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Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution

D. A. LOVEJOY*†, W. H. FISCHER‡, S. NGAMVONGCHON*, A. G. CRAIG‡, C. S. NAHORNIAK§, R. E. PETER§, J. E. RIVIER‡, AND N. M. SHERWOOD*

*Department of Biology, University of Victoria, Box 1700, Victoria BC, V8W 2Y2, Canada; ‡The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037; and §Department of Zoology, University of Alberta, Edmonton AB, T6G 2E9, Canada

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ABSTRACT **In vertebrates, gonadotropin-releasing hormone (GnRH) belongs to a family of decapeptides characterized by the conservation of residues 1, 2, 4, 9, and 10. In the jawed vertebrates only positions 5, 7, and 8 in the GnRH molecules vary. We have now purified two forms of GnRH from the brains of spiny dogfish (*Squalus acanthias*) by using reverse-phase high-performance liquid chromatography. The primary structures were established by automated Edman degradation and mass spectral analysis. The distinct structure of the first form (dogfish GnRH) is pGlu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2 (pGlu represents pyroglutamyl). The second peptide is identical to a form of GnRH originally isolated from chicken brains (chicken GnRH-II; pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2) and is widespread throughout the vertebrates. We are aware of no other species of cartilaginous fish in which the primary structures of two forms of GnRH have been determined. The presence of chicken GnRH-II in dogfish supports the idea that chicken GnRH-II is the oldest GnRH to evolve in jawed vertebrates. With the addition of the dogfish GnRH structure to the family, two main structural branches of GnRH can be delineated. The physiological effects of dogfish GnRH included the release of not only gonadotropin but also growth hormone from goldfish pituitary fragments.**

Changes in the structure of a peptide during evolution offer a logical starting point for structure–function studies. In theory, a large number of combinations (2×10^{11}) are possible for a peptide such as gonadotropin-releasing hormone (GnRH) that is 10 amino acids long. However, phylogenetic studies of structure can be used to predict which sequences or substitutions will be physiologically active. Indeed, Folkers and associates in 1986 (1) took this approach by designing various analogs of GnRH based on the sequences of four known vertebrate GnRHs. They synthesized 10 new peptides by substituting other naturally occurring L amino acids for those in positions 5, 7, and 8, the variable positions in the native GnRHs. Five of these synthetic peptides had activity equivalent to or greater than mammalian GnRH for the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) under at least one set of *in vivo* assay conditions in rats. Moreover, Folkers *et al.* (1) predicted that one or more of these agonists would be found in nature.

We have screened a number of vertebrate species to determine the immunological and HPLC elution pattern for GnRH in the brain (2). A limited number of GnRH forms occur in these species. However, dogfish brains appeared to contain a previously unidentified form of GnRH (3, 4). We now show that one of the GnRH structures, which we have isolated from dogfish brains, is identical to the sequence of one of the analogs previously described by Folkers and colleagues (1). Further, a second form of GnRH present in dogfish brains is identical to one of the known vertebrate GnRHs (chicken GnRH-II).

MATERIALS AND METHODS

Collection of Specimens. Spiny dogfish (*Squalus acanthias*) of both sexes and all stages of maturity were collected by trawling near Vancouver Island (Sidney, B.C., Canada) between December 1985 and January 1986, and by gill netting at the mouth of the Big Qualicum River, Vancouver Island, during June 1990. The brains were stored at −80°C until needed.

Extraction. Brains (n = 450; 2026 g total) in five batches of about 400 g each were pulverized in a Waring blender containing liquid nitrogen. The extraction procedure was described previously (4). The extracts were concentrated, centrifuged at 10,000 × g, and decanted. The supernatant was diluted with water and pumped through a column of eight Sep-Pak C_{18} cartridges connected in tandem. Each of the five columns was connected to the HPLC and the contents were eluted with an acetonitrile gradient that increased from 5% to 80% (vol/vol) reservoir B at 1%/min (see Table 1). Four sets of fractions containing immunoreactive GnRH were identified with antiserum GF-4 at a concentration of 1:2000 as described (4).

