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The Occurrence and Distribution of GnRH in the Brain of Atlantic Hagfish, an Agnatha, Determined by Chromatography and Immunocytochemistry

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In Atlantic hagfish (*Myxine glutinosa*), chromatographic and immunocytochemical evidence showed that the brain contains a gonadotropin-releasing hormone (GnRH)-like molecule that is closely related to lamprey GnRH-III. The chromatographic studies (HPLC) used specific antisera directed against mammalian GnRH and lamprey GnRH-I. In addition to these antisera, other specific antisera were tested in immunocytochemical studies, including chicken GnRH-I, chicken-GnRH-II, salmon GnRH, and lamprey GnRH-III. Using a lamprey GnRH-I antiserum, an early eluting GnRH form coeluted on HPLC with lamprey GnRH-III standard and an unknown form coeluted with the chicken GnRH-II standard. Radioimmunoassay of brain tissue detected GnRH immunoreactivity only when the lamprey GnRH-I antibody was used. A GnRH-like immunoreactivity was also obtained immunocytochemically in the neurohypophysis with the use of antisera against chicken GnRH-II, salmon GnRH, lamprey GnRH-I, and lamprey GnRH-III. These studies indicate that, contrary to earlier reports, hagfish do have a GnRH-like molecule that is more closely related, in terms of immunological determinants, to lamprey GnRH-III, than to other currently known vertebrate GnRH molecules. © 1995 Academic Press, Inc.

The primary structures of three previously unknown vertebrate gonadotropin-releasing hormones (GnRHs) have been elucidated recently. This expands the vertebrate GnRH molecular family to eight (Fig. 1). Included in this family are the structures of GnRHs of three primitive fish species, an agnathan, the sea lamprey *Petromyzon marinus* (lamprey GnRH-I and -III) (Sherwood *et al.*, 1986; Sower *et al.*, 1993); an elasmobranch, the spiny dogfish shark *Squalus acanthias* (dogfish GnRH and chicken GnRH-II) (Lovejoy *et al.*, 1992); and a holocephalan, the ratfish *Hydrolagus colliei* (chicken GnRH-II) (Lovejoy *et al.*, 1991).

The hagfish is a modern representative of the oldest class of vertebrates. The presence of GnRH in the hagfish brain has been a topic of disagreement. Immunoreactive (ir) GnRH was reported in brains of the hagfishes *Eptatretus hexamtrema* (King and Millar, 1980) and *E. stouti* (Jackson, 1980) using radioimmunoassay and chromatographic techniques. However, others, using similar techniques, could not detect GnRH in the Pacific hagfish *E. stouti* (Sherwood and Sower, 1985). In immunocytochemical studies, ir-GnRH was not detected in several species of hagfish brain by Nozaki and Kobayashi (1979) and Crim *et al.* (1979). Nozaki *et al.* (1984) hypothesized that hagfish may contain an ir-GnRH that is not detected by current methods or antibodies or that hagfish may lack GnRH. Further studies are required to verify whether there is a GnRH-like molecule in hagfish brain. Thus, the present study used newly available an-

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	1 2 3 4 5 6 7 8 9 10
Lamprey-III	pGlu-His-Trp-Ser-His-Asp-Trp- Lys- Pro-Gly-NH_2
Lamprey-I	pGlu-His-Tyr-Ser-Leu-Glu-Trp -Lys- Pro-Gly-NH_2
Dogfish	pGlu-His-Trp-Ser-His-Gly- Trp- Leu- Pro-Gly-NH_2
Catfish I	pGlu-His-Trp-Ser-His-Gly- Leu- Asn- Pro-Gly-NH_2
Salmon	pGlu-His-Trp-Ser-Tyr-Gly- Trp- Leu- Pro-Gly-NH_2
Chicken-II	pGlu-His-Trp-Ser-His-Gly- Trp- Tyr- Pro-Gly-NH_2
Chicken-I	pGlu-His-Trp-Ser-Tyr-Gly- Leu- Gln- Pro-Gly-NH_2
Mammal	pGlu-His-Trp-Ser-Tyr-Gly- Leu- Arg- Pro-Gly-NH_2

FIG. 1. Primary structures of the eight known vertebrate GnRH molecules. Underlined amino acids represent differences in the GnRH forms compared to lamprey GnRH-III.

tibodies to lamprey GnRH (GnRH-III), as well as other GnRH antibodies, to characterize the immunoreactivity of GnRH in the brain of the Atlantic hagfish.

