Functional characterization and kinetic studies of an ancestral lamprey GnRH-III selective type II GnRH receptor from the sea lamprey, *Petromyzon marinus*

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Abstract

The recently cloned lamprey GnRH receptor was shown to have several unique features, including the longest intracellular C-terminal tail (120 amino acids (aa)) of any previously described GnRH receptor. In the current study, a series of experiments were performed examining cAMP responses, binding kinetics, whole cell competitive binding assays and internalization studies of the lamprey GnRH receptor using a series of three C-terminal tail truncations (80 aa, 40 aa and 0 aa) to better describe the functional significance of this unique vertebrate GnRH receptor. Activation of the lamprey GnRH receptor was shown to stimulate cAMP production in a dose-dependant manner when treated with either lamprey GnRH-I ($\text{LogEC}_{50} -6.57 \pm 0.15$) or lamprey GnRH-III ($\text{LogEC}_{50} -8.29 \pm 0.09$). Truncation analysis indicated that the membrane proximal 40 aa of the lamprey GnRH receptor C-terminal tail contain a motif required for cAMP accumulation. Saturation binding assays using the wild type and truncated lamprey GnRH receptors revealed that all of three truncated lamprey GnRH receptors were capable of binding lamprey GnRH-I. Competitive, intact cell-binding assays suggested that the lamprey GnRH receptor is lamprey GnRH-III selective, based on the observed pharmacological profile: lamprey GnRH-III (Inhibitory constant (K_i) 0.708 ± 0.245 nM) = chicken GnRH-II (K_i 0.765 ± 0.160 nM) > mammalian GnRH (K_i 12.9 ± 1.96 nM) > dAla⁶Pro⁹NEt mammalian GnRH (K_i 21.6 ± 9.68 nM) > lamprey GnRH-I (K_i 118.0 ± 23.6). Finally, the lamprey GnRH receptor was shown to undergo rapid ligand-dependant internalization, which was significantly diminished in the tail-less truncated form. We have shown from our current and our previous structural studies that this unique lamprey GnRH receptor shares several characteristics of both type I and type II GnRH receptors which suggests that this receptor has retained ancestral characteristics that can provide insight into the function and evolution of the vertebrate GnRH receptor family.

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Introduction

The vertebrate hypothalamic–pituitary–gonadal axis is regulated by gonadotropin-releasing hormone (GnRH), a decapeptide hormone that is produced and released from the hypothalamus. At the anterior pituitary, GnRH action is mediated through high affinity binding with the GnRH receptor, a class A or rhodopsin-like seven transmembrane G-protein coupled receptor (GPCR) (Millar *et al.* 2004). The GnRH receptor is unique among all GPCRs in that the type I mammalian GnRH receptors lack the highly conserved intracellular carboxy-terminal (C-terminal) tail, which has been shown to be a vital structural element required for several key functions, such as G-protein coupling and second messenger activation, ligand binding, cell surface expression and ligand-dependant internalization (Koenig & Edwardson 1997, Heding *et al.* 1998, Blomenrohr *et al.* 1999, Bockaert *et al.* 2003, Ronacher *et al.* 2004). Based simply on the presence or absence of a C-terminal tail,

the GnRH receptors can be divided into two groups; the type I tail-less GnRH receptors, which have only been identified in mammals and the C-terminal tail-containing type II GnRH receptors, which have been identified across the vertebrate lineage (Okubo *et al.* 2001, Ikemoto *et al.* 2004, Silver *et al.* 2005).

GnRH receptor signaling has been characterized in several systems, and is primarily thought to function through the Inositol 1,4,5-triphosphate (IP_3) second messenger pathway; however, type II GnRH receptors and in some cases type I GnRH receptors have been shown to also activate cAMP signaling (Arora *et al.* 1998, Stanislaus *et al.* 1998, Grosse *et al.* 2000, Liu *et al.* 2002, Oh *et al.* 2005). The presence or absence of the C-terminal tail in type II and type I GnRH receptors respectively could possibly explain the signaling disparity between the two groups, where, for example, histidine–phenylalanine–arginine–lysine (an HFRK) motif in the membrane proximal region of bullfrog type II GnRH receptors was recently shown to be required for cAMP

signaling (Oh *et al.* 2005). Rapid, ligand-dependant internalization resulting in desensitization and/or signal switching is another key functional difference between type I and type II GnRH receptors; this has been described and attributed to the presence or absence of specific Ser/Thr residues located throughout the C-terminal tail (Blomenrohr *et al.* 1999, Willars *et al.* 1999, Pawson *et al.* 2003, Ronacher *et al.* 2004).

