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General and Comparative Endocrinology 139 (2004) 85–94
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Communication in Genomics and Proteomics

Cloning and analysis of the lamprey GnRH-III cDNA from eight species of lamprey representing the three families of Petromyzoniformes

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Received 7 April 2004; revised 16 July 2004; accepted 21 July 2004

Abstract

The lamprey, which are divided into three families, including the Petromyzonidae, Geotriidae, and Mordaciidae, have been shown to regulate the reproductive axis through a functional hypothalamic–pituitary–gonadal axis. To date, two forms of gonadotropin-releasing hormone (GnRH) have been identified in the sea lamprey (*Petromyzon marinus*), lamprey GnRH-I (decapeptide and cDNA) and lamprey GnRH-III (decapeptide), both of which have been shown to be expressed in the preoptic-anterior hypothalamic region and both forms have been demonstrated to regulate reproductive function (i.e. steroidogenesis and gametogenesis). The objective of this study was to isolate the cDNA encoding the prepro-lamprey GnRH-III from eight species of lamprey using a PCR based subcloning procedure. A degenerate primer designed to the lamprey GnRH-III decapeptide was used to amplify the 3′ end of each transcript, while gene specific primers were used to amplify the 5′ ends. Phylogenetic analysis using the prepro-lamprey GnRH-III amino acid sequences was performed, in which the lamprey GnRH-III sequences divided into three groups, supporting the current view of the lamprey lineage at the family level. Finally, a phylogenetic analysis of these newly identified deduced amino acid sequences together with 64 previously described GnRH sequences suggests that the lamprey GnRHs are unique, as they group together separately from the three previously described paralogous lineages of the GnRH family.

Keywords: Gonadotropin-releasing hormone; GnRH; Lamprey; Molecular evolution

1. Introduction

Gonadotropin-releasing hormone (GnRH) is the central regulator of the hypothalamic–pituitary–gonadal axis, and therefore reproductive function, in all vertebrates. To date 24 isoforms of GnRH have been identified, 14 from vertebrates and 10 from invertebrates, two of which were identified in the sea lamprey (*Petromyzon marinus*), lamprey GnRH-I and -III (Sherwood et al., 1986; Sower et al., 1993). In addition, the cDNA encoding lamprey GnRH-I has been identified in the sea lamprey (Suzuki et al., 2000). Lamprey are the earliest evolved vertebrates for which two forms of GnRH have been clearly demonstrated as functional neurohormones mediating the pituitary–gonadal axis (Sower and Kawauchi, 2001). Both lamprey GnRH-I and -III were shown to be expressed in the preoptic-anterior hypothalamus and the posterior hypothalamus of the adult sea lamprey (Nozaki et al., 2000). Lamprey GnRH-III is considered the most active form during sea lamprey maturation based on the relative number of lamprey GnRH-III producing neurons which is larger than lamprey GnRH-I producing neurons during the larval and

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doi:10.1016/j.ygcen.2004.07.011

adult stages (Nozaki et al., 2000; Tobet et al., 1995) and that lamprey GnRH-III was more potent compared to lamprey GnRH-I in inducing spermiation in male sea lamprey (Deragon and Sower, 1994; Sower, 2003). Neither lamprey GnRH-I nor -III peptides have been isolated from any other species of lamprey, although both forms have been detected in the brain and hypothalamus using immunocytochemistry and HPLC coupled with radioimmunoassay in the pouched lamprey (*Geotria australis*), Australian lamprey (*Mordacia mordax*), silver lamprey (*Ichthyomyzon unicuspis*), and western brook lamprey (*Lampetra richardsoni*) (Eisthen and Northcutt, 1996; Sower et al., 2000).

In vertebrates, it is now known that two or three forms of GnRH can be expressed within the brain of a single species (Dubois et al., 2001, 2002; Miyamoto et al., 1984; White and Fernald, 1998; White et al., 1994, 1998). A proposed relationship of these GnRH forms was recently described based on phylogenetic analysis, location of expression within the brain and general associated function (Fernald and White, 1999). In this model, the GnRH family was divided into three paralogous lineages, starting with GnRH-I (also known as mammalian GnRH and its orthologs), which is expressed in the hypothalamus and is the central regulator of the pituitary–gonadal axis. GnRH-II (also known as chicken GnRH-II) is expressed in the midbrain and is generally considered to have a neuromodulatory function (Fernald and White, 1999; Parhar, 2002). Finally, GnRH-III (also known as salmon GnRH), which is only found in the teleosts, is expressed in the telencephalon and is also believed to have a neuromodulatory function (Fernald and White, 1999; Parhar, 2002). Parhar recently modified this scheme by the addition of a fourth family that consists of medaka and seabream GnRH, which formerly would have been considered GnRH-I (Parhar, 2002). This modification is supported by the fact that the cells producing medaka and seabream GnRH do not share the same developmental origin as the other hypothalamic forms, although they do function in a similar manner and group together phylogenetically. These models that describe the molecular phylogeny of the GnRH family are incomplete since only a limited number of the known GnRH sequences were used.

Interest in the evolution of reproductive mechanisms has led researchers to study lamprey, which are one of the two extant representatives of the most ancient class of vertebrates, the agnathans, which diverged from the main line of vertebrate evolution approximately 450 million years ago. The divergence of the lamprey lineage is believed to have occurred between two proposed early genome duplications (Ohno, 1970), making the lamprey an important model for evolutionary biology (Kuratani et al., 2002). Despite this interest in the lamprey, very little is known about the lamprey phylogeny. In the 1970s, a lamprey phylogeny was proposed based primarily on the size, shape, and distribution of dentition (Hubbs and Potter, 1971; Potter and Hilliard, 1987). Based on the morphology of their teeth, the lamprey were divided into three families: the Petromyzonidae, which are found in the northern hemisphere, also referred to as the holarctic species, and the two southern hemisphere families, Geotriidae and Mordaciidae. The Petromyzonidae consists of six genera: *Ichthyomyzon, Petromyzon, Caspiomyzon, Eudontomyzon, Tetrapleurodon*, and *Lampetra*. Notably the *I. unicuspis* is believed to be the most closely related to the ancestral stock of lamprey due to its simple tooth shape and distribution (Hubbs and Potter, 1971). The Geotriidae and Mordaciidae each consist of only one genus, *Geotria* and *Mordacia* respectively (Hubbs and Potter, 1971). This overall division into three families is supported by sequence analysis of the primary structure of insulin, where the *M. mordax* sequence is more similar to that of the holarctic sequences than to the *G. australis* sequence (Conlon, 2001; Conlon et al., 2001). One of the divisions of lampreys has since been supported by an analysis of mitochondrial genes exclusively within the Lampetra, reconfirming the species designation within the this family (Docker et al., 1999), however, further analysis of other characters is needed to better our understanding of the lamprey phylogeny.

