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Distribution of lamprey gonadotropin-releasing hormone-III (GnRH-III) in brains of larval lampreys (*Petromyzon marinus*)

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Abstract. Two immunoreactive forms of gonadotropin-releasing hormone (GnRH), lamprey GnRH-I and lamprey GnRH-III, were found in neurons in larval sea lampreys (*Petromyzon marinus*). Using antisera preferentially directed against either lamprey GnRH-I or -III, dense reaction product was seen in cell bodies in the rostral hypothalamus and preoptic area. Reaction product was also dense in fibers to and within the neurohypophysis, in addition to numerous fibers which projected caudally, beyond the neurohypophysis through the mesencephalon. The majority of immunoreactive GnRH was lamprey GnRH-III, and when lamprey GnRH-I was seen, it was in cells that appeared to contain both forms of GnRH. A small number of cells found in the caudal hypothalamus contained only immunoreactive lamprey GnRH-III, and these may constitute a functional subgroup within the population of GnRH neurons. In animals undergoing metamorphosis there was a large increase in reaction product in all GnRH-containing cells and fibers. A striking change within the distribution of GnRH cells was localized to a distinct group of GnRH-immunoreactive cells (GnRH-I and -III) in the ventral anterior hypothalamic area. These cells were minimally detectable in larvae, but during metamorphosis became densely filled with immunoreactive product in perikarya and distal processes. The results are consistent with the hypothesis that lamprey GnRH-III is an important form of GnRH during the maturation of GnRH cells and fibers, and further indicates that these cells have attained their normal positions in the preoptic area and hypothalamus before metamorphosis.

Key words: Preoptic area – Hypothalamus – Brain development – Metamorphosis – *Petromyzon marinus* (Cyclostomata)

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Introduction

For virtually all vertebrates the secretion of the decapeptide gonadotropin-releasing hormone (GnRH) is a key neuroendocrine function of the hypothalamus for regulating the pituitary-gonadal axis and ensuring reproductive competence. Although the GnRH decapeptide has been found to differ by up to 5 amino acids from lamprey to mammals (Sherwood et al. 1986; Sower et al. 1993), essential molecular features of the N and C terminals, and the length of the peptide, have been conserved (Sherwood et al. 1993). Similarly, the distribution of cells containing GnRH has retained certain features among those vertebrates that have been studied. In the central nervous systems of vertebrates, GnRH is found generally in neurons that are dispersed widely across the basal forebrain (e.g., King et al. 1982; Witkin et al. 1982; Goldsmith et al. 1983; King and Anthony 1984). In contrast, in lampreys, GnRH neurons are more restricted spatially than in other vertebrate species (King et al. 1988). GnRH-immunoreactive neurons in adults (Crim et al. 1979; King et al. 1988) and in late larval (Crim et al. 1979; Wright et al. 1993) lamprey have been noted to form a single bilateral dense arc along the third ventricle in the rostral hypothalamus and preoptic area.

In various vertebrate species, multiple forms of GnRH have been found to coexist in the same brains (Calvin et al. 1993; Sherwood et al. 1993), usually in different cells (see Lepretre et al. 1993). A new form of lamprey GnRH (referred to as lamprey GnRH-III) was sequenced recently after isolation from extracts of adult lamprey brains (Sower et al. 1993). Radioimmunoassay and HPLC data from extracts of brains of lampreys during their metamorphosis from larvae (ammocoetes) to adults suggest that the levels of lamprey GnRH-III (identified previously as a second GnRH-like molecule) may actually be higher than lamprey GnRH-I at early stages of development (Youson and Sower 1991). The present experiments were conducted to characterize the anatomical distribution of lamprey GnRH-III in larval lamprey brains. In the course of characterizing the distribution of

GnRH neurons in larval lampreys, we developed procedures with increased sensitivity for the immunocytochemical detection of GnRH in larval lamprey brains. The increased sensitivity has allowed us to determine how much of the lamprey GnRH cell and fiber system is in place during larval life. Using the sensitivity of the current methods, much of the GnRH system appears to be in place before metamorphosis begins, at least by the time the larvae are 67–150 mm long.

