

# The Distribution of Lamprey GnRH-III in Brains of Adult Sea Lampreys (*Petromyzon marinus*)

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In the sea lamprey, *Petromyzon marinus*, two forms of GnRH, lamprey GnRH-I and -III, have been demonstrated to be neurohormones regulating the pituitary-gonadal axis. The objective of the present study was to determine the distribution of lamprey GnRH-III in the brains of adult sea lampreys and to compare it to the distribution of lamprey GnRH-I. For this purpose, two kinds of immunostaining were employed: one was a single immunostaining by one of two GnRH antibodies using two successive sections; the other was double immunostaining of a single section. A dense accumulation of neuronal cells immunoreactive (ir) to antisera against either lamprey GnRH-I or -III was found in the arc-shaped preoptico-anterior hypothalamic area. Additional smaller numbers of irGnRH cells were found in the periventricular zone of the posterior hypothalamus. In the above-mentioned locations, the distribution of both irGnRH-I and -III cells was intermixed and very similar, but the cells exhibiting GnRH-III immunoreactivity were distinctly different from those exhibiting GnRH-I immunoreactivity. The relative numbers of irGnRH-III cells were larger than those of irGnRH-I cells in the preoptico-anterior hypothalamic area, and more than 90% of GnRH cells in the posterior hypothalamus were irGnRH-III cells. Both irGnRH-I and -III cells projected their fibers primarily into the neurohypophysis. The relative densities of the accumulated irGnRH-III fibers were similar to

those of irGnRH-I fibers in the anterior neurohypophysis but higher than those of irGnRH-I fibers in the posterior neurohypophysis. The present study provides further immunocytochemical data to the already compelling physiological evidence that indicates that both lamprey GnRH-I and -III act through the hypothalamic-pituitary-gonadal axis to modulate reproductive processes in the sea lamprey. © 2000 Academic Press

## INTRODUCTION

The primary molecular structure of gonadotropin-releasing hormone-III (lamprey GnRH-III) recently has been determined in sea lampreys, *Petromyzon marinus*, as pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH<sub>2</sub> (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 vertebrate GnRH family than lamprey GnRH-I. Lampreys are the earliest vertebrates for which there are demonstrated functional roles for multiple GnRH neurohormones involved in pituitary-regulated reproductive activity.

In the lamprey, GnRH-like neurons, identified by immunocytochemistry, project their fibers primarily into the neurohypophysis from the preoptic region (Crim, 1981; Crim *et al.*, 1979; Nozaki and Kobayashi, 1979; Nozaki *et al.*, 1984; Wright *et al.*, 1994). These studies used antibodies directed toward mammalian

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GnRH or lamprey GnRH-I. Similar to these other studies, immunoreactive cells, identified by use of antisera to lamprey GnRH-I, which is directed against both lamprey GnRH-I and -III, project their fibers primarily into the neurohypophysis from the preoptic area in adult *Ichthyomyzon unicuspis* (Eisthen and Northcutt, 1996). Distribution of lamprey GnRH-III in adult lampreys has not yet been reported.

In larval lampreys, lamprey GnRH-III was found to be the primary form of GnRH in premetamorphic GnRH-containing neurons in an immunocytochemical study (Tobet *et al.*, 1995). GnRH-stainable cell bodies were restricted primarily to a small region within the anterior hypothalamus and preoptic area. These cell bodies gave rise to numerous immunoreactive fibers which, in part, filled the neurohypophysis. It was suggested that, in the larval stage, the majority of the immunoreactive (ir) GnRH is lamprey GnRH-III, indicating that perhaps GnRH-III is the more active form during reproductive maturation (Tobet *et al.*, 1995). Further study is needed to determine whether there are differences in relative potencies and functions of lamprey GnRH molecules in sea lampreys in different reproductive stages. Accordingly, the objective of this study was to characterize the distribution of lamprey GnRH-III in brains of adult sea lampreys and to compare it to the distribution of lamprey GnRH-I.

## MATERIALS AND METHODS

### Animals

Adult sea-run sea lampreys were collected in a trap located at the top of the salmon ladder at the Coheco River in Dover, New Hampshire in May and June during their upstream spawning migration from the ocean. The lampreys were transported to the freshwater fish hatchery at the University of New Hampshire and maintained in an artificial spawning channel supplied with flow-through water from a nearby stream-fed reservoir at an ambient temperature range of 13–20°C, under a natural photoperiod.

### Tissue Preparations

Twelve animals of both sexes were killed by decapitation. After rapid removal of the dorsal fibrocranium

and exposure of the dorsal surface of the brain, the dissected brain and the attached pituitary were removed and immersed in Bouin–Hollande sublimate solution (Romeis, 1948) for about 24 h. The fixed tissues were dehydrated through a series of increasing concentrations of ethanol. Deposits of mercuric chloride were removed by treatment of tissues with iodine–potassium iodide in 90% ethanol for 24 h. Tissues were embedded in paraplast, and serial sagittal or transverse sections of 5 to 6 µm were mounted on glass slides.

### Immunocytochemistry

The following antisera were used: (1 and 2) anti-lamprey GnRH-III, lot Nos. 3951 (working dilutions, ×6000) and 3952 (working dilutions, ×20,000), raised in different rabbits by S.A.S.; and (3 and 4) anti-lamprey GnRH-I, lot No. 1467 (working dilution, ×7000) provided by Dr. Judy King, and lot No. 21-134 (working dilution, ×10,000) raised by S.A.S.