Purification. The fractions containing immunoreactive GnRH were injected onto a Supelco C_{18} analytical column (Table 1). The mobile phase included 0.1 M heptafluorobutyric acid (HFBA) as a counter ion. The elution program consisted of a gradient of 5–80% reservoir B (80% acetonitrile in 0.1 M HFBA) at 1%/min. The immunoreactive GnRH material that eluted in the first and last set of fractions was stored, as it contained insufficient material for purification. The immunoreactive GnRH represented by the two middle peaks was selected for purification.

In the next stage of purification, 0.25 M triethylammonium formate, pH 2.5, was used as a counter ion (Table 1). The same gradient of 1%/min beginning at 5% acetonitrile was used. The immunoreactive fractions were resolved on a Vydac diphenyl column using 0.05% trifluoroacetic acid and 80% acetonitrile. A 0.5%/min gradient was used, starting at 5% acetonitrile (Table 1).

Amino Acid Composition and Sequence Analysis. Aliquots (150 μl) from each purified GnRH were used for amino acid composition analysis. HPLC fractions without immunoreactive GnRH were either used as a blank or spiked with 200 pM synthetic mammalian GnRH and used as a standard. The amino acid composition was analyzed by using the

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Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; pGlu, pyroglutamyl residue.

†To whom reprint requests should be addressed.

Table 1. Purification protocol for dogfish GnRH

Purification stage	Column	Buffer* (reservoir A)	Gradient† (reservoir B)
Sep-Pak	C_{18} Sep-Paks in tandem (eight)	0.05% TFA (pH 2.2)	1%/min
HPLC 1	Supelco C_{18} analytical	0.1 M HFBA (pH 2.0)	1%/min
HPLC 2	Supelco C_{18} analytical	0.13 M TEAF (pH 2.5)	1%/min
HPLC 3	Supelco C_{18} analytical	0.13 M TEAP (pH 6.5)	0.5%/min
HPLC 4	Vydac diphenyl analytical	0.05% TFA (pH 2.2)	0.5%/min

*TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; TEAF, triethylammonium formate; TEAP, triethylammonium phosphate.
†In programs incorporating either TFA or HFBA, 80% acetonitrile was mixed with the appropriate buffer; the other systems had 100% acetonitrile in reservoir B.

HCl/phenol hydrolysis procedure described previously (5).

An additional purification step was used before proceeding with the sequence analysis. A 100-μl aliquot from each sample was concentrated to one third in a Savant concentrator and the volume was adjusted to 85 μl with 0.5% acetic acid. This material was eluted from a Vydac C_{18} microbore column by using a gradient of 1% reservoir B per min and a flow rate of 0.2 ml/min. Sequence analysis was attempted on approximately 70% of this purified sample to determine if the amino terminus was blocked.

A mixture containing 20 μg of pyroglutamyl aminopeptidase (Boehringer Mannheim) in 10 μl of *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) buffer, pH 8, with 10 mM EDTA (ethylenediaminetetraacetate), 5 mM dithiothreitol, and 5% (vol/vol) glycerol was added to an evaporated 150-μl aliquot of each sample of GnRH. The mixture was incubated for 30 min at 37°C. The GnRH-(2–10) fragment was separated from the undigested peptide and enzyme mixture by using the same HPLC program. The peak containing the digested peptide was sequenced in the manner described previously (5).

Mass Spectral Analyses. A sample representing about 200 ng of either GnRH was added to a glycerol and 3-nitrobenzyl alcohol matrix in a ratio of 1:1. The mass spectra were measured by using a JEOL JMS-HX110 double-focusing mass spectrometer fitted with a Cs^+ gun. An accelerating voltage of 10 kV and a Cs^+ gun voltage of 25 kV were employed. An accelerating/electric field voltage scan from m/z 1100 to 1500 (60-sec scan slope) at a nominal resolution of 3000 was performed first with the sample and subsequently to measure CsI calibrant peaks. The mass accuracy of this scan was ±20 ppm.

Peptide Synthesis. Solid-phase synthesis of the peptides, based on the deduced structure, was carried out on a methylbenzhydrylamine (MBHA) resin by using previously established methods (6). The peptides were deprotected and cleaved from the support with hydrofluoric acid. After purification of the synthetic peptides with reverse-phase HPLC (7), the structures were confirmed by mass spectral and amino acid composition analyses.

Physiological Studies. The *in vitro* activity of synthetic dogfish GnRH was assessed by perifusion of goldfish pituitary fragments as previously described (8). Growth hormone (GH) and LH were measured in the perfusate by using radioimmunoassays for carp GH (8) and LH (gonadotropin II) (9).

