ELSEVIER

Regulatory Peptides 68 (1997) 189–195

REGULATORY PEPTIDES

Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain

James F.F. Powell[a,e], Emily M. Standen[a], Joachim Carolsfeld[a], Maria I. Borella[b], Ruth Gazola[b], Wolfgang H. Fischer[c], Minkyu Park[c], A. Grey Craig[c], Carol M. Warby[a], Jean E. Rivier[c], Maura V. Val-Sella[d], Nancy M. Sherwood[a,*]

[a]*Department of Biology, University of Victoria, Victoria, B.C., Canada V8W 2Y2*
[b]*Departamento da Histologia e Embriologia, Instituto de Ciências Biomedicas, Universidade de Saõ Paulo, 05.0880, Saõ Paulo, Brazil*
[c]*The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA, 92037, USA*
[d]*Departamento da Fisiologia Geral, Universidade de Saõ Paulo, 05.0880, Saõ Paulo, Brazil*
[e]*Current Address: Aquametrix Research 204-2527 Beacon Ave., Sidney, B.C., Canada V8L 1Y1*

Received 20 September 1996; revised 2 December 1996; accepted 3 December 1996

Abstract

Perchlike fish are a vast group of advanced teleosts. The species examined to date have three forms of gonadotropin-releasing hormone (GnRH) within a single species, but the origin of the third GnRH peptide is unknown. In this study, the primary structure of three GnRH peptides is determined from the brain of the pacu, *Piaractus mesopotamicus*, an example of a teleost that is less advanced than the perchlike fish. The GnRH was purified from pacu brain extracts using high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The three forms identified by chemical sequencing and mass spectrometry are sea bream GnRH (pGlu–His–Trp–Ser–Tyr–Gly–Leu–Ser–Pro–Gly–NH_2, 1113.4 Da); chicken GnRH-II (pGlu–His–Trp–Ser–His–Gly–Trp–Tyr–Pro–Gly–NH_2, 1236.6 Da); and salmon GnRH (pGlu–His–Trp–Ser–Tyr–Gly–Trp–Leu–Pro–Gly–NH_2, 1212.3 Da). In addition, the number of forms of GnRH in the brains of male and female fish was determined separately. The same three forms of GnRH were present in the brains of both sexes as determined by antisera cross-reactivity and elution position from the HPLC column. The results indicate that the pacu brain has the identical forms of GnRH identified in perchlike fish and hence, the origin of three forms occurred earlier in evolution than previously thought.

Keywords: Neuropeptide; Reproduction; Brain; Pituitary; Teleost

1. Introduction

It is commonly accepted that there are at least two forms of gonadotropin-releasing hormone (GnRH) in the brains of teleost fishes [see [1]]. One form of GnRH known as chicken GnRH-II (cGnRH-II) is present in the brains of all teleost species studied to date [see [2]]. In addition, a second form of GnRH is present, but the structure varies depending on the species: many teleosts have salmon GnRH (sGnRH), but three species of catfish have catfish GnRH (cfGnRH) [3–5], and eels, *Anguilla anguilla* [6] have mammalian GnRH (mGnRH). Together, these data are strong evidence that cGnRH-II and at least one other form of GnRH are present in the teleost brain.

Additionally, in the brains of the most advanced fish, the perchlike fish, three forms of GnRH have been identified by primary structure [7,8], cDNA coding [9,10] or antisera cross-reactivity coupled with elution position from high-

*Corresponding author.

0167-0115/97/$17.00
PII S0167-0115(96)02119-2

performance liquid chromatography (HPLC) [11,12]. These forms have been identified as cGnRH-II, sGnRH and a novel form called sea bream GnRH (sbGnRH).

The three forms of GnRH in the brain, at least in the cichlid *Haplochromis burtoni,* have distinct locations [10] and hence, may have different functions. Chicken GnRH-II is in the midbrain of *H. burtoni* and all bony fish tested with specific antisera [13–17] and in situ hybridization [10,17]. The use of both in situ hybridization, to show that the cGnRH-II cell bodies are exclusively in the midbrain [10], and retrograde labelling from the pituitary, to show that cell bodies in the midbrain are not labelled [18], suggests that cGnRH-II does not control the release of the gonadotropins from the pituitary, at least for the cichlid, *H. burtoni*. Additionally, analysis of pituitaries from the same cichlid shows that cGnRH-II is not detectable [8]. In contrast, sGnRH-producing neurons are located in the terminal nerve and sbGnRH is located in the preoptic nucleus [10] and the pituitary [8] of *H. burtoni.* Retrograde staining shows that the preoptic GnRH neurons innervate the pituitary and hence are thought to control pituitary secretions of gonadotropins [18].