In preliminary immunocytochemistry and preabsorption tests using paraffin sections of sea lamprey pituitaries, positive reaction to anti-lamprey GnRH-III, No. 3952, was abolished by the preabsorption with lamprey GnRH-III but did not change by the preabsorption with lamprey GnRH-I (Table 1). On the other hand, positive reaction to No. 3951 was largely reduced by the preabsorption with lamprey GnRH-I, as well as the complete elimination by the preabsorption with lamprey GnRH-III (Table 1). Thus, it was found that antibody No. 3952 exhibits a specific staining reaction to lamprey GnRH-III, whereas antibody No. 3951 crossreacts with lamprey GnRH-I. Positive reaction to both lots of anti-lamprey GnRH-I, No. 1467 and No. 21-134, was abolished by the preabsorption with lamprey GnRH-I (Table 1). Following the preabsorption with lamprey GnRH-III, positive reaction to antibody No. 1467 was slightly reduced, whereas positive reaction to antibody No. 21-134 was completely abolished (Table 1). Thus, it was found that antibody No. 1467 exhibits a slight crossreactivity to lamprey GnRH-III, whereas antibody No. 21-134 reacts to both GnRH-I and -III. Accordingly, in the present study, antibody No. 1467 preabsorbed with lamprey GnRH-III (50 µg/ml antibody at working dilution) and antibody No. 3952 were used for the demonstration of GnRH-I and GnRH-III, respectively.

TABLE 1

Effects on Immunostaining of Preabsorption of Anti-lamprey GnRHs with Lamprey GnRH-I and -III in the Lamprey Brain

Antibodies to	Lot no.	Obtained from	Without preabsorption	Preabsorption with	
				1 GnRH-I	1 GnRH-III
1-GnRH-I	1467	King	+++	– (Abolished)	++ (Reduced)
1-GnRH-I	21-134	Sower	+++	– (Abolished)	– (Abolished)
1-GnRH-III	3952	Sower	+++	+++ (Not changed)	– (Abolished)
1-GnRH-III	3951	Sower	+++	+ (Reduced)	– (Abolished)

Note. +++, Intense immunoreaction; ++, moderate immunoreaction; +, faint immunoreaction; –, no immunoreaction.

Since distributions of both irGnRH-I and -III cells overlapped and were mixed and very similar in the brain (see Results), two kinds of immunostaining procedures were employed: one was ordinary single immunostaining using two successive sections each exposed to both antibodies; the other was double immunostaining of a single section. The immunostaining was performed by use of a Vectastain avidin-biotin peroxidase complex (ABC) Elite kit or a Vectastain avidin-biotin alkaline phosphatase complex (ABC-AP) kit, and the procedures have been described elsewhere (Nozaki *et al.*, 1999). The procedures of double immunostaining of a single section were, in brief, as follows: sections were deparaffinized in xylene, hydrated in a graded ethanol series, and washed in phosphate-buffered saline (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.5; PBS). All procedures were performed at room temperature, and incubations with antibodies or staining reagents of kits were performed in closed humid chambers. First, the tissue sections were incubated for 10 min in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activities and then washed in PBS. To reduce nonspecific staining, sections were treated with 0.5% casein in PBS and normal goat serum for 30 min each. One of the anti-lamprey GnRH antibodies, either lot No. 1467 preabsorbed with lamprey GnRH-III or lot No. 3952, was applied to the sections for 2 h, and the biotinylated secondary antibody solution and ABC reagent were each applied for 2 h. The order of the addition of the primary antibodies was alternated in adjacent sections. The final reaction product was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 10 mM Tris-HCl containing 0.003% hydrogen peroxide and washed in running water. Sections were then incubated with the remaining

anti-lamprey GnRH antibody (lot No. 1467 preabsorbed with lamprey GnRH-III or lot No. 3952) for 2 h, washed in PBS, and incubated with the biotinylated secondary antibody solution for 45 min. After washing in PBS, sections were treated with DAB in 10 mM Tris-HCl containing 0.003% hydrogen peroxide for 20 min, followed by washing in running water and PBS. Sections were incubated with ABC-AP for 45 min and then washed in PBS. The reaction product of ABC-AP was visualized with Vector red (Vector Laboratories) and washed in running water. The sections were then counterstained with hematoxylin, washed in running water, dehydrated through an increasing concentration of ethanol series, and mounted in Entellan (Merck).

To confirm the specificity of the immunostaining, the following control staining procedures were done: (1) replacement of primary antibodies with normal rabbit serum and (2) absorption of primary antibodies with synthetic lamprey GnRH-I (Peninsula Laboratories; 50 µg/ml antisera at working dilutions) or lamprey GnRH-III (generously provided by Dr. Russell Doolittle; 50 µg/ml antisera at working dilutions).

The nomenclature employed in this study followed that of Nozaki and Gorbman (1986) and was essentially the same as that of Heier (1948) and Nieuwenhuys (1977).