As an agnathan, the oldest extant lineage of vertebrates, the sea lamprey has become a model system for analysis of the evolution of the neuroendocrine regulation of reproduction (Sower 2003). The lamprey expresses two forms of GnRH, lamprey GnRH-I and lamprey GnRH-III, both of which are produced in the hypothalamus and have been shown to regulate the reproductive axis (Sherwood *et al.* 1986, Sower *et al.* 1993, Deragon & Sower 1994, Suzuki *et al.* 2000, Silver *et al.* 2004). Recently, a GnRH receptor cDNA was cloned from the sea lamprey, *Petromyzon marinus*; this contained a C-terminal tail of 120 amino acids (aa), the longest of any previously described GnRH receptor to date (Silver *et al.* 2005). To better describe the lamprey GnRH receptor, a series of functional and pharmacological assays was performed in the current study, including examination of cAMP responses, analysis of receptor binding kinetics, whole cell competitive binding assays and internalization studies using a series of C-terminal tail truncations to better our understanding of the significance of certain regions of the lengthy C-terminal tail, as well as functional aspects of the receptor. The lamprey GnRH receptor was shown to stimulate the cAMP signaling system in a dose-dependant manner, which, through mutagenesis studies, was shown to depend on the presence of the C-terminal tail. The C-terminal tail was also shown to be required for rapid ligand-dependant internalization, binding affinity and, to some degree, cell surface expression. Finally, pharmacological profiling, in conjunction with these and previous efficacy data has confirmed that the lamprey GnRH receptor is lamprey GnRH-III selective. These data indicate that the lamprey GnRH receptor shares several characteristics of both type I and type II GnRH receptors and likely retains ancestral characteristics of the vertebrate GnRH receptor family.

Materials and methods

Cell culture, construct development and transfection

COS7 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in 5% CO₂. The coding regions of the wild type and mutant lamprey GnRH receptor (GenBank Accession Number AF439802) (see Fig. 1)

were inserted into the pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA, USA). The lamprey GnRH receptor open reading frames (ORFs) were amplified via PCR using the Advantage2 PCR system (CLONTECH, Mountain View, CA, USA) with the lamprey GnRH receptor ORF 5' (5'-CAC CAT GGA ACC CAT CAA CAT GAA CAT GAC-3') combined with either the lamprey GnRH receptor ORF 3' (to produce the wild type ORF: 5'-TCA GAT GCAGCA GCT TTC AGG ACA TAC GAG AG-3'), lamprey GnRH receptor 80 aa 3' (to produce the lamprey GnRH receptor with an 80 aa C-terminal tail: TCA-TGC-CGC-TCT-GTT-CAC-GGG-GAC), lamprey GnRH receptor 40 aa 3' (to produce the lamprey GnRH receptor with a 40 aa C-terminal tail: TCA-ACT-CCG-CAC-GGA-CGA-GGC-CGA) or the lamprey GnRH receptor 0 aa 3' (to produce the tail-less lamprey GnRH receptor: TCA-CGC-CGC-GAA-CAC-GCC-GTA-GAT). The day prior to transfection, 5×10^5 cells were seeded in 60 mm culture plates. Transfection was performed using 5 µg vector and 15 µl Lipofectamine (Invitrogen) in 2.4 ml total volume in Opti-MEM-I (Invitrogen) per culture. Transfection efficiency was optimized using a lacZ insert, while transcription consistency was measured using RT-PCR after transfection with either the wild type or mutant lamprey GnRH receptors.

Lamprey GnRH receptor cAMP assay

The day after transfection, cells were trypsinized, 96-well plates were seeded with 5×10^4 cells/well and cultures were grown overnight. On day 3, cells were stimulated with either control (ID buffer – 1.0 mM 3-isobutyl-1-methylxanthine in DMEM), lamprey GnRH-I (American Peptide Company, Sunnyvale, CA, USA) or lamprey GnRH-III (American Peptide Company) in ID buffer (concentrations ranging from 10^{-4} M to 10^{-10} M for dose-response analysis or 10^{-5} M for the C-terminal tail functional analysis) for 1 h at 37 °C. Treatments were performed in triplicate and cells transfected with blank vector were used as negative controls. cAMP assays were performed using the BioTrak enzymeimmunoassay system (Amersham), according to the manufacturer's instructions. Analyses were performed using Prism (GraphPad, San Diego, CA, USA).

