

Primary Structure of a Novel Gonadotropin-Releasing Hormone in the Brain of a Teleost, Pejerrey*

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ABSTRACT

The neuropeptide GnRH is the major regulator of reproduction in vertebrates acting as a first signal from the hypothalamus to pituitary gonadotropes. Three GnRH molecular variants were detected in the brain of a fish, pejerrey (*Odontesthes bonariensis*), using chromatographic and immunological methods. The present study shows that one form is identical to chicken GnRH-II (sequence analysis and mass spectrometry) and the second one is immunologically and chromatographically similar to salmon GnRH. The third form was proven to be

a novel form of GnRH by isolating the peptide from the brain and determining its primary structure by chemical sequencing and mass spectrometry. The sequence of the novel pejerrey GnRH is pGlu-His-Trp-Ser-Phe-Gly-Leu-Ser-Pro-Gly-NH₂, which is different from the known forms of the vertebrate and protochordate GnRH family. The new form of GnRH is biologically active in releasing gonadotropin and GH from pituitary cells in an *in vitro* assay. (*Endocrinology* **142**: 1453–1460, 2001)

GnRH is a key neurohormone for reproduction in all groups of vertebrates. This decapeptide was originally isolated from mammals as the hypothalamic hormone that regulates the reproductive system by stimulating the release of gonadotropins from the anterior pituitary gland. To date, 13 distinct GnRH forms have been sequenced from vertebrate and protochordate nervous tissue (1–3). Evidence based on indirect chromatographic and immunological techniques suggests the existence of additional GnRH forms in different groups of vertebrates (4–6).

All family members are decapeptides with a conserved structure: the N terminus (pyroglutamyl residue) and C-terminus (amidated glycine) are conserved, in addition to positions 4 and 9. A mammalian form of GnRH (mGnRH) was originally isolated from porcine and ovine brains (7, 8). Also, in mammals a novel guinea pig (gp) GnRH was identified and sequenced from nervous tissue (2). Two other GnRH forms were isolated first from chicken brains: chicken GnRH-I (cGnRH-I) and chicken GnRH-II (cGnRH-II) (9–11). Additional GnRH peptides have been isolated from brains of salmon (sGnRH) (12), lamprey (lGnRH-I and lGnRH-III) (13, 14), catfish (cfGnRH) (15), dogfish (dfGnRH) (16), herring (hrGnRH) (3), and seabream (sbGnRH) (17). Two more peptides in tunicates

(tGnRH-I and tGnRH-II) were characterized from protochordate nervous tissue (18).

In all bony fish species examined to date, it is well documented that at least two GnRH variants coexist in the brain of a single species. cGnRH-II is universally distributed in bony fish (1). In addition to cGnRH-II, mGnRH is found in lobe-finned fish (19), the ancient ray-finned bony fish that evolved before the teleosts (20, 21), and in teleosts that evolved early such as Pantodon (22) and eels (23). All other teleosts studied to date have salmon GnRH with the exception of catfish, which has cfGnRH (15). The remaining forms of GnRH in bony fish are the third forms found in addition to cGnRH-II and sGnRH in some teleosts. The third forms include hrGnRH and sbGnRH. Also, there is chromatographic and immunological evidence that there may be other variants in the brain of some fishes (24–27).

The form of GnRH isolated and sequenced from herring has been found only in that species (3), whereas sbGnRH has been isolated and sequenced from seabream (17, 28), pacu (29), and two cichlids (tilapia and the African cichlid, *Haplochromis burtoni*) (30, 31). Also, the form of GnRH found in the pejerrey, *Odontesthes bonariensis*, was reported to coexist with sGnRH and cGnRH-II based on RP-HPLC/RIA studies (6). The pejerrey fish was originally found only in the inland waters of the Province of Buenos Aires in Argentina. Later, this fish was introduced into many water bodies around the world for game fishing and aquaculture (32).

The main objective of this study was to determine the primary structure of the form of GnRH in pejerrey that had been predicted to be novel by indirect methods. The structure is characterized by RP-HPLC elution position in comparison with synthetic standards, cross-reactivity with antisera,

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chemical sequencing, and mass spectral analysis. Also, the biological activity of the novel GnRH is assessed *in vitro*.

Materials and Methods

Collection of brains

Whole brains ($n = 450$), some with attached pituitaries, weighed a total of 51.6 g. The tissue was collected during the spawning season from both sexes of adult pejerrey (*Odontesthes bonariensis*; order: Atheriniformes) in the Province of Buenos Aires, Argentina (Salada Grande and Chascomús Ponds). The tissues were removed from freshly killed fish, placed immediately on dry ice, transported to the laboratory in Buenos Aires, and stored at -70°C . Frozen brains were then transported on dry ice to the Department of Biology at the University of Victoria, Canada.

Peptide extraction

Frozen pooled brains and pituitaries were powdered in a Waring blender with liquid nitrogen. Extraction of peptides was done in an acetone/1 M HCl mixture (100:3, vol/vol) for 3 h as previously described (13). The insoluble material was reextracted in acetone/0.01 M HCl (80:20, vol/vol) for 5 min and refiltered. The combined filtrates were defatted with petroleum ether (bp 30–60 C) for five successive times using a ratio of 5 parts filtrate to 1 part petroleum ether (13). Then the final aqueous phases were concentrated to less than 1 ml using a vacuum concentrator.