## RESULTS

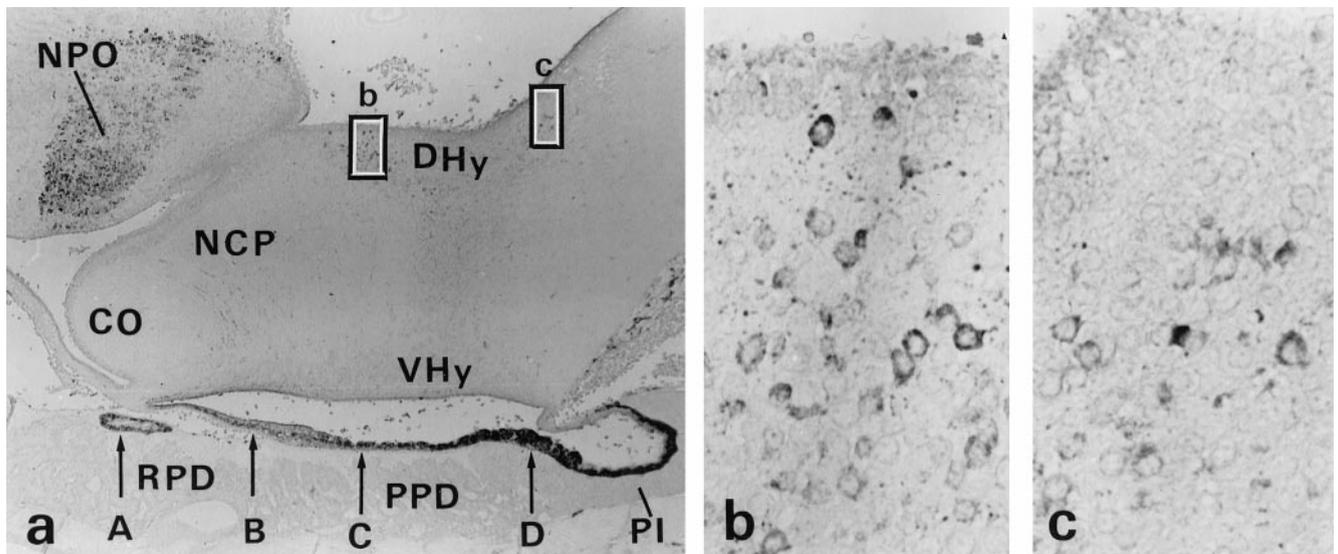
A dense accumulation of neuronal cells immunoreactive to antisera against either lamprey GnRH-I or -III was found in the arc-shaped area extending from the nucleus preopticus (NPO) and the nucleus commissurae post-opticae (NCP) to the dorsal hypothalamus,

which begins just rostral-dorsal to the optic chiasm and extends dorso-caudally over the dorsal part of the anterior hypothalamus (Figs. 1a and 1b). In transverse sections through the NPO and the NCP, most irGnRH cells to either anti-lamprey GnRH-I or -III were found together in a neuron-rich zone just below the ependyma of the third ventricle (Figs. 2a–2i and 3a–3f). Thus, the distribution of both irGnRH-I and -III cells overlapped and were mixed and very similar in the NPO and the NCP (Figs. 2a–2i and 3a–3f). The staining intensities of irGnRH cells to either anti-GnRH-I or -III varied among cells: some cells were stained intensely, whereas staining in others was weak or faint (Figs. 2a–2i and 3a–3f). When two successive transverse sections were stained with either anti-lamprey GnRH-I or -III, it was found that, in most cases, cells stained with anti-GnRH-I and those stained with anti-GnRH-III distinctly reacted to only one of the two antibodies (Figs. 2a–2i). However, for some irGnRH cells, it was difficult to determine whether they were reactive selectively to only one of two antibodies, since the stained cells were small and about the same thickness as the section itself (Figs. 2a–2i). When double immunostaining of GnRH-I and -III in a single section was performed, cells exhibiting GnRH-III immunoreactiv-