METHODS AND MATERIALS

Animals

Adult Atlantic hagfish (*Myxine glutinosa*) were collected in traps in the Gulf of Maine 10 miles east of the Isle of Shoals, New Hampshire. For immunocytochemical study, 12 animals of both sexes were killed by decapitation following anesthetization with ethyl *m*-aminobenzoate methanesulfonate (MS222). After rapid removal of the dorsal fibrocranium and exposure of the dorsal surface of the brain, the dissected brain and the attached pituitary were immersed in Bouin–Hollande sublimate solution (Romeis, 1948) for 24 hr. The fixed tissues were dehydrated through a series of increasing concentrations of ethanol. Deposits of mercuric chloride were removed by treatment with iodine–potassium iodide in 90% ethanol. Tissues were embedded in Paraplast and serial sagittal sections of 6 μm were mounted on glass slides.

For chromatographic and radioimmunoassay studies, whole brains were dissected immediately following decapitation and frozen on dry ice and stored at -80° until extracted and assayed. The mean weight of the brains was 0.22 g (10 hagfish).

Immunocytochemistry

Immunocytochemical staining was performed by use of a Vectastain avidin–biotin peroxidase complex (ABC) Elite kit. The staining precedures have been described elsewhere (Saga *et al.*, 1993). The following rabbit antisera were used (1) anti-mammalian GnRH, Lot UZ-8, obtained from Miles Yeda (working dilution, × 3000); (2) anti-chicken GnRH-1, Lot 1665, provided by Dr. Judy King (working dilution, × 1500); (3) anti-chicken GnRH-II, Lot 675, provided by Dr. Judy King (working dilution, × 1000); (4) anti-salmon GnRH, Lot 432, provided by Dr. Judy King (working dilution, × 1500); (5) anti-lamprey GnRH-1, Lot 1467, provided by Dr. Judy King (working dilution, × 2000); (6 and 7) anti-lamprey GnRH-III, Lot 3951 and 3952, raised in different rabbits in this laboratory (working dilution, × 4000). In a preliminary series of immunocytochemistry tests, these antisera were applied to paraffin sections of the pituitaries of a variety of species, including rats, Japanese quail, rainbow trout, and sea lampreys. All of the above-listed antisera yielded excellent specific GnRH immunoreactions in the neuronal elements of the median eminence (data not shown). Moreover, in the tests on sections of sea lamprey brain, all of the listed antisera gave an intense immunoreaction in the preoptico-neurohypophysial GnRH neuronal system. Thus, it was apparent that all of these antisera reacted with lamprey GnRHs. Subsequent preabsorption tests revealed that GnRH-positive reactions to anti-lamprey GnRH-III (3952) were specific for lamprey GnRH-III. All the remaining antibodies exhibited cross-reactivity, more or less, to both lamprey GnRH-1 and lamprey GnRH-III (data not shown).

To confirm the specificity of the immunostaining, the following control procedures were done: (1) replacement of primary antibodies with normal rabbit serum and (2) absorption of primary antibodies with synthetic lamprey GnRH-I (Peninsula laboratories, 20 μg/0.3 ml antisera at working dilutions) or with lamprey GnRH-III (generously provided by Dr. Russell Doolittle, 20 μg/0.3 ml antisera at working dilutions).

Extraction and HPLC

Frozen brains were extracted following the procedures described by Yu *et al.* (1987) and Fahien and Sower (1990). The HPLC procedure followed the methods previously described by Fahien and Sower (1990) and Calvin *et al.* (1993). Briefly, the extract was filtered using an ARCO LC 13 (0.45 μm) filter and then injected into a 20-μl loop on a Perkin–Elmer HPLC System with a Percosphere 3CR C18 (0.46 × 8.3 cm) reverse-phase column.

Synthetic mammalian GnRH, chicken GnRH-I and -II, salmon GnRH, and lamprey GnRH-I and -III standards were chromatographed in parallel on the same HPLC system.