To address the question of the molecular relationship within the GnRH family, as well as the phylogeny of the lamprey lineage we have isolated the cDNA encoding the prepro-lamprey GnRH-III from eight different species of lamprey representing all three families of the Petromyzoniformes. These sequences were used in a phylogenetic analysis with other known prepro-GnRH sequences published on GenBank, or solely with each other to address each question, respectively. Our analysis suggests that the lamprey GnRH forms are unique as they grouped together, yet separately from the three previously described paralogous lineages of GnRH. The analysis within the lamprey lineage supports the phylogeny based on dentition at the family level, although ultimately additional traits need to be considered.

2. Materials and methods

2.1. Animal collection and handling

Eight species of lamprey were used in this study, which were collected from both North America and Australia in accordance to UNH IACUC animal care guidelines. Experts in the field, using local fish guides, verified the identity of each species. Species collection sites and tissues dissected are described in Table 1. In

Table 1
Summary of tissue collection

Species	Collection location	Collectors	Tissues collected
P. marinus	Cocheco River, NH	Sower lab	Brain, pituitary, testis, ovary, liver, kidney, heart, muscle, and eye
L. appendix	Oyster River, NH	Sower lab	Brain
L. tridentatus	Washington	Craig Robinson (USGS)	Brain, testis, ovary, liver, and muscle
L. richardsoni	Washington	Craig Robinson (USGS)	Brain, ovary, liver, and muscle
I. Unicuspis	Great Lakes Region	Sid Morkert (US FWLS)	Brain and pituitary
I. Fossor	Great Lakes Region	Paul Sullivan (DFO)	Brain, pituitary, testis, ovary, kidney, and liver
G. australis	Tasmania, Australia	Sower and Colleagues	Brain, pituitary, testis, kidney, liver, muscle, heart, and eye
M. mordax	Tasmania, Australia	Sower and Colleagues	Brain and ovary

Tissues were collected from both North America, including six species from the Petromyzonidae, and Australia, including one species from both the Geotriidae and Mordaciidae. Obtained tissues were subject to availability.

all cases except one, the dissected tissue was immediately frozen in either liquid nitrogen or dry ice, then stored at −80 °C. The tissues collected from *G. australis* were immediately submersed in 1 mL of RNAlater (Ambion, Austin, TX) at room temperature. Samples were then stored at −20 °C until used, at which point the RNAlater was poured off the tissue.

2.2. RNA isolation

Total RNA was isolated using the Tri-Reagent (Molecular Research Center, Cincinnati, OH), an isothyocyanate–phenol–chloroform extraction method, with a glass homogenizer. RNA was isolated using 1 mL Tri-Reagent per 100 mg of tissue. Yield was determined by spectrophotometry in MilliQ H_2O or phosphate buffer at pH 8.1.

2.3. cDNA synthesis

First strand cDNA synthesis was done using the First Strand cDNA Synthesis kit by Amersham–Pharmacia Biotech (Buckinghamshire, England). First strand synthesis used 5 μg of total RNA and was catalyzed using the Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase with a *Not*I-dT_{18} primer per manufacturer's instructions. The single strand cDNA was amplified via the polymerase chain reaction (PCR), as described below, or used to make second-strand cDNA via the Gubler–Hoffman technique (Gubler and Hoffman, 1983), using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA).

2.4. Degenerate PCR

Amplification of the 3′ end of the lamprey GnRH-III cDNA from all eight species was performed by PCR using single stranded brain cDNA (ss cDNA) as template with the lamprey3–1 degenerate primer (GAR-CAY-TGG-TCN-CAC-GAT-TGG) paired with the *Not*I primer. The lamprey3–1 primer was designed based on the known decapeptide sequence of lamprey GnRH-III, with consideration of the specific differences to lamprey GnRH-I decapeptide. Promega PCR components were used and the cycling was performed with an Eppendorf Master Gradient thermal cycler under the following PCR parameters: 95 °C for 2 min followed by 35 cycles of 95 °C 30 s, 60 °C for 1 min, and 72 °C for 1 min, and finished with a 5 min 72 °C incubation and 4 °C hold. Samples were analyzed by electrophoresis using 2% agarose gels stained with ethidium bromide. The pGEM-T Easy Vector system (Promega, Madison, WI) was used to clone amplified products, which were then sequenced at the Huntsman Cancer Institute DNA Sequencing Facility at the University of Utah.

2.5. 5′-Rapid amplification of cDNA ends

5′-RACE was performed using the Marathon cDNA Amplification Kit (Clontech). A partially double stranded DNA adapter was ligated onto both ends of the synthesized double stranded cDNA (see above), to which a specific primer was used in combination with a gene specific sense or anti-sense primer [*P. marinus:* GGCGCTCTCGAGGAACTTCTCG; *I. unicuspis:* CG CGTGCCCTGTTCGTGACCAATAA; *I. fossor:* CA GGGTTCGTGTCACGTGGCGCTCT; *L. tridentatus:* CCTACACACAGCCACTCTGGGACACGC; *L. richardsoni:* CGTCACAGACCACAGCGAGGGCATT; *L. appendix:* GACCCCTGCTGGAGGAGCTTGAG GC; *G. australis:* GGCTCTCGCTGGACGGGTTCG; and *M. mordax*: CTGCGAGAGGTAACTGAGGA GGTC]. All reactions were cycled using the Eppendorf Master Gradient thermal cycler under the following parameters: 95 °C for 2 min followed by 5 cycles of 95 °C for 15 s, 2 °C above primer specific annealing temperature for 5 min, 5 cycles of 95 °C for 15 s, primer specific annealing temperature 5 min, 10 cycles of 95 °C for 15 s, primer specific annealing temperature less 2 °C for 5 min, 15 cycles of 95 °C for 15 s, primer specific annealing temperature less 4 °C for 5 min, and finished with a final 5 min 72 °C incubation and 4 °C hold. Amplified 5′ ends were cloned and sequenced as described above.