Materials and methods

Animals

Larval lampreys (ammocoetes), approximately 67–150 mm long, of *Petromyzon marinus* were collected by electrofishing several streams in northern Michigan (landlocked variety, in the spring of 1992) and in southern New Hampshire (anadromous variety, over a period of several months in the spring and summer of 1993). The New Hampshire lampreys were maintained in a fresh water flow-through system supplied with either 12°C well water, or ambient local reservoir water, under natural photoperiod; yeast was provided 3 times per week. Lamprey in Canada were housed in fiber-glass aquaria containing a layer of 5–10 cm of river silt and flow-through, dechlorinated water at 17–20°C under normal lighting. Forty animals were processed for immunocytochemical analyses. Four of the 40 animals had commenced metamorphosis, and changes in gill structure implied that they were at least stages 4 or 5 (Youson and Potter 1979).

Antibodies

Anti-peptide antibodies were generated toward lamprey GnRH-I (Sherwood et al. 1986; antiserum 1467 provided by Dr. Judy King; antiserum 21–134, Calvin et al. 1993) or lamprey GnRH-III (Sower et al. 1993; antiserum 3952 and 3951, generated by S.A.S.). Antisera 1467 was diluted 1/1 000 or 1/2 000. Antiserum 21–134 was diluted 1/4 000. Antiserum 3951 and 3952 were used at dilutions ranging from 1/3 000 to 1/5 000. Antisera were diluted in 1.0% BSA/0.05 M phosphate-buffered saline (PBS)/0.1% Triton X-100 (pH 7.4). Negative controls included omission of primary antisera, and preabsorption of each antiserum with 50 μM of lamprey GnRH-I decapeptide (Peninsula Laboratories) or lamprey GnRH-III decapeptide (generously provided by Dr. Russell Doolittle). No reaction product was noted if primary antisera were omitted. Results of antisera preabsorptions are given below.

Immunocytochemistry

Lamprey ammocoetes 67 to 150 mm long were anesthetized with tricaine methanesulfonate (MS222, 0.1%). Animals were decapitated and the head area was opened to allow diffusion of different aldehyde fixatives: Bouin-Hollande sublimate (BHS; Nozaki et al. 1985), acrolein (King et al. 1988), or 4% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (Tobet et al. 1993), all at 4°C. Brains were fixed overnight in the same fixative before transfer to 25% sucrose (w/v), 0.1 M phosphate buffer.

Cryostat sections (40–50 μm thick) of heads with brains and olfactory organs were placed sequentially into plastic containers with nitex mesh bottoms to facilitate changes in antibodies and buffer solutions. Immunocytochemical procedures were adapted from those used previously (Tobet et al. 1993), and optimized for maximal sensitivity using all GnRH antisera in larval lampreys. Briefly, sections were washed in 0.05 M phosphate-buffered saline (PBS; pH 7.5), and then pretreated sequentially with glycine (0.1 M), sodium borohydride (0.5%), and 5.0% normal goat serum (NGS) with 1.0% hydrogen peroxide and the Vector avidin/biotin blocking reagents all in PBS at 4°C. Tissues fixed in BHS were also treated overnight with potassium iodide in 90% ethanol to remove mercuric chloride. Antibodies were incubated with tissue sections over one or two nights at 4°C. Following primary antibody incubations, sections were washed with PBS containing 1.0% NGS at RT and then incubated with goat anti-rabbit biotinylated secondary antibodies (Vector Laboratories) in NGS/PBS for 2 h. After further washing, sections were incubated with Vectastain ABC reagent for 1 h (Vector Laboratories). Additional washes were done using TRIS-buffered-saline (TBS; 0.05 M, pH 7.4, @RT) A dark grey/black reaction product was produced using 0.025% 3,3′-diaminobenzidine (DAB) with 0.2% nickel ammonium sulfate in TBS as substrate with 0.02% hydrogen peroxide for 5 min (Tobet et al. 1993).