Radioimmunoassay

Radioimmunoassay (RIA) was performed as previously described by Stopa *et al.* (1988) and Fahien and Sower (1990) using synthetic mammalian GnRH or lamprey GnRH as the radio-iodinated tracer and stan-

dard. The antisera were used at dilutions of 1:100,000 of mammal RIA (R1245) and 1:25,000 for lamprey RIA (1467). The lamprey GnRH antibody (1467) binding ranged between 33 and 58%. The mammalian GnRH antibody (R1245) binding ranged between 39 and 44%. Antiserum R1245 has a specificity with cross-reactivities of 65, 19.5, 4.16, and <0.00001% for chicken GnRH-I, salmon GnRH, chicken GnRH-II, and lamprey GnRH-I, respectively (Calvin *et al.*, 1993). Antiserum 1467 has cross-reactivities of 7.3% with lamprey GnRH-III and less than 0.03, 0.02, and 0.01% cross-reactivity for chicken GnRH-II, mammalian GnRH and chicken GnRH-I, respectively (Sower *et al.*, 1993).

RESULTS

Immunocytochemistry

The results of the immunostaining and preabsorption tests are summarized in Table 1. As shown, a moderately intense immunoreaction was observed in the neurohypophysis with the use of either of the two lots of anti-lamprey GnRH-III (Fig. 2). A weak, but consistently positive immunoreaction was found in the neurohypophysis with the use of anti-chicken GnRH-II, anti-salmon GnRH, and both lots of anti-lamprey GnRH-I. For all antibodies listed above, an accumulation of immunoreactive fibers was found in the outer layer of the dorsocaudal wall of the neurohypophysis (Fig. 2). A few GnRH-positive fibers were also found in the outer layer of the rostroventral wall of the neurohypophysis. None of the antibodies listed above gave a positive reaction in other areas of the brain. No positive reaction was observed in the brain, including the neurohypophysis, with the use of anti-mammalian GnRH or anti-chicken GnRH-I.

As shown in Table 1, most GnRH antibodies yielded a positive reaction in the hagfish neurohypophysis. Preabsorption with either lamprey GnRH-I or lamprey GnRH-III resulted in elimination of the positive reaction. However, in the use of one of the anti-lamprey GnRH-III antibodies (Lot 3952), the positive reaction was eliminated only by the preabsorption with lamprey GnRH-III, although preabsorption with lamprey GnRH-I resulted in a reduction of the intensity of positive reaction.

RIA and Chromatography

The elution times of fractions from the hagfish brains were compared to elution times of six different synthetic GnRH standards in an identical HPLC system as determined by radioimmunoassay of each fraction. The elution profiles of three stan-

TABLE 1

EFFECTS ON IMMUNOSTAINING OF PREABSORPTION OF VARIOUS ANTI-GNRHS WITH LAMPREY GNRH-I AND LAMPREY GNRH-III IN HAGFISH NEUROHYPOPHYSIS

			Immunoreactivity		
				Preabsorption with	
Antibodies to	Lot	Obtained from	Without preabsorption	*l* GnRH-I	*l* GnRH-III
m GnRH[a]		Miles-Yeda	–[b]		
c GnRH-I	1665	King	–		
c GnRH-II	675	King	+	– (abolished)	– (abolished)
s GnRH	432	King	+	– (abolished)	– (abolished)
l GnRH-I	1467	King	+	– (abolished)	– (abolished)
l GnRH-I	21–134	Sower	+	– (abolished)	– (abolished)
l GnRH-III	3951	Sower	+ +	– (abolished)	– (abolished)
l GnRH-III	3952	Sower	+ +	+ (reduced)	– (abolished)

[a] The prefixes *m*, *c*, *s*, and *l* indicate that the original antigens were mammalian, chicken, salmon, or lamprey GnRHs, respectively.

[b] + +, moderate immunoreaction; +, faint immunoreaction; –, no immunoreaction.