2.6. Phylogenetic analysis

The DNAStar suite by Lasergene was used for sequence analysis (EditSeq) and alignments (MegAlign). Two alignments of the GnRH deduced amino acid precursors (including the signal peptide, GnRH decapeptide, dibasic cleavage site, and GnRH associated peptide) were constructed using ClustalW. The first alignment contained the eight lamprey GnRH-III precursors (or prepro-GnRH) with 64 GnRH precursors representing type I, II, and III GnRHs as well as the octopus GnRH and two tunicate prepro-GnRHs. This analysis was rooted using the prepro-octopus GnRH protein; the tunicate forms were not used as the root due to alignment difficulties. The second alignment contained only the eight lamprey GnRH precursors. Both were analyzed using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0beta10, and trees were constructed using the neighbor joining method.

2.7. Reverse transcriptase PCR

RNA extracts collected from all tissues were treated with RQ1-RNase free-DNase (Promega). Fifty nanograms of DNase free RNA was used in each reaction using the AccessQuick RT-PCR System (Promega) with a 0.1 μM final primer concentration in a 25.5 μL final reaction volume. The primers used were designed to amplify an approximately 400–500 bp product [*P. marinus* 5′: CTGGAATCATCACAGAAGCCACACT, 3′: TCTAAGAGACGTCACAGACCACAGC; *I. unicuspis* 5′: GTGTCGCTGACGCACACACAGCAGT, 3′: TCATGTTGACGATACGCTGAGCGGC; *I. fossor* 5′: CACACTCGGCTGCTTGTAGACAT, 3′: AGCGGCGATGAAGAATTAAATAAAC; *L. tridentatus* 5′: CGGTGGTTTATTTTCTCAACAGACC, 3′: TCTAAGAGACGTCACAAACCAGAGC; *L. richardsoni* 5′: GAAACAAACAGATTCCTCTCCGAGC, 3′: CGTTGATTATCTTCGTCTGCAGCTT; *L. appendix* 5′: CTGGAATCATCACAGAAGCCACACT, 3′: CTGAGCGGCGATGAAAAATTAAATA; *G. australis* 5′: ACAACTTTATTCACGGACAACACCC, 3′: AGATTGTGAGCTACCTCTCGCAGAA; and *M. mordax* 5′: ACACGTGTTGAGACGATGGAGAAAT, 3′: GATACACCTTGCAGGAATCATCACC]. Thermal cycling was performed using an Eppendorf Master Gradient thermal cycler using the following parameters: 48 °C for 45 min, 95 °C for 2 min followed by 30 cycles of 95 °C for 15 s, primer specific annealing temperature for 1 min and 72 °C for 1 min, and finishing with a 5 min 72 °C incubation and 4 °C hold. Samples were analyzed by electrophoresis using 2% agarose gels stained with ethidium bromide and were visualized using the Molecular Imager FX (Bio-Rad, Hercules, CA).

3. Results

3.1. Cloning of the lamprey GnRH-III cDNAs

The initial products amplified by degenerate PCR encoded from the mature GnRH decapeptide through the poly-adenylation sequence. These sequences were then used to develop the gene specific primers that were used for 5′-RACE, which amplified products from the 5′ untranslated region (UTR) through the mature GnRH decapeptide. In all, eight cDNAs were cloned that encoded the conserved tripartite structure of the prepro-GnRH protein, including a signal peptide (Sig), mature lamprey GnRH-III decapeptide, and dibasic cleavage site, followed by the GnRH associated peptide (GAP), which are flanked by untranslated regions on either side.

The eight lamprey GnRH-III cDNAs were used to construct a percent identity matrix (Table 2). This matrix shows the high percent identity (∼90%) between the Petromyzonidae sequences, and most notably the relatively low percent identity between the two southern hemisphere sequences, the *G. australis* and *M. mordax* (48.5%), is given in bold. The encoded precursor proteins were compared by domain,

Table 2
Lamprey GnRH-III cDNA percent identity matrix

	cDNA percent identity matrix							
	I. f	*I. u*	*L. a*	*L. r*	*L. t*	*P. m*	*G. a*	*M. m*
I. Fossor	X	88.8	89.5	90.1	88.0	89.0	54.3	49.2
I. Unicuspis		X	95.4	90.8	91.6	95.4	59.2	53.9
L. appendix			X	92.3	93.1	93.9	57.3	52.6
L. richardsoni				X	91.5	90.7	56.3	54.2
L. tridentatus					X	92.8	59.2	57.1
P. marinus						X	60.9	53.6
G. australis							X	**48.5**
M. mordax								X

The eight lamprey GnRH-III cDNAs were used to construct a percent identity matrix. As expected, there is a relatively high percent identity between the holarctic sequences (low 90 s). Most notably the percent identity between the sequences from the two southern hemisphere families is relatively low (48.5).

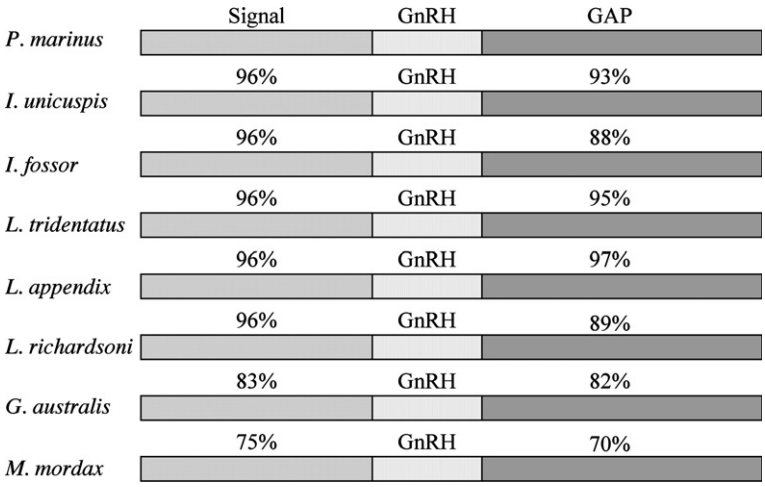

Fig. 1. Lamprey GnRH-III protein precursor domain conservation. The signal peptide (grey crisscross) and GAP (solid grey) regions of the lamprey GnRH-III precursors were compared relative to the *P. marinus*. Both regions are highly conserved within the Petromyzonidae, but are less conserved between the three families.

which showed a high level of sequence conservation relative to the *P. marinus* (Fig. 1). The signal peptide and GAP region are highly conserved within the Petromyzonidae, but are less conserved compared between families.