RP-HPLC purification

The brain extract was filtered and applied to a series of 10 Sep-Pak cartridges (Waters Corp., Milford, MA) in step 1 and eluted with mobile phases A (0.5% trifluoroacetic acid, TFA) and B (0.5% TFA-80% acetonitrile, ACN). Sixty fractions of 1 ml were collected and an aliquot of 5 μl taken for RIA. Immunoreactive GnRH fractions were pooled, concentrated under vacuum and injected onto a Supelco (Oakville, Ontario, Canada; Supelcosil LC-18) analytical column using a Beckman (Mississauga, Ontario, Canada) 166 model Liquid Chromatograph. The sample was applied at the beginning of a 10 min isocratic period of 17% ACN in 0.25 M triethylammonium formate TEAF (pH 6.5); then ACN was increased to 24% over a 7-min period and held isocratically for 43 min. The flow rate was kept at 1 ml/min and 1 ml fractions were collected (13). Aliquots of 10 μl from each fraction were used for RIA to determine immunoreactive GnRH (ir-GnRH). Eluted ir-GnRH fractions were pooled from this step and designated as peak I, II, or III. The three ir-GnRH peaks were selected for further purification. Three successive

RP-HPLC steps were performed. Column types, solvents and conditions are summarized in Fig. 1. Each injection of the tissue extract was preceded by a blank run, in which the mobile phase was injected. The blank fractions were radioimmunoassayed under the same conditions as the samples to ensure that the column was not contaminated. Fresh standards were chromatographed onto a different column to compare the elution position of each ir-GnRH peak.

RIA measurement

Aliquots of 10 μl from fractions collected at each successive step in the RP-HPLC purification were assayed for ir-GnRH by methods previously described (13). Two different antisera, GF-6 (1:25,000 final dilution) and PBL 49 (1:150,000 final dilution, kindly provided by Dr. W.W. Vale), were used in an assay with mGnRH as radioiodinated hormone and standard. This assay was heterologous for cGnRH-II, sGnRH, and the novel pejerrey (pj) GnRH. Cross-reactivities of these antisera were reported in Lescheid *et al.* (33) and Montaner *et al.* (34). The initial RP-HPLC step (Fig. 2, box B) was assayed by both GF-6 and PBL 49; each antiserum detected the same three peaks and the values measured between antisera were comparable. RP-HPLC purification of GnRH in subsequent steps (Fig. 2, boxes C–E) was followed using antiserum GF-6.

The RIA detection limit for GtH-II was better than 0.3 ng/ml and for GH was 2 ng/ml. Basal GtH-II secretion from this study averaged 263 ± 58 ng/ml and GH secretion averaged 988 ± 93 ng/ml. Appropriate dilutions of the samples were made to ensure that the values were within the useful range of the assays. The RIA for GtH-II had 4.9% interassay variability as determined by the coefficient of variation (CV) of GtH-II values at 50% displacement. Within assay variability was 11% from the same assays as determined by the average CV of quadruplicate determinations of a 5 ng/ml GtH-II standard in each of the assays. For the GH assays, the interassay variability is 6.6% and the intraassay variability is 9.4%, as determined from the average CV of quadruplicate determinations of a 20 ng/ml GH standard in each assay.

Characterization of the primary structure

An aliquot of the peptide purified by RP-HPLC on a diphenyl column was injected into a microbore C-18 column using 0.05% TFA and ACN for elution. Fractions were collected and analyzed with a Bruker Reflex time-of-flight instrument using an accelerating voltage of 31 kV and a reflectron voltage of 30 kV (100 MHz digitizer). The sample was applied to a thin layer of α -cyano-4-hydroxy cinnamic acid, allowed to dry, and rinsed with water before analysis. Sequencing was initially attempted on 10% of each sample. Failure of this sequencing indicated a blocked N

FIG. 1. Steps of GnRH purification from pejerrey brains. Each line indicates the sequential RP-HPLC system and elution program. The mobile phase at each step was a mixture of A and B as outlined in the third column. ACN, Acetonitrile; TFA, trifluoroacetic acid; TEAF, triethylammonium formate; TEAP, triethylammonium phosphate.

Step	Column			Mobile Phase		Sequential elution conditions		
	Type	Packing	Size(cm)	A	B	Initial %B	Final %B	Time (min)
1	Supelco	C18	0.45x25	0.25 M TEAF (pH 6.5)	100%	17	17	10
					ACN	17	24	7
						24	24	43
2	Supelco	C18	0.45x25	0.13 M TEAP (pH 2.5)	100%	17	17	10
					ACN	17	24	7
						24	24	43
3	Supelco	C18	0.45x25	0.05 M TFA (pH 2)	80% ACN/	5	5	5
					0.05% TFA	5	20	10
						20	50	30
						50	100	10
						100	100	15
4	Vydac	Diphenyl	0.45x25	0.05 M TFA (pH 2)	80% ACN/	5	5	5
					0.05% TFA	5	20	10
						20	50	30
						50	100	10
						100	100	15