Whole cell lamprey GnRH receptor saturation binding assay

Saturation binding assays were performed using adherent, intact cells with ¹²⁵I-labeled lamprey GnRH-I. ¹²⁵I-Labeled lamprey GnRH-I was iodinated using a modification of the chloramine-T method, which was purified as described by Stopa *et al.* (1988). Note that lamprey GnRH-III was not used as the radioligand since

Figure 1 Lamprey GnRH receptor structure and truncation demarcation. The lamprey GnRH receptor C-terminal tail of 120 aa is the longest of any known GnRH receptor. Several putative phosphorylation sites are located within the C-terminal tail, which are shown highlighted in gray. Black lines represent cutoff points within the sequence used to develop vectors containing C-terminal tail truncations to include the wild type (120 aa tail), 80 aa tail, 40 aa tail or tail-less (0 aa tail) mutants. TM, transmembrane; EC, extracellular; IC, intracellular.

it cannot be iodinated due to the lack of a Tyr residue. For saturation binding assays, the day after transfecting COS7 cells with either the wild type or mutant lamprey GnRH receptors, 1×10^5 cells were seeded into 24-well plates in 500 μ l medium; these were grown for 2 days at 37 °C in 5% CO₂. Cells were then washed once in 500 μ l assay buffer (25 mM HEPES-modified DMEM with 0.1% BSA), followed by incubation with increasing concentrations of ¹²⁵I-labeled lamprey GnRH-I (1–100 nM), in 200 μ l total volume of assay buffer, for 3.5 h on ice at 4 °C. Non-specific binding (NSB) was determined using cells incubated with both ¹²⁵I-labeled lamprey GnRH-I and 10 μ M cold lamprey GnRH-III. After 3.5 h, cells were quickly washed twice with 500 μ l ice-cold PBS and cells were examined using an inverted microscope to ensure that no cells were lost. Cells were solubilized with 300 μ l 0.5 M NaOH, 0.1% SDS, and bound ¹²⁵I-labeled lamprey GnRH-I was counted using a γ -counter. All total binding samples were run in triplicate, NSBs were run in duplicate, and each independent experiment was repeated two to three times. Data were analyzed using Prism (GraphPad).

Competitive binding analysis

Competitive binding properties of lamprey GnRH-I, lamprey GnRH-III, chicken GnRH-II (Peninsula Laboratories, San Carlos, CA, USA), mammalian GnRH (Peninsula Laboratories) and dAla⁶Pro⁹NET mGnRH (Peninsula Laboratories) were performed using COS7 cells transfected with the wild type lamprey GnRH receptor. Cells were prepared as described above; however, the ¹²⁵I-labeled lamprey GnRH-I concentration was held constant at 10 nM, with either assay buffer (total binding) or increasing concentrations of cold competing ligand in assay buffer (ranging from 10^{-13} M to 10^{-6} M) in 200 μ l total volume for 3.5 h on ice in the 4 °C incubator. Cells were washed and processed as described above. All samples were run in triplicate, in three independent experiments. Data were analyzed using Prism (GraphPad).

Lamprey GnRH receptor internalization assay

Internalization of ¹²⁵I-labeled lamprey GnRH-I was performed based on the acid-wash method, as previously

Figure 2 cAMP dose-response curve. The lamprey GnRH receptor was shown to activate the cAMP signaling system in a dose-dependant manner. Lamprey (L) GnRH-III was a significantly more potent activator of the lamprey GnRH receptor relative to lamprey GnRH-I. A representative curve from three independent experiments is shown; LogEC₅₀ is shown as means $\pm$ S.E.M.; $n=3$.

described (Hazum *et al.* 1983, Pawson *et al.* 1998, King *et al.* 2000). Briefly, COS7 cells transfected with the wild type lamprey GnRH receptor or C-terminal tail truncated mutants were seeded (1×10^5 cells) in 24-well plates in 500 μ l medium, and were grown for 48 h. Cells were incubated with 10 nM ¹²⁵I-labeled lamprey GnRH-I on ice at 4 °C for 3.5 h. Cells were then immediately brought to 37 °C for increasing periods of time. At the end of each time-point, cells were placed on ice and washed twice with 500 μ l ice-cold PBS. Acid-sensitive (surface bound) ¹²⁵I-labeled lamprey GnRH was washed away by the addition of 0.3 ml acid solution (150 mM NaCl, 50 mM acetic acid, pH 2.8) for 12 min. The acid wash was removed, and acid insensitive (internalized) ligand was recovered using solubilizing reagent (0.5 M NaOH with 0.1% SDS). Both acid-sensitive and -insensitive binding was quantified with a γ -counter, and percent internalization was determined based on comparison of internalized ligand to total cell-associated ligand (internalized+surface bound). NSBs were determined using non-transfected cells. Treatments were performed in triplicate, and independent experiments were run three times for each receptor construct. Data were analyzed using Prism (GraphPad).