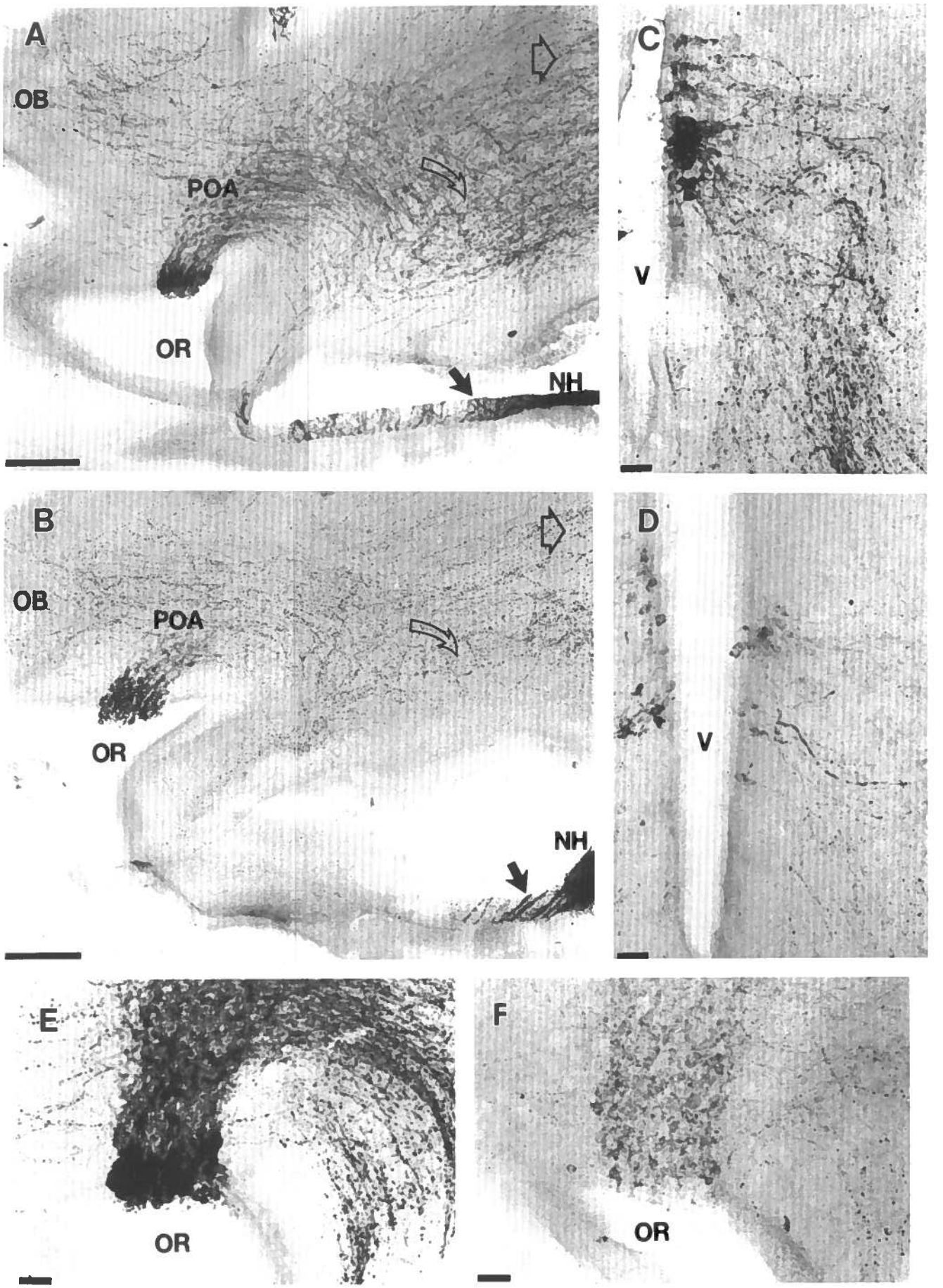
Results

Cells and fibers containing immunoreactive (ir) GnRH were detected in the brains of all larval and metamorphic lampreys in the preoptic area and hypothalamus. Fibers containing irGnRH were detected coursing through the olfactory bulb, preoptic area, hypothalamus, and further caudally through the midbrain and beyond (Figs. 1, 2). Of the 3 fixatives used, BHS provided for the most sensitive detection of lamprey GnRH by all 4 antisera (Figs. 1–4). Acrolein fixation produced excessive background reactivity for antisera 3951 and 3952. Although acrolein has been used successfully with antiserum 1467 in adult lamprey (King et al. 1988), in larvae, high backgrounds were produced. Acrolein fixation was compatible with antiserum 21–134 (Fig. 5). Immersion fixation in 4% paraformaldehyde/0.2% glutaraldehyde was inadequate for these studies.