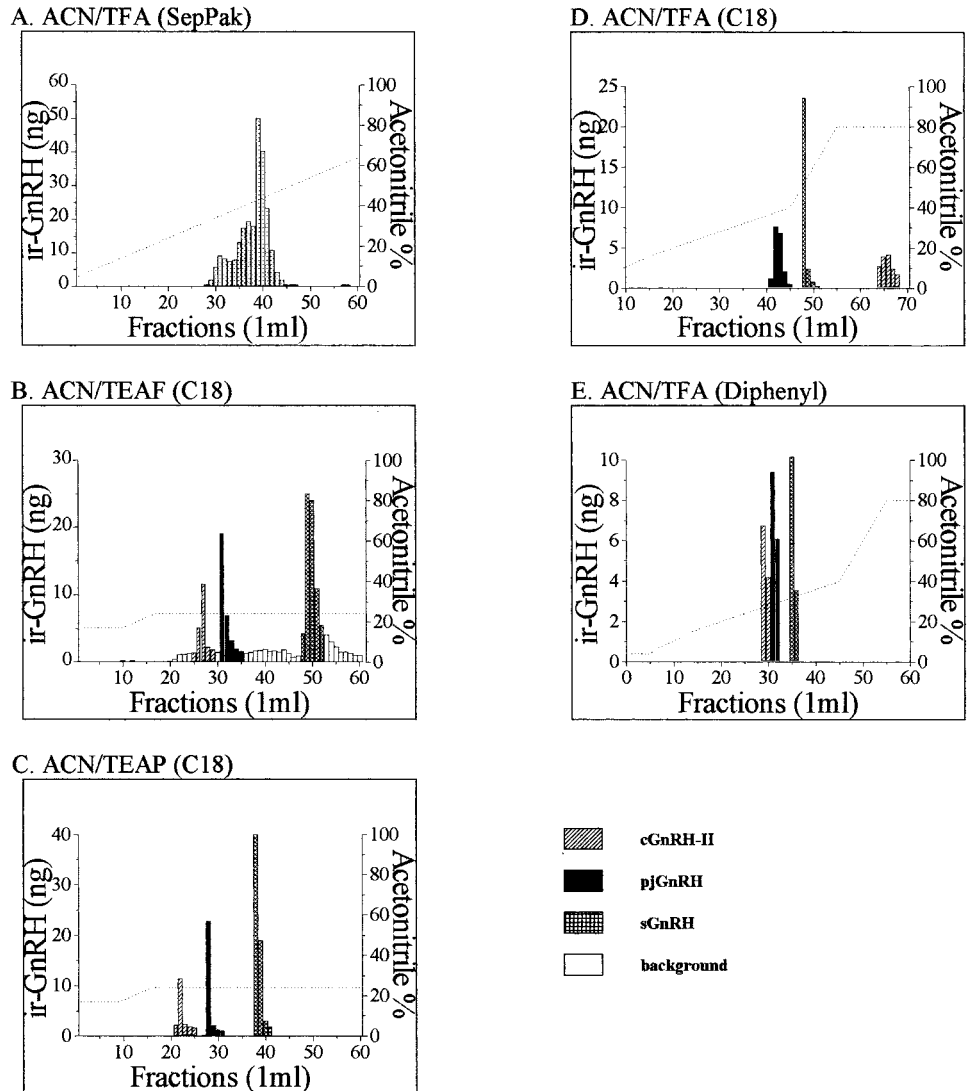


FIG. 2. Elution of ir-GnRH fractions during chromatographic purification from pejerrey brains. A, Prepurification: ACN/TFA (Sep-Pak); B, Step 1: ACN/TEAF, pH 6.5; C, Step 2: ACN/TEAP, pH 2.5; D, Step 3: ACN/TFA, pH 2.0; E, Step 4: ACN/TFA, pH 2.0. Immunoreactive fractions are indicated by bars. Different bar graph patterns indicate separate HPLC purification of the fractions. Graphs C, D, and E are composites of three HPLC runs. Final step with microbore HPLC is not shown. Dotted lines indicate ACN concentration of the mobile phase.

terminus. Subsequent sequencing was carried out on the remaining material after digestion with pyroglutamate aminopeptidase and microbore HPLC purification as detailed earlier (35). This was followed by RP-HPLC separation and sequence analysis by automated Edman degradation on a PE-ABI Procise 494 protein sequencer.

Synthesis of synthetic GnRH peptides

The peptides for (pj)GnRH, sGnRH, and cGnRH-II were synthesized using a solid phase method on a methylbenzhydrylamine resin as described (36). Boc strategy was used with the following protecting groups: pyro-Glu(carbobenzyloxy), Boc-His(tosyl), Boc-Ser(benzyl) and Boc-Tyr(2-bromocarbobenzyloxy). GnRHs were deprotected and cleaved from the solid support with hydrofluoric acid. After purification with reverse-phase HPLC in two solvent systems (37), the structure was confirmed by mass spectral and amino acid composition analyses.

Bioactivity of GnRH peptides

Synthetic peptides for pjGnRH, sGnRH, and cGnRH-II were tested for release of pituitary hormones in primary cultures of dispersed goldfish pituitary cells using 2-h static incubations. Goldfish pituitaries were used because many GnRH forms release goldfish pituitary hormones (3, 16) and assays for pituitary hormones are not available for pejerrey. Pituitary cells from both male and female goldfish were prepared by trypsinization as previously described (38). Cells were cultured over-

night at a density of 0.25×10^6 cells per ml per well in 24-well culture plates at 28 C under 5% CO₂ and saturated humidity. Culture medium 199 with Earl's salts was supplemented with 25 mM HEPES, 2.2 g/l NaHCO₃, 1% horse serum, 100,000 U/liter penicillin, and 100 ng/liter streptomycin, pH adjusted to 7.2 with NaOH (Life Technologies, Inc., Grand Island, NY). Before the experiment, cells were washed with 1 ml testing medium (medium 199 containing Hanks' salts, 0.1% BSA, 2.2 g/liter NaHCO₃, 100,000 U/liter penicillin and 100 mg/liter streptomycin, pH 7.2) and allowed to rest in the incubator for at least an hour. The cells were then washed again with 1 ml fresh testing medium and concentrated GnRH solutions added (1 μ l per ml) in distilled deionized water to achieve the final desired concentration. All treatments were done in triplicate or quadruplicate. Cells were then returned to the incubator for the duration of secretion testing (2 h). At the end of the testing period, medium (800 μ l) was collected from each well. All experiments were repeated two times with cells prepared from sexually regressed and recrudescence stages. All samples were stored at -20 C until gonadotropin-II (GtH-II) contents were quantified by RIA validated for measurements of goldfish gonadotropin-II (LH-like) (39, 40) and GH (41). The GtH-II assay does not cross-react with mammalian gonadotropins or with salmonid or carp GtH-II; the GH assay does not cross-react with goldfish PRL or with other GH or PRL preparations (39-41). The sensitivity of the assays as estimated by 90% B/Bo values was lower than 0.16 ng/ml for GtH-II and 2.5 ng/ml for GH. Results are normalized in that they are expressed as a percentage of basal release

(unstimulated controls) and as pooled data (mean \pm SEM). Statistical analyses of hormone responses were performed using ANOVA followed by Dunnett's posthoc test. Differences were considered significant at $P < 0.05$.

Results

Initial analysis

To determine the elution position of cGnRH-II and sGnRH synthetic standards, these peptides were chromatographed in a different column from that used for purification in RP-HPLC. The elution position of the standards was tested by RIA; cGnRH-II eluted in fraction 26 and sGnRH in fractions 48–49.

Screening of GnRH from brain extract using Sep-Pak cartridges revealed one extended ir-GnRH area between fractions 10–45 (Fig. 2A). These fractions were pooled, concentrated and used for subsequent steps of purification.