Results

Lamprey GnRH receptor cAMP activation

Both lamprey GnRH-I and lamprey GnRH-III stimulated a significant response in cAMP accumulation, in a dose-dependant manner, in COS7 cells that were transiently transfected with the lamprey GnRH receptor (Fig. 2). The LogEC₅₀ (represented as means $\pm$ S.E.M.; $n=3$) of lamprey GnRH-III (-8.47 ± 0.046) was

Figure 3 Effect of lamprey GnRH receptor C-terminal tail truncations on cAMP signaling. cAMP accumulation assay using wild type (WT) and mutant lamprey GnRH receptors containing C-terminal tail truncations treated with a maximum dose (10^{-5} M) of either lamprey (L) GnRH-I or lamprey GnRH-III are shown relative to fold stimulation. Lamprey GnRH-III stimulates a greater magnitude of cAMP signaling compared with lamprey GnRH-I in the wild type receptor. Truncation of the C-terminal tail reduced cAMP accumulation, which was not recovered in the tail-less mutant form.

significantly ($P<0.0001$) lower than the LogEC₅₀ of lamprey GnRH-I (-6.59 ± 0.082). This approximately 50-fold difference suggests that the presently cloned lamprey GnRH-R is lamprey GnRH-III selective. Cells transfected with blank pcDNA3.1 vector did not respond to treatment with either lamprey GnRH-I or lamprey GnRH-III (data not shown).

Consistent with this difference in LogEC₅₀, lamprey GnRH-III stimulated a larger magnitude of cAMP accumulation, compared with lamprey GnRH-I, when the wild type or mutant lamprey GnRH receptors were treated with a maximum dose (10^{-5} M) (Fig. 3 or Table 1). Stimulation of the wild type lamprey GnRH receptor led to a 1.68 ± 0.073 -fold increase in response to lamprey GnRH-I and a 2.11 ± 0.162 -fold increase in response to lamprey GnRH-III. Serial truncation of the lamprey GnRH receptor led to a drastic decrease in the magnitude of cAMP accumulation, whereas the stimulation of the 80 aa C-terminal tail resulted in 1.26 ± 0.204 - and 1.36 ± 0.117 -fold increases in response to lamprey GnRH-I and lamprey GnRH-III respectively. Further truncation of the C-terminal tail to 40 aa resulted in a recovery of cAMP accumulation (1.77 ± 0.242 -fold for lamprey GnRH-I and 2.19 ± 0.262 -fold increases for lamprey GnRH-III); however, the tail-less mutant showed a drastic reduction in ability to activate the cAMP system (1.26 ± 0.056 -fold increase in response to lamprey GnRH-I and 1.30 ± 0.059 -fold increase in response to lamprey GnRH-III).

Pharmacological characterization

Binding of ¹²⁵I-labeled lamprey GnRH-I to intact, adherent COS7 cells transfected with the lamprey

Table 1 Lamprey (L) GnRH receptor C-terminal tail truncations: function and pharmacology. Values are means±S.E.M.

Construct	cAMP accumulation		Binding kinetics	
	LGnRH-I	LGnRH-III	K _d (nM)	B _{max} (fmol/well)
Wild type (120 aa tail)	1.68±0.073	2.39±0.162	31.1±10.3	394.6±52.1
80 aa tail	1.26±0.204	1.36±0.117	51.7±18.4	276.1±46.9
40 aa tail	1.77±0.242	2.19±0.262	85.4±5.3	1906±67.1
Tail-less	1.26±0.056	1.30±0.059	72.3±19.8	774.5±140.9

GnRH receptor was saturable with a B_{max} of 394.6 fmol/well and a K_d of 31.1 nM. This relatively high K_d was expected, given that this receptor is lamprey GnRH-III selective. Binding of ¹²⁵I-labeled lamprey GnRH-I to the C-terminal tail truncated mutant lamprey GnRH receptors was also saturable (see Fig. 4 or Table 1), whereas the 80 aa tail mutant had a B_{max} of 276 fmol/well and a K_d of 51.7 nM, the 40 aa tail mutant had a B_{max} of 1906 fmol/well and a K_d of 85 nM, while the 0 aa tail-less mutant had a B_{max} of 775 fmol/well and a K_d of 72 nM. C-terminal tail truncations resulted in an increase in cell surface expression of the 40 aa C-terminal tail (483.0% of wild type) and tail-less mutants (196.4% of wild type), while the 80 aa C-terminal tail mutant expression decreased (69.9% of wild type). In all cases, the truncations resulted in an increase in K_d, 80 aa tail (166.2% of wild type), 40 aa tail (273.3% of wild type) and tail-less (231.5% of wild type).