Antibody specificity

Preabsorption experiments indicated that antisera could selectively detect lamprey GnRH-III, but not necessarily lamprey GnRH-I. Liquid phase preabsorption of antiserum 3952 with 50 μM of lamprey GnRH-III decapeptide completely eliminated reaction product in lamprey brain sections (not shown), whereas liquid phase preabsorp-

Fig. 1A–F. Photomicrographs illustrating the effect of liquid phase preabsorption of antisera directed toward lamprey GnRH-I or III. In **A** and **B** parasagittal sections from young ammocoetes were reacted with antisera 3952. In **B** the antisera was preabsorbed with 50 μM lamprey GnRH-I. In **C–F**, sections (coronal in **C** and **D**; parasagittal in **E** and **F**) were reacted with antisera 1467. In **D** the antisera was preabsorbed with lamprey GnRH-III and in **F** the antisera was preabsorbed with lamprey GnRH-1. The *curved open arrows* in **A** and **B** indicate the direction of fibers from the arc of cells in the preoptic area (*POA*) towards the neurohypophysis (*NH*). The *straight open arrows* indicate those fibers directed caudal to the hypothalamus towards the brainstem. *OB* Olfactory bulb; *OR* optic recess; *V* third ventricle. *Scale bars*: **A** and **B** 100 μm. *Scale bars*: **C–F** 20 μm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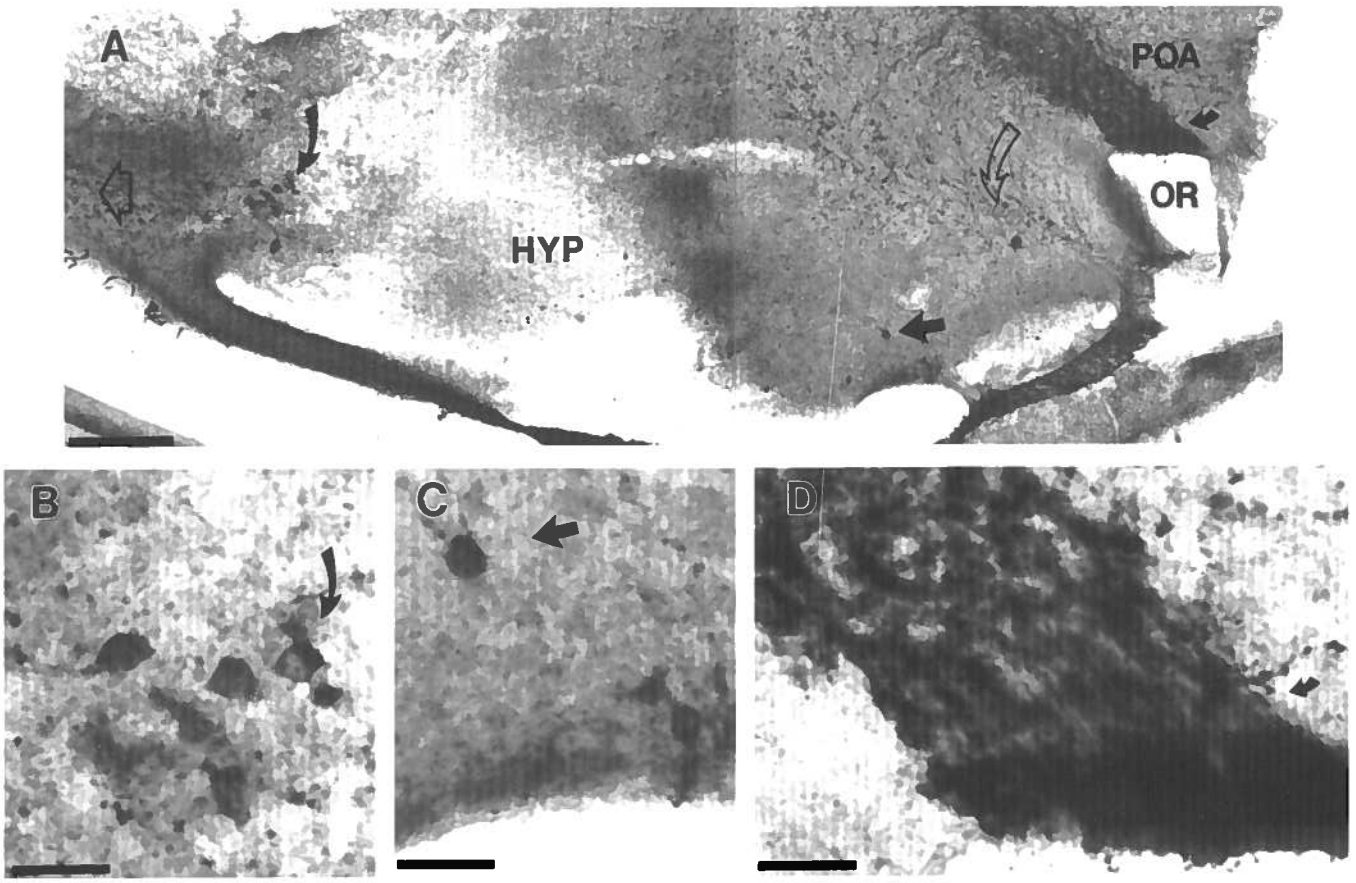