RESULTS

Purification and Sequence Analysis. Recovery of immunoreactive GnRH for the two forms of GnRH in the final purification stage was 662 and 726 ng, respectively. This represented a recovery of about 20% from the post-Sep-Pak fractions. Amino acid composition of the HPLC fraction spiked with mammalian GnRH revealed that the background contamination ranged between 6% and 26% of the concentration of the constituent residues of the GnRH peptide. The analysis of the late-eluting peak indicated that eight residues were present, suggesting a GnRH molecule with two tryptophan residues. The presence of two histidines and a single leucine was not the same as for other known forms of GnRH. Composition data for the early-eluting peak indicated the presence of a contaminating peptide. Thus additional purification was carried out. It was not possible to sequence the peptide present in aliquots of either peak, suggesting a blocked amino terminus. Digestion of both forms of GnRH with pyroglutamyl aminopeptidase yielded fragments that were eluted about 1.5 min before the intact peptide (Fig. 1).

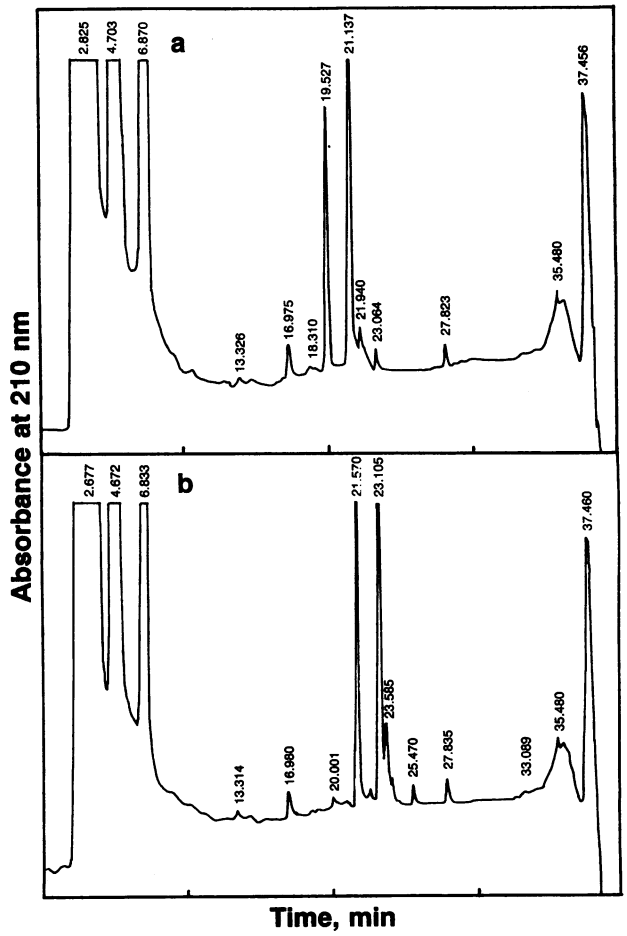

FIG. 1. HPLC purification of the GnRH-(2–10) fragments after cleavage of pyroglutamic acid from GnRH. (*a*) Early-eluting GnRH. The peak at 19.527 min represents the cleaved fragment; the peak at 21.137 is the native GnRH molecule. (*b*) Late-eluting GnRH. The peak at 21.570 min represents the cleaved fragment; the peak at 23.105 is the native GnRH molecule.