The *P. marinus* lamprey GnRH-III cDNA (AY052628) is 718 bp in length and includes an open reading frame (ORF), $bp_{107–388}$, which encodes a 93 amino acid (aa) peptide (Sig_{24aa}, GAP_{56aa}). The *L. appendix* lamprey GnRH-III cDNA (AY307176) is 722 bp in length and contains an ORF, $bp_{111–389}$, which encodes a 92 aa peptide (Sig_{24aa}, GAP_{55aa}). The *L. tridentatus* lamprey GnRH-III cDNA (AY307178) is 732 bp in length and contains an ORF, $bp_{121–399}$, which encodes a 92 aa peptide (Sig_{24aa}, GAP_{55aa}). The *L. richardsoni* lamprey GnRH-III cDNA (AY307177) is 710 bp in length and contains an ORF, $bp_{110–388}$, which encodes a 92 aa peptide (Sig_{24aa}, GAP_{55aa}). The *I. unicuspis* lamprey GnRH-III cDNA (AY307176) is 723 bp in length and contains an ORF, $bp_{114–392}$, which encodes a 92 aa peptide (Sig_{24aa}, GAP_{55aa}). The *I. fossor* lamprey GnRH-III cDNA (AY307175) is 728 bp in length and contains an ORF, $bp_{108–386}$, which encodes a 92 aa peptide (Sig_{24aa}, GAP_{55aa}). The *G. australis* lamprey GnRH-III cDNA (AY307172) is 774 bp in length and contains an ORF, $bp_{124–408}$, which encodes a 94aa peptide (Sig_{25aa}, GAP_{56aa}). The *M. mordax* lamprey GnRH-III cDNA (AY307173) is 666 bp in length and contains an ORF, $bp_{85–363}$, which encodes a 92 aa peptide (Sig_{24aa}, GAP_{55aa}).

3.2. Phylogenetic analysis

The first alignment used included the eight newly discovered deduced amino acid lamprey GnRH-III precursor (prepro-GnRH) sequences with 64 other sequences from GenBank. These additional sequences were selected such that representatives from the different classes of vertebrates were included for each GnRH type where possible. Trees were constructed using the neighbor joining method with the prepro-octopus GnRH (AB037165) sequence as an out-group (Fig. 2). The produced tree clearly shows the lamprey GnRH forms grouping together, yet outside of the other previously described groups of GnRH (I, II, and III). Phylogenetic analysis of the eight lamprey GnRH-III precursors shows the sequences are divided into three groups, shown in Fig. 3, which is a neighbor joining tree overlaid with bootstrap values (1000 replicates). This analysis, where branch length represents sequence distance, clearly shows the sequences are grouped corresponding to the Petromyzonidae, Geotriidae or Mordaciidae, and furthermore, the separation of the Geotriidae and Mordaciidae into two distinct families is supported as the *G. australis* and *M. mordax* sequences are approximately equally removed from each other as they are from the Petromyzonidae sequences.

3.3. RT-PCR

Analysis of tissue specific expression of lamprey GnRH-III was limited to the available tissues from each species. In all species examined the lamprey GnRH-III amplicon was visualized in the brain. Of the four pituitary samples, *P. marinus*, *I. fossor*, *I. unicuspis*, and *G. australis*, lamprey GnRH-III expression was only visualized in the *P. marinus* and *I. unicuspis*. Lamprey GnRH-III expression in the gonads was only seen in the ovary of the *L. tridentatus*, which was verified by sequence analysis (Fig. 4). Expression was not seen in any of the other tissues examined. Negative controls, which were identical to the experimental reactions in setup and

