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Evolution of a Neuropeptide Family: Gonadotropin-Releasing Hormone[1]

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SYNOPSIS. Gonadotropin-releasing hormone (GnRH), a small peptide in the brain, is essential for reproduction. It is now clear that GnRH is part of a family of closely related molecules. The primary structure has been identified for 4 GnRH molecules: mammalian, chicken I, chicken II and salmon. During evolution the molecule has been conserved in length, terminal amino acid structure, 70–90% of amino acid sequence and the His^2-Trp^3 residues, which are important in the release of gonadotropin. Alterations have occurred in positions 5, 7 and 8, regions thought to be involved in receptor binding. The receptors for GnRH have apparently evolved also in that the mammalian and avian receptors vary considerably in their ability to bind different GnRH molecules. Other GnRH family members have been distinguished indirectly by chromatographic or immunological means; 3 different GnRH-like molecules are present, respectively, in lamprey, sturgeon and salmon (a second form). Several GnRH-like molecules including those in chondrichthyes have not yet been distinguished from the proposed salmon II molecule. The lamprey GnRH-like molecule may be a nodal point in the analysis of the ancestral molecule; hagfish do not contain a detectable GnRH molecule. The elucidation of the GnRH precursor molecule in human placenta showed the presence of a 53-amino-acid gene-related peptide of unknown function, but did not reveal the basis for expression of multiple GnRH forms in many nonmammalian species. GnRH has a variety of novel functions in addition to release of gonadotropin from the pituitary. During evolution certain functions such as those in the retina and sympathetic ganglia have apparently disappeared in amniotes, but GnRH placental functions have appeared in mammals.

INTRODUCTION

Two papers, published in 1971 and 1972, established the primary structure of gonadotropin-releasing hormone (GnRH) in pigs and sheep (Matsuo *et al.*, 1971; Burgus *et al.*, 1972). This completed a 25-year search for a brain factor controlling release from the pituitary of the reproductive hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Green and Harris (1947) had hypothesized that such a factor existed. However, the structural identification of this small protein was difficult since only nanogram amounts exist in the mammalian brain. In the decade following the discovery of the structure of mammalian GnRH, this neuropeptide was actively studied in many vertebrates. The synthetic form effectively induced a number of reproductive events, but some scientists expressed doubt that the mammalian form of GnRH was identical to the native form in other vertebrates. Indeed, in 1982 to 1984, the primary structures for three distinct GnRH molecules in nonmammalian vertebrates were published, establishing a phylogenetic family of GnRH molecules.

This paper considers the diversity in vertebrate GnRH molecules in terms of both structure and function. This biochemical approach to the evolution of the nervous system leads to the question of an ancestral molecule in vertebrates or invertebrates. Finally, the recently identified precursor molecule for mammalian GnRH and the genetic expression of multiple forms of GnRH in one organism are examined.

IDENTIFIED MEMBERS OF THE GNRH FAMILY

The primary structures of 4 GnRH molecules are known (Fig. 1). The structure of mammalian GnRH was determined to be identical in sheep and pig hypothalamus and human placenta (Matsuo *et al.*, 1971; Burgus *et al.*, 1972; Tan and Rousseau, 1982). The amino acid sequence was established for salmon from whole brains (Sherwood *et al.*, 1983) and for chicken I and chicken II GnRH from hypothalamic tissues (King and Millar, 1982*a*, *b*; Miyamoto *et al.*, 1982, 1983, 1984; Millar and King

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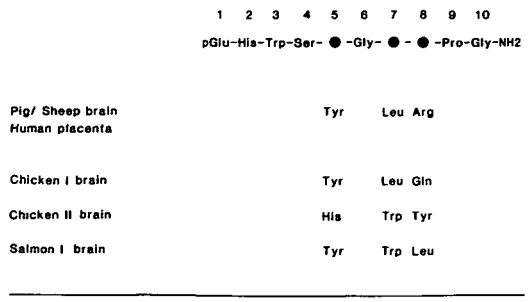

FIG. 1. The primary structure of the 4 identified forms of GnRH.

1983*b*). The major form of GnRH in rat and bullfrog brains has the same amino acid composition as mammalian GnRH, although the amino acid sequence has not been determined (Böhlen *et al.*, 1981; Rivier *et al.*, 1981). On the basis of these structures, obtained from 3 classes of vertebrates, it is clear that much of the molecule has been stable during 400 million years of evolution. Conserved are the length of the molecule, terminal amino acid structure and at least 70–90% of the amino acid sequence. This conservation of structure is not surprising given the short length of the molecule and importance of its function in relation to survival. Reproduction does not occur if the GnRH molecule is missing genetically (Cattanach *et al.*, 1977) or is blocked by immunoneutralization (Arimura *et al.*, 1973).

