Published in final edited form as:

Gen Comp Endocrinol. 2010 May 1; 166(3): 498–503. doi:10.1016/j.ygcen.2009.12.001.

A role of Histidine¹⁵¹ in the lamprey Gonadotropin-Releasing Hormone Receptor (IGnRHR-1) : Functional insight of diverse amino acid residues in the position of Tyr of the DRY motif in GnRHR from an ancestral Type II receptor

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Abstract

The highly conserved DRY motif located at the end of the third transmembrane of G protein-coupled receptors has been described as a key motif for several aspects of GPCR functions. However, in the case of the vertebrate gonadotropin-releasing hormone receptor (GnRHR), the amino acid in the third position in the DRY motif is variable. In the lamprey, a most basal vertebrate, the third amino acid of the “DRY” in GnRHR is His, while it is most often His/Gln in the type II GnRHR. To investigate the functional significance of the substitution of DRY to DRH in the lamprey(I)GnRHR, second messenger signaling, ligand binding and internalization of the wild-type and mutant IGnRH receptors were characterized with site-directed mutagenesis. Treatment of the DRE¹⁵¹ and DRS¹⁵¹ mutant receptors with lamprey GnRH-I significantly reduced inositol phosphate compared to wild-type (DRH¹⁵¹) and DRY¹⁵¹ receptors. The logIC₅₀ of wild-type receptor (−9.554±0.049) was similar to the logIC₅₀ of DRE¹⁵¹, DRS¹⁵¹ and DRX¹⁵¹ mutants, yet these same mutants were shown to significantly reduce cell surface expression. However, the DRY¹⁵¹ mutant compared to the wild-type receptor increased cell surface expression, suggesting that the reduction of IP production was due to the level of the cell surface expression of the mutant receptors. The rate of internalization of DRX¹⁵¹ (35.60%) was reduced compared to wild-type and other mutant receptors. These results suggest that His¹⁵¹ of the lamprey GnRH receptor may play a critical role in the retention of a certain level of cell-surface expression for subsequent cellular second messenger events.

Keywords

GnRH receptor; DRY motif; receptor expression; signaling; site-directed mutagenesis; lamprey

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1. Introduction

The hypothalamic-pituitary (HP) system is considered to be a vertebrate innovation and seminal event that emerged prior to or during the differentiation of the ancestral agnathans, reviewed in Sower et al., 2009. In spite of the very diverse patterns of life cycles and reproductive strategies and behaviors, this endocrine system is remarkably conserved throughout the gnathostome line-ages. Lampreys as basal vertebrates are the earliest evolved vertebrates for which there are demonstrated functional roles for two possibly three gonadotropin-releasing hormones (GnRHs) that act via the hypothalamic-pituitary-gonadal axis controlling reproductive processes (Kavanaugh et al., 2008). To date, the biochemical, molecular, immunocytochemical and functional studies on the structure and function of the GnRHs in lamprey have established that similar to all other vertebrates, the lampreys have a hypothalamic-pituitary-gonadal axis and that there is a high conservation of the mechanisms of GnRH action (Sower et al., 2009). From recent data, we propose a modified paradigm in that the neuroendocrine control of reproduction and thyroid functions in an Agnathan, the sea lamprey, exhibits an overlapping, simplified organization represented by one and possibly two glycoprotein hormones putatively interacting with two glycoprotein receptors, a gonadotropin-like receptor and a thyroid stimulating hormone -like receptor (Sower et al., 2009). This modified paradigm now includes the agnathans and can serve as a model for analysis of the evolutionary mechanisms leading to emergence of the highly specialized Gnathostome endocrine axes.