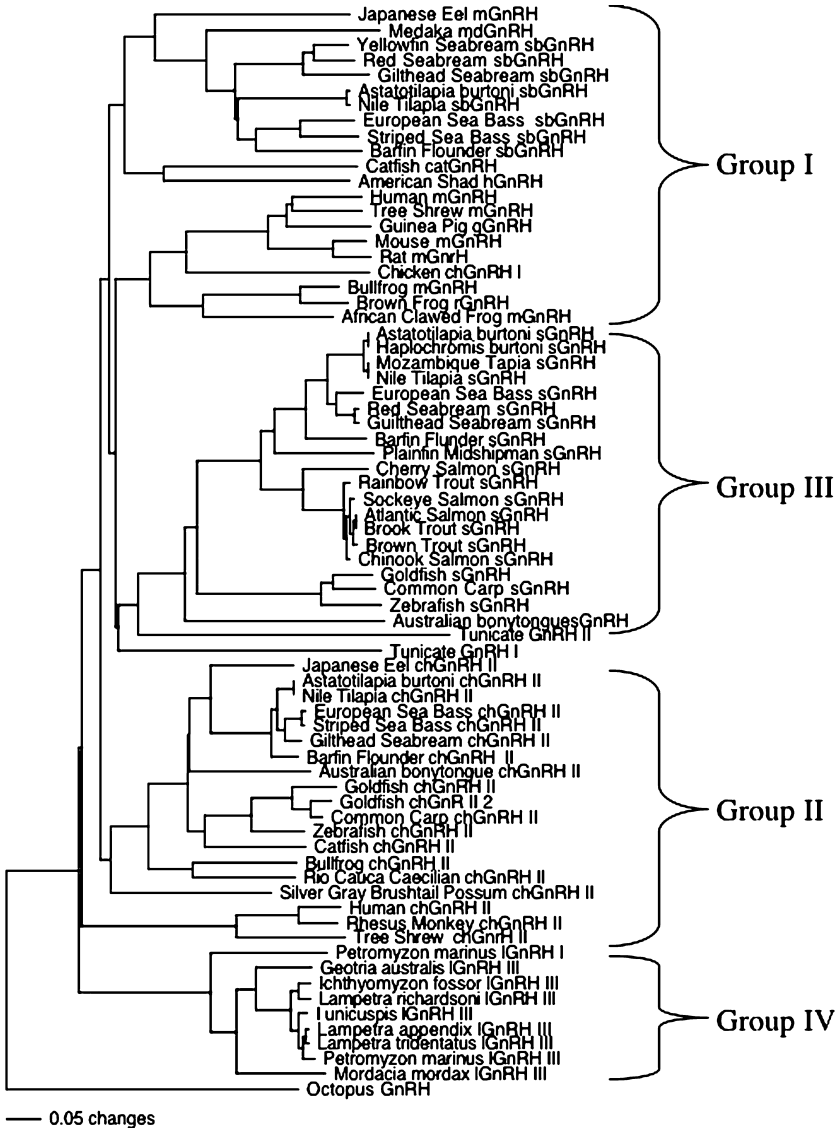

Fig. 2. Phylogenetic analysis of all GnRH precursors. The neighbor joining method was used to construct this phylogenetic tree of the deduced amino acid prepro-GnRH precursors (signal peptide, GnRH, dibasic cleavage site, and GAP), which is rooted with octopus GnRH. The lamprey GnRH forms group separately from the previously described type I, II, and, III GnRHs, which suggests they form a unique lineage of the GnRH family. m, mammalian; cat, catfish; h, herring; g, guinea pig; ch, chicken; r, rana; md, medaka; sb, seabream; s, salmon; and l, lamprey.

cycling but lacked RNA template, did not show any bands (data not shown).

4. Discussion

We have cloned the full-length cDNA encoding the deduced prepro-lamprey GnRH-III precursor from eight species of lamprey. Based on our phylogenetic analysis of 72 GnRH precursors, including the lamprey GnRH-III precursor sequences, along with data from previous immunocytochemical and functional studies, we propose that the lamprey GnRH isoforms constitute a fourth group of the GnRH family of peptides. In addition, our data showed that the lamprey GnRH-III precursors of the two southern hemisphere species are highly divergent from the lamprey GnRH-III of the holarctic species, and to each other, which supports the lamprey phylogeny based on dentition (Hubbs and Potter, 1971).

Although the application of molecular phylogenetic analysis to reconstruct species phylogeny is prone to conflicting results, it is widely accepted as an invaluable addition to anatomical, physiological, and behavioral analyses (Brocchieri, 2001; Lio and Goldman, 1998; Slowinski and Page, 1999). In our analysis, a molecular phylogenetic approach was used to assess the previously proposed phylogeny of Petromyzoniformes (Hubbs and Potter, 1971), which was based primarily on the size, shape, and distribution of dentition. Based on this

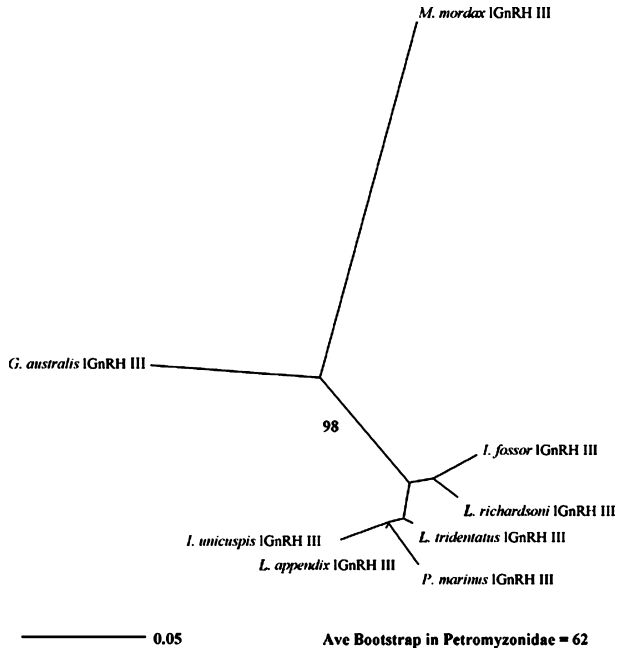

Fig. 3. Phylogenetic analysis of the prepro-lamprey GnRH-III precursors. The neighbor joining method was used to construct this unrooted phylogenetic tree, where branch length represents sequence dissimilarity, of the eight prepro-lamprey GnRH-III precursors (signal peptide, GnRH, dibasic cleavage site, and GAP). The topology of the tree is divided into three groups corresponding to the three families of lamprey, the Petromyzonidae, Geotriidae, and Mordaciidae. The analysis was resampled with 1000 bootstrap replicates, which were averaged for the Petromyzonidae group for logistical purposes. This tree supports the lamprey phylogeny based on dentition at the family level, which is a strong division as the bootstrap value of the internal branch between the three groups is 98.

scheme, it is proposed that the lamprey lineage is divided into three families, including the Petromyzonidae, or holarctic species, and the two southern hemisphere families, which include the Geotriidae and Mordaciidae. This model has been supported by sequence analysis of the primary structure of the insulin molecule, which suggested that the *M. mordax* insulin was more closely related to the insulin of the holarctic species than to the *G. australis* insulin, although no molecular phylogenetic analysis has been performed to date to re-examine this relationship (Conlon, 2001; Conlon et al., 2001). Our phylogenetic analysis of the cloned lamprey GnRH-III precursors, using the neighbor joining method, in which the tree is divided into three groups corresponding to the Petromyzonidae, Geotriidae, and Mordaciidae, supports the previously described lineage based on dentition at the family level. Furthermore, this tree, which shows the distances between taxa as a reflection of dissimilarity, confirms the tripartite division of the Petromyzoniformes in which *M. mordax* lamprey GnRH-III and *G. australis* lamprey GnRH-III sequences are approximately equally removed from holarctic clade as they are from each other. However, the internal grouping within the Petromyzonidae did not match the arrangement of the phylogeny based on dentition, in which the *Ichthyomyzon* lamprey GnRH-III sequences and *Lampetra* lamprey GnRH-III sequences would be expected to form groups together, but were rather mismatched in our analysis. Nonetheless, our analysis has provided the first supporting evidence of the phylogeny based on dentition at the family level. Further analysis on the internal grouping would need to be verified using additional traits, such as a more standard molecular marker and/or additional anatomical/physiological/behavioral traits. Concerning the bootstrap values, although there was an average bootstrap value of 62 within the Petromyzonidae sequences, the most critical internal branch, which divides the three groups of sequences into Geotriidae, Mordaciidae, and Petromyzonidae was 98, and therefore we are only drawing conclusions at the family level.

