loss of binding (12). Future mutagenesis studies may shed light on the question which particular part(s) of the intracellular C-terminal tail are pivotal for receptor functioning. C^{339} and C^{341} are interesting candidates, since homologous C residues in other GPCRs have been shown to anchor the C-terminus via palmitoylation into the plasma membrane (13,14).

D^{90} in TMD 2. Neither the ND nor the NN mutant were able to bind the endogenous ligand cGnRH-II. Even sGnRHa, the ligand for which the catfish GnRH-R has the highest affinity (11), was hardly bound by these mutants. Therefore D^{90} in TMD 2 seems to be critical for agonist binding of the catfish GnRH-R. Yet, this residue is more likely to be involved in proper receptor folding than in direct interaction with the ligand. In most other GPCRs a highly conserved D is the homologous residue in TMD 2. This D is usually not directly implicated in ligand binding. Replacement of this residue in different GPCRs resulted in a variety of functional effects (2). Hence, in this respect, the catfish GnRH-R appears to be more akin to other GPCRs than to its mammalian homologues.

D^{321} in TMD 7. The DN configuration was the only mutant catfish GnRH-R that showed characteristics

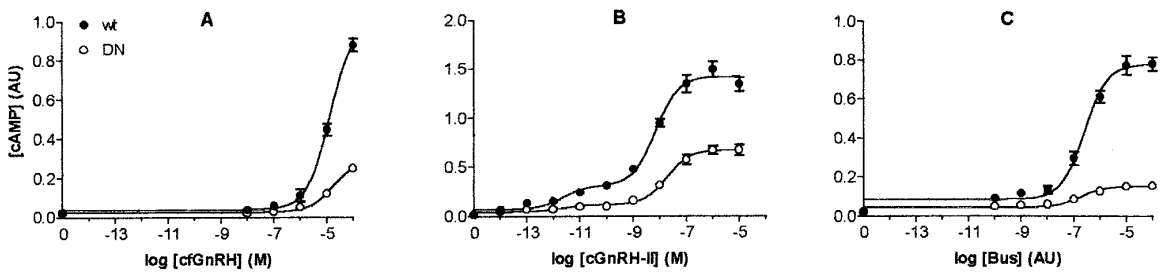

FIG. 4. cAMP response of wild-type (●) and DN mutant (○) catfish GnRH-Rs to cfGnRH (A), cGnRH-II (B) and Bus (C). cAMP concentrations were measured indirectly in a colorimetric assay, which detects reporter-gen-β-galactosidase expression (5). Optical densities were related to the optical density of forskoline stimulation. Data are represented as means ± SEM of triplicate determinations from a representative experiment out of three independent experiments.

TABLE 2

Characteristics of Agonistic Potencies of cfGnRH, cGnRH-II, and Bus at Wild-Type and Mutant Catfish GnRH-Rs Transiently Expressed in 293T Cells

	Wild-type		DN mutant	
Agonist	EC_{50} (nM)	E_{max} (AU)	EC_{50} (nM)	E_{max} (AU)
cfGnRH	1219 ± 531	0.90 ± 0.06	19901 ± 1571*	0.25 ± 0.02*
cGnRH-II	0.23 ± 0.23		0.14 ± 0.11	
	10.5 ± 6.5	1.32 ± 0.08	31.99 ± 11.09	0.62 ± 0.05*
Bus	147 ± 57	0.69 ± 0.05	247 ± 40	0.15 ± 0.02*

Note. Data shown are means ± SEM of three independent experiments. *Indicates a significant difference ($P < 0.05$) compared to the wild-type receptor. Agonistic effects were measured as the accumulation of cAMP.

similar to the wild-type receptor, including the previously reported (5) biphasic response to cGnRH-II, for which we have no explanation yet. The lower capacity of this mutant to generate cAMP upon GnRH-stimulation does not appear to be related to decreased receptor expression at the cell surface, since specific binding of [^{125}I]-sGnRHa to cells transfected with the DN mutant was even higher than to cells transfected with the wild-type receptor. D^{321} possibly determines the efficacy of receptor coupling to various G-proteins. Awara *et al.* (4) proposed a role of the homologous D^{318} residue in the murine GnRH-R for determining the coupling route to intracellular processes. They demonstrated that $D^{87}N^{318}$ and $N^{87}N^{318}$ mutant receptors led to increased cAMP production, but did not change inositol phosphate production in transfected lactotropic GH_3 cells, whereas the wild-type murine GnRH-R ($N^{87}D^{318}$) generated inositol phosphate, but not cAMP upon stimulation with GnRH. Thus, depending on the amino acid residue at this position, either G_q or G_s was activated.

Proximity of TMD 2 to 7. Mutagenesis studies suggested that N^{87} in TMD 2 and D^{318} in TMD 7 of mammalian GnRH-Rs interact via hydrogen bonding, indicating that TMD 2 and TMD 7 are in close proximity (2). $D^{87}D^{318}$ mutant mouse and rat GnRH-Rs have been shown to lose the ability to bind GnRH (2,3). According to the model of spatial relationship between TMD 2 and TMD 7 (2), the two negatively charged D residues would repulse each other resulting in a disturbed receptor conformation and a loss of binding capacity. The functional, wild-type catfish GnRH-R, however, harbors D residues at both sites. Assuming that this non-mammalian receptor also displays spatial proximity between TMD 2 and 7, alternative residues may be involved in mediating this proximity. N^{315} and N^{317} in TMD 7 are candidates, which might function as hydrogen donors to D^{90} in TMD 2. Jagerschmidt *et al.* (15) have reported that the mutant $D^{100}D^{391}$ cholecystokinin B receptor displays wild-type ($D^{100}N^{391}$) properties and proposed that there is no interaction between these two negatively charged residues. Furthermore, Sealfon *et al.* (16) created a functional $D^{120}D^{376}$ mutant serotonin 5-HT 2A receptor and suggested that protonation of one of the D side chains in the vincinity of an already negatively charged D could restore the hydrogen-bonding relationship to that found in the wild-type serotonin 5-HT 2A receptor. A similar protonation might occur in the catfish GnRH-R, allowing an interaction between D^{90} and D^{321} in a way that TMD 2 and 7 come into close proximity.

Conclusion. None of the mutants partially mimicking the situation in mammalian GnRH-Rs (ND and C-terminally truncated mutant) bound GnRHs (including mammalian GnRH analogue) or coupled to cAMP. However, the combination of both types of mutations still has to be tested. We conclude that the catfish GnRH-R differs in its structure-function properties from its mammalian homologues, since it needs its intracellular C-terminal tail as well as D^{90} in TMD 2 for functioning, and is in this respect more akin to other GPCRs than to mammalian GnRH-Rs. From an evolutionary point of view, it will be interesting to analyse the corresponding features in GnRH-Rs from other fishes and from submammalian tetrapod vertebrates.

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