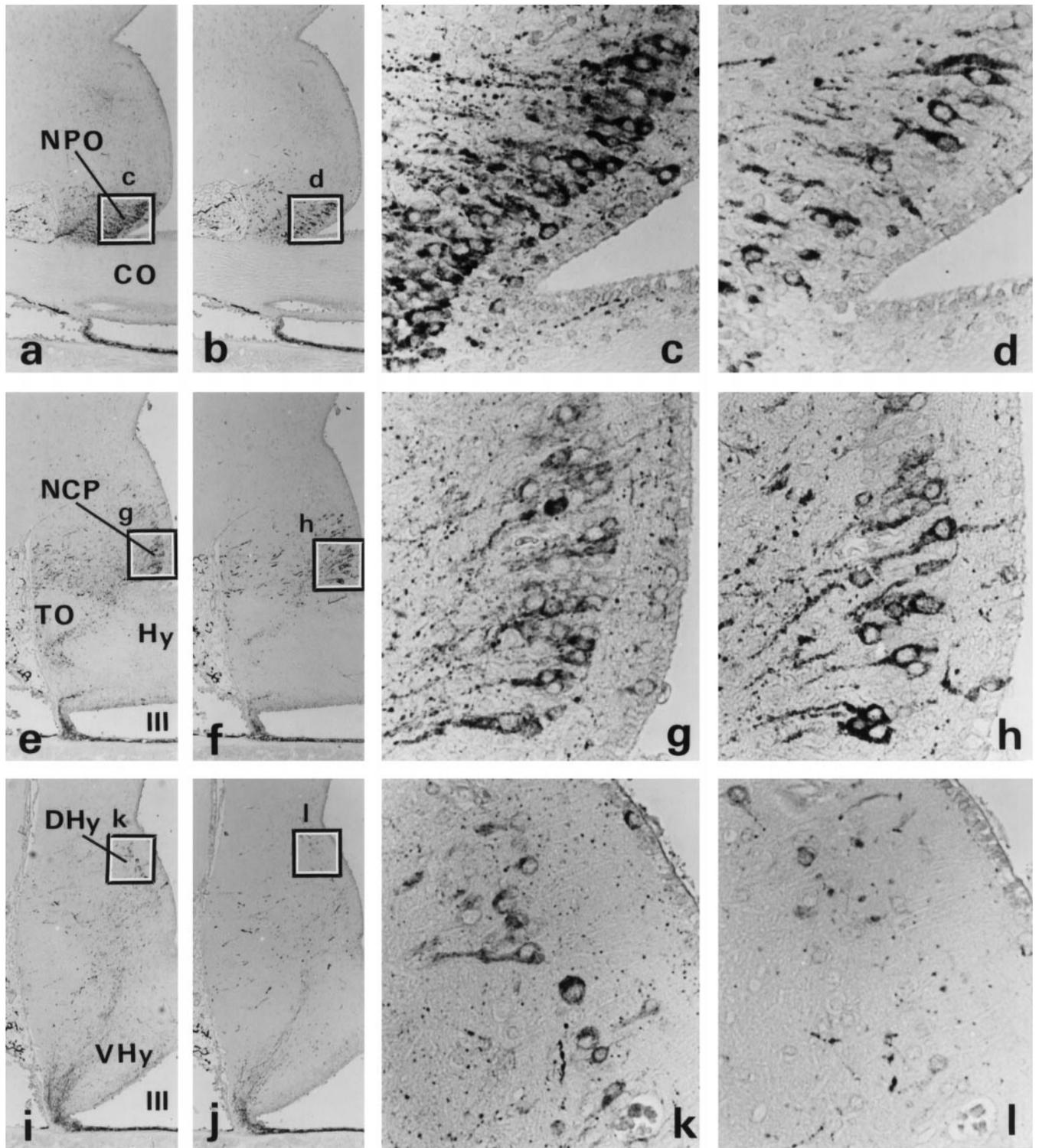
ity were distinctly different from those exhibiting GnRH-I immunoreactivity in the brain (Figs. 3a–3f): none of the irGnRH cells exhibited both immunoreactivities of GnRH-I and -III. The relative numbers of irGnRH-III cells were larger than those of irGnRH-I cells in the NPO and the NCP (Figs. 2a–2i and 3a–3f).

Additional smaller numbers of irGnRH cells were found in the periventricular zone of the dorsal and ventral parts of the posterior hypothalamus (Figs. 1c, 3g and 3h). The distributions of both irGnRH-I and -III cells were also mixed and very similar, but irGnRH-I cells were very few in number (less than 10%) in the posterior hypothalamus (Figs. 3g and 3h).