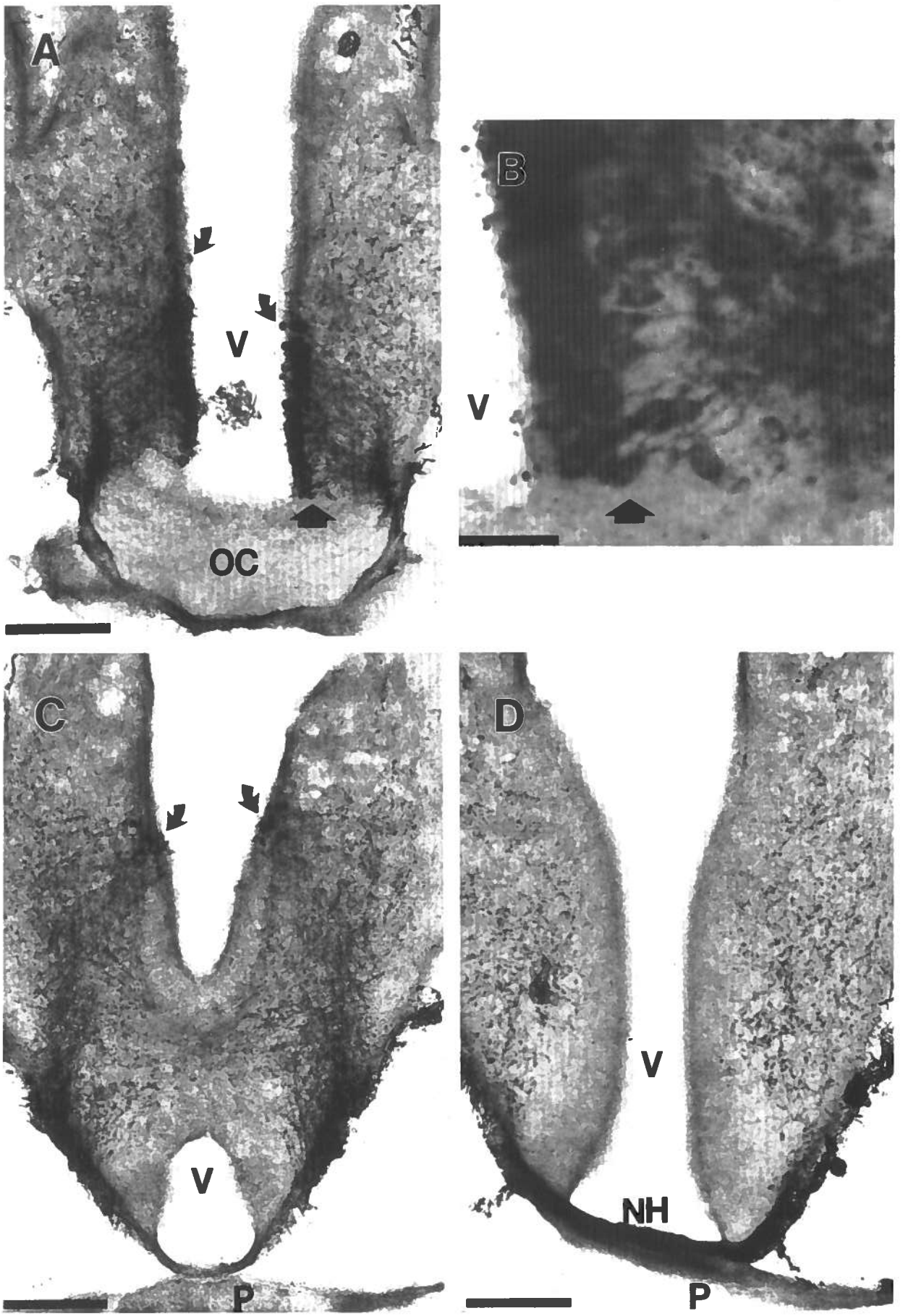
Fig. 2A–D. Photomicrographs from a parasagittal section from a young ammocoete reacted with antiserum 3952 illustrating the relationship of three different cell groups to each other. The cells shown at higher magnification in **C–D** are taken from the section shown in **A**. The *solid arrows* indicate the locations from which the higher magnification photomicrographs were taken. The *curved open arrow* in **A** indicates the direction of fibers from the arc of cells in the preoptic area (*POA*) towards the neurohypophysis. The *straight open arrow* indicates those fibers directed caudal to the hypothalamus towards the brainstem. *HYP* Hypothalamus; *OR* optic recess. *Scale bar*: **A** 100 μm. *Scale bars*: **B–D** 20 μm