In other groups of fish in which only two forms of GnRH have been detected, cGnRH-II-producing cell bodies are located in the midbrain, whereas the second form is located in both the terminal nerve region and the preoptic area of the brain. Examples of fish with this pattern of GnRH distribution are sturgeon, *Acipenser baeri* [14], salmon, *Oncorhynchus masou* [13] and catfish, *Clarias gariepinus*, [17].

The origin of the third form of GnRH is examined in the present study. An earlier study on sabalo, *Prochilodus lineatus*, [19] and preliminary work reported here for our study on pacu show that these fish, which are both in the same fish order of Characiformes, have three forms of GnRH. Characiform fishes are less advanced compared to perchlike fishes. Pacu branched from the basal teleost lineage at about the same time as primitive salmonids. However, the early history of the perchlike fishes and their relationship to basal teleosts is less clear [20]. Hence, pacu represents an interesting taxon for study of the origin of the third form of GnRH.

In the present experiments, GnRH is examined from the brain of the pacu, *Piaractus mesopotamicus*, a freshwater fish native to the Paranah River Basin in Brazil. Firstly, the elution position of immunoreactive GnRH (irGnRH) from HPLC eluates was used to determine the number of forms present. Female and male fish were investigated separately. Secondly, the three areas that were identified by HPLC to contain distinct forms of irGnRH were purified from a large number of pacu brains to obtain the amino acid sequence and molecule mass. The presence of three forms of GnRH in single species in two divergent fish groups has evolutionary implications for establishing the origin of the third form.

2. Materials and methods

2.1. Collection of brains

In the preliminary investigation, brains were collected and pooled from mature male ($n = 20$) and female ($n = 13$) pacu (*Piaractus mesopotamicus*) during the 1989 spawning season. Tissues were immediately placed on dry ice, stored frozen, then shipped on dry ice to the University of Victoria where they were placed in a -80°C freezer. In the large scale purification protocol in 1994, brains and pituitaries (total weight = 165 g) from mature male and female fish were removed, pooled and frozen. Frozen tissues were shipped as above.

2.2. Extraction of peptides

The same extraction method was used for both the preliminary investigation and purification protocol. Frozen brains wrapped in foil were pulverized with a mallet and then powdered in a Waring Blendor in the presence of liquid nitrogen. The powdered material was treated as described earlier [21]. Briefly, the material was added to 1 N HCl/acetone (3:100 v/v), stirred for 3 h and filtered (≠l Whatman) in a Buchner funnel. The residue was re-extracted with 0.01 N HCL/acetone (1:5 v/v). Acetone, lipids and other hydrophobic substances were removed from the combined filtrates by five successive extractions using petroleum ether (4:1, v/v, filtrate to petroleum ether). The final aqueous phase was reduced in volume in a vacuum centrifuge.

2.3. Preliminary investigation of irGnRH

Chromatography of pacu brain extracts from separate sexes was carried out with a Varian 5000 HPLC and detector. A Supelco C_{18} column (0.4×25 cm) was repeatedly washed overnight with increasing and decreasing gradients of solvents A and B (see below), then equilibrated to the initial conditions of 17% solution B (acetonitrile) and 83% solution A (0.25 M triethylammonium formate, TEAF, at pH 6.5) at a flow-rate of 1 ml/min. A blank run of 800 μl solution A was applied to the column and fractions collected for RIA. Extract from female brains was divided into three aliquots of 800 μl; each was applied through a 1 ml injection loop to the column at two minute intervals. After 10 min at initial conditions, an increasing gradient of 1% per minute solution B was used for 7 min, then held isocratically at 24% for 48 min. Fractions of 1 ml were collected for 65 min and dried aliquots (100 μl) were assayed for irGnRH. The column was again repeatedly washed, followed by a blank run. Extract from male brains was applied (4×800 μl) as for female brains. Immediately after the male brain extract was eluted from the HPLC column, the column was

equilibrated to the starting conditions. Five synthetic GnRH standards (lamprey GnRH-I, mammalian GnRH, sGnRH, chicken GnRH-I and cGnRH-II) were prepared as a mixture of 200 ng each in a total volume of 800 μl of water and applied to the column. Synthetic sea bream GnRH (sbGnRH) was not available to add to the mixture for this preliminary study, but the elution position was determined after we had identified sbGnRH in the latter part of the study. HPLC fractions for blanks, standards and extracts were assayed using antisera raised against GnRH.