The evolutionary changes have occurred in the C-terminal amino acids in positions 5, 7 and 8. This part of the molecule appears to be important for receptor binding. Affinity for the mammalian GnRH receptors is reduced by the substitutions found in chicken and salmon GnRH. Salmon and chicken I GnRH show 5% or less of the affinity of mammalian GnRH for rat pituitary membrane homogenates (Millar and King, 1983*a*, *b*; Milton *et al.*, 1983; Sherwood *et al.*, 1983; Hasegawa *et al.*, 1984). And yet chicken I and mammalian GnRH are equally effective in binding to membrane receptors in chicken pituitary cells (Millar and King, 1983*a*). The binding studies imply that the mammalian and chicken receptors are different. The potency of GnRH molecules follows a similar pattern. Salmon and chicken I GnRH release 3% or less LH or FSH relative to mammalian GnRH in cultured mammalian pituitary cells; chicken II GnRH, however, releases 32% LH and 41% FSH compared with mammalian GnRH (Millar and King, 1983*a*, *b*; Milton *et al.*, 1983; Sherwood *et al.*, 1983; Hasegawa *et al.*, 1984; Miyamoto *et al.*, 1984). The binding of chicken II to mammalian pituitary cells is likely to be higher compared with salmon and chicken I. In cultured chicken pituitary cells, chicken I GnRH was either equipotent (Millar and King, 1983*a*, *b*; Milton *et al.*, 1983) or 2.7 times more potent (Hasegawa *et al.*, 1984) in releasing LH compared with mammalian GnRH. Other molecules with neutral or basic residues in position 8 of mammalian GnRH are biologically effective in the chicken system (King *et al.*, 1983).

Milton and associates (1983) argue that the chicken receptor is "promiscuous" in binding chicken and mammalian GnRH, but the mammalian receptor is "stringent" in recognizing GnRH. However, the chicken II data suggests the mammalian GnRH receptor may be somewhat less strict than previously thought. If the receptors have coevolved with GnRH molecules, then much of the loss of biological potency of the nonmammalian GnRH molecules in a mammalian system may depend primarily on their lack of ability to bind to the receptor. Receptor binding must precede LH or FSH release.

The part of the molecule which has not changed during evolution is the N-terminal residues. Amino acids in positions 2 and 3, His^2-Trp^3, appear to be essential for the release of LH and FSH from the pituitary (Burgus *et al.*, 1973; Schally and Coy, 1977; Vale *et al.*, 1977, 1981). Analogs with substitutions in positions 2 and 3 bind competitively to the pituitary receptors, but lack GnRH activity. It is known that the maximum release and rate of release of LH and FSH in rat pituitary cells is equal for mammalian and chicken I GnRH if equipotent solutions (40 × chicken I relative to mammalian) are applied. Hasegawa and colleagues argue that "the difference in their biological potencies arises from the differences in their binding affinities for rat pitu-

itary membrane receptors." Hence positions 2 and 3 may be critical for functional effects and amino acids in other positions for conformation, receptor binding and resistance to enzymatic breakdown.

FURTHER STRUCTURAL DIVERSITY IN THE GNRH FAMILY

Indirect evidence suggests that several more GnRH family members may exist. The amino acid sequence has not been determined, but high performance liquid chromatographic (HPLC) and immunological evidence suggests that several distinct GnRH molecules exist in Pisces.

The first of these molecules is found in lamprey brain. The lamprey is a representative of the oldest known class of vertebrates, Agnatha. These primitive, jawless vertebrates do not have a hypothalamopituitary portal system. However, GnRH was identified in their brains by immunological studies (Crim *et al.*, 1979; Nozaki and Kobayashi, 1979). Figure 2 shows that an extract made from lamprey (*Petromyzon marinus*) brain elutes from an HPLC column in the same position as synthetic mammalian and native rat GnRH. The lamprey GnRH-like activity is widely separated from synthetic or native salmonid (trout in this example) GnRH in our isocratic HPLC system. A further similarity between the lamprey and mammalian GnRH molecules is that they produce parallel inhibition of the binding of tracer to antiserum in a radioimmunoassay (Sherwood and Sower, 1985). In spite of these similarities, lamprey and mammalian GnRH can be distinguished immunologically. Two other antisera detect mammalian GnRH, but not the lamprey molecule. The hydrophobicity of both peptides was identical in our HPLC conditions suggesting that the amino acid substitution(s) which distinguish the molecules have similar hydrophobic properties. Also, a substitution in the C-terminal region of the molecule in lamprey compared with mammalian GnRH is likely because the unreactive antisera are directed toward that region.

A second GnRH-like molecule, which is not yet fully characterized, exists in sturgeon (*Acipenser transmontanus*) brain. The

