GnRH NEURONS
GENE TO BEHAVIOR

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EVOLUTION OF GnRH IN
FISH OF ANCIENT ORIGINS

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Chapter 2
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INTRODUCTION

A key neuroendocrine function of the hypothalamus is the release of the decapptide, gonadotropin-releasing hormone (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 and two for invertebrates. Included in this family are the structures of GnRHs of three fish species of ancient origin, an agnathan, the sea lamprey, *Petromyzon marinus*, (lamprey GnRH-I and -III)\(^{34,64}\); an elasmobranch, the spiny dogfish shark, *Squalus acanthias*, (dogfish GnRH and chicken GnRH-II)\(^{38}\); and a holocephalan, the ratfish, *Hydrolagus colleii*, (chicken GnRH-II)\(^{37}\).

This chapter will summarize the most recent information on the
structure and function of GnRH in the agnathans. Agnathans are of particular importance in understanding hypothalamic-pituitary relationships since they represent one of the oldest lineage of vertebrates which evolved over 500 million years ago. The agnathans are classified into two groups, myxinoids (hagfish) and petromyzonids (lamprey). Lampreys are the earliest vertebrates for which there are demonstrated functional roles for multiple GnRH neurohormones involved in pituitary-reproductive activity. Both lamprey GnRH-I and -III have been shown to induce steroidogenesis and spermatiation and/or ovulation in adult sea lampreys. In lampreys undergoing metamorphosis, there was a demonstrated increase of brain lamprey GnRH-I and -III which coincided with the acceleration of gonadal maturation. In immunocytochemistry studies, both lamprey GnRH-I and -III immunoreaction were found in the cell bodies in the rostral hypothalamus and preoptic area in larval and adult sea lamprey (Nozaki, Gorbman, and Sower, unpublished). Tobet et al. have suggested that in the larval stage, most of the irGnRH is lamprey GnRH-III, indicating that GnRH-III may be the more active form during gonadal maturation. These data suggest that the structure and function of the GnRHs in vertebrates appear to be highly conserved throughout vertebrate evolution.

In Atlantic hagfish, chromatographic and immunocytochemical evidence showed that the neurohypophysis contains a gonadotropin-releasing hormone (GnRH)-like molecule that is closely related to lamprey GnRH-III. In other immunocytochemical studies using the Pacific hagfish, Eptatretus stouti, Braun et al. have suggested that there are two GnRH systems in hagfish, one system which is widely diffuse throughout the brain and the other restricted to the preoptic-neurohypophysial system. Thus, modern hagfish may have retained one or more of the early or stem GnRH forms. Chicken GnRH-II now has been structurally sequenced in representative species of five of the seven classes of vertebrates. 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 two single base mutations.

1 LIFE HISTORIES

Modern vertebrates are classified into two major groups, the gnathostomes (jawed vertebrates) and the agnathans (jawless vertebrates). The agnathans are divided into two groups, myxinoids (hagfish) and petromyzonids (lamprey); while, the gnathostomes constitute all the other living vertebrates, including the bony and cartilaginous fishes and the tetrapods. Forey and Janvier have hypothesized from their phylogenetic analysis that modern lampreys are more closely related to gnathostomes than
they are to hagfish (Fig. 2-1). The hagfishes are considered the most primitive vertebrates known, living or extinct.

1.1 Reproductive Cycle of the Hagfish

The endocrinology and physiology of reproduction of the hagfishes are probably the least understood of all the vertebrates\textsuperscript{16}. Many of the unknown factors include the age of reproduction, whether ovogenesis is arrhythmic or cyclic or time of year of reproduction. \textit{Eptatretus burgeri} is the only known species of hagfish that has a regular annual reproductive cycle and undergoes an annual migration\textsuperscript{34}. The reproductive patterns of all other hagfish are unknown. \textit{Myxine glutinosa} live in deep water of 100-300 m on soft muddy bottoms. These hagfish were once considered functional hermaphrodites with their single unpaired sex organ developing sperm only in the posterior portion, then eggs later in the anterior portion. Subsequent investigations have shown that the hagfish are not hermaphrodites, but that the hagfish gonads undergo differentiation into male and female gonads\textsuperscript{16}. Hagfish reproduce by laying large oval eggs, enclosed in a tough shell with threads at each end which act as anchors in the mud.

1.2 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. The parasitic lampreys are generally anadromous. All larval lampreys, called ammocoetes, live in fresh water as burrowing organisms in the bottoms of streams or lakes. In the parasitic sea lamprey, sexual maturation is a seasonal, synchronized process. The sea lampreys begin their lives as freshwater ammocoetes, which are blind, filter feeding larvae\textsuperscript{22}. After approximately five to seven years in freshwater streams, metamorphosis occurs and the ammocoetes become free swimming, sexually immature.