2.4. Purification: Sep-Pak HPLC

A column of 10 Sep-Pak cartridges (C_{18}, Waters, Milford, CT) connected in series was primed with 100% methanol and washed with 6 ml Milli-Q water. The extract from 165 g of brains was diluted to one liter with Milli-Q water and pumped through the column using a peristaltic pump at a flow-rate of 1.5 ml/min. The cartridge column was connected to a Beckman 125 HPLC with initial conditions of 95% solution A (0.05% trifluoroacetic acid in water) and 5% solution B (80% acetonitrile diluted with 20% solution A) at a flow-rate of 1 ml/min. Then, an increasing gradient of 1% solution B per minute was applied to the cartridge column for 60 min. Fractions of l ml were collected for 60 min and 10 μl from each fraction was assayed for GnRH-like immunoreactivity.

2.5. Further purification of GnRH

Three successive HPLC programs were used after Sep-Pak HPLC. Table 1 describes the column type, solvents and ion-pairing agents; the latter includes TEAF, TFA and TEAP [22]. Aliquots of 10 μl were used to identify the fractions containing immunoreactive GnRH (irGnRH) for further purification.

2.6. Radioimmunoassay (RIA)

Aliquots from HPLC fractions were assayed for irGnRH by RIA as previously described [21]. Antiserum GF-4 (raised against sGnRH) was used in a final dilution of 1:50,000 resulting in 22–32% binding of ^{125}I-mGnRH. Antiserum BLA-4 (raised against lamprey GnRH-I) was used in a final dilution of 1:10,000 resulting in 9–17% binding of ^{125}I-mGnRH. Antiserum B-6 (1:5000 final dilution) was raised against mammalian GnRH (mGnRH) and resulted in binding of 52% with ^{125}I-mGnRH. Cross-reactivity studies show that antisera GF-4 and BLA-4 together recognize the 9 known vertebrate GnRHs: mGnRH, cGnRH-I, cGnRH-II, sGnRH, cfGnRH, sbGnRH, dogfish GnRH and lamprey GnRH-I and -III [[23] and unpublished observations]. In contrast, antiserum B-6, although a polyclonal antibody, is specific for mGnRH and does not cross-react with the other eight known forms of GnRH in vertebrates as listed above [[23] and unpublished observations]. Limits of detection for assays (B/Bo = 80%) averaged 10.4 pg for GF-4, 47.6 pg for Bla-4, and 9.4 pg for B-6. Fractions with high irGnRH (tracer binding, B/Bo, of 20% or less) were serially diluted 1:2 and reassayed. The value closest to 50% tracer binding is reported. Only antiserum GF-4 was used in the purification of irGnRH from pacu brains.

2.7. Characterization of primary structure

Firstly, sequence analysis was attempted on 10% of the purified sample. The lack of sequence data suggested that the peptide possessed a blocked N terminus. Secondly, 50% of the sample was dried and digested with calf liver pyroglutamyl aminopeptidase (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Aliquots containing approximately 250 ng of pacu GnRHs were concentrated in a vacuum centrifuge. Reaction buffer (10 μl) containing 100 mM TES (N-tris-(hydroxymethyl)methyl-2-aminoethane-

Table 1
Steps for the purification of pacu GnRH after Sep-Pak HPLC. Solvent and column types are listed for each successive step. Immunoreactive areas identified by radioimmunoassay were reduced in volume, combined and applied to the next step of purification