tion with lamprey GnRH-I decapeptide had no discernible blocking effect (Fig. 1A, B). Immunoreactivity using antiserum 3951, was similarly blocked by preabsorption with lamprey GnRH-III decapeptide, but it was also slightly reduced following preabsorption with lamprey GnRH-I decapeptide. Immunoreactivity using antiserum 21–134 was completely eliminated following preabsorption with either lamprey GnRH-I or -III decapeptides. Immunoreactivity using antiserum 1467 was significantly reduced, but not eliminated, following liquid phase preabsorption with either lamprey GnRH-III (Fig. 1C, D) or lamprey GnRH-I (Fig. 1E, F). Preabsorption of antiserum 1467 with the combination of 50 μM lamprey GnRH-I and 50 μM lamprey GnRH-III decapeptides completely eliminated all cellular reaction product, but left a minimal number of immunopositive fibers (not shown).

Lamprey GnRH-III using antisera 3952 or 3951

In sagittal sections of premetamorphic ammocoetes, the majority of lamprey irGnRH-III cells in the forebrain formed an arc of cells which began in the rostral, ventral midline, above the optic chiasm and arched caudally and dorsally over the rostral hypothalamus towards the surface of the telencephalic lobe (Figs 1A, B, 2). In contrast to the fusiform appearance of irGnRH cells in mammals, lamprey irGnRH-III cells in larval lampreys were small and rounded, or cuboidal in shape (Figs. 1–3). Immunoreactive cells appeared more pear-shaped in metamorphic animals and fibers were more easily traced from their cells of origin (Fig. 4). In the coronal plane, irGnRH cells in ammocoetes and metamorphic lamprey were located in or near the walls of the third ventricle and fibers extended first laterally, and then ventrally, me-