GnRH is a central regulator of reproductive function in vertebrates and acts via the hypothalamic-pituitary-gonadal axis. Its function is mediated through a pituitary GnRH receptor (GnRHR), a class A 7-transmembrane G protein-coupled receptor (GPCR). One of the remarkable characteristics of vertebrate GnRHRs is the absence or presence of the intracellular C-terminal tail. The receptors are generally categorized as two different types: type I receptor, lacking the intracellular C-terminal tail, or type II receptor, retaining the C-terminal tail (Millar et al., 2004). The other notable characteristic of GnRHRs is the variation of a DRY motif of GPCRs that is a highly conserved amino acid triplet at the end or junction of the third transmembrane domain and the second intracellular loop. DRY has been described as a key motif for several aspects of GPCR functions including receptor activation, ligand binding and G-protein coupling (Rovati et al., 2007). There are variable substitutions of the third amino acid in the “DRY” motif of GnRHRs from different classes of vertebrates. This region potentially contributes to GnRHR function. In many cases, type I receptor DRY motif is substituted with DR‘S’, while type II has DR‘H/Q’ (Fig. 1). To date, there are few reports about the functional significance of the Ser in DRS of type I receptors (Arora et al., 1997; Arora et al., 1995; Byrne et al., 1999). Thus, the functional significance of this variation of the DRY motif, particularly the type II GnRHR, is not established.

In the sea lamprey, *Petromyzon marinus*, one of only two extant representatives of the oldest lineage of vertebrates, agnathans, a functional type II GnRH receptor was cloned from the pituitary (Silver et al., 2005). The lamprey GnRH receptor (lGnRHR-1) was shown to activate both the cAMP and IP signaling systems; however, the IP system was activated at an approximately 10 fold lower concentration to both lamprey GnRH-I and lamprey GnRH-III, and was also activated to a greater magnitude of approximately 4.5 fold, compared to ~1.7 fold (lamprey GnRH-I) or ~2.1 fold (lamprey GnRH-III) (Silver and Sower, 2006). These responses of IP₃ and cAMP signaling systems are similar to the type I and type II GnRH receptors from different vertebrate species (Arora et al., 1998; Grosse et al., 2000; Liu et al., 2002; Oh et al., 2005; Stanislaus et al., 1998). In addition, the lGnRHR-1 retains conserved structural features and amino acid motifs of other known GnRH receptors. An HFRK motif in the membrane proximal region of the bullfrog type-II GnRH receptor-1 was shown to be required for cAMP signaling, but not for IP signaling (Oh da et al., 2005).

Similarly a homologous motif, HVRR, was shown also to be required for cAMP signaling in the lGnRHR-1 (Silver and Sower, 2006). In the lamprey, the third amino acid residue of the DRY motif in GnRH receptor is “His” instead of “Tyr” and in the gnathostome type II GnRH receptors, the third amino acid residue is most often His/Gln instead of Tyr. These identified conserved motifs in the lamprey GnRH receptor are thought to be evolutionarily conserved throughout vertebrates. Thus, the lGnRHR-1, an ancestral form of vertebrate GnRHRs, is a critical model in our understanding of the structural and functional evolution of GnRHR. To investigate the functional significance of the substitution of DRY to DRH in the lGnRHR-1, second messenger signaling, ligand binding and internalization of the wild-type and mutant lGnRHRs were characterized with site-directed mutagenesis. The His¹⁵¹ of DRH was substituted with Tyrosine (DRY¹⁵¹ mutant), Serine (DRS¹⁵¹ mutant), Glutamate (DRE¹⁵¹ mutant), or point deletion (DRX¹⁵¹ mutant).