Fig. 2-1: A phylogenetic tree modified from Forey and Janvier\textsuperscript{13}. 
lampreys, which migrate to the sea or lakes\textsuperscript{21}. During the approximately 15 month long parasitic sea phase, gametogenesis progresses. In males, spermatogonia proliferate and develop into primary and secondary spermatocytes, in females, vitellogenesis occurs\textsuperscript{21}. 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.

2 STRUCTURAL AND IDENTIFICATION STUDIES

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, *Petromyzon marinus* (lamprey GnRH-I and -III\textsuperscript{54,64}; an elasmobranch, the spiny dogfish shark, *Squalus acanthias*, (dogfish GnRH and chicken GnRH-II)\textsuperscript{38}; and a holocephalan, the ratfish, *Hydrolagus colliei*, (chicken GnRH-II)\textsuperscript{37}. A taxonomic distribution of the known primary structures are shown in Fig.2-2.

Three distinct GnRHs exist in lampreys\textsuperscript{54,64}. Previous studies had led to the identification of two molecular forms of gonadotropin-releasing hormone (GnRH-I and -II) in the brain of the sea lamprey\textsuperscript{54}. Analysis of these two forms yielded the primary structure of GnRH-I and the amino acid composition of GnRH-II\textsuperscript{54}. A third molecular form of GnRH (lamprey GnRH-III) from the brains of the sea lamprey was isolated which is distinctly different from GnRH-I and -II\textsuperscript{64}. The primary structure of lamprey GnRH-III is pGlu-His-

![Fig. 2-2: A taxonomic distribution of the primary structures of GnRHs identified in vertebrates. M, mammalian GnRH; CI, chicken GnRH-I; SB, seabream GnRH; C, catfish GnRH; S, salmon GnRH; CII, chicken GnRH-II; D, dogfish GnRH; LIII, lamprey GnRH-III; LI, lamprey GnRH-I.](image-url)
Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH₂. In these studies, lamprey GnRH-II was more hydrophobic than lamprey GnRH-I or -III and differed from lamprey GnRH-I by three residues (Ile, Phe, and His instead of Glu, Lys, and Tyr) and from lamprey GnRH-III by three residues (Ile, Phe and Leu instead of Asp, Lys and Trp). The primary structure of lamprey GnRH-III differs by three amino acids, compared with lamprey GnRH-I. Lamprey GnRH-III is more closely related to the other members of the GnRH family than is lamprey GnRH-I. Lamprey GnRH-III has 80% sequence 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-I⁶⁴. In all GnRH peptides, certain regions of the molecule have been highly conserved, including the NH₂-terminal, pGlu¹-His² and Ser⁴, and the COOH-terminal. These regions and the length of the molecule have remained unchanged during the 500 million years of evolution of the lamprey group. The conservation of the NH₂- and COOH-termini suggests these regions are significant for conformation, receptor binding, resistance to enzymatic degradation and in receptor-mediated events required for gonadotropin release ⁴⁰.

Based on the known sequences, a phylogenetic tree is presented in Fig. 2-3. It is proposed that there are three sets of GnRH molecules with 9 single-base changes in genetic codons not including position 8⁶⁴. Mammal GnRH and chicken GnRH-I can be converted from each other by single-base changes. The conversion of lamprey GnRH-III to chicken GnRH-II would require a minimum of two base changes. Lamprey GnRH-I is far removed from the other peptides by a minimum of 5 to 7 base changes and is considered the outlier and closer to the ancestral molecule. However, based on the structure of

![Phylogenetic tree of GnRH](image)

Fig. 2-3: A vertebrate phylogenetic GnRH tree adapted from Sower et al.⁶⁴. One arrow signifies one base change. Lamprey GnRH-I is considered to be the outlier.
lamprey GnRH-III and that position 8 has experienced multiple changes, it is predicted that there are at least two other GnRH forms because of at least one non-connected residue in position 5 between Leu and His\textsuperscript{64}. In addition, these authors predict that one of the unidentified GnRH forms would have a His in the 8th position\textsuperscript{44}. Miyamoto et al.\textsuperscript{41} and Lovejoy et al.\textsuperscript{37} have suggested that chicken GnRH-II has occurred at a much earlier stage of evolution compared to the other forms (except lamprey GnRH-I). Chicken GnRH-II was structurally characterized in the ratfish (class Chondrichthyes) which diverged from the line of evolution about 400 million years ago\textsuperscript{37}. This information combined with the structure of lamprey GnRH-III and dogfish GnRH suggest that these three molecules are more closely related to the ancestral molecule(s). Thus, Sower et al.\textsuperscript{64} predict that Leu\textsuperscript{5} evolved first followed by a conversion to Tyr then to His or that His\textsuperscript{5} and Leu\textsuperscript{5} evolved separately.