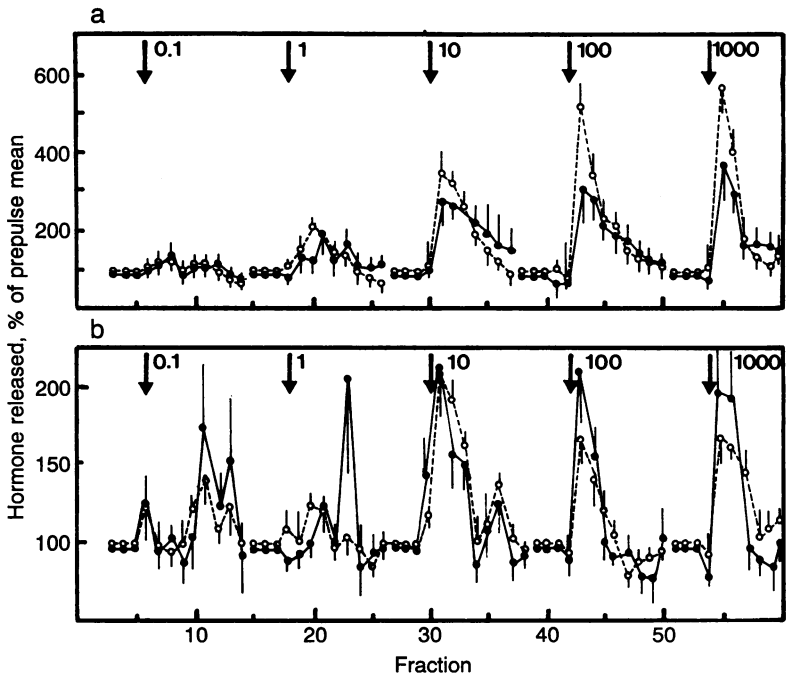

FIG. 2. Release of LH (*a*) and GH (*b*) from perifused goldfish pituitary fragments by synthetic dogfish (●) and salmon (○) GnRH. The release of LH and GH is expressed as a percent of the mean hormone concentration in the three fractions immediately preceding the GnRH pulse. This mean was defined as 100%. The arrows denote each pulse. Goldfish pituitary fragments were perifused with the appropriate concentration (nM) of either synthetic dogfish or salmon GnRH for 2 min every 60 min. Five-minute fractions were collected. GH and LH were measured in each fraction by using carp GH and LH radioimmunoassays.

Edman degradation of the peptide fragments yielded the following sequences:

Fragment I His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly
Fragment II His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly

Mass Spectral Analyses. An elemental composition of $C_{60}H_{70}N_{17}O_{13}$ with a monoisotopic protonated mass ($M + H^+$) of 1236.53 atomic mass units (amu) was obtained for the early-eluting GnRH. The late-eluting GnRH had an elemental composition of $C_{57}H_{72}N_{17}O_{12}$ and a monoisotopic protonated mass of 1186.55 amu. The theoretical molecular masses for the early- and late-eluting GnRHs for the amidated sequence based on Edman degradation were 1236.53 and 1186.38 amu, respectively. The mass of each ion was consistent with the amidated carboxyl-terminal sequences predicted from Edman degradation.

Physiological Studies. Dogfish GnRH stimulated the release of LH in a dose-dependent manner at concentrations ranging from 1 to 1000 nM (Fig. 2). Both dogfish GnRH and salmon GnRH were effective at physiological concentrations in releasing LH. GH secretion from the goldfish pituitary was also stimulated by physiological concentrations of dogfish GnRH (Fig. 2). The release of GH occurred at concentrations ranging from 1 to 1000 nM.

DISCUSSION

The brain of the spiny dogfish contains a form of GnRH with a unique amino acid sequence in addition to a form of GnRH originally isolated from chicken brains. The late-eluting GnRH (Fig. 1), which we call dogfish GnRH hereafter, has the primary structure pGlu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2 (pGlu, pyroglutamyl residue). The primary structure of the early-eluting GnRH is identical to chicken GnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2). The proof of structural identity of these peptides included Edman degradation to determine the sequence of the GnRH-(2–10) fragment. The amino-terminal pyroglutamic acid was identified by specific cleavage with pyroglutamyl aminopeptidase. The amidated carboxyl terminus was established by mass spectrometry. Amino acid composition analysis was used to corroborate the findings that were based on Edman degradation and mass spectral analysis.

The identification of dogfish GnRH expands the GnRH family to seven members (Fig. 3). It is clear in these peptides that only positions 5, 7, and 8 have changed in the last 500 million years. In preparing synthetic GnRH analogs, Folkers and his colleagues (1) made substitutions in positions 5, 7, and 8. They found five of these analogs were biologically active in rats and predicted that one or more would be found in nature. The dogfish GnRH is identical to one of their synthetic GnRHs. This analog was active in releasing both LH and FSH in a rat. Compared with mammalian GnRH, dogfish