Step	Column Type	Solvent A	Solvent B	Program
1	C_{18}	0.25 M TEAF (triethylammonium formate)	Acetonitrile	0 min: 17% B 10–17 min: 17–24% B 17–65 min: 24% B
2	C_{18}	0.25 M TEAP (triethylammonium phosphate)	Acetonitrile	5 min: 5% B, 5–15 min: 5–20% B 15–55 min: 20–40% B
3	C_{18}	0.05% TFA in water (trifluoroacetic acid)	80% Acetonitrite 0.05% TFA in 20% water	0–5 min: 5% B, 5–60 min: 5–55% B
4	Phenyl	0.05% TFA in water	80% Acetonitrile 0.05% TFA in 20% water	0–5 min: 5% B, 5–15 min: 5–20% B 15–55 min: 20–40% B

sulphonic acid, (pH 8.0)), l0 mM EDTA, 5 M dithiothreitol, 5% glycerol and 40 μg of lyophylized pyroglutamyl aminopeptidase was added and the solutions incubated at 37°C for 30 min [24]. Thirdly, the digested GnRH was separated from the mixture using a Hewlett Packard HP 1090L HPLC fitted with a Vydac C_{18} microbore column. The initial solvent mixture passed through the column was 95% solution A (0.05% trifluoroacetic acid in water) and 5% solution B (90% acetonitrile diluted with 10% solution A) to which the digested peptides were added. The rate of increase of solution B was from 5% to 40% in 30 min. Fractions were collected as peaks of the chromatograph and saved for peptide sequencing. Finally, the digested peptide was sequenced using an Applied Biosystems Protein Sequencer (Model 470A) equipped with an on-line phenylthiohydantoin analyzer.

2.8. Mass spectrometry

A sample of the purified, undigested peptide was analyzed on a Bruker Reflex time-of-flight mass spectrometer with matrix assisted laser desorption. An accelerating voltage of 31 kV and a reflectron potential of 30 kV were employed. The sample was applied to a thin film of α-cyano-4-hydroxy cinamic acid, dried and rinsed with deionized water. An accelerating/electric field voltage scan from m/z 1100 to m/z 1500 was done. The mass accuracy of the measurement was $+/-500$ ppm.

3. Results

3.1. Preliminary investigation

Immunoreactive GnRH in female and male brain extracts was detected in HPLC fractions that eluted in three distinct areas (Fig. 1). These areas were identical with the elution positions of standards for chicken GnRH-I (later shown to be identical to the sbGnRH position), cGnRH-II and sGnRH. A small amount of irGnRH material eluted between the cGnRH-II and sGnRH standards, but did not correspond to the elution position of any of the synthetic standards.

The amount of irGnRH eluting in the first area, which corresponds to the elution position of cGnRH-I is shown in Fig. 1. Both antisera GF-4 and BLA-4 detected irGnRH in male and female eluates. sbGnRH was not available at the time of the present pacu investigation, but we found that sbGnRH elutes in the same position as cGnRH-I under similar conditions [8]. Antiserum B-6 did not detect irGnRH in any fractions assayed (Fig. 1).

For the second irGnRH area, which corresponds to the elution position of the cGnRH-II standard, GF-4 detected irGnRH in both the HPLC fractions eluting from female and male brain extracts. BLA-4, which has less than 1% cross-reactivity with cGnRH-II [23], barely detected irGnRH in either the female or male HPLC eluates.

The third area of irGnRH eluted from the HPLC column in the same position as sGnRH. Both female and male HPLC eluates had irGnRH that was detected by antisera GF-4 and BLA-4, but not by B-6 (Fig. 1). Finally, the area of irGnRH elution that did not correspond to known GnRH standards, was detected in small amounts by antisera GF-4 and BLA-4 in female and male pacu brain extract (Fig. 1).

3.2. Purification of pacu GnRH

Immunoreactive GnRH eluted from the Sep-Pak column in a broad area. The Sep-Pak HPLC fractions that contained irGnRH were pooled and further purified by the TEAF-HPLC method (Table 1). Four areas of irGnRH were detected in the same positions as shown in Fig. 1 for the preliminary investigation. The areas were labelled Pl, P2, P3 according to elution position and then each area was sequentially purified (Table 1). The area of irGnRH that eluted between the cGnRH-II and sGnRH standards was not purified in the present study because irGnRH eluting in the same position was identified as sGnRH by sequence identification in our earlier studies on tilapia (*Oreochromis niloticus*) and salmon (*Oncorhynchus keta*) [unpublished results]. It appears that a small amount of sGnRH changes elution position during the purification procedure without changing its primary sequence. At the end of step 4, 1190 ng of P1, 102 ng of P2 and 183 ng of P3 irGnRH were recovered from the phenyl HPLC column.