The GnRH family of peptides is a highly conserved group of neurohormones that has been subject to intense investigation since the first primary structure of GnRH was identified in the early 1970s (Burgus et al., 1972; Matsuo et al., 1971). As more sequences have been identified and more species representing different classes of vertebrates have been investigated it has become clear that multiple forms of GnRH are expressed within the brain of a single species (Dubois et al., 2002; King and Millar, 1995; White et al., 1998; White and Fernald, 1998). An earlier analysis considering phylogenetic analysis of 23 GnRH transcripts, location of expression within the brain, and function demonstrated that the GnRH family was composed of three paralogous lineages (Fernald and White, 1999), which included GnRH-I (hypothalamic releasing form: mammalian GnRH and orthologs), GnRH-II (midbrain neuromodulatory form: chicken GnRH-II in all vertebrates but lamprey), and GnRH-III (telencephalic neuromodulatory form: salmon GnRH, found only in teleosts). It was later suggested that a fourth lineage of GnRH consisted of medaka and seabream GnRH, which is based exclusively on the difference in origin of the cell bodies that produce these forms of GnRH compared to the other type I GnRHs (Parhar, 2002). Neither of these analyses considered the lamprey GnRH forms of GnRH in their models, and as such we have re-evaluated these groupings using the deduced amino acid sequences from the eight cloned lamprey GnRH-III cDNAs, the previously described prepro lamprey GnRH-I protein (Suzuki et al., 2000), and the known distribution and origin of the GnRH lineages. Our phylogenetic analysis confirms Fernald and Whites division of the GnRH family, but shows the medaka and seabream forms of GnRH grouping with the type I GnRHs, which conflicts with Parhar's model. Additionally, the lamprey GnRH forms group together separately from the three previously described lineages of GnRH, and as such we suggest that

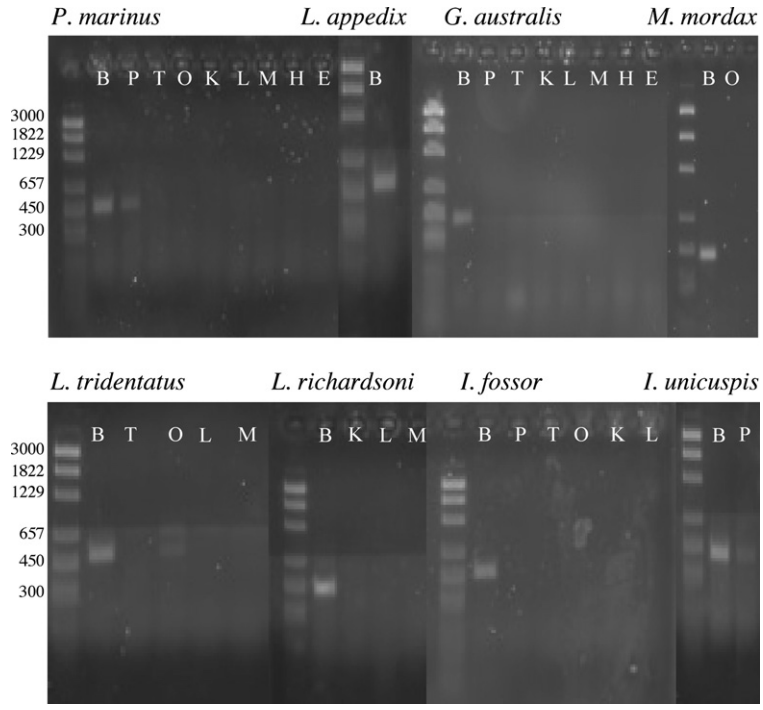

Fig. 4. Tissue specific expression of the lamprey GnRH-III transcript. Tissue specific expression of the lamprey GnRH-III transcript was determined using RT-PCR. Products were analyzed by gel electrophoresis using the PGem7 ladder (in lane one of each gel), which contains molecular weight markers of 3000, 1822, 1229, 657, 450, and 300 bp. B, brain; P, pituitary; T, testes; O, ovary; K, kidney; L, liver; M, muscle; H, heart; and E, eye.

they, and not medaka and seabream GnRH, form the 4th lineage of GnRH (see Table 3). Our assertion is based directly on our phylogenetic analysis which is supported by immunocytochemical and functional data on lamprey GnRH. The origin of the cells that produce the lamprey GnRH forms, which were shown to arise from cells in the proliferative zones of the diencephalon (Tobet et al., 1993, 1997), differs from the origin of the type I GnRHs, which arise from cells in the olfactory placode (Norgren and Gao, 1994; Schwanzel-Fukuda, 1999; Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). In addition, both lamprey GnRH-I and -III differ from the type I GnRH lineage as they are both distributed within the preoptic-neurohypophyseal system of the lamprey as demonstrated using immunocytochemistry in the *P. marinus* (Nozaki et al., 2000), *G. australis* (Sower et al., 2000), *I. unicuspis* (Eisthen and Northcutt, 1996), *L. richardsoni* (Crim, 1985), and *L. tridentatus* (Crim et al., 1979), and both are active in the regulation of the reproductive axis in the *P. marinus* (Deragon and Sower, 1994). To ultimately understand the molecular evolution of the GnRH family additional sequence data, distribution, functional, and physiological data are needed across vertebrates and invertebrates.