The sixth position is considered critical for function of the molecule. Conformation energy analysis of mammal GnRH suggests that the termini of the molecule are proximal\textsuperscript{42} and that the GnRH interacts with the receptor in the folded conformation due to the 6-7\beta turn\textsuperscript{67}. Interestingly, both lamprey GnRH-I and -III are the only molecules to have substitutions in the sixth position, Glu\textsuperscript{6} and Asp\textsuperscript{6}, respectively. Glu appears to have evolved first with a single base change to Asp followed by a single base change to Gly. Sower et al.\textsuperscript{66} have tested the biological activity of Gly\textsuperscript{6} lamprey GnRH in lampreys and determined that the substitution of Gly for Glu inhibited the biological activity. These data would suggest that the receptor requirements for GnRH are different in the lampreys from those in other vertebrates.

The most variable position is position eight. Of the GnRH peptides, only lamprey GnRH-I and -III, tunicate and salmon and dogfish GnRH have the same amino acid in the eighth position, Lys\textsuperscript{8} and Leu\textsuperscript{8}, respectively. Lamprey GnRH-I and -III and tunicate-I have Lys\textsuperscript{8}, suggesting Lys\textsuperscript{8} may have occurred in the ancestral molecule or that given the substitutions in the sixth position of Glu and Asp, Lys\textsuperscript{8} may reflect changes in receptor molecules that are different in lampreys compared to other vertebrates\textsuperscript{64}.

Several evolutionary models for GnRH have been proposed including those of Sherwood and colleagues and Millar and King. As discussed in Chapter One of this volume, Sherwood and colleagues propose a new scheme for the evolution of GnRH in light of recent identification of tunicate GnRH-I and -II. However, before a complete analysis of phylogenetic trees of GnRH can be done, there needs to be more information on the identification and function of GnRHs in representative species; there needs to be full identification of novel GnRH forms in vertebrates and protochordates; and the cDNAs need to be sequenced in more species, particularly the early evolved vertebrates and the protochordates.
3 FUNCTIONAL STUDIES

3.1 Physiological Studies

Until recently, there was little evidence for a regulatory influence of the hypothalamus on the pituitary-gonadal axis in Agnathans. Using synthetic lamprey GnRH-I and its analogs in earlier studies, the first evidence of neuroendocrine control of reproduction in lampreys was obtained\textsuperscript{52}. Investigations of the role of GnRH in reproductive processes have been impeded by the lack of a purified gonadotropin that can be used in assays to measure pituitary function. However, the biological activity of lamprey GnRH-I or -III has been assessed by steroidogenesis or gametogenesis in \textit{in vitro} and \textit{in vivo} studies\textsuperscript{9,56-60,63,64} (Fig. 2-4). Other studies have shown that there are seasonal correlations between changes in brain GnRH and gametogenic and steroidogenic activity of the gonads in adult male and female sea lampreys\textsuperscript{2,12}. Lamprey GnRH-III is also considered a neurohormone involved in reproduction based on its ability to stimulate steroidogenesis and gametogenesis in adult sea lampreys\textsuperscript{9,64} and of the occurrence of this peptide in lampreys undergoing different stages of metamorphosis coinciding with the acceleration of gonad maturation\textsuperscript{74}.

Sower\textsuperscript{57} demonstrated that lamprey GnRH-I stimulated plasma progesterone and estradiol in adult male sea lampreys after single and two successive injections of lamprey GnRH-I. In this same study, lamprey GnRH-I was determined to induce spermiation in adult male sea lampreys compared to controls after four successive injections of lamprey GnRH-I. Lamprey GnRH-III was also shown to stimulate plasma concentrations of both progesterone and estradiol in the adult male lamprey after a single injection of lamprey GnRH-III, and induce spermiation after four successive injections of lamprey

Fig. 2-4: \textit{In vivo} biological activity of lamprey GnRH-I or -III in adult female sea lamprey. Plasma estradiol levels (ng/ml) of female lampreys injected with saline (control) or lamprey GnRH-I or -III at 0.1 mg/g. Mean + SE (n=10). * Denotes significance at P<0.05. (Modified from Sower et al.\textsuperscript{64}).
GnRH-III. In both studies, neither lamprey GnRH-III nor lamprey GnRH-I appeared to produce a dose-related response in plasma levels of estradiol and progesterone. The percent spermiation data demonstrate that the injection of adult male sea lampreys with lamprey GnRH-III induced a higher percent spermiation after days 16 and 21, indicating that lamprey GnRH-III may be more potent as a neurohormone than lamprey GnRH-I in the adult male sea lamprey. This is supported by the fact that lamprey GnRH-III brain content concentration was determined to be three times greater than that of lamprey GnRH-I. However, until the release rates of lamprey GnRH-I and lamprey GnRH-III are known, and gonadotropins can be directly measured, the differences in potency of lamprey GnRH-I and -III can only be inferred.