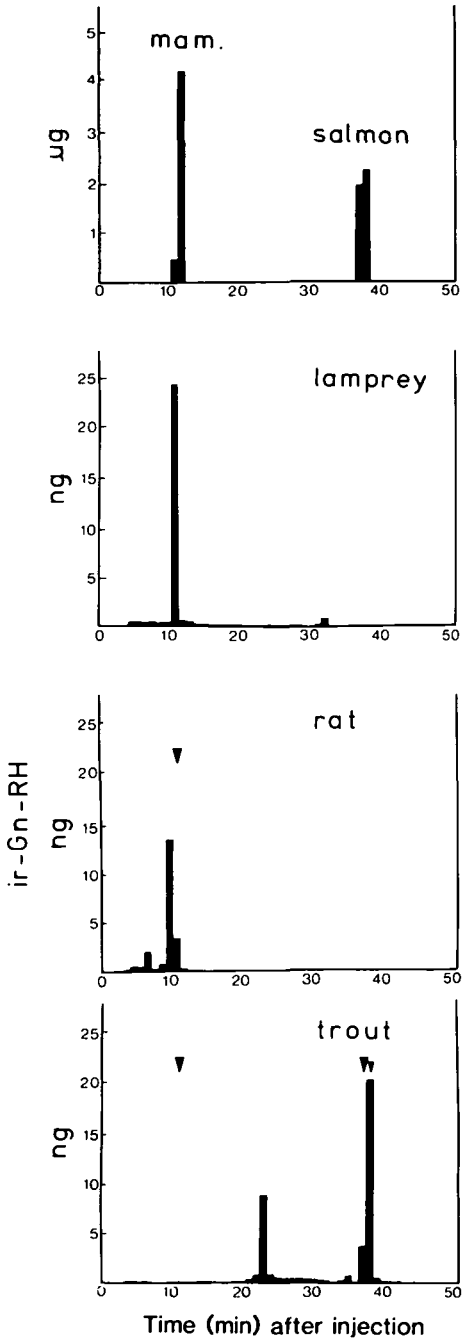

FIG. 2. Reverse-phase HPLC of lamprey brain extract containing immunoreactive gonadotropin-releasing hormone (ir-GnRH) is shown in the second figure from the top. The elution pattern of synthetic mammalian (mam.) and synthetic salmon GnRH are shown in the top figure. The arrows in the lower figures mark the elution of the standards run with rat and trout brain extracts. This figure is reproduced in part from Sherwood and Sower (1985) with permission.

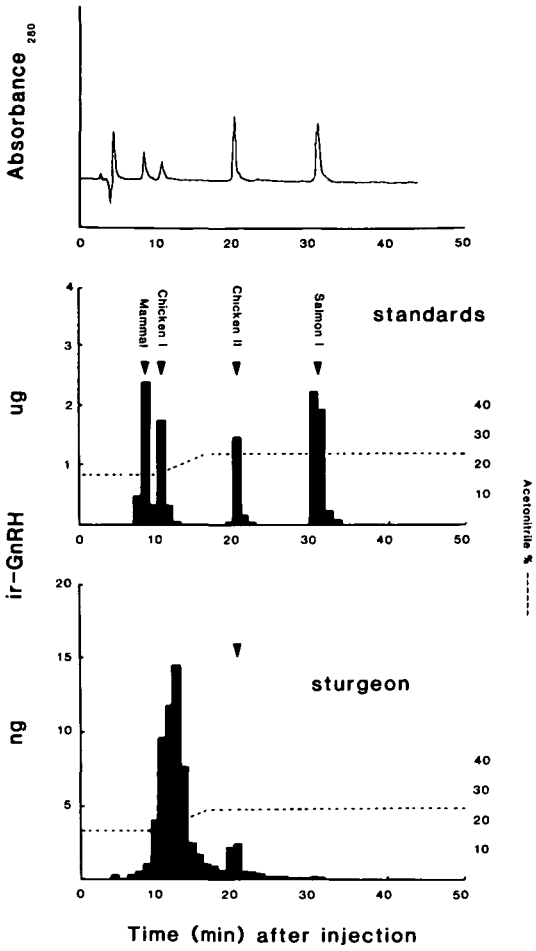

FIG. 3. Reverse-phase HPLC of sturgeon brain extract containing immunoreactive (ir) GnRH is shown in the bottom figure. The elution pattern of synthetic mammalian, chicken I, chicken II and salmon I are shown in the top and middle figures. The top figure shows the absorbance at 280 nm for the 4 standards. The middle figure shows the immunoreactive GnRH for the standards. The percent acetonitrile in the mobile phase is shown by a dotted line. The arrow in the bottom figure shows the elution time of chicken II GnRH (Sherwood, Carolsfeld, and Doroshov, unpublished).

sturgeon, a primitive bony fish, is distinct from the teleosts. Figure 3 shows the HPLC evidence which distinguishes sturgeon GnRH compared with lamprey, mammalian, chicken I, chicken II and salmon GnRH (Sherwood, Carolsfeld, and Doroshov, unpublished). The sturgeon molecule is unusual in its response to antibody B-6. Only mammalian and sturgeon GnRH-like molecules are recognized by B-6; the other GnRH-like peptides including chicken I and II are not. This suggests the change in the sturgeon molecule occurs in the N-terminal amino acids (possibly 2–5). The recognition of sturgeon extract by antiserum R-42, a conformational antibody, strongly supports the idea that sturgeon GnRH-like molecule has the same termini as mammalian, chicken and salmon GnRH. Injection of synthetic mammalian GnRH into sturgeon results in ovulation and an increase in plasma gonadotropin (Barannikova *et al.*, 1982).

The final molecule(s) which may be added to the GnRH family is found in several Pisces. I have classified several GnRH-like molecules as a single molecule labelled salmon II on the baiss of their known chromatographic and antigenic properties; further studies may show that more than one molecule exists. These GnRH-like molecules from the various species elute at the same time in our isocratic HPLC system; the molecules are recognized by antiserum R-42, but not by antiserum B-6. Figure 2 shows that this second form of GnRH in trout elutes from HPLC at a position intermediate to synthetic mammalian and salmon GnRH. This GnRH-like molecule(s) is also found in two elasmobranchs: dogfish shark, *Squalus acanthias,* and ratfish, *Hydrolagus colliei* (Fig. 4; Sherwood and Carolsfeld, unpublished). It occurs as a small second peak in sturgeon (Fig. 3) and as a second form in several teleosts: salmon (*Oncorhynchus keta*), trout (*Salmo gairdneri*), milkfish (*Chanos chanos*), mullet (*Mugil cephalus*) (Sherwood *et al.*, 1984), goldfish (*Carassius auratus*) (Sherwood and Harvey, 1986) and herring (*Clupea harengus pallasi*; Fig. 4; Sherwood and Carolsfeld, unpublished).