3.3. Sequence identification and mass spectral analysis

The initial aliquot of 10% of each purified form of irGnRH could not be sequenced with Edman degradation, indicating a blocked N terminus. However, pyroglutamyl aminopeptidase digestion resulted in a fragment that was sequenced. The deduced sequences are shown in Table 2 and include a pyroglutamyl residue in the first amino acid position along with the remaining sequence for the purified material isolated from the pacu brains. P1, P2 and P3 are identified as sbGnRH, cGnRH-II and sGnRH, respectively. The mass for each purified intact peptide is also shown.

4. Discussion

The three forms of GnRH in the brain of the pacu are sbGnRH, cGnRH-II and sGnRH as determined by HPLC elution position in comparison with synthetic standards, antisera cross-reactivity, primary sequence identification and mass spectral analysis. These three GnRH forms are identical to the forms that were isolated earlier from the brains of perchlike fish (sea bream [7], *H. burtoni* [8,10] and tilapia [unpublished observation] as shown in Fig. 2.

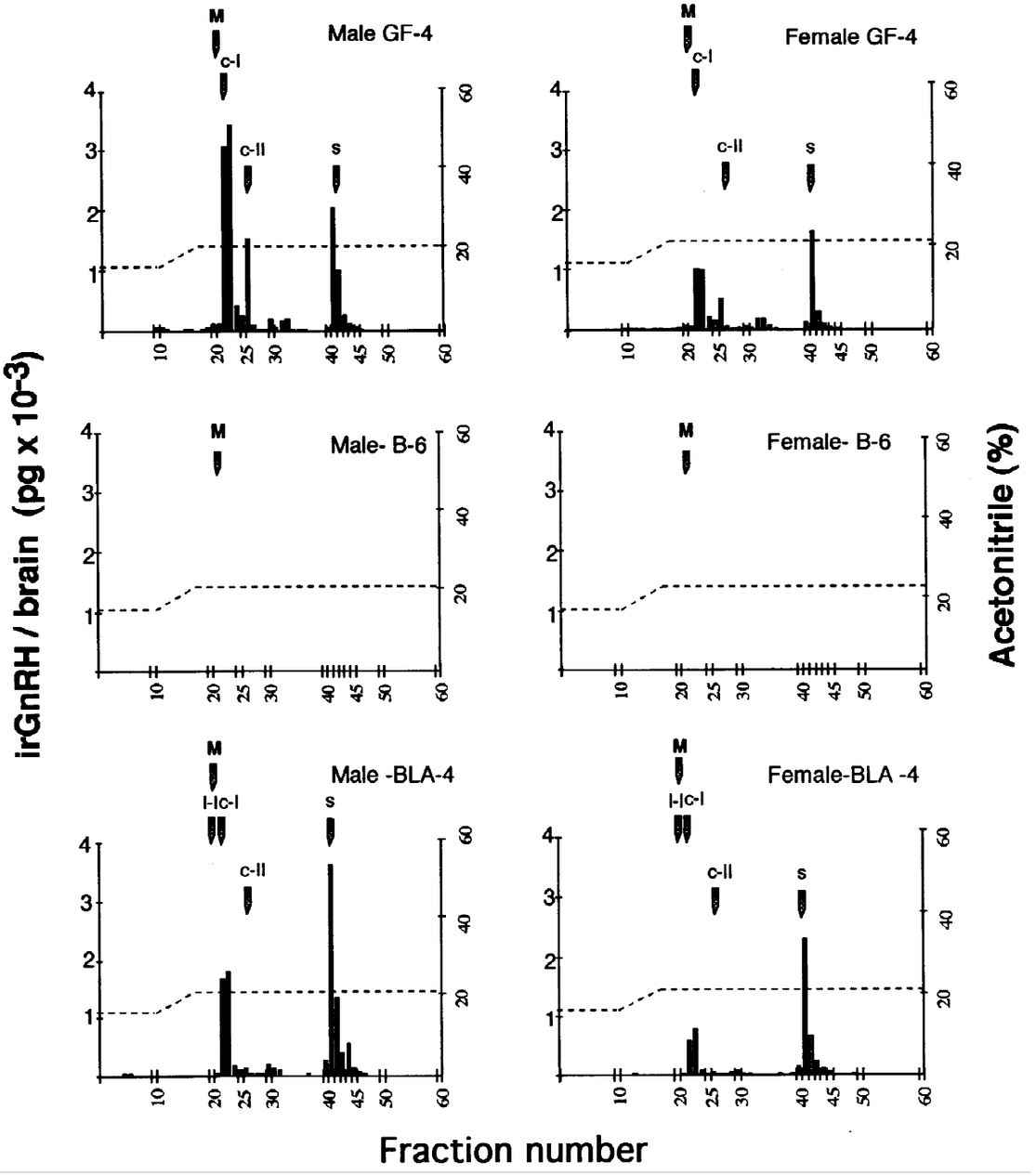

Fig. 1. Immunoreactive GnRH in extracts from male and female brains of pacu fish after elution from HPLC. HPLC fractions from extracts of male brains were tested with three antisera and are shown on the left side, whereas results from females are shown on the right. The elution position of synthetic GnRH peptides are shown at the top of each graph. The percent acetonitrile in the HPLC mobile phase is shown as a dashed line with values on the right of the graphs. Antiserum GF-4 detects many forms of GnRH, whereas B-6 detects only mammalian GnRH. Antiserum BLA-4 also detects several forms of GnRH, but not cGnRH-II. M = mammalian GnRH; c-I = chicken GnRH-I; c-II = chicken GnRH-II; s = salmon GnRH; 1-I = lamprey GnRH-I.