COS7 cells transfected with the wild type lamprey GnRH receptor were used in a series of competitive binding assays in order to describe the binding affinity of different GnRH isoforms. Assays using intact, adherent

cells were performed holding ¹²⁵I-labeled lamprey GnRH-I constant at 10 nM with increasing concentrations of cold competitor, ranging from 10⁻¹³ M to 10⁻⁶ M. These competitive binding analyses indicated that the wild type lamprey GnRH receptor is lamprey GnRH-III selective (Fig. 5), with a pharmacological profile of lamprey GnRH-III (K_i=0.708 ± 0.245 nM)= chicken GnRH-II (K_i=0.765 ± 0.160 nM)>mammalian GnRH (K_i=12.9 ± 1.96 nM)>dAla⁶Pro⁹NEt mammalian GnRH (K_i=21.6 ± 9.68 nM)>lamprey GnRH-I (118.0 ± 23.6) (see Table 2).

Lamprey GnRH receptor internalization

Internalization of ¹²⁵I-labeled lamprey GnRH-I was used to characterize the effect of C-terminal tail length on the rate of ligand-dependant internalization, which is described as the percent of total cell-associated ligand, and fit using a single component exponential equation (Y=Y_{max}(1 - e^{kt}); y= % internalized, k= % internalized/min and t=time in min (see Fig. 6). The wild type lamprey GnRH receptor was rapidly internalized in

Figure 4 Saturation binding analysis. Saturation binding analysis of ¹²⁵I-labeled lamprey GnRH-I using adherent, intact COS7 cells transfected with the wild type lamprey GnRH receptor, 80 aa tail, 40 aa tail or tail-less mutants. Data are shown as means±S.E.M. (n=3), representing two to three independent experiments.

Figure 5 Lamprey GnRH receptor pharmacological profile. Competitive binding analysis of ¹²⁵I-labeled lamprey GnRH-I using intact adherent COS7 cells transfected with the wild type lamprey GnRH receptor incubated with increasing concentrations of lamprey (L) GnRH-III, lamprey GnRH-I, mammalian (m) GnRH, dAla⁶Pro⁹NEt mammalian GnRH or chicken (ch) GnRH-II. Data shown as means±S.E.M. (n=3) of % maximum binding demonstrate a binding preference for lamprey GnRH-III, which was equal to chicken GnRH-II> mammalian GnRH>dAla⁶Pro⁹NEt mammalian GnRH>lamprey GnRH-I.

Table 2 Summary of lamprey (L) GnRH receptor efficacy and pharmacological profiling. Values are means±S.E.M.

GnRH form	Lamprey GnRH receptor characterization		
	LogEC ₅₀	LogIC ₅₀	Ki (nM)
IGnRH-III	-8.29±0.09	-8.99±0.0922	0.708±0.245
IGnRH-I	-6.57±0.15	-6.81±0.116	118.0±23.6
chGnRH-II	—	-8.96±0.0713	0.765±0.16
mGnRH	—	-7.79±0.147	12.9±1.96
mGnRH _a	—	-7.68±0.116	21.6±9.68

mGnRH_a, DALa⁶Pro⁹NEt mammalian GnRH; ch, chicken; m, mammalian.

response to treatment with 10 nM ¹²⁵I-labeled lamprey GnRH-I. Within the first 10 min at 37 °C, approximately 63% of the cell-associated radioligand was found in the intracellular fraction. Lamprey GnRH receptor mutants with C-terminal tail truncations were used to identify regions containing motifs that are required for rapid ligand-dependant internalization. Truncation of the C-terminal tail to 80 aa or 40 aa had no effect on either the rate or extent of internalization when compared with wild type. The tail-less lamprey GnRH receptor, however, showed a marked reduction in ligand-dependent internalization, with a Y_{max} of 28.9%, a drastic reduction compared with the wild type or other truncated mutants.