Additional forms of GnRH may be present in the brains of the fish mentioned above. The amounts are small judged by HPLC immunoreactive peaks. It remains to be shown by amino acid composition studies that these additional forms are distinct from the dominant forms.

EVOLUTION AND THE ANCESTRAL FORM

The phylogenetic pattern for neuropeptides such as GnRH must be determined

from structural changes in molecules from living organisms since soft tissues like brain do not fossilize. This limitation is perhaps balanced by the simplicity of the GnRH molecule. Not only can the basic molecule be clearly identified by structure and function, but a very limited number of changes appear to have occurred in the vertebrates. Variation of the molecule within individuals of a species has not been detected unlike the variation which occurs in anatomical or behavioral characteristics. Additionally, the presence of multiple forms of GnRH, some of which may be common to different classes of vertebrates, may help to establish the evolutionary thread for GnRH in vertebrates.

The phylogenetic distribution of different GnRH molecules is shown in Figure 5. The molecules for which the primary structure is not yet known are in lower case: a is lamprey; b is salmon II; and c is sturgeon GnRH-like molecule. For identified GnRH molecules, the letters are in capitals: D is salmon I; E is mammalian/amphibian; F is chicken I; and G is chicken II GnRH. Hagfish GnRH is represented by a zero (0) as we were unable to detect any GnRH activity in hagfish brains with 6 antisera directed against different parts of the mammalian molecule (Sherwood and Sower, 1985). This includes antiserum R-42, which we found recognizes all other forms of GnRH listed above (a-G). In hagfish other workers did not detect a GnRH-like molecule (Crim *et al.*, 1979; Nozaki and Kobayashi, 1979) or detected only picogram amounts in whole brains (Jackson, 1980; King and Millar, 1980). The salmon II molecule (b) as mentioned above is hypothesized to be present in ratfish, dogfish, sturgeon and 6 teleosts because these GnRH-like peptides have not been distinguished by our chromatographic or immunological methods to date.

The urodeles (salamanders) and anurans (frogs) both contain the mammalian form of GnRH as the dominant form in their brains. Additionally, we found that small amounts of two peptides which are immunologically and chromatographically identical to salmon I and II are present in brains of certain larval, neotenic (*Ambystoma gracile*) and adult (*Taricha granulosa*) salamanders and adult frogs (*Rana pipiens; Hyla regilla*) (Sherwood *et al.*, 1986). A late-eluting form of GnRH activity, possibly salmon I GnRH, was also reported to be present in small amounts in the brains of tadpole and adult bullfrog (Branton *et al.*, 1982). The most parsimonious interpretation of the presence of both mammalian- and salmon-like GnRH in anurans and urodeles is that a common phylogenetic ancestor also possessed the two forms of GnRH. The mammalian form of GnRH may have been present in labyrinthodont amphibians and crossopterygian fishes. The salmon forms of GnRH may have been present in chondrichthian (salmon II) or osteichthian

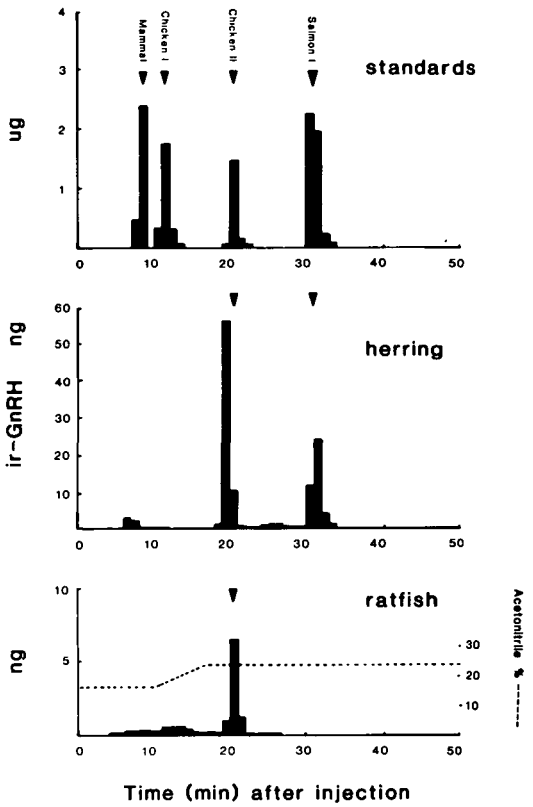

FIG. 4. Reverse-phase HPLC elution pattern of immunoreactive (ir) GnRH in herring brain extract (middle figure) and ratfish brain extract (lower figure). The elution of synthetic mammalian, chicken I, chicken II and salmon I GnRH are shown in the upper figure. The percent acetonitrile in the mobile phase is shown by a dotted line. The arrows in the figures show the elution time of the appropriate synthetic GnRH standard (Sherwood and Carolsfeld, unpublished).