2. Materials and methods

2.1. Site-directed mutagenesis

The point mutations were introduced into the pcDNA3.1 vectors (Invitrogen, Carlsbad, CA, USA) including the full coding sequence of lGnRHR-1 by Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using mutagenic primers as follows: DRY¹⁵¹ mutant was amplified using the sense primer, 5'-GCTTGGACCGCTACTCGGCCATCCTC -3' and the antisense primer, 5'-GAGGATGGCCGAGTAGCGGTCCAAGC -3'. The sense primer, 5'-GCTTGGACCGCGAGTCGGCCATCCTC -3' and the antisense primer, 5'-GAGGATGGCCGACTCGCGGTCCAAGC -3' were used for DRE¹⁵¹ mutant, the sense primer, 5'-GCTTGGACCGCTCATCGGCCATCCTC -3' and the antisense primer, 5'-GAGGATGGCCGATGAGCGGTCCAAGC -3' were for DRS¹⁵¹ mutant, and the sense primer, 5'-CAGCTTGGACCGCTCGGCCATCCTCA -3' and the antisense primer, 5'-TGAGGATGGCCGAGCGGTCCAAGCTG -3' were for DRX¹⁵¹ mutant. The underlined nucleotides indicate the sequences encoding the substituted amino acids (Fig. 2). PCR was performed with 2 ng of the pcDNA3.1 including the full coding sequence of lGnRHR-1 as the template at 95°C for 30 sec as an initial denaturation followed by 18 cycles of 95°C for 30 sec as denaturation, 55°C for 1min as annealing and 68°C for 6 min as extension. PCR product was subsequently digested by Dpn I restriction enzyme (4 U/PCR mixture) at 37°C for 1h and directly transformed into OneShot Top 10 chemically competent E.coli (Invitrogen, Carlsbad, CA, USA). The desired mutations of the insert sequences were confirmed by DNA sequencing.

2.2. Cell culture and transfection

COS7 cells were maintained in 10% fetal bovine serum in DMEM (Invitrogen) at 37°C in 5% CO₂ before transfection as previously described (Silver et al., 2005). Each 24 µg of construct DNA: DRH¹⁵¹ (wild-type), DRY¹⁵¹, DRE¹⁵¹, DRS¹⁵¹ and DRX¹⁵¹ mutants, or non-insert vector was diluted in 1.5 ml of Opti-MEM (Invitrogen) and mixed gently, while 60 µl of Lipofectamin 2000 (Invitrogen) was diluted in 1.5 ml of Opti-MEM for each construct DNA and incubated for 5 min at room temperature. After the 5 min incubation, the diluted DNA and the diluted Lipofectamine were combined, mixed gently and incubated for 20 min at room temperature. After the 20 min incubation, each DNA/Lipofectamine/Opti-MEM complex was added into 2.2×10^6 cells cultured with 10% fetal bovine serum in DMEM in 10-cm culture plate and incubated for 24 h at 37°C in 5 % CO₂.

2.3. Inositol phosphate assay

The inositol phosphate assay (IP assay) was performed as previously described (Silver et al., 2005). Transfected cells were trypsinized and seeded in 12-well plates (1.0×10^5 cells per

well) and cultured in 10% fetal bovine serum in DMEM at 37°C in 5% CO₂. Briefly, after the adhesion of the cells (overnight culture), cells were washed with PBS and incubated in 1 ml of Medium 199 (Invitrogen) containing 2% fetal bovine serum and 2μCi Myo [2-³H]inositol (Amersham Pharmacia, Buckinghamshire, UK) per well for 18h. After the 18h incubation, cells were washed and pre-incubated. After pre-incubation, cells were incubated in 1 ml of IP buffer containing concentrations of lGnRH-III (10⁻⁶ to 10⁻¹⁴M) per well. The stimulations were stopped and the plates were incubated on ice for 30 min. The extracts from the cells were transferred, neutralized with 5M KOH and 20% perchloric acid and incubated. The supernatant of the extracts were transferred after centrifugation at 5000 rpm at 4°C. Total inositol phosphate was isolated from the extracts by ion exchange chromatography with AG1×8 resin (BioRad, Hercules, CA, USA) and measured. Treatments with each concentration of lGnRH-III were run in duplicate and the data were fitted with sigmoidal dose-response curves by Prism 4 (GraphPad, San Diego, CA, USA).