Discussion

The GnRH receptor from the sea lamprey, *Petromyzon marinus*, a member of the oldest extant lineage of vertebrates, the agnathans, was used in a series of functional and pharmacological experiments in order to investigate and better define how the function of the GnRH receptor family has evolved across vertebrates. Stimulation of the lamprey GnRH receptor was shown to activate cAMP production; however, this activation was shown to require the first 40 aa of the C-terminal tail. Since the truncated tail-less mutant lamprey GnRH receptor was capable of ligand binding we propose that Gα_s coupling requires a motif within the first 40 aa of the C-terminal tail. Pharmacological profiling indicated that the lamprey GnRH receptor is lamprey GnRH-III selective, with an equal affinity for lamprey GnRH-III and chicken GnRH-II. The wild type, 80 aa C-terminal tail mutant and 40 aa C-terminal tail mutant lamprey GnRH receptors were shown to undergo rapid, ligand-dependant internalization, but the tail-less mutant was not, indicating a motif within the first 40 aa that is required for this process. This unique lamprey GnRH receptor has high affinity for both lamprey GnRH-III

Figure 6 Internalization profile. ¹²⁵I-labeled lamprey GnRH-I mediated internalization of the wild type lamprey GnRH receptor, 80 aa tail, 40 aa tail or tail-less mutants in transiently transfected COS7 cells. Treatments were brought to steady state on ice and then rapidly brought to 37 °C for increasing periods of time. Percent internalization was calculated as a measurement of internalized radioligand relative to the total cell-associated radioligand. Data are shown as means±S.E.M. (n=3), representing two to three independent experiments.

and chicken GnRH-II and ligand-binding activity when truncated to a tail-less form.

Unique among the approximately 1000 GPCR encoding genes in the human genome (Fredriksson *et al.* 2003), the type I GnRH receptor lacks an intracellular C-terminal tail, which is thought to be involved in G-protein coupling, cell surface expression and internalization (Blomenrohr *et al.* 1999, Vrecl *et al.* 2000, Ronacher *et al.* 2004). Interestingly, tail-less or type I GnRH receptors have only been identified in mammals, while type II GnRH receptors, which contain C-terminal tails, have been cloned from species across the vertebrate lineage, suggesting a recent, rapid evolutionary history (Sealfon *et al.* 1997, Millar *et al.* 2004). A comparative analysis of GnRH receptors across the vertebrate lineage can provide significant insight into the molecular evolution of this receptor family. In this light, the GnRH receptor from the sea lamprey, *Petromyzon marinus* (Silver *et al.* 2005), provides an ideal model to analyze basal, or ancestral-like functions and functional elements that are involved in ligand binding, signaling and internalization.

Pituitary GnRH receptors are thought to primarily signal through Gα_{q/11}, resulting in the stimulation of the IP₃ second messenger system; however, Gα_s activation and cAMP signaling has been reported as well (Arora *et al.* 1998, Stanislaus *et al.* 1998, Grosse *et al.* 2000, Liu *et al.* 2002, Oh *et al.* 2005). G-protein coupling to type I GnRH receptors clearly occurs within the intracellular loops (ILs), where several motifs have been identified that may be involved in G-protein coupling (see Table 3). For instance, the DRxxxI/VxxPL motif in IL2 and a conserved Ala residue in IL3 have been linked to Gα_{q/11}

Table 3 GnRH receptor (R) domains involved in G-protein coupling

Receptor	G-protein	Region involved in G-protein coupling	Reference
Mouse GnRH-R	Gα _s	IL1 (K71LKSR75; L58; L80)	Arora <i>et al.</i> (1998)
Mouse GnRH-R	Gα _q /11	IL2 (L147)	Arora <i>et al.</i> (1995)
Mouse GnRH-R	Gα _q /11	IL2 (DRS)	Arora <i>et al.</i> (1997) and Kitanovic <i>et al.</i> (2001)
Mouse GnRH-R	Gα _q /11	IL3 (Leu237)	Chung <i>et al.</i> (1999)
Rat GnRH-R	Gα _q /11 and Gα _s	IL3	Ulloa-Aguire <i>et al.</i> (1998)
Rat GnRH-R	Gα _q /11	C-terminus (F325S326L327)	Brothers <i>et al.</i> (2002)
Human GnRH-R	Gα _q /11	IL3 (A261)	Myburgh <i>et al.</i> (1998)
Bullfrog GnRH-R1	Gα _s	C-T (membrane proximal HFRK)	Oh <i>et al.</i> (2005)
Lamprey GnRH-R	Gα _q /11	IL1, 2 or 3 (activated by tail-less mutant)	Silver <i>et al.</i> (2005)
Lamprey GnRH-R	Gα _s	C-T (within first 40 aa)	Presented here

