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A NEW FAMILY MEMBER FOR GONADOTROPIN-RELEASING HORMONE

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ABSTRACT

The two living representatives of the most ancient vertebrates, Agnathans, are lamprey and hagfish. Using immunological methods, we identified gonadotropin-releasing hormone (GnRH)-like molecules in the lamprey brain, but not hagfish. The lamprey GnRH was detected poorly by antisera directed at the C-terminus, suggesting that a C-terminal amino acid substitution may have occurred in the lamprey molecule compared with mammalian GnRH. In spite of this, lamprey and mammalian GnRH-like molecules have the same retention time on an isocratic HPLC system and parallel inhibition of mammalian ^{125}I-GnRH in a radioimmunoassay. The lamprey GnRH-like molecule has a distinct HPLC elution pattern compared with dogfish shark, salmon, trout and probably birds. Thus lamprey GnRH represents another member of the growing family of GnRH molecules. Additionally, lamprey GnRH may be a stem molecule in the vertebrate evolution of GnRH.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH), a peptide synthesized in the brain, stimulates the pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH), which subsequently affect the gonads. The identified members of the GnRH family are decapeptides (Table 1). Mammalian GnRH in sheep and pig brain and in human placenta is identical (1,2,3). Substitutions in positions 7,8 (Salmon), 8 (Chicken I) or 5,7,8 (Chicken II) have been identified (4,5,6,7). An identical amino acid composition exists for adult amphibian and mammalian brain GnRH (8). However, the ancestral molecule for the GnRH family of peptides remains unknown. For this reason, we have studied the jawless lamprey and hagfish, the only two living representatives of the most ancient vertebrates, Agnathans. We have used immunological and high pressure liquid chromatographic (HPLC) methods to show that a GnRH-like molecule is present in the lamprey brain only (9) and that its structure is distinct from the identified GnRH molecules.

METHODS AND MATERIALS

Tissues. Whole brains of landlocked sea lampreys Petromyzon marinus and hagfish Eptatretus stouti were collected and immediately placed on dry ice. The brains were subsequently stored at -20°C. Adult male and female lamprey brains (N = 20) were obtained at Hammond Bay Biological Station, Millersburg, Michigan in June, 1983. Six additional male (44 cm in length, 210 g in weight, average) brains were obtained in Aug., 1983. Juvenile hagfish (N = 40; 6-30 cm in length) were collected at Bamfield, B.C., Canada. Lamprey larvae (N = 16) were collected in the early stages of metamorphosis (7 cm in length, 350 mg in body weight).

Tissue extraction. Frozen brains of lamprey or hagfish were powdered and added to an acetone: 1 N HCl (100:3) mixture. The tissue was re-extracted in acetone: 0.01 N HCl (80:20) and defatted with petroleum ether. Further extraction details are given in reference 10. The final aqueous phase was evaporated in a vacuum concentrator to about 1 ml, then filtered (0.45 um; Millipore).

Frozen brains of ammocoetes (larvae) were placed in 1 ml of 0.1 N HCl, immediately boiled for 10 minutes and sonicated for 15 seconds. The extract was spun at 10,000 rpm for 30 min., decanted and dried in a vacuum concentrator. The rehydrated extract containing 16 larval brains was diluted in a 1:2 series for radioimmunoassay; the tube with the highest concentration of tissue contained the equivalent of 4 ammocoete brains.

Radioimmunassay (RIA). Tissue extracts were screened in an RIA as previously described (4). We assumed that if the unknown GnRH-like peptides shared an identical antibody binding site with mammalian GnRH, then the unknowns could be detected in a heterologous RIA with antisera to the mammalian peptide. Antisera were used with specificities for different regions of the mammalian GnRH molecule: the C-terminal amino acids (antisera B-6, CRR11B73, D-185, A-772), the N-terminal amino acids (L-49) and the entire molecule (R-42). Final dilutions of the antisera were 1:250,000 (R-42), 1:5,000 (B-6), 1:50,000 (CRR11B73), 1:750,000 (D-185), 1:42,850 (A-772) or 1:5,000 (L-49) resulting in 29-78% binding of ^{125}I-GnRH. RIA sensitivities are shown in Figure 1. The antisera were gifts of Drs. T. Nett (R-42), Y. Chen and V. Ramirez (CR11B73), W. Dermody (D-185), A. Arimura (A-772) and W. Vale (L-49) or were prepared by L. Eiden and N. Sherwood (B-6). Synthetic mammalian GnRH was used as the standard and iodinated tracer.