2.4. Receptor binding assessment

The ligand-binding assay was performed as previously described (Silver and Sower, 2006). Briefly, transfected cells were seeded and after two days, cells were washed with assay buffer (25 mM HEPES-modified DMEM with 0.1% BSA) and then incubated with assay buffer containing 1 nM ¹²⁵I-labeled lamprey GnRH-I and decreasing concentrations of unlabeled lGnRH-III (10⁻⁶ to 10⁻¹²M) for 3.5 h on ice at 4°C. After incubation, cells were washed and solubilized. All total binding and NSB samples were run in triplicate, and each independent experiment was repeated at least three times. The data were fitted with one-site competition binding curves by Prism 4 (GraphPad).

2.5 Receptor-internalization assay

Transfected cells were seeded in 24-well plates at 1.5 × 10⁵ cells per well and maintained for 48 h as previously described (Silver and Sower, 2006). After 48 h, cells were incubated with 1 nM ¹²⁵I-labeled lamprey GnRH-I at 4°C for 3.5 h and then incubated at 37°C for various periods of time. Subsequently, cells were washed twice for 20 min to remove surface bound ¹²⁵I-labeled lamprey GnRH-I (Silver and Sower, 2006). Acid resistant (internalized) ligand was solubilized in 0.5 M NaOH. Nonspecific binding was determined by cells transfected with non-insert vector or non-transfected cells at each time point. Surface bound and internalized ligands were counted by γ-counter. The treatments were run in triplicate. The data were fitted with one phase exponential association curves by Prism 4 (GraphPad).

3. Results

3.1. IP response of the DRY¹⁵¹, DRS¹⁵¹, DRE¹⁵¹ mutants, or wild type (DRH¹⁵¹)

All mutant receptors showed dose-response effects to lGnRH-III from 10⁻⁶ to 10⁻¹⁴M and with different Log EC₅₀ values (Fig. 3). Log EC₅₀ (mean ± SEM) of DRE¹⁵¹ mutant (-11.06 ± 0.17) and DRS¹⁵¹ mutant (-10.95 ± 0.16) were higher than that of wild-type (-11.39 ± 0.21), while maximal IP production (mean ± SEM) of DRE¹⁵¹ mutant (1166 ± 42.75) and DRS¹⁵¹ mutant (1458 ± 48.67) was lower than that of wild-type (1794 ± 71.59). On the other hand, log EC₅₀ of DRY¹⁵¹ mutant (-12.32 ± 0.27) was lower than that of wild-type, while the maximal IP production of DRY¹⁵¹ mutant (1808 ± 79.52) was comparable to that of wild-type.

3.2. Ligand binding of the DRY¹⁵¹, DRS¹⁵¹, DRE¹⁵¹, DRX¹⁵¹ mutants, or wild type

Ligand-binding affinity between all receptors was similar (Fig. 4). The logIC₅₀ of wild-type receptor (-9.554±0.049) was similar to the DRY¹⁵¹ mutant (-9.422±0.050), DRE¹⁵¹ mutant (-9.426±0.072), DRS¹⁵¹ mutant (-9.433±0.067) and DRX¹⁵¹ mutant (-9.528±0.065).

However, ^{125}I -IGnRH-I binding varied between wild-type and mutant receptors. The level of the specific binding (%WT) of DRY 151 mutant was significantly higher (164%) than WT, and DRE 151 (12%), DRS 151 (29%) and DRX 151 (14%) mutants were significantly lower than WT (Fig 5).

3.3. Wild-type and mutant lGnRHRs internalization

To investigate the effects of His 151 point mutations of lGnRHR-1 on endocytotic trafficking in ligand-dependent manner, internalization assays were performed with ^{125}I -IGnRH-I. Wild-type and mutant receptors were rapidly internalized ($t_{1/2} = 7.5\text{--}11$ min) and each reached a plateau within 60 min. Y max of mutant receptors (DRY $^{151} = 77.19\%$, DRE $^{151} = 70.13\%$, DRE $^{151} = 71.65\%$) except for DRX 151 was similar to that of the wild-type receptor (77.36%) (Fig. 6). The rate of internalization of DRX 151 (35.60%) was reduced compared with wild-type and the other mutant receptors.