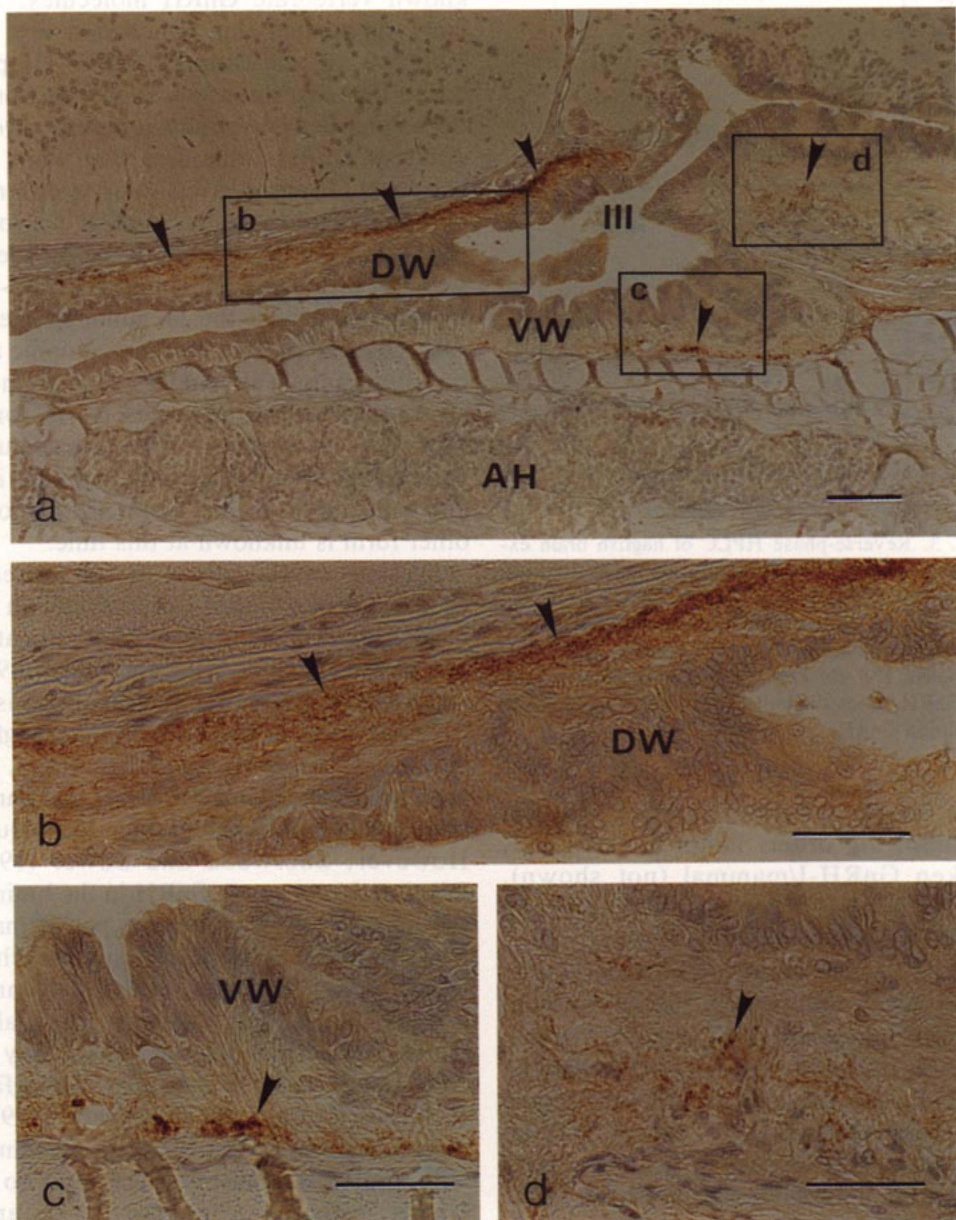

FIG. 2. Nearly midsagittal section of the hagfish neurohypophysis stained with anti-lamprey GnRH-III (Lot 9351). The anterior end is to the right. The areas outlined by rectangles are enlarged and shown in b, c, and d. (a–c) An accumulation of GnRH-immunoreactive fibers (arrowheads) terminating in the external layer of the dorsal and ventral walls (DW and VW) of the neurohypophysis. AH, adenohypophysis; III, third ventricle; Scale bar: (a) 100 μm (×98); (b, c, and d) 50 μm (×315).