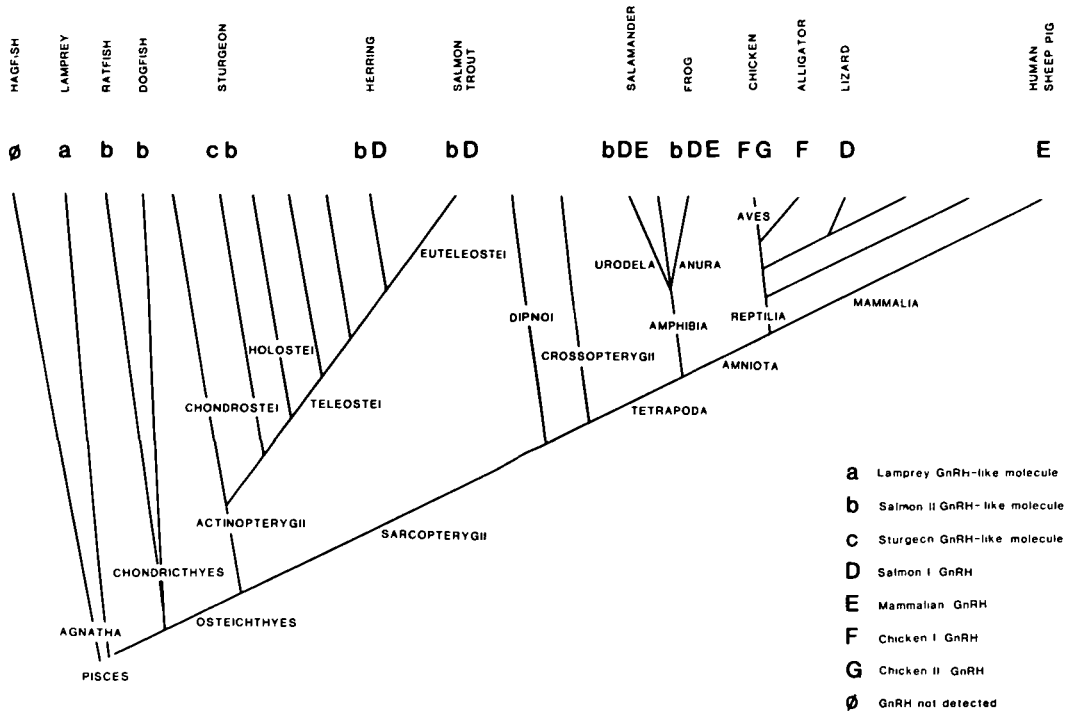

FIG. 5. A phylogenetic diagram showing the presence of GnRH-like peptides. The lower case letters (a, b, c) represent GnRH molecules which have been identified by indirect methods such as chromatography and immunoassay. The upper case letters (D, E, F, G) are for GnRH molecules with known primary structure. The animals listed at the top of the diagram are the representatives of the various subdivisions in which the peptide was studied. The presence of salmon II GnRH-like molecule in several groups is based on the fact that the peptides have not yet been differentiated by chromatographic or immunological methods.

(salmon I and II) ancestors. We cannot rule out the possibility that salmon I (D) evolved twice, first in teleosts and a second time in amphibian ancestors.

In reptiles, Millar (personal communication) has found a form of chicken I and chicken II-like GnRH in the brain of an alligator and salmon I in the brain of a South African lizard. The presence of chicken forms of GnRH in an alligator is not surprising in that the class Aves is thought to have separated from the reptilian stem. The subclass Archosauria of the reptiles is closest to this separation point and includes the order Crocodilia containing alligators. The presence of salmon I-like GnRH molecule in a lizard suggests that this form of GnRH may have existed in the ancestors of bony fish, amphibians and reptiles. In snakes (*Naja naja*) and turtles (*Sternotherus odoratus*), neither synthetic mammalian and chicken I GnRH nor their agonists produced a significant increase in plasma gonadotropin or steroids (Licht *et al.*, 1984).

Finally, the molecule labelled salmon II elutes from HPLC very close to chicken II; both are recognized by antiserum R-42, but not by B-6. Figure 4 shows that ratfish and herring GnRH-like activity elute at the same time as synthetic chicken II; Figure 3 also shows the second peak of sturgeon GnRH-like activity elutes near chicken II GnRH. It is too early to speculate whether these molecules are identical. It is critical to determine the amino acid sequence of salmon II to 1) verify that salmon II is distinct from salmon I and not a byproduct, and 2) determine whether salmon II is identical to ratfish, dogfish and possibly chicken II GnRH-like molecules. Perhaps a true cladistic diagram can be drawn after the identity of lamprey, sturgeon and salmon II GnRH molecules are known.

It is of considerable interest to speculate on the nature of an ancestral molecule. To date, amino acid substitutions have been identified only in positions 5, 7 and 8. Hence the ancestral molecule may have been very similar to the identified forms. This is like the story of oxytocin and vasopressin in which amino acid substitutions have occurred only in positions 3, 4 and 8 in this nonapeptide (Acher, 1981). In both GnRH and oxytocin/vasopressin, the peptide length and termini have remained stable.

The structure of the lamprey molecule may provide a nodal point in the analysis of an ancestral molecule. The HPLC elution position of lamprey GnRH suggests similarity to the amphibian/mammalian molecule; the amino acid substitution(s) may be between residues of similar hydrophobicity. Again the amino acid sequence is needed.

The lack of detection of a reproductive peptide in hagfish brains highlights the importance of the lamprey molecule as related to an ancestral form of GnRH. Although vertebrate reproductive peptides may have arisen later than the hagfish ancestor, it is also possible that hagfish have a degenerate nervous system that no longer has a GnRH-like peptide. The adult hagfish (*Eptatretus stouti*) lives in constant darkness and temperature in deep water; environmental cues acting on the nervous system may not be important. A continuous breeder, the hagfish can release mature eggs many months after hypophysectomy (Matty *et al.*, 1976; Gorbman, 1980). Another possibility is that hagfish contain a GnRH-like molecule that is structurally distinct from other vertebrate molecules as shown by the immunological studies mentioned above.