4. Discussion

DRY is a highly conserved amino acid triplet of GPCRs and has been described as a key motif for several functions of GPCR (Rovati et al., 2007). It has been shown that the critical residues for receptor functions of the DRY motif of GPCRs are the first two residues, Asp and Arg, rather than the third residue, Tyr (Flanagan, 2005; Rovati et al., 2007). The GnRH receptor is a class A rhodopsin-like GPCR and one of the receptors which has been characterized physiologically and pharmacologically in mammals and also some nonmammalian vertebrates because of its key role in reproductive function through vertebrates. The third amino acid residue in the DRY motif is variable in the vertebrate gonadotropin-releasing hormone receptor. In the lamprey, a most basal vertebrate, the third amino acid residue of the DRY motif in the GnRH receptor is "His" instead of "Tyr". In type II GnRH receptors, the third amino acid residue is most often His/Gln instead of Tyr. This is the first study that has examined the third amino acid substitution in the DRY/H/Q of any type II GnRH receptors. Treatment of the DRE 151 and DRS 151 mutant receptors with lamprey GnRH-I significantly reduced inositol phosphate compared to wild-type (DRH 151) and DRY 151 receptors. Subsequent studies using ligand binding and receptor expression were analyzed with wild-type and mutant receptors. The binding affinity using log IC $_{50}$ was similar between wild-type and mutant receptors, which indicates that ligand binding affinity didn't influence the change of IP response. However, the level of cell-surface expression of the receptors labeled with ^{125}I -GnRH-I was different between wild-type and mutant receptors. Since the competitive binding assays showed that the ligand binding affinity between all mutant and wild-type receptors was not significantly different, the total binding of ^{125}I -GnRH-I to the wild-type and mutant lGnRHRs could be directly compared as the receptor expression level. DRE 151 , DRS 151 and DRX 151 mutants significantly reduced cell surface expression while DRY 151 mutant increased cell surface expression when compared to the wild-type receptor, suggesting that the reduction of IP production was due to the level of the cell surface expression of the mutant receptors. In addition, the level of internalization was similar between wild-type and mutant receptors except for the DRX 151 mutant. The DRX 151 mutant induced a decrease in internalization as well as a reduced cell surface expression, suggesting ligand-independent receptor expression, i.e. such as receptor trafficking from the endoplasmic reticulum (ER).

In general, point mutations lead to misfolding that can affect the cell surface expression of proteins involving GPCRs (Duvernay et al., 2005; Garriga et al., 1996; Sitia and Braakman, 2003). It has been described that most of the point mutations in GnRH receptor lead to a decrease in receptor expression including the natural mutations which are linked to human disease, hypogonadotropic hypogonadism (Conn et al., 2007; Millar et al., 2004; Rispoli and Nett, 2005). According to several reports from several types of GPCR, Asp and Arg of DRY