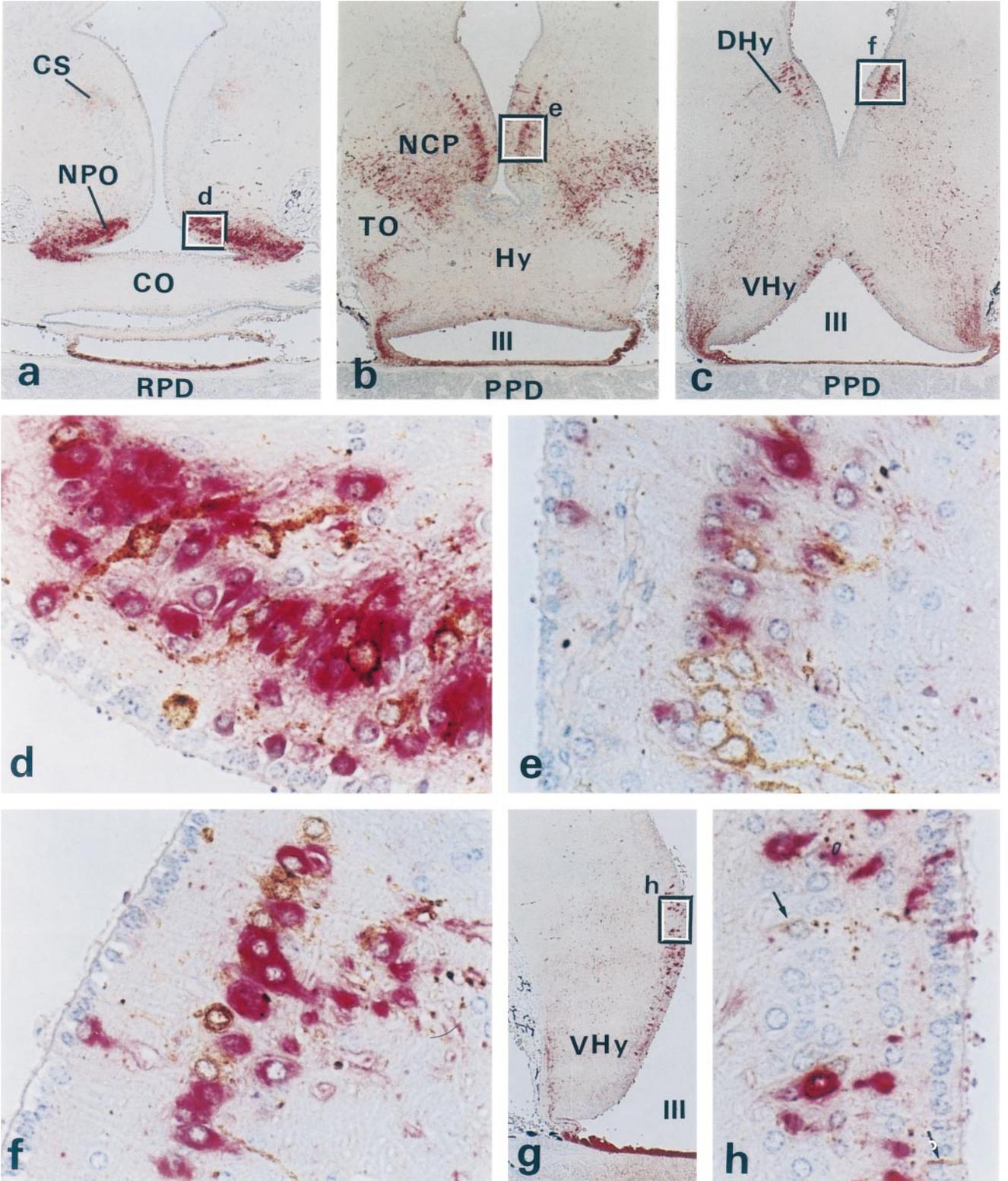
GnRH-immunoreactive nerve fibers, bearing either irGnRH-I or -III, originating from cells in the arc-shaped preoptico-anterior hypothalamic area, projected ventro-caudally toward the base of the brain, condensing in the ventral hypothalamus. The fibers formed a dense bundle or tract, the preoptico-hypophysial GnRH tract. Thus, the distributions of GnRH-I and -III immunoreactive fibers largely overlapped and were very similar in the hypothalamus (Figs. 2a–2i and 3a–3f). Accordingly, it was difficult to determine whether each group of irGnRH fibers was stained positively by only one of the two GnRH



**FIG. 1.** (a) A sagittal section of the hypothalamo-hypophysial region of the adult sea lamprey stained with anti-lamprey GnRH-III. A to D with arrows represent transverse levels shown in Figs. 2 to 4. The areas outlined by rectangles are enlarged and shown in b and c. CO, chiasma opticum; CS, corpus striatum; DHy, dorsal hypothalamus; Hy, hypothalamus; NCP, nucleus commissurae postopticae; NPO, nucleus preopticus; PI, pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis; TO, tractus opticus; VHx, ventral hypothalamus. III, third ventricle. a,  $\times 44$ ; b and c,  $\times 430$ .



**FIG. 2.** Three sets of two successive transverse sections through the levels of A (a and b), B (e and f), and C (i and j) in Fig. 1 of the adult sea lamprey. Sections a, e, and i were stained with anti-lamprey GnRH-III, whereas sections b, f, and j were stained with anti-lamprey GnRH-I. The areas outlined by rectangles in a, b, e, f, i, and j are enlarged and shown in c, d, g, h, k, and l, respectively. See Fig. 1 for abbreviations. a, b, e, f, i, and j,  $\times 44$ ; c, d, g, h, k, and l,  $\times 330$ .