GnRH purification

The first RP-HPLC purification step revealed three main ir-GnRH peaks (Fig. 2B). The first ir-GnRH peak (peak I, fractions 25–29) eluted as observed for the synthetic cGnRH-II standard. The second ir-GnRH peak (II, fractions 31–35) did not coelute with any of the known GnRH forms. The third ir-GnRH peak (III, fractions 48–52) eluted in the same position as synthetic sGnRH. All immunoreactive fractions were pooled for individual peaks and used for subsequent steps of purification.

In the second RP-HPLC purification step (TEAP step), a single ir-GnRH peak eluted from each peak of immunoreactive material applied from the previous run (Fig. 2C). Peak I eluted in fractions 21–25 (as did the cGnRH-II standard); peak II eluted in fractions 28–31 and peak III eluted in fractions 38–41 (as did the sGnRH standard). Then the ir-GnRH fractions from the second HPLC step of purification were concentrated in a vacuum centrifuge and reinjected into a C-18 column for the third step of the purification protocol (TFA step). Three different immunoreactive fractions corresponding to peaks I, II, and III eluted between fractions 64–68, 41–44, and 48–51 (Fig. 2D).

Each peak was concentrated and subjected to the last step of purification (Fig. 2E). The three ir-GnRH peaks eluting from the diphenyl column in positions 29–30, 31–32, and 35–36, were used for sequence analysis.

Sequence and mass spectral analysis

The retention of the second ir-GnRH fraction on RP-HPLC was different from that of any known form of GnRH. Initial attempts to analyze the peptide by chemical sequence analysis failed due to a blocked N terminus. When the peptide was reanalyzed after treatment with pyroglutamyl aminopeptidase, the following sequence was obtained: His-Trp-Ser-Phe-Gly-Leu-Ser-Pro-Gly. The intact molecule mass of the purified peptide as determined by MALD mass spectrometry was 1097.8 (M+H⁺) (Fig. 3), which is in good agreement with the theoretical mass of 1097.52 kDa for the sequence of pGlu-His-Trp-Ser-Phe-Gly-Leu-Ser-Pro-Gly-NH₂. The isolated native peptide was found to elute at the same percentage of ACN as the identical synthetic peptide.

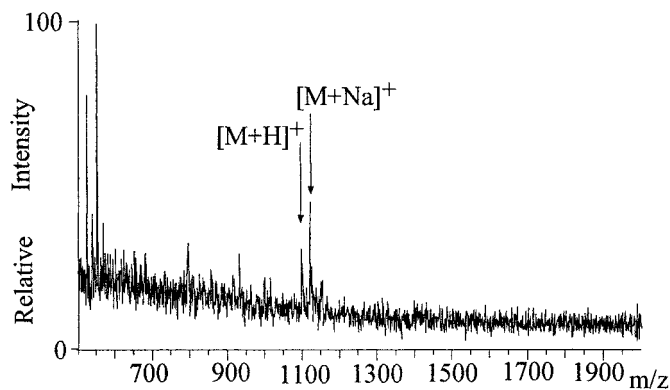


FIG. 3. MALD Mass Spectrum of purified immunoreactive pjGnRH from pejerrey brain.

The purified ir-GnRH fraction I was identified as cGnRH-II by protein sequencing and mass spectrometry. The amino acid sequence of the pyroglutamyl aminopeptidase-treated GnRH peak was determined to be His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly. The monoisotopic molecule mass measured by electrospray was 1236.6 (data not shown), comparable to the cGnRH-II theoretical mass of 1236.53 kDa.

The ir-GnRH peak III showed the same immunological characteristics and retention time as sGnRH in all RP-HPLC systems used. However, the amount was not enough to determine the sequence.

Bioactivity of peptides: GtH-II and GH release

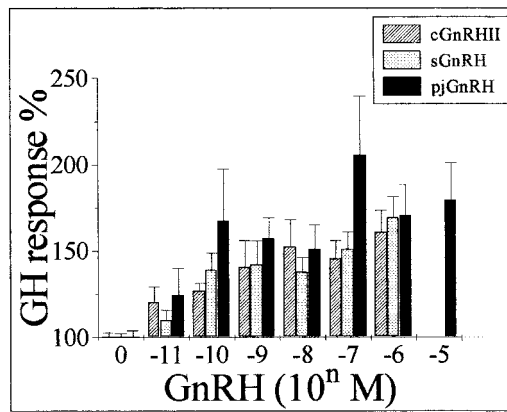
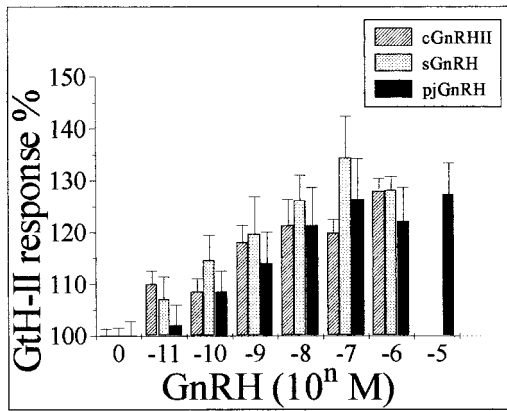
The release of GtH-II and GH from goldfish pituitary cells after the addition of synthetic forms of pjGnRH, cGnRH-II or sGnRH is shown in Fig. 4. Similar to cGnRH-II and sGnRH, pjGnRH released GtH-II and GH from dispersed goldfish pituitary cells *in vitro*. The lowest dose at which GnRH resulted in a significant release of GtH-II compared with the control was 10^{-11} M for cGnRH-II and 10^{-10} M for sGnRH and pjGnRH. The maximal GtH-II response was not different when the three GnRHs were compared.

cGnRH-II and sGnRH induced release of GH to the medium as in previous studies (3, 38). Also, pjGnRH induced GH release. The minimal effective dose for GH release was 10^{-10} M for all three GnRHs. The maximal response for pjGnRH was at 10^{-7} M.