	1	2	3	4	5	6	7	8	9	10
GnRH-3 (CHICKEN I)	pGLU	HIS	TRP	SER	TYR	GLY	LEU	GLN	PRO	GLY-NH_2
GnRH-1 (MAMMAL)	pGLU	HIS	TRP	SER	TYR	GLY	LEU	ARG	PRO	GLY-NH_2
GnRH-7 (CATFISH)	pGLU	HIS	TRP	SER	HIS	GLY	LEU	ASN	PRO	GLY-NH_2
GnRH-4 (CHICKEN II)	pGLU	HIS	TRP	SER	HIS	GLY	TRP	TYR	PRO	GLY-NH_2
GnRH-6 (DOGFISH)	pGLU	HIS	TRP	SER	HIS	GLY	TRP	LEU	PRO	GLY-NH_2
GnRH-2 (SALMON)	pGLU	HIS	TRP	SER	TYR	GLY	TRP	LEU	PRO	GLY-NH_2
GnRH-5 (LAMPREY)	pGLU	HIS	TYR	SER	LEU	GLU	TRP	LYS	PRO	GLY-NH_2

FIG. 3. Comparison of the primary structures of the seven known forms of GnRH. The sequence of each peptide has been compared with that of chicken GnRH-II, since this form of GnRH appears to be the oldest in the jawed vertebrates. Position 8 is the most variable, and position 7 is either Leu or Trp. With the exception of lamprey GnRH, only His or Tyr occurs in position 5. Lamprey GnRH has 60% sequence identity with salmon GnRH, chicken GnRH-II, and catfish GnRH even though the lineage leading to the lamprey has been phylogenetically isolated from the jawed vertebrates for about 600 million years.

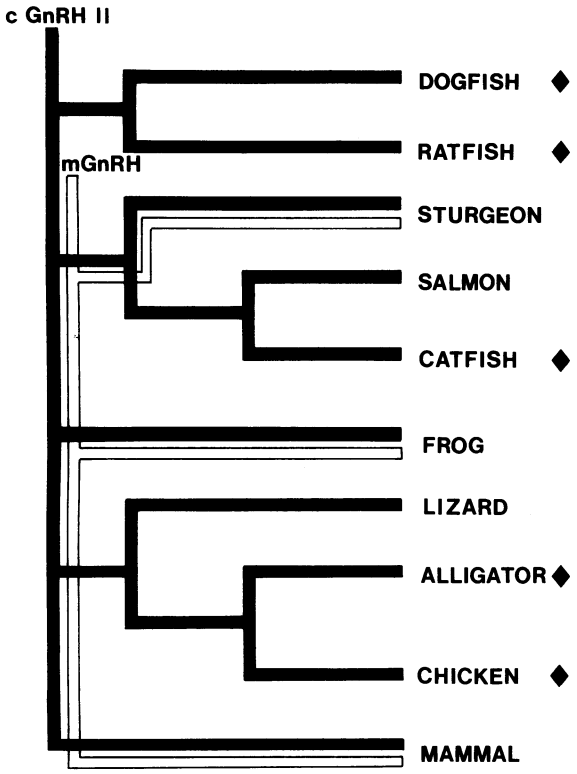

FIG. 4. Distribution of two forms of GnRH in vertebrates. Radiation of chicken GnRH-II (c GnRH II) in the vertebrates is shown by the black bars. The presence of chicken GnRH-II has been confirmed by sequence analysis in five species from three vertebrate classes. Chromatographic and immunological evidence suggests this peptide is also present in amphibians and mammals. Species in which chicken GnRH-II has been sequenced are shown by ◆. The presence of mammalian GnRH (mGnRH) is shown by open bars. The mammalian GnRH structure has been deduced from amino acid sequencing data in mammals, from amino acid composition data in amphibians, and from chromatographic and immunological evidence in the primitive bony fish (sturgeon).

GnRH released an equivalent amount of FSH and about 40% the amount of LH in one set of assay conditions.

The discovery that dogfish GnRH at physiological concentrations stimulates GH as well as gonadotropin release helps to explain the coordination of reproduction and growth by neuropeptides. Marchant and her associates (8) have shown that both mammalian and salmon GnRH stimulate release of GH from goldfish pituitary fragments. Moreover, the release of GH in response to GnRH may occur not only in goldfish but also in humans, as reported for some pathological states (10, 11).