are critical for cell surface expression due to the structural instability, impairment of the association with β -arrestin and internalization (Alewijnse et al., 2000; Hawtin, 2005; Wilbanks et al., 2002). Additionally, pharmacopone, which rescues misfolded mutant proteins to correctly route in the cells, was shown to function in an Arg mutant of the V2 vasopressin receptor suggesting that DRY is important for folding and cell surface trafficking from the ER (Conn et al., 2007). The significance of Tyr residue of DRY was unclear from this vasopressin receptor study. In the present study, the lamprey GnRHR and three of the four mutants of lamprey GnRHR all induced a decrease in receptor expression except the mutant DRY¹⁵¹ which induced an increase in receptor expression. Other studies on the DRS motif in mammalian type I GnRHRs revealed that Ser could be involved in ligand binding and receptor internalization events (Arora et al., 1997; Arora et al., 1995; Byrne et al., 1999). Substituting Ser with Tyr increased both the ligand binding affinity and internalization (Arora et al., 1995) but substituting with Ala had no effect (Arora et al., 1997). In our study, substituting His with Tyr increased the cell-surface expression of the receptor but had no effect on ligand binding affinity and did not increase internalization. Thus, mammalian type I and lamprey type II GnRH receptors can both accept the substitution of Tyr and retain the functionality of the original residues which imply Tyr could be primordial through vertebrate GnRHRs, although the specific functions are different between the two types of receptors. These differences between the mammalian and lamprey GnRH receptors may represent a different functionality between type I and type II receptors, which are representative of two evolutionarily distant receptors. For instance, there is not an intracellular c-terminal tail in type I receptor so that the receptor cannot be rapidly internalized. This phenomenon implies that different mechanisms of desensitization in the mammalian type I GnRH receptors compared to the type II GnRH receptors have evolved over the course of the evolution of vertebrates (McArdle et al., 1999; McArdle et al., 2002). The rate of internalization of type II GnRH receptors is much greater compared to type I GnRH receptors suggesting the c-terminal tail is essential for the rapid internalization (Pawson et al., 1998), and seems to be maximized at the biological threshold. This suggests that the type II GnRH receptor mutants may not be capable of augmenting internalization. In addition, Byrne et al (1999) suggested that the third amino acid position in DRY and the phosphorylation in the site are not essential for internalization. This would support the result of our study that the rate of internalization of the DRE¹⁵¹, DRS¹⁵¹ and DRY¹⁵¹ mutants was similar to that of wild-type because the c-terminal tail was more essential for internalization, although DRX¹⁵¹, point deletion of His¹⁵¹ deletion in DRH, decreased the rate of internalization compared to other mutants and the wild-type receptors. DRX¹⁵¹ mutant possesses Ser¹⁵² next to Arg¹⁵⁰ (Fig. 2) so that DRX¹⁵¹ could compensate for the lack of His¹⁵¹ with Ser¹⁵² and maintain the same amino acid triplet as DRS¹⁵¹. However, the rate of internalization of DRX¹⁵¹ was different from DRS¹⁵¹, suggesting that the conformational change of the receptor caused by His¹⁵¹ might be linked to the internalization event.

The third amino acid of the DRY motif is highly conserved among most of GPCRs but not in the GnRH receptors. Although there are some exceptions in that DRY is found in a few vertebrate and invertebrate GnRH receptors (Hauser et al., 1998; Ikemoto and Park, 2005). Lampreys have two other GnRH receptors that have not yet been published. One of the novel lamprey GnRH receptors (lGnRHR-3, accession number [DQ915103](#)) has DRY instead of DRH. The experimental evidence from the current study shows that substitution of DRH with DRY maintains the receptor functions. In examining this motif across invertebrate and vertebrate GnRH receptors, it is suggested that DRY could be an ancestral motif of an ancestral GnRHR since it is also highly conserved in the other types of GPCRs. This motif in GnRHR receptors has perhaps been subjected to selection pressures leading to variable amino acid residues including S, H or Q in the third position of DRY motif during the course of GnRHR molecular evolution.

In summary, the mutation of His¹⁵¹ of the lamprey GnRH receptor altered the cell-surface receptor expression as well as second messenger signaling and internalization. These results suggest that His¹⁵¹ of the lamprey GnRH receptor may play a critical role in the retention of a certain level of cell-surface expression for subsequent cellular second messenger events. Further studies of the lamprey GnRH receptor(s) that have retained key ancestral residues and motifs will be necessary in furthering our understanding of the evolution of the structure and function of the GnRH receptor in vertebrates.

Acknowledgments

This research was supported by a grant of NIH [5R21RR024477-02]. This is Scientific Contribution Number 2386 from the New Hampshire Agricultural Experiment Station. We thank Bernadine Schultz, Mike Wilmot and Allisan Aquilina-Beck for their assistance with iodination. We also thank Dr. Mihael Freamat and Scott I. Kavanaugh for their useful advice and suggestions on the experimental techniques.