Discussion

The present results prove that there are three forms of GnRH in the pejerrey brain and that one form is a novel GnRH. The primary structure of the novel form was determined to be: pGlu-His-Trp-Ser-Phe-Gly-Leu-Ser-Pro-Gly-NH₂. Previous indirect evidence based on chromatographic and immunological data had suggested that three forms might be present (6). The novel form in the present paper is termed pejerrey (pj)GnRH in keeping with the tradition that GnRH peptides are named for the species in which they are first described. Analysis of the primary structure for pjGnRH, especially the modified terminal residues, show that pjGnRH is a member of the GnRH family (Fig. 5), but is the first GnRH form to have Phe in position 5.

Proof of the structural identity of the pjGnRH amidated



	cGnRH-II	sGnRH	pjGnRH
GtH-II	0-10-11-9-7-8-6	0-11-10-9-8-6-7	0-11-10-9-8-6-7-5
GH	0-11-10-9-7-8-6	0-11-8-10-9-7-6	0-11-8-9-10-6-5-7

FIG. 4. Comparison of cGnRH-II, pjGnRH, and sGnRH to stimulate the release of (A) gonadotropin-II (GtH-II) and (B) GH from dispersed goldfish pituitary cells. GtH-II and GH secretions are expressed as a percentage of the basal mean \pm SEM. For GnRH peptides, doses resulting in responses that are not different from one another are identified by the same underscore (by ANOVA followed by Dunnett's posthoc test with significant differences at $P < 0.05$).

decapeptide included: Edman degradation to determine the sequence of the GnRH fragment (2–10), specific cleavage with pyroglutamyl aminopeptidase to identify the NH₂-terminal pyroglutamyl acid and mass spectrometry to determine the intact molecule mass. The primary structure for cGnRH-II isolated from pejerrey brain was established with the same techniques; sGnRH was identified by chromatographical and immunological methods as its elution position is distinct and insufficient material was available.

Teleost fishes are known to lack a functional hypothalamo-hypophysial portal system and to have pituitary cells that are directly innervated by GnRH nerve terminals (42). In a previous paper (6), we showed the presence of only one ir-GnRH peak (having pjGnRH characteristics) in pejerrey pituitary extracts. This fact strengthens the idea that pjGnRH has a hypophysiotropic role. In the present study, all three forms

	1	2	3	4	5	6	7	8	9	10
mGnRH	p-Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH ₂
gpGnRH	p-Glu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH ₂
sbGnRH	p-Glu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH ₂
pjGnRH	p-Glu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly-NH ₂
hrGnRH	p-Glu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly-NH ₂
cfGnRH	p-Glu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH ₂
cGnRH-I	p-Glu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH ₂
sGnRH	p-Glu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH ₂
cGnRH-II	p-Glu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH ₂
dfGnRH	p-Glu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH ₂
lGnRH-III	p-Glu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH ₂
IGnRH-I	p-Glu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH ₂
tGnRH-I	p-Glu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH ₂
tGnRH-II	p-Glu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH ₂

FIG. 5. Comparison of the known GnRH structures with mGnRH. Boxed residues indicate amino acids changes.

of GnRH were shown to release both GtH-II and GH from fish pituitary cells in culture. The purpose of the assay was to establish that the novel pjGnRH form is biologically active and to determine its relative potency. For release of GtH-II, the ED₅₀ estimate of potency for cGnRH-II is shifted one order of magnitude to the left relative to that for sGnRH and pjGnRH. For GH secretion, the ED₅₀ estimates for the three GnRHs did not differ greatly (less than 0.3 orders of magnitude difference between the three). The goldfish assay system has become the standard one to test fish GnRH bioactivity because so few teleost assays (and none for pejerrey) exist for pituitary hormones.

It is important to consider the effects of negative controls in the goldfish assay system because all three pejerrey GnRHs released both GtH-II and GH. Previous results indicate that the goldfish GtH-II and GH cell secretion assay to various neuroendocrine factors is selective and specific. For example, a related naturally occurring GnRH peptide (seabream GnRH) did not stimulate GtH-II release but was effective in stimulating GH secretion (3). Similarly, GnRH analogs that differentially inhibit the ability of sGnRH to stimulate GtH-II and GH secretion from the goldfish pituitary have been identified (43). In addition, dopamine stimulates GH secretion, but inhibits GtH-II release, whereas somatostatin inhibits GH, but not GtH-II secretions (44). Taken together, these and other data (including the demonstrated presence of GnRH receptors on goldfish gonadotropes and somatotopes (45), strongly suggest that the ability of pjGnRH to elevate GtH-II and GH secretion from goldfish pituitary cells in this study is due to specific effects on the pituitary cells.

In pejerrey fish, ir-GnRH fibers reach all three areas of the adenohypophysis: rostral pars distalis, proximal pars distalis, and pars intermedia as revealed by immunocytochemistry (46). The specific form of GnRH in these fibers could not be determined by immunocytochemistry, but these fibers were in close association with cells expressing PRL, GtHs, GH, and somatolactin (SL). In addition, the presence of GnRH receptors was reported in cells expressing GtH, GH, PRL, and SL using a combination of autoradiography and immunohistochemistry on dispersed pejerrey pituitary cells (47). These data suggest that GnRH may play a broad role in regulating pituitary cell function in this as in other teleost fishes (30).

In considering evolutionary changes in the structure of the GnRH molecule, there is a general agreement that two genomic duplications occurred between protochordates and the ancestral bony fish. A third duplication may have occurred early in teleost evolution (48). One of these ancestral forms became the highly conserved cGnRH-II, whereas other forms gave rise to further GnRH peptides (1, 49). In this context, knowledge of the primary structure of pjGnRH, allows us to modify the scheme for the evolution of GnRH (1, 3) within teleost fish (Fig. 6). This scheme takes into consideration the decapeptide sequence, the distribution of the GnRH forms among the bony fishes, the distinct location of different GnRH forms in the brain (50), and the minimum number of amino acids changes.