The presence of GnRH-like molecules was determined in male and female mature pacu using HPLC and RIA. We found that the same three forms of GnRH are present in both sexes. The areas of irGnRH from the HPLC column for both males and females eluted in the same positions as the irGnRH that we identified by primary structure in the second part of the study (Fig. 1, Table 2). The amount of immunoreactive material per brain showed more sbGnRH, cGnRH-II and sGnRH in the male brain compared with the female brain (Fig. 1), but the functional significance is unknown. The absence of mGnRH in pacu brains was confirmed with antiserum B-6, which cross-reacts with the mGnRH standard, but not with the three GnRH forms reported here (Fig. 1).

Table 2
Sequence and mass of the three purified GnRH peptides from pacu

Area	Sequence	Mass	GnRH
P1	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Ser–Pro–Gly–NH2	1113.4 Da	sbGnRH
P2	pGlu–His–Trp–Ser–His–Gly–Trp–Tyr–Pro–Gly–NH2	1236.6 Da	cGnRH-II
P3	pGlu–His–Trp–Ser–Tyr–Gly–Trp–Leu–Pro–Gly–NH2	1212.3 Da	sGnRH

Chemical sequence was determined after digestion of each peptide with pyroglutamyl aminopeptidase.
Intact mass was determined by mass spectrometry.

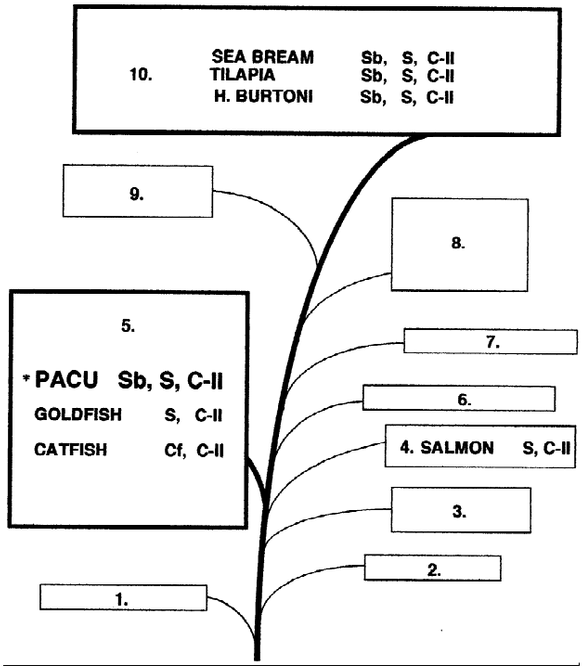

Fig. 2. Diagram of the major groups of living teleosts with the names of fish from which all detectable forms of GnRH have been sequenced. Forms of GnRH that have been detected using only RIA–HPLC methods are not shown. sb = sea bream GnRH; s = salmon GnRH; c-II = chicken GnRH-II; cf = catfish GnRH. Numbers in each box represent teleost superorders or infradivisions: 1. Osteoglossomorpha (bony tongued fish), 2. Elopomorpha (eels), 3. Clupeomorpha (herring), 4. Protacanthopterygii (salmonids), 5. Ostariophysi, 6. Stenopterygii (e.g. lightfish and dragon fish), 7. Scopelomorpha (e.g. lantern fish and greeneyes), 8. Paracanthopterygii (e.g. cod and hake), 9. Atherinomorpha (e.g. medaka, molly), 10. Percomorpha. Diagram based on [20,25].

sbGnRH is present not only in pacu, but also in the brains of several species of perchlike fish: snook [11], sea bream [7], sunfish, cichlids [8,10] and rockfish [12]. This suggests that sbGnRH was present in stem line ancestors for both pacu and the perchlike groups before they diverged.