Plasma levels of estradiol and progesterone have been used as indicators of reproductive activity in response to lamprey GnRH injections in both male and female lampreys. Previous physiological studies in male lampreys and the demonstrated absence of androgen receptors in the lamprey testis suggest that testosterone may not have a role during the final spermatogenic phases in adult male lampreys. As reviewed in Sower, estradiol appears to be associated with reproductive activity in male sea lampreys. The role of progesterone in male reproductive activity has yet to be determined, although plasma progesterone was demonstrated to be significantly higher in males compared to females during final reproductive stages.

Gonadotropin(s) in lampreys have yet to be identified. The purification of lamprey gonadotropin(s) has been very difficult due to the size of the pituitary and particular difficulties associated with purification. Evidence from physiological and immunocytochemical studies strongly suggest the presence of a gonadotropin-like molecule in lampreys indicating that a reasonably typical pituitary-gonadal relationship exists in this group. In an attempt to identify a GTH homologue in the pituitary glands of adult sea lampreys, a novel homodimeric glycoprotein which was called nasohypophysial factor, NHF, was instead isolated and sequenced. In addition, other lamprey pituitary hormones, adrenocorticotropic (ACTH) and melanotrops (MSH A and B) have been isolated, purified and sequenced. While these previous attempts have isolated additional pituitary factors, a gonadotropin-like molecule has remained elusive. Recent experiments suggest the promise of molecular approaches to isolate such a molecule, and provide additional tools to study regulation.

3.2 Feedback Studies

In mammals, it has been well documented that a positive feedback by estradiol occurs at the time prior to ovulation, when it triggers the preovulatory surge of luteinizing hormone (LH). In lampreys, evidence appears to indicate
a similar type of hormonal signal. In earlier studies, significant changes of estradiol$^{14,61}$ and GnRH$^2$ have been shown to occur during the final gonadal maturation of adult female lampreys. In these same studies, a general increase in plasma estradiol and GnRH occurs during the weeks preceding ovulation. The greatest increase occurs immediately before ovulation, followed by a decline at ovulation. These changes in hormonal patterns suggest that a similar preovulatory positive feedback occurs in lampreys similar to that in other vertebrates. Autoradiography studies by Kim et al.$^{28,29}$ have demonstrated the presence of estrogen target cells in the forebrain of river lampreys, *Ichthyomyzon unicuspis*, and in the larval sea lamprey, *Petromyzon marinus*, respectively. These data, combined with the earlier studies, suggest that a steroid feedback action on the brain of lampreys influences GnRH release and/or affects reproductive behavior.

To test this hypothesis, the effects of estradiol on levels of brain gonadotropin-releasing hormone (GnRH) in adult female sea lamprey were investigated during two successive reproductive seasons(Sower, unpublished). At each of three times during the reproductive season, adult female sea lampreys were injected with a microcapsule preparation containing either no steroid (control) or estradiol at 1 mg/ml. Estradiol significantly elevated brain concentrations of GnRH compared to controls when the lampreys were one week from ovulation, but had no effect three to seven weeks before ovulation (Fig. 2-5). Plasma estradiol concentrations were significantly higher in the estradiol-treated lampreys compared to controls at all sampling times. In addition, plasma estradiol increased dramatically in the estradiol-treated lampreys as the reproductive season progressed. The results of this study suggest that there may be a positive feedback effect of estradiol at a time just prior to ovulation. This is similar to the pattern of estradiol action established in mammals.

![Fig. 2-5: Mean brain GnRH (ng/mg brain) of control or microencapsulated-estradiol treated female lampreys. Brain samples were taken 48 hrs following injection at each of three times during the reproductive season.](image-url)
3.3 Structure-Activity Studies

The lamprey pituitary is able to discriminate between variants of the GnRH molecule, since it has been demonstrated that the mammalian GnRH antagonist ([Ac3Pro1,4FDPhe2, D-Trp3,6]-GnRH and a lamprey GnRH analog ([D-Phe2,6,Pro3]-lamprey GnRH (a putative antagonist), significantly inhibited ovulation62,63. A mammalian [DAla6,Pro9 NEt] GnRH has been shown to be a potent stimulator of gametogenesis and steroidogenesis in the lamprey60. In the adult male sea lamprey, administered lamprey GnRH resulted in the elevation of plasma progesterone and stimulation of spermatiation compared to a reduction in plasma progesterone and inhibition of spermatiation occurred following injection of [D-Phe2,6,Pro3]-lamprey GnRH37. These studies showed that variations in the lamprey GnRH molecule do effect the function of this molecule.