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		TM3	DRY motif	
Type II	Lamprey	GEFACRLMFLRLLLAMYSSAFITVVISLDRHSAILNPLGIG	161	
	Catfish1	GDAMCKLMCFLKLFAMHSAAFILVVVSLDRHHAILHPLDTL	153	
	Catfish2	GDGLCKLLSFLKLFAMQASAFILVVISLDRQHAILHPLDTL	151	
	Medaka1	GDAACRFLMFLKLQAMYSACFVTVVISLDRQSAILRPLSIS	159	
	Medaka2	GDALCKLLCFLKLFAMHASAFILVVITLDRQHAILHPLDAL	159	
	Medaka3	GDLACRLLMFMKLVAMYSACFVTVVISLDRQSAILNPLGIS	148	
	GoldfishA	GDGLCKLLCFLKLFAMQTSAFILVVISLDRHHAILHPLDSL	142	
	GoldfishB	GNAMCKNLCFLKLFAMHSAAFILVVVSLDRHHAILHPLEAL	152	
	Pufferfish1-1	GDLACRILMFLKLQAMYSACFVTVVISLDRQWAVLHPLAVV	147	
	Pufferfish1-2	GDLACRALMFLKLQAMYSACFVTVVISLDRQSAILHPLAIT	160	
	Pufferfish1-3	GDAACRLMFLKLQAMYSACFVTVVISLDRQSAILNPLGIS	148	
	Pufferfish2-1	GDVMCKLMCFLKLFAMHSAAFILVVVSLDRYRAILHPLDSL	137	
	Pufferfish2-2	GDALCKLLCFLKLFAMYASAFILVVISLDRHHVILQPLNSI	145	
	Bullfrog1	GDIACRILMFLKLLSMYSACFVTVVISLDRQSAILNPLAIN	157	
	Bullfrog2	DEISCKILNFGKLFAMYSAAFLVVVISLDRHWAILYPLSFT	146	
	Bullfrog3	GDVACRILMFLKLQAMYSACFVTVVISLDRHAAAILNPLGIG	168	
	Chicken1	GDLCKLLNFLKLFAMYAAALVLVVISLDRHAAVLQP--FA	148	
	Chicken2	GDLACRLMFLRLLLAMYSAFVTVVISLDRQAAILRPLAIA	175	
Type I	RhesusMonkey2	EDIACRTLMFLKLQAMYSAAFLPVVIGLDRQAAVLNPLGSR	149	
	Cow	GELLCKVLSYLKLFSMYAPAFMMVVISLDRSLAITRPLAVK	150	
	Sheep	GELLCKVLSYLKLFSMYAPAFMMVVISLDRSLAITRPLAVK	150	
	Pig	GEFLCKVLSYLKLFSMYAPAFMMVVISLDRSLAITRPLAVK	150	
	Dog	GEFLCKVLSYLKLFSMYAPAFMMVVISLDRSLAITRPLAMK	149	
	Horse	GELLCKVLSYLKLFSMYAPAFMMVVISLDRSLAITRPLAVK	150	
	Rat	GEFLCKVLSYLKLFSMYAPAFMMVVISLDRSLAVTQPLAVQ	150	
	Mouse	GEFLCKVLSYLKLFSMYAPAFMMVVISLDRSLAITQPLAVQ	150	
	Human	GELLCKVLSYLKLFSMYAPAFMMVVISLDRSLAITRPLALK	150	

Figure 1.

Sequence alignment of the proximal region of TM3 and DRY motif in GnRHR from representative vertebrate species. The alignment was arranged with the entire amino acid sequences of the receptors based on Clustal W. The accession numbers for species are followed : Lamprey ([AAQ04564](#)), Catfish1 ([O42329](#)), Catfish2 ([AAM95605](#)), Medaka1 ([NP_001098352](#)), Medaka2 ([NP_001098392](#)), Medaka3 ([NP_001098393](#)), GoldfishA ([AAD20001](#)), GoldfishB ([AAD20002](#)), Pufferfish1-1 ([BAE45695](#)), Pufferfish1-2 ([BAE45697](#)), Pufferfish1-3 ([BAE45699](#)), Pufferfish2-1 ([BAE45701](#)), Pufferfish2-2 ([BAE45704](#)), Bullfrog1 ([AAG42575](#)), Bullfrog2 ([AAG42949](#)), Bullfrog3 ([AAG42574](#)), Chicken1 ([NP_989984](#)), Chicken2 ([NP_001012627](#)), RhesusMonkey2 ([NP_001028014](#)), Cow ([NP_803480](#)), Sheep ([NP_001009397](#)), Pig ([NP_999438](#)), Dog ([NP_001003121](#)), Horse ([NP_001075305](#)), Rat ([NP_112300](#)), Mouse ([NP_034453](#)), Human ([NP_000397](#)).