It is generally accepted that the fish group (Ostariophysi), which includes pacu, catfish and goldfish (Fig. 2), separated from the teleost stem line some time close to the emergence of the salmonids [20,25]. Indeed, the pacu has sGnRH and cGnRH-II in its brain (Fig. 2) as do goldfish [16,26] and salmon [27]. Further, the presence of sbGnRH in the brains of pacu and the perchlike fishes suggests that this GnRH form evolved early among the teleost fishes.

In sabalo (*Prochilodus lineatus*), which is in the same order of fish (Characiformes) as pacu, three forms of GnRH were detected using HPLC and RIA in a protocol very similar to that reported here [19]. Two of the forms eluted from the HPLC column in positions that corresponded to the elution position of the cGnRH-II and sGnRH standards. The earliest eluting irGnRH area corresponds to the elution position of sbGnRH [8] and the area from which sbGnRH was purified and identified in the present study. This supports the evidence that the three forms of GnRH reported here are in more than one species of Characiformes, and are in at least two orders of fishes (Characiformes and Perciformes). However, the third form of GnRH has not been detected to date in other species or orders of fish using HPLC-RIA methods.

The most logical explanation for the presence of three forms of GnRH in pacu is that a gene duplication event occurred in an ancestral teleost. This ancestor was common to both the orders of Characiformes (pacu) and Perciformes (sea bream) fishes. This would account for the presence of the same three forms of GnRH in divergent species. The point of gene duplication is not known, but probably occurred before the emergence of perchlike fish in the Upper Cretaceous Period about 100 million years ago [20]. The appearance of a third form of GnRH in single species of teleosts clearly is not a recent event.

Acknowledgments

We thank the Canadian Medical Research Council and National Institutes of Health for financial assistance. JFFP and JC were supported by British Columbia Science Council Fellowships.

References

[1] Sherwood, N.M., Lovejoy, D.A. and Coe, I.R., Origin of mammalian gonadotropin-releasing hormones. Endocr. Rev., 14 (1993b) 241–254.
[2] Sherwood, N.M., Parker, D.B., McRory, J.E. and Lescheid, D.W. Molecular evolution of growth hormone-releasing hormone and gonadotropin-releasing hormone. In: Fish Physiology Vol. XIII (N.M. Sherwood and C.L. Hew, eds.) Academic Press, San Diego., (1994) 3–66.