C-T, C-terminal tail; C-terminus refers to the C-terminal amino acids of the tail-less type GnRH receptors.

coupling (Arora *et al.* 1995, Myburgh *et al.* 1998), while a BBxxB (where B is any basic amino acid) in IL1 was shown to be required for Gα_s coupling (Arora *et al.* 1998). Furthermore, the presence or absence of the C-terminal tail in the type II or type I GnRH receptors could possibly explain the signaling disparity between the two groups, whereas an HFRK motif in the membrane proximal region of the bullfrog type II GnRH receptor-1 was recently shown to be required for cAMP signaling, but not for Inositol phosphate (IP) signaling (Oh *et al.* 2005). In the present study, lamprey GnRH receptor was shown to activate the cAMP signaling system, in a dose-dependent manner, in transiently transfected COS7 cells. Lamprey GnRH-III was a more potent activator of this system compared with lamprey GnRH-I, which supports the previous hypothesis, based on IP activation (Silver *et al.* 2005), that the lamprey GnRH receptor is lamprey GnRH-III selective. These data have several interesting implications. The lamprey GnRH receptor activates both the cAMP and IP signaling systems; however, the IP system is activated at an approximately 10-fold lower concentration of both lamprey GnRH-I and lamprey GnRH-III, and is also activated to a greater magnitude of approximately 4.5-fold, compared with ~1.7-fold (lamprey GnRH-I) or ~2.1-fold (lamprey GnRH-III) (see Fig. 3) accumulation of cAMP. Not unexpectedly, truncation of the lamprey GnRH receptor C-terminal tail interfered with cAMP signaling; this is partially recovered by the 40 aa tail mutant, and lost again in the tail-less mutant form. The exact nature of GPCR/G-protein coupling is still in question since no conserved motifs that can be generally used to define G-protein specificity have been identified, nor has any particular domain been shown to be required. These current data indicate that a motif within the first 40 aa of the lamprey GnRH receptor is involved in the Gα_s coupling, which we propose to be the 'HFRK'-like motif

(histidine–valine–arginine–arginine (HVRR) in lamprey) located within the membrane proximal region of the C-terminal tail. Furthermore, this region contains a BBxxB (B is any basic amino acid), which has been shown to be involved in Gα_s coupling in type I GnRH receptors, in which case this motif is located in the first IL (Arora *et al.* 1998).

The pharmacological profile of the lamprey GnRH receptor, as shown in Fig. 5 and Table 2, confirms the hypothesis that the lamprey GnRH receptor is lamprey GnRH-III selective, and furthermore supports the hypothesis that the lamprey expresses at least one additional, lamprey GnRH-I selective receptor, as previously described based on quantitative *in vitro* autoradiography (Knox *et al.* 1994) and IP signaling efficacy (Silver *et al.* 2005). The equivalent affinity for lamprey GnRH-III and chicken GnRH-II for this ancestral vertebrate GnRH receptor implies that perhaps they represent ancestral forms of GnRH, as previously suggested (Sherwood *et al.* 1997, Sower 1997); nonetheless, more data would be required to confirm this hypothesis.

Efficacy data on their own can be difficult to interpret and misleading; however, in conjunction with binding affinity studies they can provide invaluable insight into the molecular mechanisms of GPCR function. The effects of lamprey GnRH receptor C-terminal tail truncations on binding affinity and cell surface expression showed that perturbation of the C-terminal tail increases K_d (reduces binding affinity) and increases the level of cell surface expression. However, there is one exception, in which the level of cell surface expression decreased in the truncated 80 aa C-terminal tail receptor. These data may explain the drastic decrease in signaling of the truncated 80 aa C-terminal tail receptor, which likely results from a combination of decreased binding affinity and cell surface expression. Alternatively, the recovery of cAMP accumulation that was seen

when the lamprey GnRH receptor with a 40 aa tail was activated may be due to the increase in cell surface expression, which compensates for the reduction in binding affinity. Finally, the fully truncated tail-less receptor was shown to maintain ligand-binding capability at a diminished binding affinity, which, despite an increase in surface expression, led to the accumulation of near basal levels of cAMP. Because the tail-less lamprey GnRH receptor mutant is known to be active in stimulating IP_3 at an equivalent magnitude compared with wild type (Silver *et al.* 2005), these data support the hypothesis that $G\alpha_s$ coupling occurs within the first 40 aa of the C-terminal tail.