As already stated, the sixth position is considered critical for function of GnRH. Conformation energy analysis of mammalian GnRH suggests that the termini of the molecule are proximal and that the GnRH interacts with the receptor in the folded conformation due to the 6-7β turn involving glycine26,42,68. Interestingly, lamprey GnRH-I and lamprey GnRH-III are the only two vertebrate molecules to have substitutions in the sixth position, Glu and Asp, respectively; all other GnRH peptides have Gly in the sixth position. If a folded conformation is necessary for lamprey GnRH to interact with its receptor, that is, if its termini are proximal, then altering amino acids at position six would likely interfere with its folding and disrupt receptor binding.

A structure-activity study of lamprey GnRH-I or analogs that were cyclized or with sixth position substitutions were done in vivo in adult female sea lampreys66. The following analogs which were tested, [D-Glu6]-GnRH-I; cyclo-[D-Glu6-Trp7-Lys8]-GnRH-I; or cyclo-[Glu6-Trp7-Lys8]-GnRH-I, significantly elevated plasma estradiol compared to controls. However, [D-Glu6]-lamprey GnRH-I was the only analog to significantly stimulate ovulation while another analog [Gly6]-lamprey GnRH-I significantly delayed ovulation. As hypothesized, these data suggest that the sixth position of lamprey GnRH is critical for function.

3.4 Receptor Studies

In lampreys, studies have shown two high affinity, specific classes of binding sites in a single vertebrate pituitary, which is in contrast to all other vertebrates in which only a single class of high affinity binding has been demonstrated33(Fig. 2-6). To date, representative species from all vertebrate classes have shown the presence of two or more forms of GnRH64. In teleosts, in particular, at least two GnRH forms have been identified in each of the
species examined. However, with the exception of goldfish, only a single class of GnRH binding sites has been demonstrated in these same teleosts: stickleback, $K_a = 0.71 \times 10^9 \text{M}^{-1}$, African catfish, $K_a = 0.66 \times 10^9 \text{M}^{-1}$, the seabream, $K_a = 7.08 \times 10^9 \text{M}^{-1}$, and winter flounder, $K_a = 2.1 \times 10^9 \text{M}^{-1}$. In the goldfish pituitary, there are only high affinity and low affinity sites with $K_d$'s of $17.6 \times 10^9 \text{M}$ and $0.02 \times 10^9 \text{M}$, respectively. Scatchard analysis of lamprey pituitary revealed two classes of high affinity binding sites with $K_d$'s of $1.5 \times 10^{12} \text{M}$ and $5 \times 10^9 \text{M}$ and $B_{max}$'s of $8.4 \times 10^{-14} \text{M}$ and $5 \times 10^{-11} \text{M}$, respectively. Binding to the GnRH receptors was saturable, reversible, tissue specific and time- and temperature-dependent. The two high affinity binding sites in the lamprey pituitary may be binding to each of the two known GnRH molecules in the lamprey. Lamprey GnRH-I and -III may bind to different classes of receptors and have different biological actions.

The displacement experiments also suggested the presence of two binding sites within the lamprey pituitary. All of the GnRH peptides and analogs tested (chicken GnRH-I, chicken GnRH-II, synthetic mammal, salmon lamprey GnRH-I, lamprey GnRH-III, DAla$^6$,Pro$^9$ NEt mammalian GnRH and DPhe$^2$,Pro$^3$ lamprey GnRH), displaced the radiolabeled ligand from the receptor except the lamprey analog and thyrotropin-releasing hormone. In vivo studies, variant analogs of the GnRH peptide have been shown to induce biological actions. In the receptor study, two of these analogs, DAla$^6$,Pro$^9$ NEt mammalian GnRH and DPhe$^2$,Pro$^3$ lamprey GnRH, were tested and shown to displace $I^{25}$-DAla$^6$,Pro$^9$ NEt mammalian GnRH. Knox et al. suggested from analysis of the displacement studies that these two analogs may be binding to site I. In goldfish, displacement analysis demonstrated that all native and synthetic GnRH forms bind to the one high affinity class of receptor sites and bioactivity occurs with the binding of the GnRH to these high affinity receptors only. Further experiments will be needed to determine the actual binding site for these analogs in lampreys.