The existence of a reproductive GnRH-like peptide in invertebrates cannot yet be ruled out. In the phylum Chordata, GnRH immunoreactivity was identified in a tunicate (*Ciona intestinalis*) by an immunocytochemical study (Georges and Dubois, 1980). In another chordate, amphioxus (*Branchiostoma belcheri* Gray), injections of a GnRH agonist or human chorionic gonadotropin caused an increase in certain sex steroids (Chang *et al.*, 1983). In invertebrates, we did not find any GnRH immunoreactivity in the prawn (*Penaeus monodon*) eyestalk or abdominal ganglion (Sherwood and Harvey, unpublished) nor in *Schistosoma mansoni* with antiserum R-42 (Sherwood, Brownstein, and Basch, unpublished). However the case for GnRH-like molecules in more primitive animals remains open. Hunt and Dayhoff (1979) noted a structural similarity between yeast α mating factor and mammalian GnRH. Six of 13 amino acids in yeast α mating factor are homologous with mammalian GnRH. Likewise there is a functional homology between the two peptides. Yeast mating factor binds to rat pituitary GnRH receptors and stimulates the release of LH from cultured rat pituitary cells (Loumaye *et al.*, 1982). The conservation of the yeast molecule is interesting in that the homology of yeast mating factor is closer to mammalian GnRH (60% of mammalian residues) than to chicken I (50%) or chicken II and salmon (40%). Also present in the yeast factor is the critical His^2-Trp^3 portion thought to be important for gonadotropin release. Likewise the yeast residues match those for positions 7 and 8 in mammalian GnRH, unlike their counterparts in salmon, chicken I or chicken II.

Genetic Expression of a GnRH Precursor and Multiple Forms

The biosynthesis of GnRH, like that of many other hormones, involves cleavage of the molecule from a larger precursor. Recently Seeburg and Adelman (1984) reported on the structure of a GnRH precursor isolated from human placenta (Fig. 6). A signal peptide of 23 amino acids precedes the mammalian GnH molecule; three amino acids, glycine-lysine-arginine, follow. Glycine is thought to be an NH_2 donor for amidation of the C-terminal end of GnRH; lysine and arginine are the proposed cleavage site. The DNA sequence shows that a 53 amino acid peptide follows the cleavage site and is terminated by another lysine-lysine cleavage site. The 53 amino acid associated peptide is of unknown function, but suggestions include a role as a carrier similar to neurophysin or a role

Human GnRH Precursor

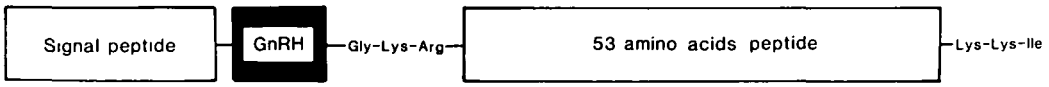

FIG. 6. Schematic representation of the precursor molecule for GnRH in human placenta. The diagram is drawn from data provided by Seeburg and Adelman, 1984. The signal peptide contains 23 amino acids.

as the releaser of FSH (Seeburg and Adelman, 1984).

Genomic analysis showed the presence of only one GnRH gene in the placenta, but closely related genes might not be seen using cDNA (complementary DNA) probes because the encoded sequence for GnRH is short. It is possible a related gene is present since Miyamoto and co-workers (1984) briefly mention their observation of the presence in rat and pig hypothalamus of two or more chromatographically distinct substances which release gonadotropins.

In submammalian tissue, the evidence is quite clear that multiple forms of GnRH can be expressed. It is possible the avian genome contains more than one gene for the expression of the two homologous forms of chicken GnRH. The structural difference in chicken I and II suggests a genomic rather than a post-translational change occurs in the molecules. It is not known if both forms of chicken GnRH are encoded in a single precursor molecule.

Multiple forms of GnRH also appear to be expressed in certain amphibian and fish brains, although the structural analysis of the molecules is not complete (Sherwood *et al.*, 1984, 1986; Sherwood and Sower, 1985). Rather, the existence of multiple forms of GnRH in bony fish, salamanders and frogs are based on chromatographic and immunological evidence. But clearly a pattern for expression of multiple forms of GnRH in one organ is emerging in several species.

In theory, point mutations or single nucleotide base changes are sufficient to explain the interchange of amino acids among mammalian, salmon I and chicken I GnRH. For example, the substitutions in position 7 involve leucine and tryptophan. Only a single change in the codon TGG (Trp^7) or TTG (Leu^7) would be needed. In fact, two changes in the codon have occurred. The analysis of the precursor for mammalian GnRH in placenta shows that two nucleotide bases have changed; Leu^7 is coded by CTG, whereas Trp^7 (salmon and chicken II GnRH) can only have the codon of TGG. The amino acid substitutions in position 8 require, in theory, a minimum of one nucleotide change among mammalian, salmon and chicken I GnRH, but a minimum of 2 base changes for the Tyr^8 in chicken II. In position 5, a minimum of one base change would be necessary for the His^5 (chicken II) and Tyr^5 (mammalian, salmon, chicken II) interchange. In view of the similar elution of salmon II and chicken II, it is noted that chicken II differs from salmon I only in 2 positions (5 and 8), whereas chicken II differs from chicken I and mammalian GnRH in 3 positions.

