EVOLUTION OF GnRH IN FISH OF ANCIENT ORIGINS

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Summary

A key neuroendocrine function of the hypothalamus is the release of the decapeptide gonadotropin-releasing hormone (GnRH) which in turn acts on the pituitary regulating the pituitary-gonadal axis for all vertebrates. Agnathans are of particular importance in understanding hypothalamic-pituitary relationships since they represent the oldest lineage of vertebrates which evolved over 550 million years ago. The agnathans are classified into two groups, myxinoids (hagfish) and petromyzonids (lamprey). Lampreys are the first vertebrates to clearly demonstrate roles for multiple GnRH molecules as neurohormones involved in reproduction. In addition, we suggest from structure-activity and receptor studies that lamprey GnRH receptor requirements for GnRH are different in the lamprey from those of all other vertebrates. This paper summarizes our current studies on the structure, function, distribution and embryonic origin of lamprey GnRH-I and -III and the evolution of GnRH systems in vertebrates.

Reproductive Cycle of the Lampreys

There are approximately 32 species of living lampreys that are classified as parasitic or nonparasitic. Lamprey spawn only once in their lifetime after which they die. Sexual maturation is a seasonal, synchronized process. The sea lampreys, *Petromyzon marinus*, begin their lives as freshwater, filter feeding larvae which burrow in the bottoms of streams (Hardisty and Potter, 1972). After approximately five to seven years in freshwater streams, metamorphosis occurs and the larvae become free swimming, sexually immature lampreys, which migrate to the sea or lakes. While the lampreys are in the parasitic sea phase, gametogenesis progresses. In males, spermatogonia proliferate and develop into primary and secondary spermatocytes and in females, vitellogenesis occurs. After approximately 15 months at sea, lampreys return to freshwater streams to spawn and undergo the final maturational processes resulting in mature eggs and sperm.

Gonadotropin-Releasing Hormone: Structure

A key neuroendocrine function of the hypothalamus is the release of the decapeptide, GnRH, which in turn acts on the pituitary regulating the pituitary-gonadal axis for all vertebrates. Currently, nine primary structures of GnRH have been determined in various representatives of vertebrates. Included in this family are the structures of GnRHs of three fish species of ancient origin, an agnathan, the sea lamprey (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).

Previous studies have led to the identification of two molecular forms of gonadotropin-releasing hormone (GnRH-I and II) in the brain of the sea lamprey. From analysis of these two forms, the primary structure of GnRH-I and the amino acid composition of GnRH-II were determined (Sherwood et al., 1986). We have now isolated a third molecular form of GnRH (lamprey GnRH-III) from the brain of this species that is different from GnRH-I and -II. We determined the primary structure of lamprey GnRH-III as pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2 (Sower et al., 1993). The primary structure of lamprey GnRH-III differs in three amino acids compared with lamprey GnRH-I. Lamprey GnRH-III is more closely related to the other members of the GnRH family than lamprey GnRH-I. Lamprey GnRH-III has 80% identity with chicken GnRH-II and dogfish GnRH; 70% identity with catfish GnRH-I, lamprey GnRH-I, and salmon GnRH; and 60% identity with mammal GnRH and chicken GnRH-I (Sower *et al.*, 1993). In all GnRH peptides, certain regions of the molecule have been highly conserved including the NH_2-terminal, pGlu[1]- His[2] and Ser[4] and the COOH- terminal. These regions and the length of the molecule have remained unchanged during 500 million years of evolution.

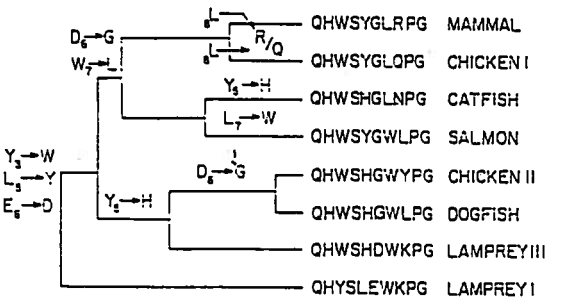

Fig. 1. Phylogenetic tree based on eight sequences of the primary structures of GnRH. One arrow signifies one base change. (Sower et al., 1993)