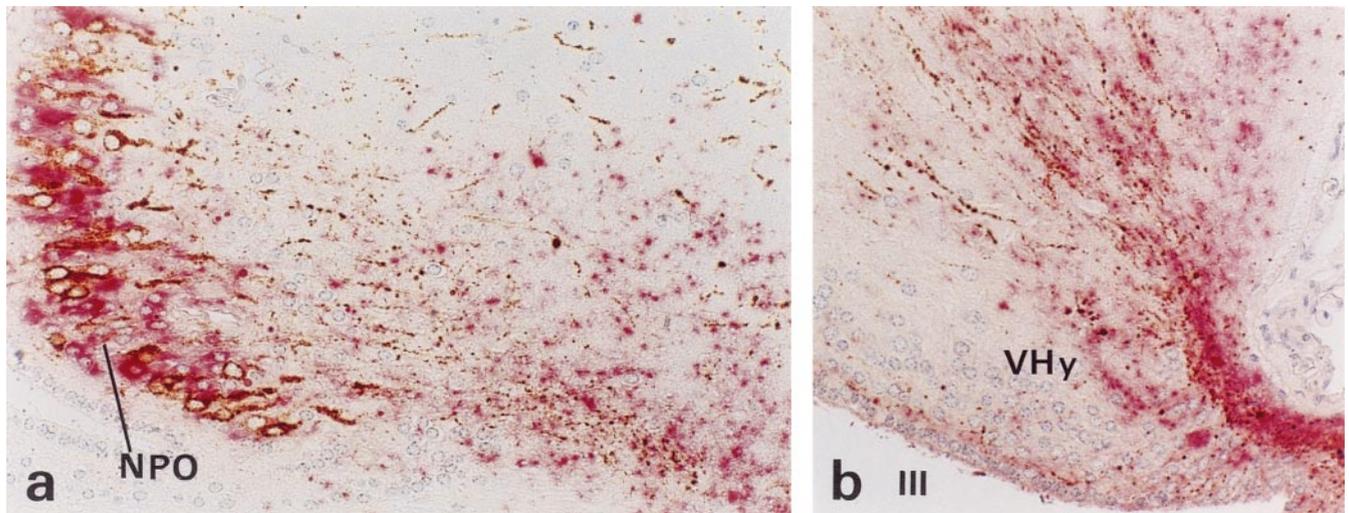


FIG. 4. Two transverse sections through the levels of A (a) and C (b) in Fig. 1 of the adult sea lamprey, showing double immunostaining of a single section. In both sections, lamprey irGnRH-III cells and fibers are shown in red, whereas lamprey irGnRH-I cells and fibers are shown in brown. NPO, nucleus preopticus; VHy, ventral hypothalamus; III, third ventricle. a and b,  $\times 260$ .

antibodies in the two successive transverse sections stained with either anti-lamprey GnRH-I or -III (Figs. 2a–2i). However, when double immunostaining in a single section was employed, irGnRH-III fibers were distinctly different from irGnRH-I fibers in the hypothalamus (Figs. 4a and 4b). The relative numbers of irGnRH-III fibers were larger than those of irGnRH-I fibers at whole rostro-caudal levels of the brain (Figs. 2a–2i, 3a–3h, 4a and 4b).

Heavy accumulations of both irGnRH-I and -III nerve terminals were found in the neurohypophysis. The relative densities of the accumulated irGnRH-III fibers were similar to those of the irGnRH-I fibers in the anterior neurohypophysis, at parallel to both the rostral and the proximal pars distalis (Figs. 5a, 5b, 5d, and 5e). On the other hand, irGnRH-III fibers were more densely accumulated in the posterior neurohypophysis, facing the pars intermedia, than irGnRH-I fibers (Figs. 5c and 5f).

Extrahypothalamic projections of irGnRH-I and -III neurons were also observed but were more diffuse and

not organized into discrete bundles. Both irGnRH-I and -III fibers were apparent in the corpus striatum, in the stria medullaris projecting to the habenula, in the olfactory stria, and in descending tracts of the brain-stem (data not shown).

## DISCUSSION

In the adult lamprey brain, cells immunoreactive to either of two GnRH antibodies, lamprey GnRH-I and -III, were found chiefly in two areas of concentration, the arc-shaped preoptico-anterior hypothalamic area and the posterior hypothalamus. Although the distributions of both irGnRH-I and -III cells are mixed and very similar, irGnRH-I and -III cells are structurally distinctly different from each other at all above-mentioned locations.

The preoptic GnRH neurons have been well documented in the lamprey brain (Crim, 1981; Crim *et al.*,

FIG. 3. Four transverse sections through the levels of A (a), B (b), C (c), and D (g) in Fig. 1 of the adult sea lamprey, showing double immunostaining of a single section. In all sections, lamprey irGnRH-III cells and fibers are shown in red, whereas lamprey irGnRH-I cells and fibers are shown in brown. The areas outlined by rectangles in a, b, c, and g are enlarged and shown in d, e, f, and h, respectively. Arrows in h show cells and fibers stained weakly with anti-lamprey GnRH-I. See Fig. 1 for abbreviations. a, b, c, and g,  $\times 47$ ; d, e, f, and h,  $\times 500$ .