HPLC. The filtered extract (800-900 µl) of each set of brains was injected through a one-ml injection loop onto a Supelco C18 Supelcosil column (0.46 x 25 cm; Supelco, Inc., Bellefonte, PA). A Varian 5000 liquid chromatograph was programmed for a flow rate of 1 ml/minute. The filtrate was applied at the beginning of a 10 minute isocratic period of 17% acetonitrile (CH_3CN) in TEAF (0.25 M formic acid, pH adjusted to 6.5 with triethylamine). CH_3CN was then increased to 24% over a 7 minute period. GnRH was eluted under isocratic conditions at 24% CH_3CN. One-ml fractions were collected and aliquots dried and assayed for ir-GnRH with antiserum R-42 and mammalian GnRH standards and tracer. Each injection of brain

extract was preceded by a blank run in which TEAF (800 µl) was injected and one-ml fractions collected under the same conditions as for the peptide. Certain fractions were assayed for ir-GnRH to determine whether previously injected peptides were being carried over to subsequent runs. Fresh standards were prepared for comparison with each brain extract. Synthetic mammalian GnRH (2.5 µg; Peninsula Laboratories Inc., San Carlos, CA) and synthetic salmon GnRH (5 µg; a gift from Dr. Jean Rivier) were mixed, diluted with TEAF to a final volume of 800 µl and injected onto the HPLC column under the conditions used for the brain extracts. About 1 year after the completion of the above experiments, 4 synthetic standards of mammalian, chicken I, chicken II and salmon GnRH were applied to the same HPLC column and under the same conditions listed above.

RESULTS AND DISCUSSION

Antiserum R-42, which recognizes the known members of the GnRH family, also detected a GnRH-like molecule in adult lamprey brain extract (Fig. 1), but not in larvae. Immunoreactive GnRH was not detected in the hagfish brain extract by any of the 6 antisera (Fig. 1); hagfish brain was tested at dilutions of the same tissue weight as lamprey brain. This confirms a study by Crim and associates in which they found GnRH in lamprey, but not hagfish brains; only one antiserum was tested (11).

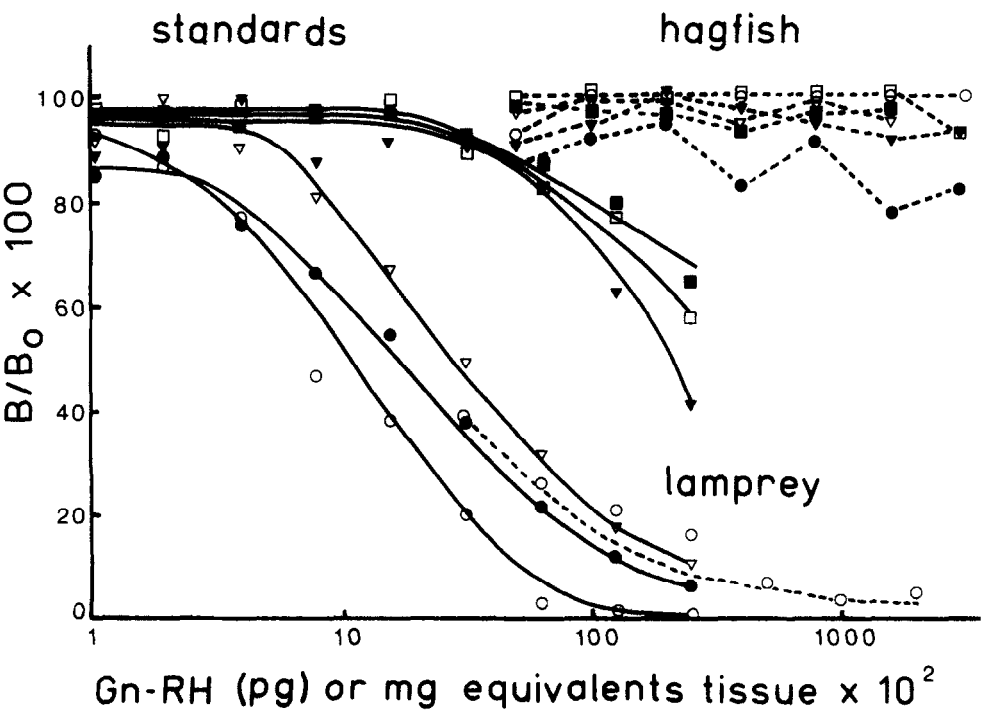

Figure 1. Displacement of mammalian ^{125}I-GnRH from various antisera (0 = R-42, ● = B-6, ▽ = CRR11B73, ▼ = D-185, □ = A-772, ■ = L-49) by synthetic