Another analog, [DAla$^6$,Pro$^9$-OH free carboxylic acid] lamprey GnRH, was unable to displace the radioactive ligand in the receptor study. In vivo studies, this same lamprey analog stimulated plasma progesterone levels but inhibited spermiation in male lampreys and inhibited ovulation in female lampreys. The apparent lack of binding or competitive inhibition of GnRH in the pituitary, and the inhibition and stimulatory biological activity of this
analog, implies that the sixth position and \( \alpha \)-amidated COOH-terminal is significant for receptor binding and in receptor-mediated events for biological activity. Previous studies have suggested that GnRH-I acts directly on the pituitary and does not directly influence steroidogenesis in lamprey gonads. In \textit{in vitro} studies, lamprey GnRH-I (range of dose: 1 to 1000 ng peptide /ml media) had little or no direct effect on estradiol or progesterone as determined from media of testes culture or ovary cultures compared to controls\(^{58}\). In addition, GnRH has not been detected in circulating plasma\(^{12,40}\). These data along with the lack of binding in the liver in the Knox et al.\(^{33}\) study suggest that GnRHs's action occurs at the pituitary. Both lamprey GnRH-I and -III are the only vertebrate GnRH molecules to have substitutions in the sixth position, Glu\(^6\) and Asp\(^6\), respectively\(^{64}\). Based on the structural and receptor studies, the receptor requirements for GnRH may be different in the lamprey from those in other vertebrates.

The highest concentration of GnRH binding sites occurred in the proximal pars distalis of the pituitary with little specific binding in the rostral pars distalis (Plate 2-1). In review of light and electron microscopy studies, both the rostral and proximal pars distalis have been shown to have Periodic acid-schiff positive staining basophilic cells that may be the gonado-
tropes\textsuperscript{11,24,35,50}. However, until the gonadotropins are purified and identified, the binding of GnRH to the putative gonadotropic cells in the proximal pars distalis is only hypothesized.

4 BRAIN-PITUITARY AND IMMUNOCYTOCHEMICAL STUDIES

4.1 Anatomical studies of hypothalamus-pituitary

Of all vertebrates, only the agnatha and teleosts lack a portal vascular system (median eminence) for transferring regulatory peptides from the brain to the adenohypophysis\textsuperscript{15}. The adaptive importance of such a portal system is that it makes possible central nervous regulation of such vital processes as reproduction by external (and internal) cycling environmental conditions. The teleosts have solved this structural problem by direct innervation of the pars distalis by appropriate neurosecretory neurons from the adjacent hypothalamus\textsuperscript{51}. The agnathans, however, have no nervous or vascular communication between the brain and neurohypophysis\textsuperscript{74}. This has led to speculation that nervous regulation of the agnathan pars distalis is by diffusion of brain peptides from the adjacent neurohypophysis, across the thin connective tissue layer that separates the neural from the glandular tissues.

Proof that diffusion is an adequate basis for brain regulation of the pars distalis has rested on such experiments as those of Nozaki et al.\textsuperscript{45} and Tsukahara et al.\textsuperscript{72}. They injected substances of varying molecular size into the third ventricles of hagfish. By use of staining procedures that revealed the positions of these substances a few minutes after injection, they showed that significant amounts of test materials diffused rapidly from the third ventricle, through the neurohypophysis, to the pars distalis. In several ways, however, it was felt that experiments with hagfish might not represent the diffusion hypothesis fairly. There is some question as to whether hagfish species have an environmentally regulated reproductive cycle\textsuperscript{16}. Indeed, it has not been established whether the hagfish pituitary contains tropic hormones of any kind\textsuperscript{39}.

Lampreys, on the other hand, are clearly seasonal and temperature-responsive in the timing of their anadromous migrations and mating and breeding\textsuperscript{21}. In addition, 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\textsuperscript{32,46,73}. In the lamprey, GnRH-like neurons identified by immunocytochemistry project their fibers primarily into the neurohypophysis from the preoptic region\textsuperscript{5,32,44,47}. In addition, Crim\textsuperscript{4} and King et al.\textsuperscript{32} using a
lamprey GnRH-I antibody showed that GnRH neurons project into the third ventricle. These authors proposed an additional route of GnRH via secretion into the third ventricle and transport by tanycytes to the adenohypophysis. If indeed, as the data indicate, GnRH does diffuse across the connective tissue to the adenohypophysis, then control by the hypothalamus is probable. Studies were done to experimentally examine the functional anatomical relationship between the hypothalamus and adenohypophysis in sea lamprey. Horseradish peroxidase (HRP), a protein that can be visualized by appropriate histochemical procedures, was injected into the third ventricle of the brain of adult lampreys. 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. These authors concluded that neurosecretory peptides like gonadotropin-releasing hormone diffuse from the brain (neurohypophysis) to the adenohypophysis, and thus regulate its secretory activity in lampreys.