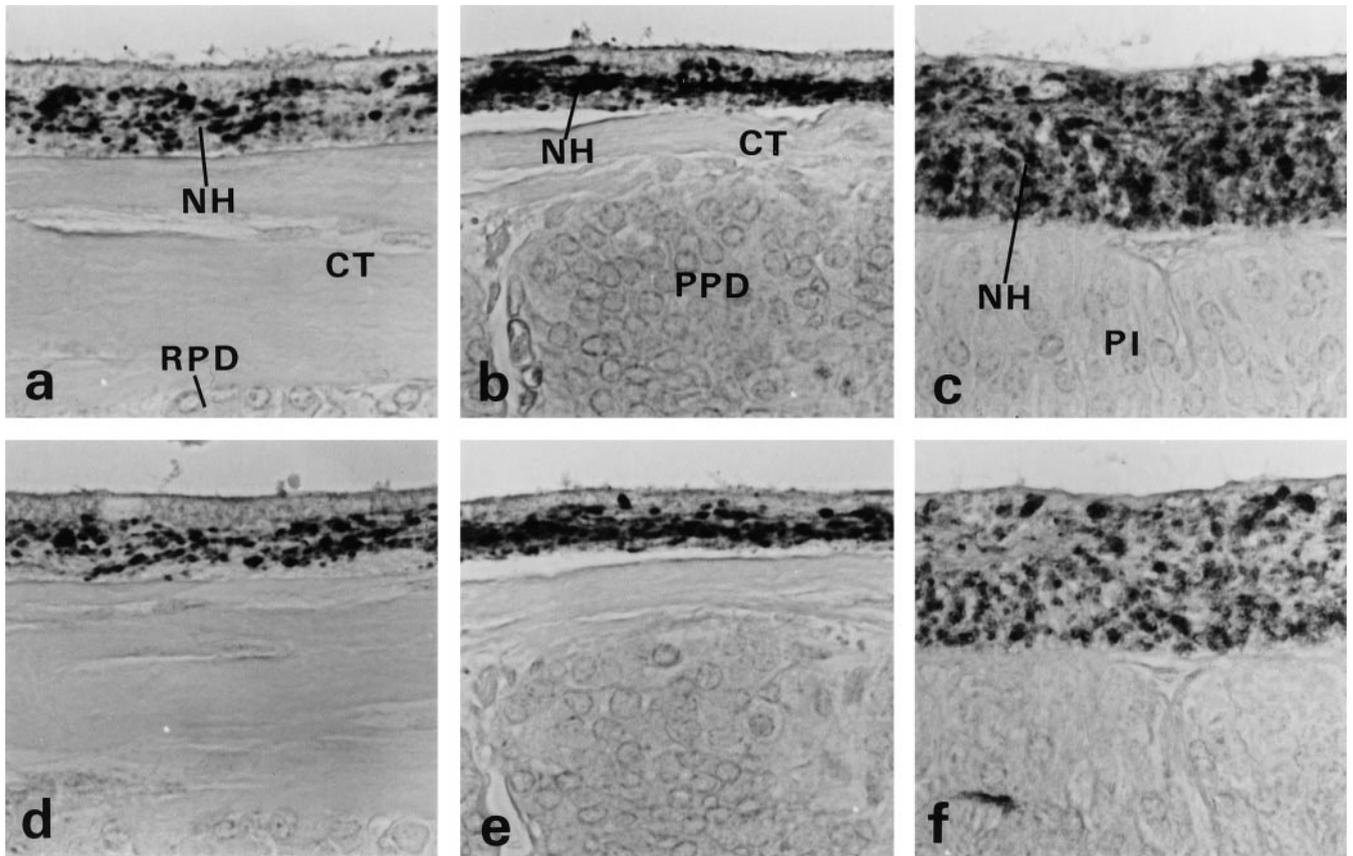


FIG. 5. Three sets of two successive transverse sections through the rostral pars distalis (RPD) (a and d), the proximal pars distalis (PPD) (b and e), and the pars intermedia (PI) (c and f) of the adult sea lamprey. Sections a, b, and c were stained with anti-lamprey GnRH-III, whereas sections d, e, and f were stained with anti-lamprey GnRH-I. CT, connective tissue; NH, neurohypophysis. a-f,  $\times 570$ .

1979; Nozaki and Kobayashi, 1979; Nozaki *et al.*, 1984; Wright *et al.*, 1994). However, most previous studies were conducted with antisera directed toward mammalian GnRH or lamprey GnRH-I, with unknown specificity to lamprey GnRH-III. Accordingly, it was difficult to know whether those studies revealed only GnRH-I or both forms of GnRH. Only recently, using antisera preferentially directed against either lamprey GnRH-I or -III, Tobet *et al.* (1995) reported that in the larval *P. marinus* the majority of irGnRH in the brain was lamprey GnRH-III, and when lamprey GnRH-I was seen, it was in cells that appeared to contain both forms of GnRH. Thus, they apparently reported the colocalization of GnRH-I and -III in the same cells in the brain of larval lampreys. It may be possible that both forms of GnRH are colocalized in the same cells in the larval lamprey, as reported by Tobet *et al.* (1995). However, in their study, a single immunostaining was performed

on the thick Cryostat sections (40–50  $\mu\text{m}$  in thickness). Even in the present study, it was difficult to determine whether irGnRH-I and -III cells were distinctly different from each other by a single immunostaining using two successive paraffin sections. Thus, further detailed studies using double immunostaining in single sections seem to be needed to determine whether both forms of GnRH are colocalized in the same cells in the larval lamprey.

In the present study, a subpopulation of irGnRH cells was found in the dorsal and ventral parts of the posterior hypothalamus, where more than 90% of irGnRH cells were irGnRH-III cells. In support of the present finding, Tobet *et al.* (1995) reported that irGnRH cells found in the posterior hypothalamus were all irGnRH-III cells in the larval *P. marinus*. IrGnRH cells in the posterior hypothalamus were not previously reported in the adult *P. marinus* using antisera

directed against GnRH-I (King *et al.*, 1988; Wright *et al.*, 1994). The difference between the present and the previous studies may be due to the very small populations of irGnRH-I cells located in the posterior hypothalamus.

Two or more forms of GnRH are present in the brains of representatives of all vertebrate classes (for review, see Sower, 1998). Moreover, different forms of GnRH molecules have been observed in different neuronal cells in all gnathostome vertebrate species thus far studied (Muske, 1997). For example, in teleosts, two or three GnRH forms have been structurally identified. A single form, chicken GnRH-II, is universally present, whereas the second or third form is one of the salmon, mammal, catfish, or seabream GnRHs (Sower, 1998). Among teleosts, tissue distributions of different forms of GnRHs in the brain are largely different in masu salmon (Amano *et al.*, 1991) and dwarf gourami (Yamamoto *et al.*, 1995): in these species only salmon GnRH cells project their fibers to the neurohypophysis. On the other hand, salmon GnRH and chicken GnRH-II cells are largely overlapping and intermixed in the goldfish (Kim *et al.*, 1995), and both cells project their fibers to the neurohypophysis. Thus, the present findings show that, in the lamprey, the distribution of irGnRH-I and -III cells are largely overlapping and intermixed, and both forms of irGnRH cells project their fibers to the neurohypophysis, which is similar to the situation seen in the teleost species that have been studied.