mammalian GnRH (standards are on the left side of graph) or by 1:2 serial dilutions of hagfish or lamprey brain extracts. The standard curves are solid lines; the single lamprey curve and six hagfish curves are dashed lines. Weight (mg) equivalents of fish brain tissue were calculated by multiplying the original frozen weight of the brains by the fraction of the original extract used in the radioimmunoassay. B/B_0 is the amount of mammalian GnRH tracer bound by antibody in the presence of unlabelled peptide, divided by the total amount of tracer bound by antibody.

Although a lamprey GnRH-like molecule was recognized by antiserum R-42, the molecule is immunologically distinct from mammalian GnRH. The lamprey peptide was detected poorly by antisera directed toward the C-terminal amino acids of mammalian GnRH. Antisera B-6 and CRR11B73 detected only 0.4% and 1.6%, respectively, of the activity seen by R-42 in a partially-purified lamprey sample. The greater sensitivity of antiserum R-42 (2-3 times at 50% B/B_0) compared with the other two antisera to mammalian GnRH does not account for the 63-211 fold increase in detection of lamprey GnRH by R-42 antiserum. Furthermore, recognition of the lamprey peptide by antiserum R-42, a conformational type antibody, shows that the lamprey GnRH-like material probably contains 10 amino acids and a C-terminal amide. Although cross-reactivity of R-42 with mammalian GnRH is unaffected by conservative amino acid substitutions in positions 2,3,5,7 or 8, neither GnRH fragments nor GnRH without the terminal amide are recognized (12,13).

In spite of the immunological distinctness, the lamprey and mammalian GnRH molecules have the same retention time in an isocratic HPLC system (Fig. 2). For comparison with other vertebrates, brain extracts of dogfish shark (Squalus acanthias), trout (Salmo gairdneri), frog (Rana pipiens) and rat were treated in the same manner. The lamprey brain extracts, injected onto an HPLC column, contained immunoreactive (ir)-GnRH which eluted within 60 seconds of synthetic mammalian GnRH standard as did frog (not shown) and rat brain. In contrast, lamprey extract had neither an immunoreactive peak that eluted with synthetic salmon GnRH at 26 minutes after the mammalian molecule nor one which eluted with major peaks of ir-GnRH from trout (10) or dogfish shark (Sherwood & Carolsfeld, unpublished) (Fig. 2). This HPLC evidence from a C-18 column supports the idea that the amino acid changes between lamprey and mammalian GnRH do not result in a marked difference in their net charge or hydrophobicity. The amino acid properties conserved in evolution tend to be hydrophobicity and molecular bulk, both properties relating to the secondary structure of the molecule (14).

Figure 2. (below) shows the reverse-phase HPLC of lamprey brain extract containing immunoreactive gonadotropin-releasing hormone (ir-GnRH). The elution pattern of synthetic mammalian (mam.) and synthetic salmon GnRH are shown in the top figure. The arrows (▼) in the lower figures mark the elution of the standards run with rat, trout or dogfish brain extracts. The trout results have been previously published (10), but are included for comparison with lamprey.