DIVERSITY OF FUNCTION

For many years a single function was ascribed to GnRH: the release of LH and FSH from the pituitary. A reassessment of this function has been necessary because of the presence of 1) multiple forms of GnRH in the brain of certain species, 2) GnRH in the brain in extrahypothalamic areas, 3) GnRH-like molecules in a number of tissues outside the CNS, including both peripheral and autonomic nervous system and nonneural tissues, and (4) specific

receptors for GnRH outside of the pituitary. Study of novel functions of GnRH is in an early stage; much of the evidence is based on the assumption that the expression of the peptide in tissues outside the preoptic-hypothalamic area means the peptide is not acting to release pituitary hormones. The expression of GnRH in different tissues may be related to the long (over 1,000 nucleotides) untranslated region preceding the DNA coding for GnRH precursor (Seeburg and Adelman, 1984).

The function of multiple forms in the brain is not clear. That the separate forms in chicken might release LH and FSH respectively, seems unlikely at this time. Chicken II is 10 times more potent compared with chicken I in releasing both LH and FSH from rat anterior pituitary cells (Miyamoto *et al.*, 1984).

The mediation of certain environmental factors on reproductive behavior and events has been suggested for the GnRH neurons near the olfactory bulbs. These GnRH neurons in the ventral telencephalon have been located by immunocytochemical methods and their location in a variety of vertebrates is reviewed by Peter (1983) and Demski (1984). This rostral GnRH system has been characterized most thoroughly in teleosts and mammals. In the rat, GnRH neurons or fibers were found along the nervus terminalis and within both the main and accessory olfactory bulbs; some GnRH fibers extended to the mucosa of the epithelium of the vomeronasal organ. Other GnRH fibers in the accessory olfactory tract made connections with the amygdala and bed nucleus of the stria terminalis (Witkin and Silverman, 1983). In goldfish, GnRH neurons in the terminal nerve are closely associated with the olfactory nerve. Cell bodies are located near the rostromedial aspect of the olfactory bulb; GnRH fibers extend rostrally within the olfactory nerve and caudally in the medial olfactory tract to various brain regions and to the optic nerves and retina (Stell *et al.*, 1984). Some preliminary results support the idea that this olfactory-related GnRH network translates olfactory signals into reproductive events in mammals (see Witkin and Silverman, 1983). Demski and Northcutt (1983) also speculate that in certain fish the terminal nerve GnRH system is important in the pheromonal triggering of sexual responses; courtship behavior is reduced if the system is damaged as is sperm release in response to electrical stimulation of the olfactory tract (see Demski, 1984). Halpern-Sebold and Schreibman (1983) argue that the sequential development of the GnRH centers in the brain from rostral (nucleus olfactoretinalis) to caudal may be related to the development and maintenance of reproduction including mediation of environmental influences in the reproductive system.

Another group of GnRH cells existing outside the preoptic-hypothalamic region is in the limbic system and midbrain (Demski, 1984; King and Anthony, 1984). Their function is unknown, but their proximity in some cases to steroid-concentrating cells suggests they may be involved in sexual behavior or motivation (Demski, 1984). It is known that GnRH can enhance mating behavior in certain animal preparations (Moss and McCann, 1973) and that GnRH antagonists can suppress mating behavior in castrated estrogen-progesterone treated female rats (see Vale *et al.*, 1981).

The presence of GnRH fibers in the retina suggests a possible neurotransmitter function. Certain GnRH neurons in the terminal nerve system in fish send axons to the retina (Demski and Northcutt, 1983; Stell *et al.*, 1984). In goldfish, application of synthetic salmon GnRH modulates the electrophysiological response in retinal ganglion cells. Stell and co-workers (1984) suggest that sex related olfactory stimuli may act through the GnRH terminal nerve to alter visual responsiveness of certain retinal neurons. In amphibians, the retina in bullfrog (*Rana catesbeiana*) contains 2 forms of GnRH; the forms elute with mammalian GnRH and with carp GnRH-like material (Eiden *et al.*, 1982). GnRH in the retina has been identified in fish (carp, goldfish and trout) and amphibians (bullfrog), but not in reptiles (turtle), birds (chicken) or mammals (rat, guinea pig and monkey) (Eiden *et al.*, 1982).

Diversity of GnRH function and form is

also expressed in the autonomic nervous system. The first indication that molecular heterogeneity of GnRH could occur within the same animal was reported for amphibians (Eiden and Eskay, 1980). Frog brain contains predominately the mammalian form of GnRH; sympathetic ganglia and adrenal glands contain a form of GnRH which is chromatographically and immunologically distinct compared with mammalian GnRH. It is speculated that the sympathetic ganglionic form of GnRH is similar to "piscine" GnRH (Eiden *et al.*, 1982). Also frog ganglionic GnRH and synthetic salmon GnRH and its analogs are more potent than their mammalian counterparts in mimicking the neurotransmitter actions of ganglionic GnRH (Jan *et al.*, 1983; Jones *et al.*, 1984) indicating a further similarity of structure. The role of a neurotransmitter is the only function suggested to date for the sympathetic form of GnRH in amphibians. Extensive evidence supports the idea that sympathetic ganglionic GnRH-like peptide mediates the late slow excitatory postsynaptic potential (EPSP) in bullfrog sympathetic ganglia (Jan *et al.*, 1979, 1983; Jan and Jan, 1982; Jones *et al.*, 1984). There is no known relationship between this function and the reproductive system.