The origin of pjGnRH could be due to nucleotide substitutions in either hrGnRH or sbGnRH because both forms of GnRH: 1) evolved before pjGnRH; 2) are a third form in the brain; and 3) have the same location and function in the brain. Both herring and pejerrey express cGnRH-II, sGnRH and a third form that is the dominant form in the pituitary. The data suggest that hrGnRH and pjGnRH are the GnRH peptides expressed by preoptic neurons that send axons to the pituitary gland (3, 6). However, the origin of hrGnRH is not yet clear. If hrGnRH is the first of the third forms of GnRH, then hrGnRH could have evolved by a gene duplication from either mGnRH (2 amino acid differences) or sGnRH (3 amino acid substitutions). In this context, both mGnRH and sGnRH were present in early teleosts (bonytongue fish and eels) that evolved before the herring, although these two peptides never appear together in the same species. The origin of the third form may be tied to the genome duplication that is thought to have occurred in early teleosts (48). RP-HPLC and RIA evidence suggest that the third form of GnRH was present already in some species of bonytongue fish (22), but direct proof is needed.

The other possible origin of pjGnRH is from the GnRH molecule that was first identified in sea bream (sbGnRH), but is present also in fish that evolved long before sea bream and pejerrey, *i.e.* pacú (29) and sábalo (27) (order Characiformes). The pjGnRH differs in only one amino acid from sbGnRH (position 5), but the distribution of sbGnRH in different fish species appears to be much broader than that of pjGnRH in studies to date. The distribution of sbGnRH in teleosts has been determined by several methods. Sequence determination was used to identify sbGnRH not only in pacú (29), but also in three perciform species, sea bream (17, 28), tilapia (30), and the African cichlid, *Haplochromis burtoni* (31, 50). Also, indirect chromatographic and immunological data give evidence that sbGnRH is the third molecular form in addition to cGnRH-II and sGnRH in other fish in the orders of Perciformes (24, 25, 26, 51), Pleuronectiformes (52), Characiformes (27), and Gonorynchiformes (25). In all cases, although not sequenced, this GnRH form followed a similar chromatographic pattern to that of sbGnRH, suggesting a wide distribution of this GnRH variant in teleost fishes. These distribution data imply a nucleotide substitution occurred only in the stem line leading to the order (Atheriniformes) in which pejerrey is found.

Although the neuroanatomical distribution of pjGnRH has not been clearly demonstrated as yet, it can be considered the releaser of pituitary hormones because it is the only GnRH form found in pituitary extracts of pejerrey (6). Our evidence shows that the newly described pjGnRH form is present in pejerrey brain together with cGnRH-II and sGnRH. pjGnRH is likely to have emerged from sbGnRH through a substitution in position 5 (Phe instead of Tyr) or less likely from herring GnRH (Phe instead of His in position 5). The phylogenetic position of pejerrey in an order of teleosts thought to have evolved between orders of fish that have sbGnRH,

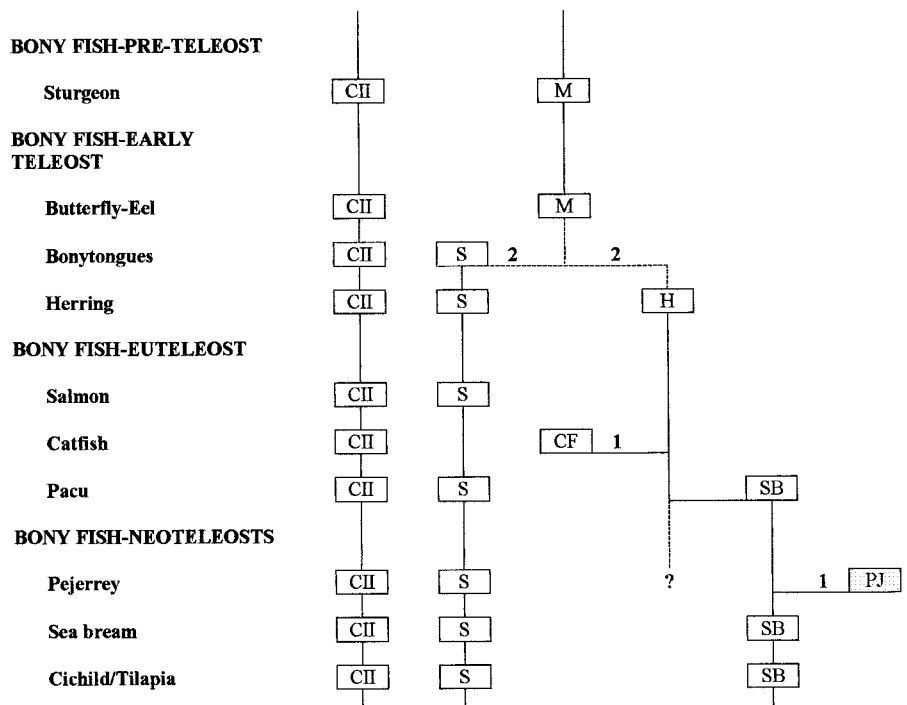


FIG. 6. Proposed evolutionary scheme showing GnRH multiplicity in teleost fish. All GnRH forms were identified by primary structure. Dotted lines indicate possible changes or represent unknown data. CII, cGnRH-II; M, mGnRH; S, sGnRH; SB, sbGnRH; CF, cfGnRH; H, hrGnRH; PJ, pjGnRH. See text for references.

adds weight to the argument that pjGnRH was due to a substitution in the sbGnRH gene.

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Note Added in Proof

It has come to our attention that a cDNA isolated from medaka encodes a form of GnRH with deduced amino acids identical to the pejerrey form reported here as a peptide. Medaka and pejerrey are in different orders of fish. The reference is Okubo K, Amano M, Yoshiura Y, Suetake H, Aida K 2000 A novel form of gonadotropin-releasing hormone in the medaka, *Oryzias latipes*. *Biochem Biophys Res Commun* 276:298–303.