![Diagram](https://via.placeholder.com/150)

Fig. 2-7: Diagrammatic representation of the three types of relationship between the brain (hypothalamus) and the adenohypophysis in vertebrates. In agnathans, the thin neurohypophysis is coextensive with a thin layer of epithelial glandular cells, making diffusion between these two structures a relatively efficient means for neurosecretory regulation of the adenohypophysis. In teleosts the many neurohypophysial axonal extensions penetrate the adenohypophysis and either directly innervate synaptically the adenohypophysial cells or end near these cells for diffusional (paracrine) action of neurosecretions. In lower vertebrates (represented at the bottom-left) an intermediate form of vascular transport via many short vessels occurs between a thin flat area of the neurohypophysis, and the coextensive relatively thin adenohypophysis. The most highly evolved median eminence is that of other vertebrates (amphibians, reptiles, birds, and mammals) usually with larger pituitaries, in which portal vessels collect neurohypophyselial blood and conduct it to and within the adenohypophysis. AH, Adenohypophysis; NH, neurohypophysis; P, portal blood vessel from vascular median eminence Modified from Nozaki et al.48.
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. These authors concluded that in the evolutionary sense there have been three types of regulation of the adenohypophysis developed in the vertebrates: the agnathan diffusional type, the teleostean direct innervationtional type, and the vascular type seen in all other vertebrates\textsuperscript{48} (Fig. 2-7). Perhaps, the principal advantage of the vascular median eminence type of control of the pars distalis by the brain is that it permitted development of larger and thicker glands as vertebrates became larger and more complicated in form as the distance between the hypothalamus and pituitary increased significantly.

4.2 Immunocytochemical Studies

4.2.1 Hagfish

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, \textit{Eptatretus hexamtrema}\textsuperscript{31} and \textit{Eptatretus stoutii}\textsuperscript{25}, using radio-immunoassay and chromatographic techniques. However, others, using similar techniques, could not detect GnRH in the Pacific hagfish, \textit{E. stoutii}\textsuperscript{52}. In immunocytochemical studies, ir-GnRH was not detected in several species of hagfish brain by Nozaki and Kobayashi\textsuperscript{44} and Crim et al.\textsuperscript{5}. Nozaki et al.\textsuperscript{47} hypothesized that hagfish may contain an irGnRH that is not detected by current methods or antibodies or that hagfish may lack GnRH. In two recent studies using newly available antibodies to lamprey GnRH (GnRH-III), as well as other GnRH antibodies, immunoreactive GnRH was detected in the brain of the Atlantic hagfish\textsuperscript{65} and Pacific hagfish\textsuperscript{3} (Fig. 2-8 and 2-9). In the

![Fig. 2-8: Nearly midsagittal section of the hagfish neurohypophysis stained with anti-lamprey GnRH-III (Lot 3951). The anterior end is to the left. An accumulation of GnRH-immunoreactive fibers (arrowheads) is shown terminating in the external layer of the dorsal and ventral walls (DW and VW) of the neurohypophysis. AH, adenohypophysis; III, third ventricle; Scale bar, 100 mm (x98). Modified from Sower et al.\textsuperscript{69}.](image-url)
Pacific hagfish studies, two GnRH systems were proposed, one system which is widely diffuse throughout the brain and the other restricted to the preoptic-neurohypophysial system\(^3\).

These two distinct GnRH systems proposed in Pacific hagfish were identified by use of a salmon GnRH antibody (PBL-49)\(^3\). This antiserum displayed a differential affinity for the two systems, indicating that the two systems differ in the amount or identity of GnRH. In an unpublished study using another salmon antiserum, King, Anthony and Sower also detected ir-salmon GnRH in the mid- and hindbrain of Atlantic hagfish. The chromatography data of Sower et al.\(^6^5\) support the evidence that there are at least two forms of GnRH in hagfish brain, although in this same study, the immunocytochemical data demonstrated the presence and distribution of only a lamprey GnRH-III-like immunoreactivity in the neurohypophysis of the hagfish. The studies in hagfish, from both HPLC-RIA and immunocytochemistry, clearly suggest the presence of a lamprey GnRH-III-like molecule in the hagfish brain\(^6^5\).

The use of lamprey GnRH antibodies in Sower et al.\(^6^5\) studies suggests 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\(^6^4\). Chicken GnRH-II has been characterized in the ratfish (Class Chondrichthyes) which diverged from the line of vertebrate evolution about 400 million years ago\(^3^7\). It has been proposed that lamprey GnRH-III, chicken GnRH-II and dogfish GnRH are relatively more closely related to the "ancestral" GnRH molecule\(^6^4\). The evidence suggests that the hagfish may have also retained one or more of these GnRH forms.
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\(^{31,38,64}\). Lamprey GnRH-III is more closely related to the other members of the GnRH family than is lamprey GnRH-I\(^6\). 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 recent studies. Chicken GnRH-II now has been structurally sequenced in representative species of five of the seven classes of vertebrates\(^{55}\). 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.