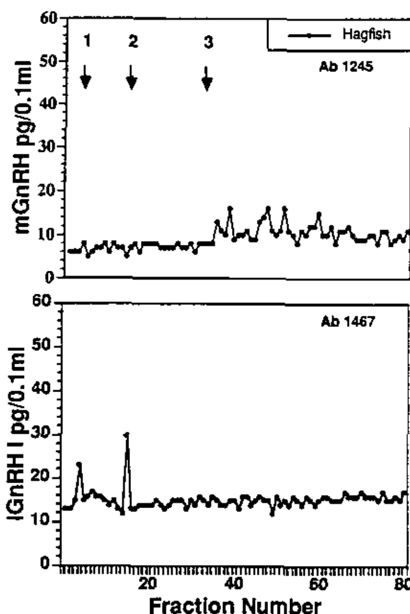

FIG. 3. Reverse-phase HPLC of hagfish brain extract and radioimmunoassay with mammalian GnRH antiserum R1245 (top) and lamprey GnRH antiserum 1467 (bottom). Arrows indicate peaks of assayed synthetic standards in the order: (1), lamprey GnRH-III, mammalian and chicken GnRH-I (not shown), lamprey GnRH-I (not shown); (2), chicken GnRH-II and (3), salmon GnRH.

dards, shown by arrows in Fig. 3, occur in the following order: lamprey GnRH-III, chicken GnRH-I/mammal (not shown), lamprey GnRH-I (not shown), chicken GnRH-II, and salmon.

In the hagfish brain, there were two ir-GnRH peaks detected by use of the lamprey GnRH antibody 1467 (Fig. 3). The major peak coeluted with synthetic chicken GnRH-II, and the early eluting form eluted with lamprey GnRH-III. There was no cross-reactivity of hagfish brain fractions with mammalian GnRH antibody 1245 (Fig. 3).

DISCUSSION

These chromatographic and immunocytochemical data clearly demonstrate that the hagfish brain contains a GnRH-like molecule that is more closely related, in terms of immunological determinants, to lamprey GnRH-III than to other currently known vertebrate GnRH molecules. The facts supporting this conclusion are: (1) both lots of anti-lamprey GnRH-III produced the most intense positive reaction in the hagfish neurohypophysis among several different GnRH-antibodies that were applied and (2) positive reactions to different GnRH-antibodies in the hagfish neurohypophysis were equally abolished by preabsorption with synthetic lamprey GnRH-III. In agreement with the immunocytochemical study, the present chromatographic and radioimmunoassay studies also demonstrated an ir-lamprey GnRH-III form. There was an additional form that coeluted with chicken GnRH-II. Whether this is a chicken GnRH-II-like molecule or some other form is unknown at this time.

Previous investigations of the presence or absence of a GnRH-like molecule in the hagfish brain have provided both negative and positive results. King and Millar (1980) and Jackson (1980) reported that there is an ir-GnRH form in the brain of two hagfish species, *E. hexamtrema* and *E. stouti*, using a mammalian GnRH antisera and immunological and chromatographic techniques. However, Sherwood and Sower (1985) failed to detect any ir-GnRH in the brain of *E. stouti* using six different types of mammalian GnRH antisera and similiar chromatographic techniques. An earlier immunocytochemical study using a mammalian GnRH antiserum did not detect any ir-GnRH in the brain of the Japanese hagfish, *E. burgeri* (Nozaki and Kobayashi, 1979) or *E. stouti* (Crim *et al.*, 1979). In agreement with previous studies, there was no ir-GnRH detected after use of an anti-mammalian GnRH antibody, in either the chromatographic or the immunocytochemical procedures in the present study. Thus, the use of lamprey GnRH antibodies clearly indicates the probability that the hagfish brain contains a GnRH-like molecule that is more similar to lamprey GnRH-III and

chicken GnRH-II than to lamprey GnRH-I. Lamprey GnRH-III has 80% molecular identity with chicken GnRH-II and dogfish GnRH (Sower *et al.*, 1993). Chicken GnRH-II has been characterized in the ratfish (Class Chondrichthyes) which diverged from the line of vertebrate evolution about 400 million years ago (Lovejoy *et al.*, 1991). It has been proposed that lamprey GnRH-III, chicken GnRH-II, and dogfish GnRH are relatively more closely related to the "ancestral" GnRH molecule (Sower *et al.*, 1993). Our results suggest that the hagfish may have also retained one or more of these GnRH forms. Testing other hagfish species under the same analytical conditions may provide additional enlightenment in this area.