[3] Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E. and Sherwood, N.M., Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain, Mol. Cell. Neurosci., 3 (1992a) 17–22.
[4] Ngamvongchon, S., Sherwood, N.M., Warby, C.M. and Rivier, J.E., Gonadotropin-releasing hormone from Thai catfish: Chromatographic and physiological studies, Gen. Comp. Endocrinol., 87 (1992b) 266–274.
[5] Bogerd, J., Li, K.W., Janssen-Dommerholt, C. and Goos, H., Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*), Biochem. Biophys. Res. Commun., 187 (1992) 127–134.
[6] King, J.A., Dufour, S., Fontaine, Y.A. and Millar, R.P., Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary, Peptides, 11 (1990) 507–514.
[7] Powell, J.F.F., Zohar, Y., Elizur, A., Park, M., Fischer, W.H., Craig, A.G., Rivier, J.E., Lovejoy, D.A. and Sherwood, N.M., Three forms of gonadotropin-releasing hormone characterized from brains of one species, Proc. Natl. Acad. Sci. USA., 91 (1994) 12081–12085.
[8] Powell, J.F.F., Fischer, W.H., Park, M., Craig, A.G., Rivier, J.E., White, S.A. Francis, R.C., Fernald, R.D., Licht, P., Warby, C. and Sherwood, N.M., Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pituitary; three forms of GnRH in brain of cichlid and pumpkinseed fish, Regul. Pept. 57 (1995) 43–53.
[9] Gothilf, Y., Elizur, A., Chow, M., Chen, T.T. and Zohar, Y., Molecular cloning and characterization of a novel gonadotropin-releasing hormone from the gilthead seabream (*Sparus aurata*), Mol. Mar. Biol. Biotechnol., 4 (1995) 27–35.
[10] White, S.A., Kasten, T.L., Bond, C.T., Adelman, J.P. and Fernald, R.D., Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide, Proc. Natl. Acad. Sci. USA, 92 (1995) 8363–8367.
[11] Sherwood, N.M., Grier, H.J., Warby, C. Peute, J. and Taylor, R.G., Gonadotropin-releasing hormones, including a novel form, in snook *Centropomus undecimalis*, in comparison with forms in black sea bass *Centropristis striata*, Regul. Pept., 46 (1993a) 523–534.
[12] Powell, J.F.F., Krueckl, S.L., Collins, P.M. and Sherwood N.M., Molecular forms of GnRH in three model fishes: rockfish, medaka and zebrafish, J. Endocrinol., 150 (1996) 17–23.
[13] Amano, M., Oka, Y., Aida, K., Okumoto, N., Kawashima, S. and Hasegawa, Y., Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon, *Oncorhynchus masou*, J. Comp. Neurol., 314 (1991) 587–597.
[14] Leprêtre, E., Anglade, I., Williot, P., Vandesande, F., Tramu, G. and Kah, O., Comparative distribution of mammalian GnRH (gonadotrophin-releasing hormone) and chicken GnRH-II in the brain of the immature Siberian sturgeon (*Acipenser baeri*), J. Comp. Neurol., 337 (1993) 568–583.
[15] Suzuki, M., Hyodo, S., Kobayashi, M., Aida, K. and Urano, A., Characterization and localization of mRNA encoding the salmon-type gonadotrophin-releasing hormone precursor of the masu salmon, J. Mol. Endocrinol., 9 (1992) 73–82.
[16] Yu, K.L., Sherwood, N.M. and Peter, R.E. Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides, 9 (1988) 625–630.
[17] Zandbergen, T., Kah, O., Bogerd, J., Peute, J. and Goos, H.J.Th., Expression and distribution of two gonadotropin-releasing hormones in the catfish brain, Neuroendocrinology, 62 (1995) 571-578.
[18] Bushnik, T.L. and Fernald, R.D., The population of GnRH-containing neurons showing socially mediated size changes project to the pituitary in a teleost, *Haplochromis burtoni*, Brain Behav. Evol., 46 (1995) 371–377.
[19] Somoza, G.M., Stefano, A., D'Eramo, J.L.. Canosa, L.F. and Friedman, O., Immunoreactive GnRH suggesting a third form of GnRH in addition to cIIGnRH and sGnRH in the brain and pituitary gland of *Prochilodus lineatus* (Characiformes), Gen. Comp. Endocrinol., 94 (1994) 44–52.
[20] Lauder, G.V. and Liem, K.F. Patterns of diversity and evolution in ray-finned fishes. In: Fish Neurobiology, (G. Northcutt and R. Davis, eds.), University of Michigan Press, Ann Arbor, 1983, pp. 1–24.
[21] Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A. and Brownstein, M.J., Primary structure of gonadotropin-releasing hormone from lamprey brain, J. Biol. Chem., 261 (1986) 4812–4819.
[22] Rivier, J. Use of trialkylammonium phosphate (TEAP) buffers in reverse phase HPLC for high resolution and high recovery of peptides and proteins, J. Liq. Chromatogr., 1 (1978) 343–367.
[23] Kelsall, R., Coe, I.R. and Sherwood, N.M., Phylogeny and ontogeny of gonadotropin-releasing hormone: Comparison of guinea pig, rat and a protochordate, Gen. Comp. Endocrinol., 78 (1990) 479–494.
[24] Fischer, W.H. and Park, M., Sequence analysis of pyroglutamyl peptides: microscale removal of pyroglutamate residues, J. Prot. Chem., 11 (1992) 366.
[25] Nelson, J.S., Fishes of the World, 2nd ed. John Wiley, New York, 523 pp.
[26] Lin, X.-W. and Peter, R.E., Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish, Gen. Comp. Endocrinol., 101 (1996) 282–296.
[27] Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J. and Vale, W. Characterization of a teleost gonadotropin-releasing hormone, Proc. Natl. Acad. Sci. USA, 80 (1983) 2794–2798.