Figure 2.

A schematic diagram of the location of the point mutations in IGnRHR-1. The rectangle shows the DRH motif and the point substitutions and deletion in IGnRHR-1 corresponding to DRY motif in GPCR. In IGnRHR-1, Tyr of DRY is substituted with His (wild-type), same as most of type II receptors, while Tyr is substituted with Ser in type I receptor. His¹⁵¹ in IGnRHR-1 was substituted with Tyr (DRY¹⁵¹), Glu (DRE¹⁵¹), or Ser (DRS¹⁵¹) or deleted (DRX¹⁵¹).

	LogEC ₅₀	Maximal IP production (dpm)
DRH ¹⁵¹ (WT)	-11.39 ± 0.21	1794 ± 71.59
DRY ¹⁵¹	-12.32 ± 0.27	1808 ± 79.52
DRE ¹⁵¹	-11.06 ± 0.17	1166 ± 42.75
DRS ¹⁵¹	-10.95 ± 0.16	1458 ± 48.67

Figure 3.

IP response of wild-type and mutant IGnRHRs. DRH¹⁵¹ (wild-type), DRY¹⁵¹, DRE¹⁵¹ and DRS¹⁵¹ showed different dose-response curves responding to IGnRH-III. The values of IP productions were shown as dpm. The data are mean ± SEM of duplicate in a representative experiment. LogEC₅₀ of DRE¹⁵¹ and DRS¹⁵¹ were higher than DRH¹⁵¹ (wild-type) while the maximal IP productions were lower than DRH¹⁵¹ (wild-type). LogEC₅₀ of DRY¹⁵¹ was lower than DRH¹⁵¹ (wild-type) while the maximal IP production was similar to DRH¹⁵¹ (wild-type).

	Log IC ₅₀	total binding ¹²⁵ I-GnRH I (dpm)
DRH ¹⁵¹ (WT)	-9.554 ± 0.049	12514 ± 174.50
DRY ¹⁵¹	-9.422 ± 0.050	14188 ± 200.10
DRE ¹⁵¹	-9.426 ± 0.072	1930 ± 33.41
DRS ¹⁵¹	-9.433 ± 0.067	4308 ± 76.83
DRX ¹⁵¹	-9.528 ± 0.065	2480 ± 40.85

Figure 4.

Ligand binding of wild-type and mutant IGnRHRs. Competitive binding assays of ¹²⁵I-IGnRHR-1 were performed with decreasing concentrations of IGnRH III. The values of radioligand binding in the graph were shown as the percentages of maximum ¹²⁵I-IGnRH binding (mean ± SEM in triplicate). The data represents one of three independent experiments with essentially same results. Log IC₅₀ of wild-type and mutant receptors were similar but total bindings of ¹²⁵I-IGnRH were varied

Figure 5.

Receptor expression of wild-type and mutant lGnRHRs. The data are shown as the percentages of wild-type binding to ^{125}I -lGnRH (1 nM) and each bar represents mean $\pm$ SEM of three independent experiments in triplicate. One-way ANOVA was performed with Bonferroni post hoc test (a, $P < 0.001$ vs WT ; b, $P < 0.001$ vs DRY)

Figure 6. Internalization of wild-type and mutant I GnRHRs. Percent internalization was expressed as the ratio of internalized radioligand to the total cell-surface radioligand at each time point. Data are shown as mean $\pm$ SEM in triplicate, representing at least two independent experiments.