References

- Sherwood NM, von Schalburg K, Lescheid DW 1997 Origin and evolution of GnRH in vertebrates and invertebrates. In: Parhar IS, Sakuma Y (eds) *GnRH Neurons. Gene to Behavior*, Brain Shuppan, Tokyo, pp 3–25
- Jimenez-Liñan M, Rubin B, King JC 1997 Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. *Endocrinology* 138:4123–4137
- Carolsfeld J, Powell JFF, Park M, Fischer WH, Craig AG, Chang J, Rivier JE, Sherwood NM 2000 A novel form of gonadotropin-releasing hormone (GnRH) in herring sheds light on evolutionary pressures. *Endocrinology* 141:505–512
- Montaner AD, Affanni JM, King JA, Bianchini JJ, Tonarrelli G, Somoza GM 1999 Differential distribution of gonadotropin-releasing hormone variants in the brain of *Hydrochaeris hydrochaeris* (Mammalia, Rodentia). *Cell Mol Neurobiol* 19:635–651
- Montaner AD, Gonzalez O, Paz DA, Affanni JM, Somoza GM 2000 Gonadotropin-releasing hormone (GnRH) variants in a lizard brain: is mammalian GnRH being expressed? *Gen Comp Endocrinol* 119:121–131
- Stefano AV, Canosa LF, D'Eramo JL, Fridman O, Affanni JM, Somoza GM 1997 GnRH molecular variants in the brain and pituitary gland of pejerrey, *Odontesthes bonariensis* (Atheriniformes). Chromatographic and immunological evidence for the presence of a novel molecular variant. *Comp Biochem Physiol* 118C:335–345
- Matsuo H, Baba Y, Nair RMG, Arimura A, Schally AV 1971 Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem Biophys Res Commun* 43:1334–1339
- Burgus R, Butcher M, Amoss M, Ling N, Monahan M, Rivier J, Fellows R, Blackwell R, Vale W, Guillemin R 1972 Primary structure of ovine luteinizing hormone-releasing factor (LRF). *Proc Natl Acad Sci USA* 69:278–282
- King JA, Millar RP 1982 Structure of chicken hypothalamic luteinizing hormone-releasing hormone I. Structural determination on partially purified material. *J Biol Chem* 257:10772–10772
- Miyamoto K, Hasegawa Y, Igarashi M, Chino N, Sakakibara S, Kangawa K, Matsuo H 1983 Evidence that chicken hypothalamic luteinizing hormone-releasing hormone is [Gln⁸]LHRH. *Life Sci* 32:1341–1347
- 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* 81:3874–3878
- Sherwood N, Eiden L, Brownstein M, Spiess J, Rivier J, Vale W 1983 Characterization of a teleost gonadotropin-releasing hormone. *Proc Natl Acad Sci USA* 80:2794–2798
- Sherwood NM, Sower SA, Marshak DR, Fraser BA, Brownstein MJ 1986 Primary structure of gonadotropin-releasing hormone from lamprey brain. *J Biol Chem* 261:4812–4819
- Sower SA, Chiang YA, Lova S, Conlon JM 1993 Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* 132:1125–1131
- Ngamvongchon S, Lovejoy DA, Fischer WH, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM 1992 Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. *Mol Cell Neurosci* 3:17–22
- Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM 1992 Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc Natl Acad Sci USA* 89:6373–6377
- Powell JFF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA, Sherwood NM 1994 Three forms of gonadotropin-releasing hormone characterized from brains of one species. *Proc Natl Acad Sci USA* 91:12081–12085
- Powell JFF, Reska-Skinner SM, Om Prakash M, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM 1996 Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc Natl Acad Sci USA* 93:10461–10464
- Joss JMP, King JA, Millar RP 1994 Identification of the molecular forms and steroid hormone response to gonadotropin releasing hormone in the Australian lung fish *Neoceratodus forsteri*. *Gen Comp Endocrinol* 96:392–400
- Lescheid DW, Powell JFF, Fischer WH, Park M, Craig A, Bukovskaya O, Barannikova IA, Sherwood NM 1995 Mammalian gonadotropin-releasing hormone (GnRH) identified by primary structure in Russian sturgeon. *Acipenser gueldenstaedti*. *Regul Pept* 55:299–309
- Sherwood N, Doroshov S, Lance V 1991 Gonadotropin-releasing hormone in bony fish that are phylogenetically ancient: reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). *Gen Comp Endocrinol* 84:44–57
- O'Neill DF, Powell JFF, Standen EM, Youson JH, Warby CM, Sherwood NM 1998 Gonadotropin-releasing hormone (GnRH) in ancient teleosts, the bony-tongue fishes: putative origin of salmon GnRH. *Gen Comp Endocrinol* 112:415–425
- King JA, Dufour S, Fontaine Y, Millar RP 1990 Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* 11:507–514
- King JA, Millar RP 1985 Multiple molecular forms of gonadotropin-releasing hormone in teleost fish brain. *Peptides* 6:689–694
- Sherwood N, Harvey B, Brownstein M, Eiden L 1984 Gonadotropin-releasing hormone (GnRH) in striped mullet (*Mugil cephalus*), milkfish (*Chanos chanos*) and rainbow trout (*Salmo gairdneri*): comparison with salmon GnRH. *Gen Comp Endocrinol* 55:174–181
- Sherwood N, Grier H, Warby C, Peute J, Taylor R 1993 Gonadotropin-releasing hormones, including a novel form, in snook *Centropomus undecimalis*, in comparison with forms in black sea bass *Centropristis striata*. *Regul Pept* 46:523–534
- Somoza GM, Stefano AV, D'Eramo JL, Canosa LF, Fridman O 1994 Immunoreactive GnRH suggesting a third variant form of GnRH in addition to cIIIGnRH and sGnRH in the brain and pituitary gland of *Prochilodus lineatus* (Characiformes). *Gen Comp Endocrinol* 94:44–52
- Gothilf Y, Muñoz-Cueto JA, Sagrillo C, Selmanoff M, Chen TT, Kah O, Elizur A, Zohar Y 1996 Three forms of gonadotropin-releasing hormone in a teleost fish: cDNA characterization and brain localization. *Biol Reprod* 55:636–645
- Powell JFF, Standen EM, Carolsfeld J, Borella M, Gazola R, Fischer WH, Park M, Craig AG, Warby CM, Rivier JE, Val-Sella M, Sherwood NM 1997 Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain. *Regul Pept* 68:189–195
- Powell JFF, Fischer WH, Park M, Craig AG, Rivier JE, White SA, Francis RCF, Fernald RD, Licht P, Warby C, Sherwood NM 1995 Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pituitary; three forms of GnRH in brain of cichlid pituitary and pumpkinseed fish. *Regul Pept* 57:43–53
- Weber G, Powell J, Park M, Fischer W, Craig A, Rivier E, Nanakorn U, Parhar I, Ngamvongchon E, Grau E, Sherwood N 1997 Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. *J Endocrinol* 155:121–132
- Strüssmann CA, Takashima F, Toda K 1996 Sex differentiation and hormonal feminization of pejerrey *Odontesthes bonariensis*. *Aquaculture* 139:31–45
- Lescheid DW, Terasawa E, Abler LA, Urbanski HF, Warby CM, Millar RP, Sherwood NM 1997 A second form of gonadotropin-releasing hormone (GnRH) with characteristics of cGnRH-II is present in the primate brain. *Endocrinology* 138:5618–5629
- Montaner AD, Somoza GM, King J, Bianchini J, Bolis C, Affanni J 1998 Chromatographic and immunological identification of GnRH (gonadotropin-releasing hormone) variants. Occurrence of mammalian and a salmon-like GnRH in the forebrain of an eutherian mammal: *Hydrochaeris hydrochaeris* (Mammalia, Rodentia). *Regul Pept* 73:197–204
- Fischer WH, Park M 1992 Sequence analysis of pyroglutamyl peptides: microscale removal of pyroglutamate residues. *J Protein Chem* 11:366
- Rivier JE, Porter J, Hoeger C, Thoebald P, Craig AG, Dykert J, Corrigan A, Perrin M, Hook WA, Siraganian RP, Vale W, Rivier C 1992 Gonadotropin releasing hormone antagonists with N-omega-triazolnitrine, -lysine, or-p-aminophenylalanine residues at position 5 and 6. *J Med Chem* 35:4270–4278
- Rivier J, McClintock R, Galyean R, Anderson H 1984 Reversed-phase high-performance liquid chromatography: preparative purification of synthetic peptides. *J Chromatogr* 288:303–328
- Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990 Use of a pituitary cell dispersion method and primary culture system