In support of the chromatographic data, the present immunocytochemical study clearly demonstrates the presence and distribution of lamprey GnRH-III-like immunoreactivity in the neurohypophysis of the hagfish. Although the chromatography indicates that there are two forms of GnRH, this was not supported by the immunocytochemical study. The following immunocytochemical data suggest the presence of only one form of GnRH-like molecule that is closely related to lamprey GnRH-III: (1) both lots of anti-lamprey GnRH-III gave the most intense immunoreaction in the neurohypophysis of the hagfish compared to the other antibodies that were tested, (2) the topographic distributions of the positive material within the neurohypophysis were quite similar among antibodies, and (3) of all antibodies which gave a positive reaction in the neurohypophysis, preabsorption with lamprey GnRH-III resulted in elimination of their positive reaction. However, since lamprey GnRH-III has 80% identity with chicken GnRH-II, it is also possible that our immunocytochemical method cannot distinguish chicken GnRH-II from lamprey GnRH-III. There is also the possibility that the GnRH-like form that coeluted with the chicken GnRH-II standard may not be chicken GnRH-II but closely related to this GnRH, or this form may not be a GnRH molecule. As was shown in an earlier study, we detected and purified somatostatin-14 from lamprey brains using the GnRH antisera 1467 which had virtually no cross-reactivity to synthetic somatostatin-14 (less than $8 \times 10^{-7}\%$ cross-reactivity) (Sower *et al.*, 1994). Thus, a peptide with apparent GnRH-like immunoreactivity was purified and sequenced and shown to be somatostatin-14. Clearly, further studies are needed to clarify whether the hagfish brain contains two forms of GnRH.

The present data, from both HPLC-RIA and immunocytochemistry, clearly suggest the presence of a lamprey GnRH-III-like molecule in the hagfish brain. Nevertheless, immunocytochemistry revealed only weak GnRH-labeling in the hagfish neurohypophysis. There are several possible explanations for the weak GnRH-labeling in the hagfish brain. The first, and most probable, explanation for this is that the molecular nature of the hagfish GnRH may differ from lamprey GnRH-III, and anti-lamprey GnRH-III sera may have a lower binding affinity to hagfish GnRH than to lamprey GnRH-III. Another possible explanation is that the weakness of GnRH labeling in the hagfish neurohypophysis may reflect particular physiological conditions of hagfish reproduction. The latter possibility may be supported by the finding that total hypophysectomy in *E. stouti* was not followed by any clear change in gonadal function (Matty *et al.*, 1976). Thus, it is possible that gonadotropic function in the hagfish is very weak or actually absent. Accordingly, hypothalamic regulation of pituitary gonadotropic function may have degenerated to some extent in the hagfish (see also Gorbman, 1988). Further studies are needed to clarify these alternative possibilities.

As stated earlier, all vertebrate groups have been found to contain two or more forms of GnRH. Prior research has led to several proposed models for the phylogeny

of the GnRH molecule (Lovejoy *et al.*, 1992; King and Millar, 1980; Sower *et al.*, 1993). Lamprey GnRH-III is more closely related to the other members of the GnRH family than is lamprey GnRH-I (Sower *et al.*, 1993). Lamprey GnRH-III, chicken GnRH-II, and dogfish GnRH occur in species representing the two oldest lineages of vertebrates. Modern hagfish may have retained one or more of these early or stem GnRH forms, as indicated in the present study. Chicken GnRH-II now has been structurally sequenced in representative species of five of the seven classes of vertebrates (Sherwood *et al.*, 1993). The structural similarity of chicken GnRH-II to lamprey GnRH-III suggests that an ancestral molecule gave rise to these two forms through gene duplication or a single base mutation.

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REFERENCES

Calvin, J. L., Slater, C. H., Bolduc, T. G., Laudano, A. P., and Sower, S. A. (1993). Multiple molecular forms of gonadotropin-releasing hormone in the brains of an elasmobranch: Evidence for ir-lamprey GnRH. *Peptides* **14**, 725–729.