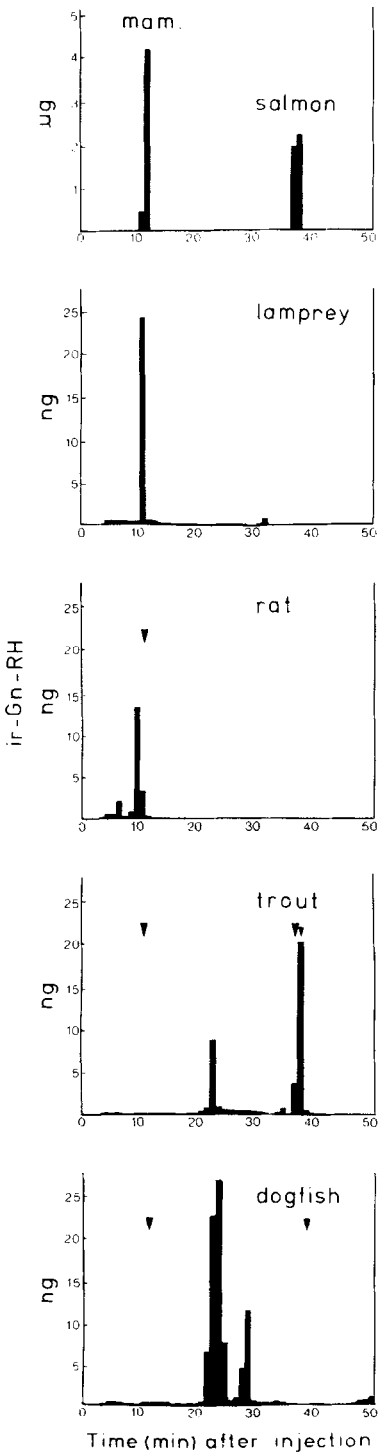

Figure 2. HPLC of brain extracts containing gonadotropin-releasing hormone.

Further information about the substitutions between lamprey and mammalian GnRH is provided by a comparison of the HPLC elution patterns of salmon, lamprey and mammalian GnRH. The difference in properties (see Table 1) of tryptophan, an aromatic amino acid, and arginine, a basic amino acid, are sufficient to separate the elution of salmon and mammalian GnRH by 26 minutes using our isocratic HPLC method. The same method also separated salmon GnRH from a synthetic peptide in which only the order of amino acids 7 and 8 was reversed (4). Thus the similarity in retention times of lamprey and mammalian GnRH underscores the idea that substitutions are between similar amino acid residues or that the sum of the substitutions produce similar HPLC mobility. Synthetic chicken I and chicken II GnRH, tested at a later time, consistently eluted at 2 and 10 minutes, respectively, after mammalian GnRH. Thus it is unlikely that lamprey and chicken GnRH are identical.

The nature of the difference between the mammalian and lamprey peptides was further characterized by RIA. Lamprey brain GnRH isolated by HPLC (fraction 11) and synthetic mammalian GnRH produced closely parallel displacement of mammalian ^{125}I-GnRH tracer as detected by antiserum R-42 (Fig. 3). The original extract of lamprey brains before HPLC also produced an inhibition curve roughly parallel to that of mammalian GnRH although fewer dilutions were available for comparison (Fig. 1).