GnRH also appears in nonneural tissues. In mammals the ovary was reported to contain a GnRH-like peptide, gonadocrinin (Ying *et al.*, 1981), although later the same investigators were unable to reproduce the original conditions for isolation of the peptide (Guillemin, 1982). However, indirect evidence supports the concept that a GnRH-like molecule may be present in the ovary. Hypothalamic GnRH does not appear to reach peripheral tissues due to its transport in the portal system and rapid degradation by the pituitary. And yet high affinity receptors for GnRH agonists exist in the ovary (Clayton *et al.*, 1979; Harwood *et al.*, 1980; Dalkin *et al.*, 1981; Pieper *et al.*, 1981). Also synthetic GnRH and its agonists inhibit gonadotropin-induced increases in estrogen and progesterone secretion both *in vitro* in cultured ovarian cells and *in vivo* in hypophysectomized rats (Clayton *et al.*, 1979; Hsueh and Erickson, 1979*a*; Harwood *et al.*, 1980).

Another peripheral tissue which may contain a GnRH-like peptide is the testes. In 1981, the seminiferous tubules from both rat and macaque were reported to "contain a factor which has LHRH (GnRH)-like receptor binding and biological activity *in vitro*, but which is immunologically distinct from native LHRH" (Sharpe *et al.*, 1981). This factor, secreted *in vitro* by cultured rat Sertoli cells, was hypothesized to exert local control on steroidogenesis. A variety of GnRH antisera have been tested against testicular GnRH. Although some are reported to cross-react, purification of the testicular molecule has not been reported to date.

The presence of GnRH specific membrane receptors in the Leydig cells of the testes supports the idea that a GnRH-like molecule acts in the tissue (Clayton *et al.*, 1980; Sharpe and Fraser, 1980; Dalkin *et al.*, 1981; Pieper *et al.*, 1981; Sharpe *et al.*, 1981, 1982). The specificity and binding of GnRH and its analogues for GnRH receptors is similar in the anterior pituitary, ovary and testes (Clayton *et al.*, 1980; Reeves *et al.*, 1980; Sharpe and Fraser, 1980; Pieper *et al.*, 1981; and see Sharpe, 1982). Indeed, prolonged administration of pharmacological amounts of GnRH or its agonists produce striking antifertility effects: a decrease in the weight of the testes, seminal vesicles and ventral prostate; degeneration of seminiferous tubules; inhibition of spermatogenesis; a decline in testicular LH/hCG receptors and testosterone secretion; inhibition of ovulation, ovum transport, ovum implantation, pregnancy, puberty, gonadotropin receptors, ovarian-dependent mammary tumorigenesis and estrogen and progesterone secretion (Auclair *et al.*, 1977; Pelletier *et al.*, 1978; Cusan *et al.*, 1979; and see Hsueh and Erickson, 1979*a*, *b*). That these GnRH effects on the gonads are at least partially independent of the action of GnRH on the pituitary gland was shown in hypophysectomized animals and in tissue cultures of gonadal cells (Clayton *et al.*, 1979, 1980; Hsueh and Erickson 1979*a*, *b*; Harwood *et*

al., 1980). In summary, the purpose of the gonadal receptors for GnRH and the structure of a GnRH-like molecule in the gonads remains to be elucidated.

The placenta story is more clear. The placenta synthesizes GnRH of identical structure to hypothalamic peptide; the content varies with the stage of pregnancy. Low affinity placental receptors for GnRH also exist (Belisle *et al.*, 1984). Synthetic GnRH incubated with placental tissues releases human chorionic gonadotropin and steroids (Khodr and Siler-Khodr, 1978; Belisle *et al.*, 1984).

The function is not apparent for the GnRH-like peptide reported to be present in pancreatic islets in rats and humans (Seppälä *et al.*, 1979; Seppälä and Wahlström, 1980*a*). Immunoreactive GnRH has also been detected in certain human tumors: in pancreatic tumors of the islets (Wahlström and Seppälä, 1979) and in certain ductal carcinomas of the mammary gland (Seppälä and Wahlström, 1980*b*).

There is clearly an evolutionary trend in the diverse functions of GnRH. Although the olfactory-related system is well developed in teleosts and mammals and probably the other less-studied vertebrates, GnRH in the retina can only be detected in fish and amphibians. Likewise GnRH in sympathetic ganglia has only been identified in amphibians and not in higher vertebrates. The appearance of GnRH in nonneural tissues and its effects on the gonads has been most thoroughly documented in mammals. There is some indication that fish and amphibian gonads are not as easily inhibited by GnRH compared with mammalian gonads. Finally, the presence of GnRH and its effects in the placenta are limited to mammals. Thus, both the novel functions and structures of GnRH clearly have evolved.

NOTE ADDED IN PROOF

The sequence of rat GnRH is now confirmed to be identical to mammalian GnRH (J. P. Adelman, A. J. Mason, J. S. Hayflick, and P. H. Seeburg. 1986. Proc. Natl. Acad. Sci. U.S.A., 83:179–183). The sequence of lamprey GnRH is pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2 (Sherwood, N. M., S. A. Sower, D. R. Marshak, B. A. Fraser, and M. J. Brownstein. 1986. J. Biol. Chem. 261:4812–4819).

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