4.2.2 Lampreys

In lampreys, there is a general pattern of GnRH distribution in brains of adult lampreys as determined by immunocytochemical studies using different GnRH antibodies including antibodies to lamprey GnRH-I and -III (Fig. 2-10). GnRH immunoreactive cells project their fibers primarily into the neurohypophysis from the preoptic area as determined by immunocytochemical studies in adult Entosphenus tridentata\(^5\), Lampetra richardsoni \(^6\), Petromyzon marinus \(^{32,44,46}\) and Ichthyomyzon unicuspis \(^{10}\). As described by King et al.\(^{32}\), immunopositive neuronal perikarya are present in an arc-shaped population extending from ventral to dorsal preoptic areas. Fibers from these cells project to the neurohypophysis via the preoptico-hypophyseal tract, but in addition also protrude into the third ventricle. Additionally, some fibers course along the external surface of the brain, and may also release GnRH in meningeal

![Diagram](image-url)

Fig. 2-10: Diagrammatic nearly-midsagittal section of the distribution of GnRH in the brain of adult lamprey as determined by immunocytochemical studies using different GnRH antibodies including antibodies to lamprey GnRH-I and -III. Perikarya, circles; Fibers, broken lines. CPD: caudal pars distalis; DT: dorsal thalamus; Hyp: hypothalamus; IPN: interpeduncular nucleus; NH: neurohypophysis; PI: pars intermedia; PON: preoptic nucleus; RPD, rostral pars distalis. Modified from Nozaki et al.\(^{47}\); King et al.\(^{32}\); Nozaki, Gorbman and Sower (unpubl.).
compartments. Unlike lampreys, when two forms of GnRH are found in other species of vertebrates, there are significant differences in their distribution as well as being widely distributed in the midbrain or extra hypothalamic regions.

In larval lampreys, both lamprey GnRH-I and -III are found in the cell bodies in the rostral hypothalamus and preoptic area\textsuperscript{68,75}. On the basis of such evidence, most immunoreactive GnRH in developing larval lampreys is of the lamprey GnRH-III type which suggests that this is the more active form during reproductive development\textsuperscript{70}. A small number of cells found in the caudal hypothalamus contain only immunoreactive lamprey GnRH-III, which may constitute a functional subgroup within the population of GnRH neurons\textsuperscript{70} (Fig. 2-11). In lampreys undergoing metamorphosis there is a large increase in reaction product in all GnRH-containing cells and fibers in the rostral and preoptic hypothalamic areas\textsuperscript{6,68,75}. In addition, there is a noted increase of immunoreactive GnRH cells in the ventral hypothalamic area in larger ammocoetes and during metamorphosis, which suggests that these cells play a unique role during metamorphosis\textsuperscript{70}.

4.3 Origin and Evolution of GnRH During Development

As stated earlier, chromatographic and immunological studies of vertebrate brain extracts have shown that there are two or more GnRH-like peptides in representative species of all vertebrate classes\textsuperscript{43}. The functional significance of multiple forms of GnRH within the brain, and in extra-hypothalamic locations within the same species, has not been elucidated with the possible exception of lampreys. The GnRHs have apparently multiple actions on phases of reproductive physiology and behavior either through pituitary or non-pituitary agents, depending on the origin of the GnRH system during development. Muske\textsuperscript{43} has proposed that gnathostome vertebrates have

![Diagram of GnRH distribution](image-url)

Fig. 2-11: Diagrammatic nearly midsagittal section of the distribution of GnRH in the brain of ammocoete as determined by immunocytochemical studies using different GnRH antibodies to lamprey GnRH-I and -III. Solid circles are cell bodies, and Xs represent GnRH-III in caudal hypothalamus. Modified from Tobet et al.\textsuperscript{70}. 
two principle GnRH systems, each with different embryonic origins expressing different molecular forms of GnRH and affecting different targets. In the vertebrates examined, neurons which contain forms of GnRH which are considered to regulate pituitary-gonadal function are thought to be derived from progenitor cells that originate in the olfactory placode, and which migrate to their definitive adult positions in the preoptic /hypothalamic areas. With some exceptions, the other GnRH system probably arises from a non-placodal origin and is involved in non-pituitary-gonadal function. Recent experiments in lampreys have characterized the earliest development of lamprey GnRH neurons and indicate the probable pathway of their migration. Mature eggs from adult sea lampreys were fertilized in the laboratory. Embryos and developing larvae were maintained in fresh flow-through well water over a sandy bottom for up to 100 days. GnRH neurons were first visualized immunocytochemically at day 22 after fertilization in the preoptic area and hypothalamus. The number of reactive neurons steadily increased with age through day 100. GnRH neurons were not seen within the olfactory system. In contrast to all other vertebrates, these authors propose that GnRH neurons in developing lampreys originate within proliferative zones of the diencephalon and not in the olfactory system. Thus, this scheme is illustrated in Fig.2-12.

![Diagram](image)

Fig. 2-12: A proposed diagram on the evolution of GnRH systems and functions in vertebrates. Modified from Sower and Tobet, unpublished.
REFERENCES