Rapid, ligand-dependant GPCR internalization is a well-established regulatory mechanism that results in receptor desensitization or alternatively facilitates signal switching from G-protein-mediated second messaging to MAP kinase signaling (McArdle *et al.* 2002). Activation of type II GnRH receptors has been extensively shown to lead to rapid internalization that can be β -arrestin dependent or independent, dynamin dependent or independent and through either clathrin coated vesicles or caveolae (Acharjee *et al.* 2002, Pawson *et al.* 2003, Ronacher *et al.* 2004, Hislop *et al.* 2005). Type I GnRH receptors have been shown to be internalized but at a significantly slower rate and to a lesser extent when compared with type II GnRH receptors (Pawson *et al.* 1998, Willars *et al.* 1999, Hislop *et al.* 2005). Addition of the C-terminal tail from the type II catfish GnRH receptor to the rat GnRH receptor was shown to result in several functional aberrations, most notably induction of rapid ligand-dependant internalization, which was shown to be reversible through C-terminal tail truncations (Lin *et al.* 1998). Furthermore, removal of the C-terminal tail from the type II chicken GnRH receptor was shown to result in an internalization profile similar to the naturally tail-less human type I GnRH receptor (Pawson *et al.* 1998). These studies provided the impetus to further define the motifs involved in this rapid internalization, leading to a series of studies indicating specific Ser/Thr moieties located in both the membrane proximal or distal regions of the C-terminal tail (Blomenrohr *et al.* 1999, Pawson *et al.* 2003, Millar *et al.* 2004, Ronacher *et al.* 2004). The 120 aa C-terminal tail of the lamprey GnRH receptor, the longest of any known GnRH receptor (Silver *et al.* 2005), contains several Ser/Thr residues located throughout the entire sequence; however, they are concentrated within the first 40 aa. The lamprey GnRH receptor was shown to be rapidly internalized in response to stimulation, whereas approximately 60% of the ligand-bound receptors were located in the intracellular space. Truncation of the C-terminal tail to 80 aa or 40 aa had no effect on internalization (rate or maximum level) but the tail-less mutant showed a drastic reduction in internalization, similar to other previously described

tail-less receptor internalization profiles (Heding *et al.* 1998, Pawson *et al.* 1998). It should be noted that these internalization profiles were generated using lamprey GnRH-I because of the inability to iodinate lamprey GnRH-III, which lacks a tyrosine residue. The use of lamprey GnRH-I in these experiments may lead to different results compared with lamprey GnRH-III stimulation of the lamprey GnRH receptor; however, these differences would likely be reflected in the magnitude of responses and not novel response profiles.

In summary, the lamprey GnRH receptor was shown to activate the cAMP signaling system, which required the first 40 aa of the C-terminal tail. Pharmacological profiling, in conjunction with efficacy data, provided evidence that the lamprey GnRH receptor is lamprey GnRH-III selective, which supports the hypothesis that the lamprey expresses a second, lamprey GnRH-I selective receptor. Truncations of the lamprey GnRH receptor's C-terminal tail were shown to reduce binding affinity, which explains their reductions in signaling capacity. Finally, the lamprey GnRH receptor underwent rapid ligand-dependant internalization, which was drastically reduced in the tail-less mutant form, suggesting that putative phosphoacceptor sites located within the first 40 aa of the C-terminal tail are required for this regulatory mechanism. Since the 40 aa C-terminal tail lamprey GnRH receptor mutant is capable of stimulating both IP_3 (Silver *et al.* 2005) and cAMP accumulation and undergoes rapid ligand-dependant internalization, given the results of these studies we propose that the extensive length of the lamprey GnRH receptor C-terminal tail may not have a functional significance with these signaling systems. However, the intact lengthy lamprey GnRH receptor C-terminal tail likely reflects an ancestral characteristic and may be required for structural stability, efficient ligand binding and/or stimulation of another unknown signaling pathway. Further detailed mutagenic studies need to be done to determine the function of the C-terminal tail of the lamprey GnRH receptor. In conclusion, the data from the current and previous studies indicate that the lamprey GnRH receptor shares several characteristics of both type I and type II GnRH receptors, suggesting that it retains ancestral characteristics of the vertebrate GnRH receptor family.

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