Gonadotropin-Releasing Hormone: Function

Until the past few years, there was little evidence for a regulatory influence of the hypothalamus on the pituitary-gonadal axis in Agnathans. Using synthetic lamprey GnRH-I and analogs in our earlier studies, these experiments provided the first evidence of neuroendocrine control of reproduction in lampreys. The biological activity of lamprey GnRH-I or -III has been assessed by steroidogenesis or gametogenesis in *in vitro* and *in vivo* studies (Review: Sower, 1990; Sower et al., 1993; Deragon and Sower, 1994). Other studies showed seasonal correlations between changes in brain GnRH and gametogenic and steroidogenic activity of the gonads in adult male and female sea lampreys

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(Fahien and Sower, 1990; Bolduc and Sower, 1992). Our recent studies indicate that lamprey GnRH-III is also a neurohormone involved in reproduction based on its ability to stimulate steroidogenesis and gametogenesis in adult sea lampreys (Sower et al., 1993; Deragon and Sower, 1994) and of the occurrence of this peptide in lampreys undergoing different stages of metamorphosis coinciding with the acceleration of gonad maturation (Youson and Sower, 1991).

The purification of lamprey gonadotropin(s) has been a very difficult project due to the size of the pituitary and difficulties associated with purification. However, all available information strongly suggests that lamprey pituitaries have a reasonably typical pituitary-gonadal axis. (see Review, Sower, 1990).

Lamprey GnRH-I and lamprey GnRH-III are the only two members of the GnRH family to have substitutions in the sixth position, Glu^6 and Asp^6, respectively; all other GnRH peptides have Gly in the sixth position suggesting a different conformational structure. Thus, a structure-activity study of lamprey GnRH-I or analogs that were cyclized or with sixth position substitutions were determined *in vivo* in adult female sea lamprey (Sower, et al., In Press). The following analogs which were tested, ([D-Glu^6]-GnRH-I; cyclo-[D-Glu^6-Trp^7-Lys^8]-GnRH-I; or cyclo-[Glu^6-Trp^7-Lys^8]-GnRH-I), significantly elevated plasma estradiol compared to controls. However, [D-Glu^6]-lamprey GnRH-I was the only analog to significantly stimulate ovulation while another analog [Gly^6]-lamprey GnRH-I significantly delayed ovulation. These data suggest that the sixth position of lamprey GnRH is critical for function.

Pituitary GnRH Receptor:

Quantitative *in vitro* autoradiography was used to characterize and localize putative gonadotropin-releasing hormone (GnRH) receptors in the anterior pituitary of the adult female sea lamprey (Knox et al., 1994). Scatchard analysis revealed two classes of high affinity binding sites with K_d's of 1.5 X 10^{-12} M and 5 X 10^{-9}M and B_{max}'s of 8.4 x 10^{-14} M and 5 x 10^{-11}M, respectively. Binding to the GnRH receptors was saturable, reversible, tissue specific and time- and temperature-dependent. Displacement studies showed that labeled peptide could be displaced by chicken GnRH-I, chicken GnRH-II, synthetic mammal, salmon lamprey GnRH-I, lamprey GnRH-III, DAla^6,Pro^9 NEt mammalian GnRH and DPhe^{2,6},Pro^3 lamprey GnRH. The proximal pars distalis region of the anterior pituitary contained most of the GnRH binding sites with slight binding in the rostral pars distalis. These data provide the first direct evidence of GnRH activity in the pituitary of an Agnathan and are the first to demonstrate that a vertebrate pituitary contains two high affinity binding sites for GnRH.