Expression of the lamprey GnRH-III mRNA was detected in the brain of all eight species, as well as the pituitary of the *P. marinus* and *I. unicuspis*. Expression was also detected in the ovary of the *L. tridentatus*, which is unique among all of the species tested. The expression pattern of lamprey GnRH-III in the *P. marinus* differs to that of the previously described lamprey GnRH-I pattern, where expression was only seen in the brain and faintly in the testis (Suzuki et al., 2000), although pituitary expression was not investigated in this study. GnRH expression in non-neural tissues is typical (Azad et al., 1991; Dong et al., 1996; White et al., 1998; Yoo

Table 3
The four putative paralogous lineages of GnRH: the GnRH family is proposed to be divided into four paralogous lineages based on phylogenetic analysis, function, neural distribution, and developmental origin

Type GnRH	Brain distribution/origin	Primary GnRH structures identified in vertebrates
GnRH-I	Hypothalamus, diencephalon/olfactory origin	Mammal GnRH in *mouse*, *primate*, *human*, *sheep*, *pig*, *eel*, *newt*, and *frog*; chicken GnRH-I in *chicken*, *lizard*; salmon GnRH in *goldfish*, *salmon*; catfish GnRH in *catfish*; dogfish GnRH in *dogfish*
GnRH-II	Midbrain/ventricular ependyma origin	Chicken GnRH-II in *primate*, *human*, *chicken*, *lizard*, *frog*, *newt*, *eel*, *goldfish*, *catfish*, *salmon*, *medaka*, *red seabream*, *tilapia*, and *ratfish*
GnRH-III	Telencephalon/olfactory origin	Salmon GnRH in *medaka*, *red seabream*, and *tilapia*
GnRH-IV	Hypothalamus, diencephalon/ventricular origin	Lamprey GnRH-I and lamprey GnRH-III in *lamprey*

et al., 2000), although it's function in tissues other than the gonad is unclear. In *P. marinus* it has been demonstrated that both lamprey GnRH-I and -III stimulates steroidogenesis at the level of the gonads, suggesting a possible paracrine and/or autocrine regulatory mechanism (Gazourian et al., 1997, 2000), although there was no lamprey GnRH-III expression detected in the *P. marinus* testis or ovary in this study.

In summary, the cDNA encoding the lamprey GnRH-III was cloned from eight species of lamprey, which was used in a series of phylogenetic analyses to address questions concerning the molecular phylogeny of the GnRH family and the lineage of the Petromyzoniformes. Based on our phylogenetic analysis using the deduced prepro lamprey GnRH-III with 64 other GnRH precursors, along with data from previous immunocytochemical and functional studies, we propose that the lamprey forms of GnRH constitute a unique lineage within the GnRH family. The phylogenetic analysis of the lamprey GnRH-III precursors supports the phylogeny based on dentition at the family level, dividing into the Petromyzonidae, Geotriidae, and Mordaciidae. The information derived from this study provides critical information on the molecular evolution of GnRH in vertebrates.

Acknowledgments

We thank Drs. Joss and Takahashi, as well as Sid Morkert, Paul Sullivan, and Craig Robinson for their assistance with animal and tissue collection. This research was supported by NSF (IBN 0090852 and 0421923 to SAS) and NSF-JSPS (INT-981528 to SAS and HK). This work was also partly supported by Grant-in-Aid for Scientific Research (No. 15370029) and Japan–US and Japan–Australia Cooperative Science programs from the Japan Society for the Promotion of Science. This is scientific contribution number 2217 from the New Hampshire Agriculture Experimentation Station.

References

Azad, N., Emanuele, N.V., Halloran, M.M., Tentler, J., Kelley, M.R., 1991. Presence of luteinizing hormone-releasing hormone (LHRH) mRNA in rat spleen lymphocytes. Endocrinology 128 (3), 1679–1681.

Brocchieri, L., 2001. Phylogenetic inferences from molecular sequences: review and critique. Theor. Popul. Biol. 59 (1), 27–40.

Burgus, R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W., Guillemin, R., 1972. Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF) (LH–hypothalamus–LRF–gas chromatography–mass spectrometry–decapeptide–edman degradation). Proc. Natl. Acad. Sci. USA 69 (1), 278–282.

Conlon, J.M., 2001. Evolution of the insulin molecule: insights into structure–activity and phylogenetic relationships. Peptides 22 (7), 1183–1193.

Conlon, J.M., Wang, Y., Potter, I.C., 2001. The structure of mordacia mordax insulin supports the monophyly of the petromyzontiformes and an ancient divergence of mordaciidae and geotriidae. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129 (1), 65–71.

Crim, J.W., 1985. Physiology of endogenous luteinizing hormone-releasing hormone in agnathan fishes and amphibians. In: Lofts, B., Holmes, W.M. (Eds.), Current Trends in Comparative Endocrinology. Hong Kong University Press, Hong Kong.

Crim, J.W., Urano, A., Gorbman, A., 1979. Immunocytochemical studies of luteinizing hormone-releasing hormone in brains of agnathan fishes. I. Comparisons of adult pacific lamprey (*Entosphenus tridentata*) and the pacific hagfish (*Eptatretus stouti*). Gen. Comp. Endocrinol. 37 (3), 294–305.

Deragon, K.L., Sower, S.A., 1994. Effects of lamprey gonadotropin-releasing hormone-III on steroidogenesis and spermiation in male sea lampreys. Gen. Comp. Endocrinol. 95 (3), 363–367.

Docker, D.F., Youson, J.H., Beamish, R.J., Devlin, R.H., 1999. Phylogeny of the lamprey genus *Lampetra* inferred from mitochondrial cytochrome *b* and ND3 gene sequences. Can. J. Fish Aquat. Sci. 56, 2340–2349.

Dong, K.W., Duval, P., Zeng, Z., Gordon, K., Williams, R.F., Hodgen, G.D., Jones, G., Kerdelhue, B., Roberts, J.L., 1996. Multiple transcription start sites for the GnRH gene in rhesus and cynomolgus monkeys: a non-human primate model for studying GnRH gene regulation. Mol. Cell. Endocrinol. 117 (2), 121–130.

Dubois, E.A., Zandbergen, M.A., Peute, J., Bogerd, J., Goos, H.J., 2001. Development of three distinct GnRH neuron populations expressing two different GnRH forms in the brain of the african catfish (*Clarias gariepinus*). J. Comp. Neurol. 437 (3), 308–320.

Dubois, E.A., Zandbergen, M.A., Peute, J., Goos, H.J., 2002. Evolutionary development of three gonadotropin-releasing hormone (GnRH) systems in vertebrates. Brain Res. Bull. 57 (3–4), 413–418.

Eisthen, H.L., Northcutt, R.G., 1996. Silver lampreys (*Ichthyomyzon unicuspis*) lack a gonadotropin-releasing hormone- and FMRF amide-immunoreactive terminal nerve. J. Comp. Neurol. 370 (2), 159–172.