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

Fahien, C. M., and Sower, S. A. (1990). Relationship between brain gonadotropin-releasing hormone and final reproductive period of the adult male sea lamprey, *Petromyzon marinus*. *Gen. Comp. Endocrinol.* **80**, 427–437.

Gorbman, A. (1988). Reproduction in cyclostome fishes and its regulation. *In* "Fish Physiology" (W.S. Hoar, D.J. Randall, and E.M. Donaldson, Eds.), Vol. IXA. Academic Press, New York.

Jackson, I. M. D. (1980). Distribution and evolutionary significance of the hypophysiotropic hormones of the hypothalamus. *Front. Horm. Res.* **7**, 35–69.

King, J. A., and Millar, R. P. (1980). Comparative aspects of luteinizing hormone-releasing hormone structure and function in vertebrate phylogeny. *Endocrinology* **106**, 707–717.

King, J. A., and Millar, R. P. (1991). Gonadotropin-releasing hormones. *In* "Vertebrate Endocrinology, Fundamentals and Biological Implications" (P.K.T. Pang and M.P. Schreibman, Eds.), Vol. 4, Part B, pp. 1–31, Academic Press, Orlando.

Lovejoy D. A., Sherwood, N. M., Fischer, W. H., Jackson, B. C., Rivier, J. E., and Lee, T. (1991). Primary structure of gonadotropin-releasing hormone from the brain of a holocephalan (Ratfish: *Hydrolagus colliei*). *Gen. Comp. Endocrinol.* **82**, 152–161.

Lovejoy, D. A., Fischer, W. H., Ngamvongchon, S., Craig, A. G., Nahorniak, C. S., Peter, R. E., Rivier, J. E., and Sherwood, N. M. (1992). Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc. Natl. Acad. Sci. USA* **89**, 6373–6377.

Matty, A. J., Tsuneki, K., Dickhoff, W. W., and Gorbman, A. (1976). Thyroid and gonadal function in hypophysectomized hagfish, *Eptatretus stouti*. *Gen. Comp. Endocrinol.* **30**, 500–516.

Nozaki, M., and Kobayashi, H. (1979). Distribution of LHRH-like substance in the vertebrate brain as revealed by immunocytochemistry. *Arch. Histol. Jpn.* **42**, 201–219.

Nozaki, M., Tsukahara, T., and Kobayashi, H. (1984). Neuronal systems producing LHRH in Vertebrates. *In* "Endocrine Correlates of Reproduction" (K. Ochiai, *et al.*, Eds.), pp. 3–27, Japan Sci. Soc., Tokyo/Springer-Verlag, Berlin.

Romeis, B. (1948). "Microskopische Technik," 15 verb. Aufl. Leibnit Verlag, Munchen.

Saga, T., Oota, Y., Nozaki, M., and Swanson, P. (1993). Salmonid pituitary gonadotrophs. III. Chronological of GTH I and other adenohypophysial hormones in the pituitary of the developing rainbow trout (*Oncorhynchus mykiss irideus*). *Gen. Comp. Endocrinol.* **92**, 233–241.

Sherwood, N. M., and Sower, S. A. (1985). A new family member for gonadotropin-releasing hormones. *Neuropeptides* **6**, 205–214.

Sherwood, N. M., Lovejoy, D. A., and Imogen, R. C. (1993). Origin of mammalian gonadotropin-releasing hormones. *Endocr. Rev.* **14**, 241–254.

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

Sower, S. A., Chiang, Y-C., and Conlon, J. M. (1994). Polygenic expression of somatostatin in lamprey. *Peptides* **15**, 151–154.

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

Stopa, E. G., Sower, S. A., Svendsen, C. N., and King, J. C. (1988). Polygenic expression of gonadotropin-releasing hormone (GnRH) in human? *Peptides* **9**, 419–423.

Yu, K. L., Nohorniak, C. S., Peter, R. E. Corrigan, A., Rivier, J.R., and Vale, W. W. (1987). Brain distribution of radioimmunoassayble gonadotropin-releasing hormone in female goldfish: Seasonal variation and periovulatary changes. *Gen. Comp. Endocrinol.* **67**, 234–246.