Brain-Pituitary Relations and Distribution of GnRH:

Unlike most vertebrates, a distinct vascular or neural link between the hypothalamus and the adenohypophysis has not been observed in either lamprey or hagfish (Gorbman, 1965). In the lamprey, the neurohypophysis and adenohypophysis are separated by avascular connective tissue (Gorbman, 1965). However, there is anatomical evidence to support the concept of hypothalamic control of adenohypophysial function by diffusion of the neurohormones from the neurohypophysis to the pars distalis of the adenohypophysis (Nozaki et al., 1984; King et al., 1988). In the lamprey, GnRH-like neurons identified by immunocytochemistry project their fibers primarily into the neurohypophysis from the preoptic region (Nozaki et al., 1984; King et al. 1988). In these same studies, GnRH was not demonstrated to be widely distributed in extra hypothalamic regions as noted for other neuropeptides within the lamprey brain. We propose in this latter study that an additional route of GnRH is via secretion into the third ventricle and transported by tanycytes to the adenohypophysis (King et al., 1988). We experimentally examined the functional anatomical relationship between the hypothalamus and adenohypophysis in sea lamprey. Horseradish peroxidase (HRP) was injected into the third ventricle of the brain of adult lampreys (Nozaki et al., 1994). Within 5 to 15 minutes HRP had passed through the neurohypophysis, which forms the floor of the third ventricle, and diffused throughout the connective tissue separating the adenohypophysial follicles from the neurohypophysis and into intracellular spaces in the adenohypophysis. We conclude that neurosecretory peptides are able to diffuse from the brain to the adenohypophysis, and thus regulate its secretory activity in lampreys. Thus, there is evidence of normal occurrence of GnRH in a part of the lamprey brain homologous with that brain region in higher vertebrates in which GnRH localization forms part of a neuroendocrine mechanism for gonadotropin secretion.

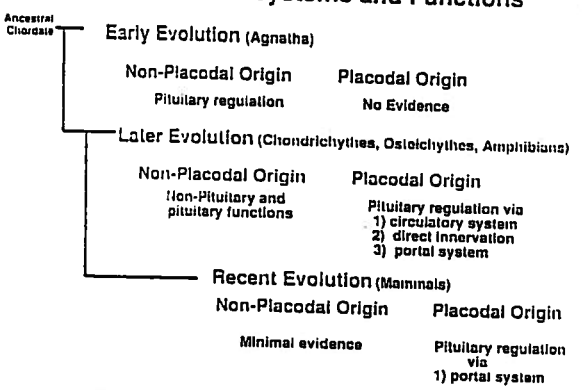
Using antibodies to the lamprey GnRH-III molecule, several immunocytochemistry studies have been completed. Both lamprey GnRH-I and -III immunoreaction were found in the cell bodies in the rostral hypothalamus and preoptic area in larval (Wright et al., 1994; Tobet et al., 1995a) and adult sea lamprey (Nozaki, Gorbman and Sower, unpublished). We have suggested that in the larval stage, the majority of the irGnRH is lamprey GnRH-III indicating that GnRH-III perhaps is the more active form during reproductive maturation (Tobet et al., 1995a).

Origin and Evolution of GnRH During Development

Chromatographic and immunological studies of vertebrate brain extracts have shown two or more GnRH-like peptides in representative species of all vertebrate classes (Muske, 1993). The functional significance of multiple forms of GnRH within the brain and in extrahypothalamic locations within the same species has not been established with the possible exception of lampreys. However, the GnRHs have apparently multiple actions on reproductive physiology and behavior either through pituitary or non-pituitary stimulation depending on the origin of the GnRH system during development. Muske (1993) has proposed that gnathostome vertebrates have two

principle GnRH systems each with different embryonic origins expressing different molecular forms of GnRH and projecting to different targets. In the vertebrates examined, neurons which contain forms of GnRH which are considered to regulate pituitary-gonadal functional are thought to be derived from progenitor cells in the olfactory placode which then will migrate to its final position in the preoptic /hypothalamic areas. With some exceptions, the other GnRH system arises from non-placodal origin and involved in non-pituitary-gonadal function. Our most recent experiments in lampreys were conducted to characterize the earliest development of GnRH neurons and determine the probable pathway of their migration (Tobet et al., 1995b). GnRH neurons were first visualized at day 22 after fertilization in the preoptic area and hypothalamus. GnRH neurons were not seen within the olfactory system. In contrast to all other vertebrates, we propose that GnRH neurons in developing lampreys originate within proliferative zones of the diencephalon and not ir the olfactory system. Thus, we propose the following hypothesis as shown in Fig. 2.

Evolution of GnRH Systems and Functions



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