- for the studies of gonadotropin-releasing hormone action in goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perfusion studies. *Gen Comp Endocrinol* 77:256–273
39. Peter RE, Nahorniak CS, Chang JP, Crim LW 1984 Gonadotropin release from the pars distalis of goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricle, additional evidence for gonadotropin-release inhibiting factor. *Gen Comp Endocrinol* 55:337–346
 40. Van der Kraak G, Suzuki K, Peter RE, Itoh H, Kawauchi H 1992 Properties of common carp gonadotropin I and gonadotropin II. *Gen Comp Endocrinol* 85:217–229
 41. Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus* L.) pituitary fragments *in vitro*. *Regul Pept* 17:41–52
 42. Peter RE, Yu KL, Marchant TA, Rosenblum P 1990 Direct neural regulation of the teleost adenohypophysis. *J Exp Zool* 4:84–89
 43. Murthy CK, Nahorniak CS, Rivier JE, Peter RE 1993 *In vitro* characterization of gonadotropin-releasing hormone antagonists in goldfish, *Carassius auratus*. *Endocrinology* 133:1633–1644
 44. Wong AOL, Chang JP, Peter RE 1993 *In vitro* and *in vivo* evidence that dopamine exerts growth hormone releasing activity in the goldfish, *Carassius auratus*. *Amer J Physiol* 264:E925–E932
 45. Cook H, Berkenbosch JW, Fernhout MJ, Yu KL, Peter RE, Chang JP, Rivier JE 1991 Demonstration of gonadotropin releasing-hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. *Regul Pept* 36:369–378
 46. Vissio PG, Stefano AV, Somoza GM, Maggese MC, Paz DA 1999 Close association among GnRH (gonadotropin-releasing hormone) fibers and GtH, GH, SL, and PRL expressing cells in pejerrey, *Odontesthes bonariensis* (Teleostei, Atheriniformes). *Fish Physiol Biochem* 21:121–127
 47. Stefano AV, Vissio PG, Paz DA, Somoza GM, Maggese MC, Barrantes GE 1999 Colocalization of GnRH binding sites with gonadotropins, somatotropin, somatolactin and prolactin expressing cells of the pejerrey, *Odontesthes bonariensis*, *in vitro*. *Gen Comp Endocrinol* 116:133–139
 48. Amores A, Force A, Yan Y-L, Joly L, Ameniya C, Frits A, Ho RK, Langeland J, Prince V, Westerfield M, Ekker M, Postlethwait JH 1998 Zebrafish hox clusters and vertebrate genome evolution. *Science* 282:1711–1714
 49. King JA, Millar RP 1997 Coordinated evolution of GnRHs and their receptors. In: Parhar IS, Sakuma Y (eds) *GnRH Neurons. Gene to Behavior*, Brain Shuppan, Tokyo, pp 51–78
 50. White SA, Kasten TL, Bond CT, Adelman JP, Fernald RD 1995 Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. *Proc Natl Acad Sci USA* 92:8363–8367
 51. Miranda LA, Montaner AD, Ansaldo M, Affanni JM, Somoza GM 1998 Characterization of brain gonadotropin-releasing hormone (GnRH) molecular variants in brain extracts from different perciform fishes from Antarctic waters. *Polar Biol* 21:122–127
 52. Idler DR, Everard BA 1987 Mammalian, salmon and chicken-like LHRH's from hypothalami of winter flounder (*Pseudopleuronectes americanus*) as evidenced by chromatographic mobility and immunoreactivity. In: Idler DR, Crim LW, Walsh JM (eds) *Proceedings of the Third International Symposium on the Reproductive Physiology of Fish*. Marine Science Research Laboratory, Memorial University of Newfoundland, Canada, p 30