Based on the present and other immunocytochemical studies, both lamprey GnRH-I and -III neurons project their fibers primarily into the neurohypophysis from the preoptico-hypothalamic region (King *et al.*, 1988; Nozaki *et al.*, 1984). This results in a heavy accumulation of both irGnRH-I and -III nerve terminals in the neurohypophysis. In these studies in lampreys, GnRH was not found to be as widely distributed in extrahypothalamic regions as other neuropeptides, such as somatostatin (Nozaki *et al.*, 1984). Thus, if the function of the GnRH is to release GTH from the adjacent adenohypophysis, it must diffuse through the connective tissue separating adenohypophysial follicles from the neurohypophysis to reach the GTH cells. Unlike most vertebrates, a distinct vascular or neural link between the hypothalamus and the pars distalis has not been observed in either

lamprey or hagfish (Gorbman, 1965). However, there is evidence to support the concept of hypothalamic control of adenohypophysial function by diffusion of the neurohormones from the neurohypophysis to the pars distalis in lampreys (King *et al.*, 1988; Nozaki *et al.*, 1994) and to the entire adenohypophysis in hagfish (Nozaki *et al.*, 1975; Tsukahara *et al.*, 1986). It is hypothesized that in lampreys neurosecretory peptides such as GnRH can diffuse from the brain (neurohypophysis) to the adenohypophysis, either directly or by an additional route via secretion into the third ventricle, to be transported by tanycytes to the adenohypophysis and regulate secretory activity. Thus, the present study provides further evidence that GnRH regulates pituitary function by a neuroendocrine mechanism.

The functional significance of the two forms of lamprey GnRH in the brain of the lamprey is currently under investigation. However, since GTH in lampreys has not yet been identified, these studies have examined indirect responses to GnRH by measurement of plasma steroids and observation on gametogenesis. Ovulatory, spermatogenic, and steroidogenic responses to lamprey GnRH-I have been well documented in adult sea lampreys (Sower, 1989, 1990, 1997, 1998; Sower *et al.*, 1987). Other studies have demonstrated 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 (Bolduc and Sower, 1992; Fahien and Sower, 1990; MacIntyre *et al.*, 2000). Lamprey GnRH-III also has been shown to be biologically active in adult female lampreys, as determined by increased levels of plasma steroids following administration (Deragon and Sower, 1994; Gazourian *et al.*, 1997; Sower *et al.*, 1993). Lamprey GnRH-III has been detected in brain tissue samples of lampreys undergoing metamorphosis (Youson and Sower, 1991) and there was an increase of brain tissue GnRH during metamorphosis which coincided with the acceleration of gonadal maturation. In a more recent study and similar to lamprey GnRH-I, lamprey GnRH-III has also been shown to elevate levels of both progesterone and estradiol in the adult male lamprey after a single injection and to induce spermiation after four successive injections of lamprey GnRH-III (Deragon and Sower, 1994). Neither lamprey GnRH-III nor lamprey GnRH-I appeared to produce a

dose-related response in levels of estradiol and progesterone. Injection of adult male sea lampreys with lamprey GnRH-III induced a higher percentage of 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 the lamprey GnRH-III brain content concentration was three times greater than that of lamprey GnRH-I (Sower *et al.*, 1993). In contrast to these findings in males, another study showed that lamprey GnRH-I and -III were equally potent in inducing ovulation and stimulating steroidogenesis in female sea lampreys (Gazourian *et al.*, 1997). In both *in vivo* and *in vitro* studies, the actions of lamprey GnRH-I and -III and analogs appear to be dependent on temperature and/or stage of reproduction, likely reflecting differences in metabolic turnover or degradation rates of GnRH, GTH, and/or their receptors (Gazourian *et al.*, 1997, 2000). However, until the release rates of lamprey GnRH-I and lamprey GnRH-III are known and GTHs can be directly measured, the relative differences in potency of lamprey GnRH-I and -III can only be inferred.

It may be assumed that the molecular evolution of two GnRH cell types in the lamprey brain must have had some adaptive value. In sea lampreys, studies have shown that there are two high-affinity, specific classes of binding sites in the pituitary (Knox *et al.*, 1994). This is in contrast to all other vertebrates, in which only a single class of high-affinity GnRH binding sites in the pituitary has been demonstrated. The proximal pars distalis region of the pituitary contains most of the GnRH binding sites (Knox *et al.*, 1994). It is hypothesized that lamprey GnRH-I and -III each has a different binding site or receptor, suggesting differential control of the pituitary GTH-like molecules. In addition, if the two GnRH cells types are not involved in gonadotropic control but are acting as neuromodulators or neurotransmitters, it will be of interest to determine whether there are brain receptors that are preferential for one GnRH molecular type. It is also possible, if the GnRH cells respond differentially to steroids, that this provides the basis for separate steroid feedback control.

In summary, the present study provides further immunocytochemical data supporting the already com-

elling physiological evidence that indicates that both lamprey GnRH-I and -III act through the hypothalamic-pituitary-gonadal axis to modulate reproductive processes in the sea lamprey.

## ACKNOWLEDGMENTS

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