Fernald, R.D., White, R.B., 1999. Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front. Neuroendocrinol. 20 (3), 224–240.

Gazourian, L., Deragon, K.L., Chase, C.F., Pati, D., Habibi, H.R., Sower, S.A., 1997. Characteristics of GnRH binding in the gonads and effects of lamprey GnRH-I and -III on reproduction in the adult sea lamprey. Gen. Comp. Endocrinol. 108 (2), 327–339.

Gazourian, L., Evans, E.L., Hanson, L., Chase, F., Sower, S.A., 2000. The effects of lamprey GnRH-I, -III and analogs on steroidogenesis in the sea lamprey (*Petromyzon marinus*). Aquaculture 188, 147–165.

Gubler, U., Hoffman, B.J., 1983. A simple and very efficient method for generating cDNA libraries. Gene 25 (2–3), 263–269.

Hubbs, C.L., Potter, I.C., 1971. Distribution, phylogeny and taxonomy. In: Hardisty, M.W., Potter, I.C. (Eds.), The Biology of Lampreys. Academic Press, London/New York, pp. 1–65.

King, J.A., Millar, R.P., 1995. Evolutionary aspects of gonadotropin-releasing hormone and its receptor. Cell. Mol. Neurobiol. 15 (1), 5–23.

Kuratani, S., Kuraku, S., Murakami, Y., 2002. Lamprey as an evo-devo model: lessons from comparative embryology and molecular phylogenetics. Genesis 34 (3), 175–183.

Lio, P., Goldman, N., 1998. Models of molecular evolution and phylogeny. Genome Res. 8 (12), 1233–1244.

Matsuo, H., Baba, Y., Nair, R.M., Arimura, A., Schally, A.V., 1971. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Commun. 43 (6), 1334–1339.

Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., Matsuo, H., 1984. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc. Natl. Acad. Sci. USA 81 (12), 3874–3878.

Norgren Jr, R.B., Gao, C., 1994. LHRH neuronal subtypes have multiple origins in chickens. Dev. Biol. 165 (2), 735–738.

Nozaki, M., Ominato, K., Gorbman, A., Sower, S.A., 2000. The distribution of lamprey GnRH-III in brains of adult sea lampreys (*Petromyzon marinus*). Gen. Comp. Endocrinol. 118 (1), 57–67.

Ohno, S., 1970. Evolution by gene duplication. Springer-Verlag, Berlin, Germany.

Parhar, I.S., 2002. Cell migration and evolutionary significance of GnRH subtypes. Prog. Brain Res. 141, 3–17.

Potter, I.C., Hilliard, R.W., 1987. A proposal for the functional and phylogenetic significance of differences in the dentition of lampreys (Agnatha: Petromyzontiformes). J. Zool. (Lond.) 212, 713–737.

Schwanzel-Fukuda, M., 1999. Origin and migration of luteinizing hormone-releasing hormone neurons in mammals. Microsc. Res. Tech. 44 (1), 2–10.

Schwanzel-Fukuda, M., Pfaff, D.W., 1989. Origin of luteinizing hormone-releasing hormone neurons. Nature 338, 161–164.

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., Brownstein, M.J., 1986. Primary structure of gonadotropin-releasing hormone from lamprey brain. J. Biol. Chem. 261 (11), 4812–4819.

Slowinski, J.B., Page, R.D., 1999. How should species phylogenies be inferred from sequence data. Syst. Biol. 48 (4), 814–825.

Sower, S.A., 2003. The endocrinology of reproduction in lampreys and applications for male lamprey sterilization. J. Great Lakes Res. 29, 50–65.

Sower, S.A., Chiang, Y.C., Lovas, S., Conlon, J.M., 1993. Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. Endocrinology 132 (3), 1125–1131.

Sower, S.A., Kawauchi, H., 2001. Update: brain and pituitary hormones of lampreys. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129 (2–3), 291–302.

Sower, S.A., McGregor, A.J., Materne, O.L., Chase, C., Potter, I., Joss, J., 2000. Evidence for lamprey GnRH-I and -III-like molecules in the brains of the southern hemisphere lampreys *Geotria australis* and *Mordacia mordax*. Gen. Comp. Endocrinol. 120 (2), 168–175.

Suzuki, K., Gamble, R.L., Sower, S.A., 2000. Multiple transcripts encoding lamprey gonadotropin-releasing hormone-I precursors. J. Mol. Endocrinol. 24 (3), 365–376.

Tobet, S.A., Crandall, J.E., Schwarting, G.A., 1993. Relationship of migrating luteinizing hormone-releasing hormone neurons to unique olfactory system glycoconjugates in embryonic rats. Dev. Biol. 155 (2), 471–482.

Tobet, S., Nozaki, M., Youson, J.H., Sower, S.A., 1995. Distribution of lamprey gonadotropin-releasing hormone-III (GnRH-III) in brains of larval lampreys (*Petromyzon marinus*). Cell Tissue Res. 279, 261–270.

Tobet, S.A., Sower, S.A., Schwarting, G.A., 1997. Gonadotropin-releasing hormone containing neurons and olfactory fibers during development: from lamprey to mammals. Brain Res. Bull. 44 (4), 479–486.

White, S.A., Bond, C.T., Francis, R.C., Kasten, T.L., Fernald, R.D., Adelman, J.P., 1994. A second gene for gonadotropin-releasing hormone: cDNA and expression pattern in the brain. Proc. Natl. Acad. Sci. USA 91 (4), 1423–1427.

White, R.B., Eisen, J.A., Kasten, T.L., Fernald, R.D., 1998. Second gene for gonadotropin-releasing hormone in humans. Proc. Natl. Acad. Sci. USA 95 (1), 305–309.

White, R.B., Fernald, R.D., 1998. Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. Gen. Comp. Endocrinol. 112 (1), 17–25.

Wray, S., Grant, P., Gainer, H., 1989. Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. Proc. Natl. Acad. Sci. USA 86 (20), 8132–8136.

Yoo, M.S., Kang, H.M., Choi, H.S., Kim, J.W., Troskie, B.E., Millar, R.P., Kwon, H.B., 2000. Molecular cloning, distribution and pharmacological characterization of a novel gonadotropin-releasing hormone ([trp8] GnRH) in frog brain. Mol. Cell. Endocrinol. 164 (1–2), 197–204.