It appears that position 5 can be occupied by either His or Tyr and position 7 by Leu or Trp without loss of biological potency (see Fig. 3). Our *in vitro* study using goldfish pituitary fragments shows that both dogfish GnRH (His^5, Trp^7) and catfish GnRH (His^5, Leu^7) (12) are equivalent to the salmon GnRH (Tyr^5, Trp^7) in releasing LH and GH. To date, these are the only two substitutions found for positions 5 (His or Tyr) and 7 (Trp or Leu) for GnRH molecules in jawed vertebrates.

The structural similarity of dogfish GnRH to chicken GnRH-II and the presence of the latter in both subclasses of the Chondrichthyes offers the possibility that dogfish GnRH arose from a duplication of the chicken GnRH-II gene. A change in two nucleotide bases is required to transform the tyrosine to a leucine codon. However, amino acid substitutions of this type are rare (13).

Chicken GnRH-II is the most widely distributed of the GnRH molecules (Fig. 4). The finding that chicken GnRH-II is present in dogfish is consistent with our finding that the same peptide exists in a related species, ratfish (5). Its presence in Chondrichthyes, Osteichthyes (14), Reptilia (15), and Aves (16) has been confirmed by amino acid sequence analysis. Additional chromatographic and immunological data indicate that it is present in all jawed vertebrate classes. Thus it appears that chicken GnRH-II has been conserved for over 400 million years.

Examination of the structure of dogfish GnRH in the context of the GnRH family suggests that GnRH evolution may follow two lines. There appear to be two natural groupings with three peptides each (Fig. 5). The GnRH molecules isolated from jawed vertebrates differ at positions 5, 7, and 8, but it is primarily the amino acids in positions 7 and 8 that determine the grouping. These molecules possess either Leu^7 and hydrophilic[8] residues or Trp^7 and hydrophobic[8] residues. The hydrophobic group contains dogfish GnRH, salmon GnRH, and chicken GnRH-II, whereas the hydrophilic group has catfish GnRH, mammalian GnRH, and chicken GnRH-I. This structural classification of GnRH molecules provides a framework within which GnRH molecules can be tested for distinct and possibly nonreproductive functional roles. Func-

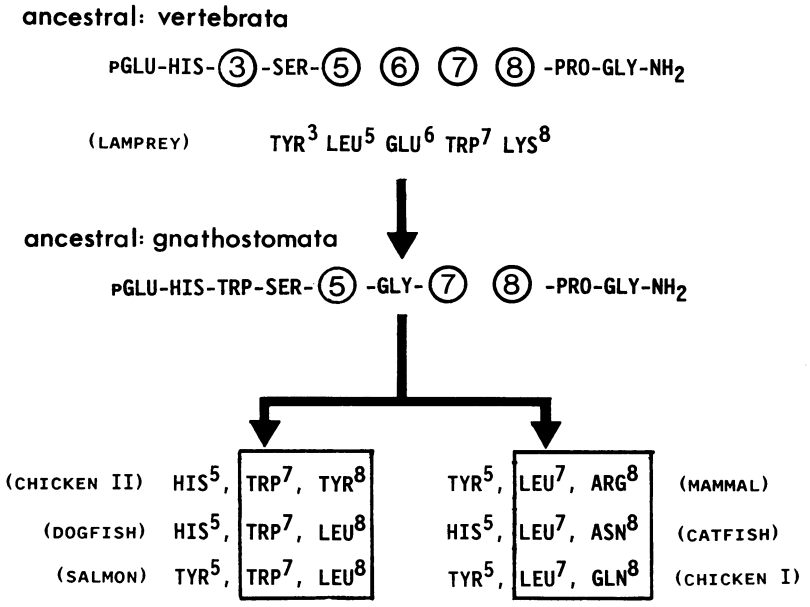

FIG. 5. Schema for the evolution of the GnRH molecule. The data suggest that a progenitor GnRH molecule with residues $pGlu^1$, His^2, Ser^4, Pro^9, Gly^{10}, and possibly Trp^7 existed before the lineage bifurcation that led to lampreys and jawed vertebrates. The existing GnRH molecules for the latter group can be classified according to their structure. One group possesses a Trp^7 and a hydrophobic[8] residue and the other is characterized by Leu^7 and hydrophilic[8] amino acids.

tional differences in the oxytocin–vasopressin family are based on a similar structural scheme (17).

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