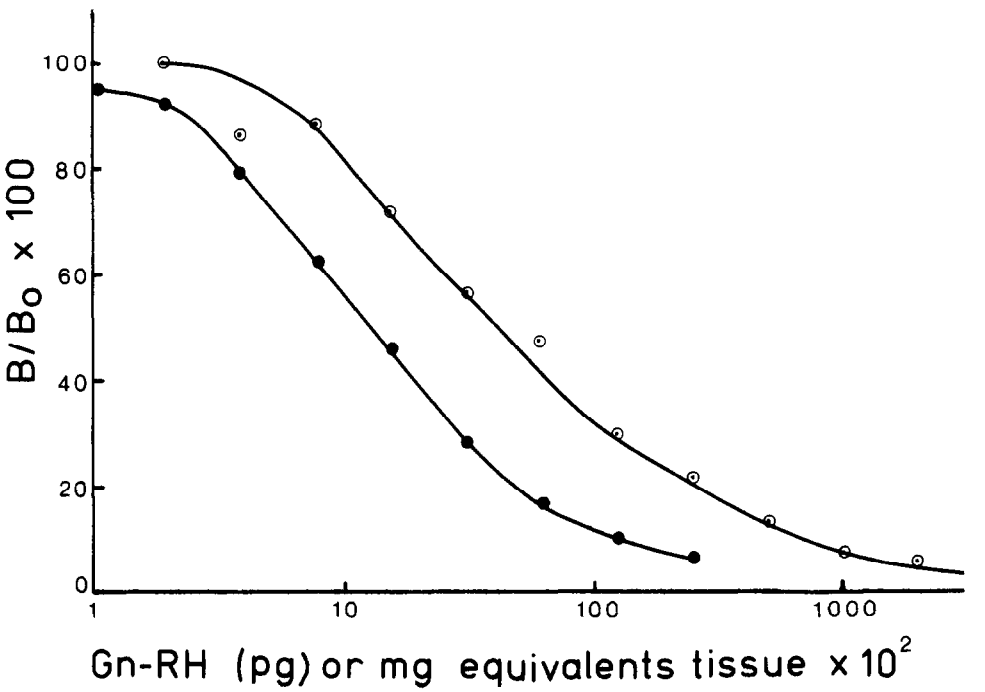

Figure 3. Displacement of mammalian ^{125}I-GnRH from antiserum R-42 by synthetic mammalian GnRH (●) or serial dilutions of lamprey brain extracts (⊙). The lamprey brain sample was derived from aliquots of HPLC fraction 11 as shown in Figure 2.

The presence of GnRH in the brains of spawning lampreys supports the idea that lampreys have brain-pituitary control of the gonads. The structure of GnRH in lamprey is not yet known and hence the biological effectiveness of the synthetic peptide cannot be tested. However, the injection of an agonist to mammalian GnRH advances ovulation in mature lamprey (15). Neural control of reproduction is understandable in that the lamprey's life cycle (metamorphosis, migration and breeding) is seasonal and appears to be triggered by environmental signals. Although the lamprey does not have a hypothalamo-hypophysial portal system, there is evidence that the neural peptide could reach the anterior pituitary by diffusing across a thin layer of connective tissue (16).

Hagfish may have evolved before brain-pituitary reproductive peptides or may have a degenerate nervous system that has lost the GnRH-like peptide. The adult hagfish, E. stouti, lives in deep water with constant darkness and temperature and may not need a neural peptide to mediate environmental cues; furthermore, E. stouti is a continuous breeder and can release mature eggs months after hypophysectomy (16, 17).

Alternatively, hagfish may contain a structurally-distinct GnRH-like molecule. Antiserum R-42 does not cross-react with hagfish brain extract, but does detect GnRH-like molecules in the nervous system of other fish (lamprey, dogfish, various teleosts), amphibians, birds and mammals (4,10,13,18,19). That other antisera directed against the N- or C-terminal portions of the mammalian GnRH molecule likewise do not cross-react with hagfish brain extracts suggests both ends of a hypothetical "hagfish molecule" are different from the mammalian molecule. Antisera used by other workers did not detect a GnRH-like molecule in Eptatretus burgeri or Eptatretus stouti or detected picogram amounts in whole brains of Eptatretus stouti or Heptratretus hexatrema (11,20,21,22). The contradictory results and small amount of GnRH make it difficult to judge from these earlier studies whether a reproductive peptide is present.

The existence of a hagfish reproductive peptide with some homology to mammalian GnRH can nonetheless not yet be ruled out. Organisms more primitive than hagfish contain a reproductive hormone with a considerable sequence homology to mammalian GnRH. The structural homology of yeast α_1-mating factor, phylogenetically the oldest reproductive hormone yet identified, and mammalian GnRH (23) is shown in Table 1. The functional homology of these two peptides is shown by the ability of yeast mating factor to specifically bind to rat pituitary GnRH receptors and to stimulate the release of luteinizing hormone from cultured rat pituitary cells (24). These similarities suggest the presence of a partially conserved molecule predating those found in vertebrates.

Table 1. Known peptides of GnRH family and yeast α_1-mating factor.

		1	2	3	4	5	6	7	8	9	10			
Mammalian GnRH		pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2			
Avian GnRH	I.	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2			
	II.	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2			
Piscine GnRH		pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2			
		1	2	3	4	5	6	7	8	9	10	11	12	13
Yeast α_1-mating factor		Trp	His	Trp	Leu	Gln	Leu	Lys*	Pro	Gly	Gln	Pro	Met	Tyr

Sequence homologies to mammalian GnRH are underlined. *Lysine (Lys) is considered homologous to arginine (Arg) in position 8 of mammalian GnRH.

Only determination of the primary structure of lamprey GnRH will help clarify the role of the lamprey peptide as an ancestral stem molecule of higher vertebrates, represented by amphibian and mammalian GnRH. Meanwhile our evidence that the lamprey GnRH-like molecule is immunologically distinct from mammalian GnRH and chromatographically distinct from other GnRH molecules clearly shows that a new member can be added to the growing GnRH family.

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