Fig. 3A–D. Photomicrographs from coronal sections from a young ammocoete reacted with antiserum 3952 illustrating the relationship of the position of the rostral cells and the dense innervation of the neurohypophysis. The cells shown at higher magnification in **B** are taken from the section shown in **A**. The *solid arrow* indicates the locations from which the higher magnification photomicrograph was taken. The *curved arrows* in **A** and **D** indicate the most dorsal cells along the third ventricle (*V*) in each section. Fiber density in the neurohypophysis (*NH*) in **D** is too dense to distinguish individual fibers. *P* Pars distalis. *Scale bars*: **A, C, D** 100 μm. *Scale bar*: **B** 20 μm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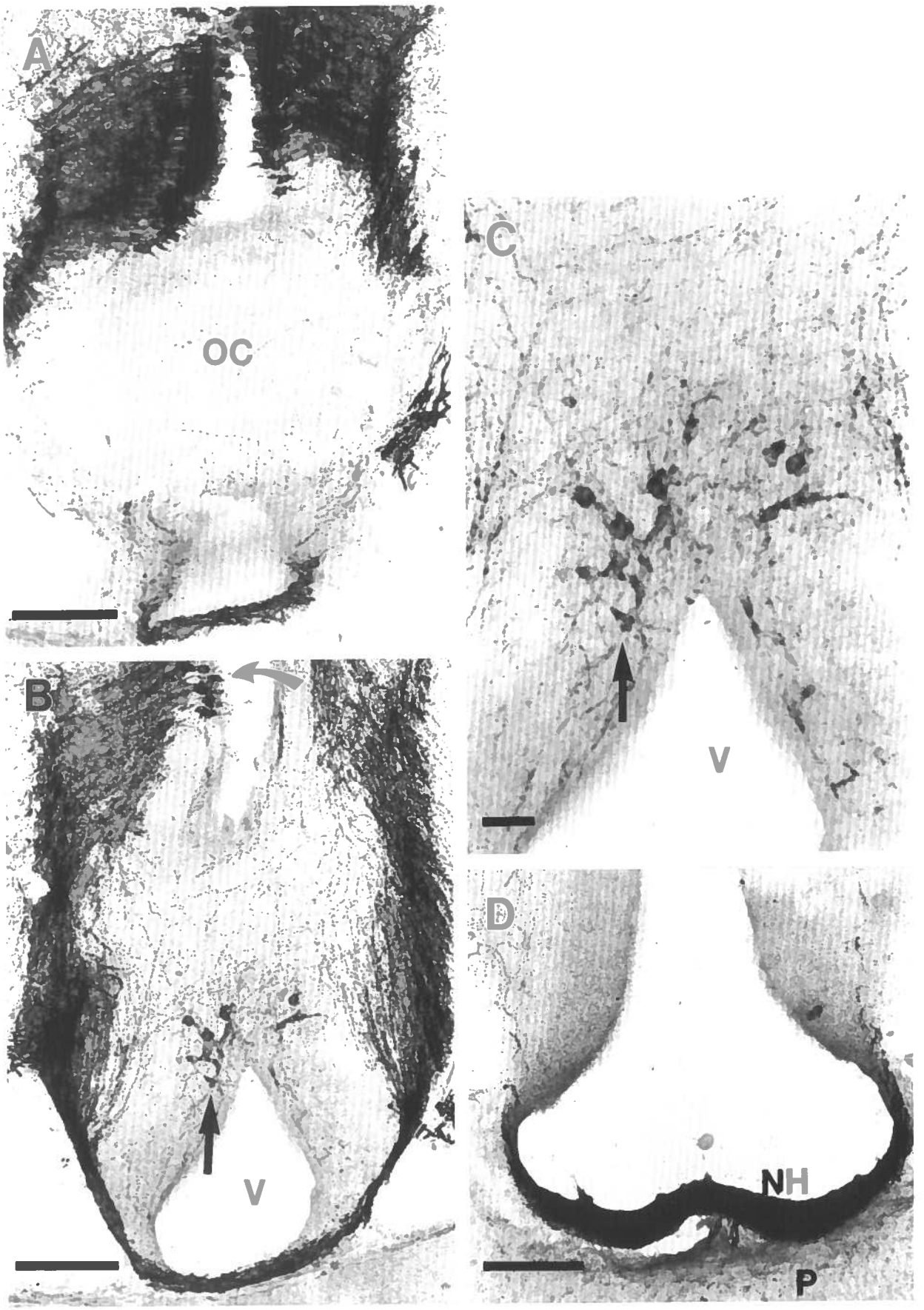
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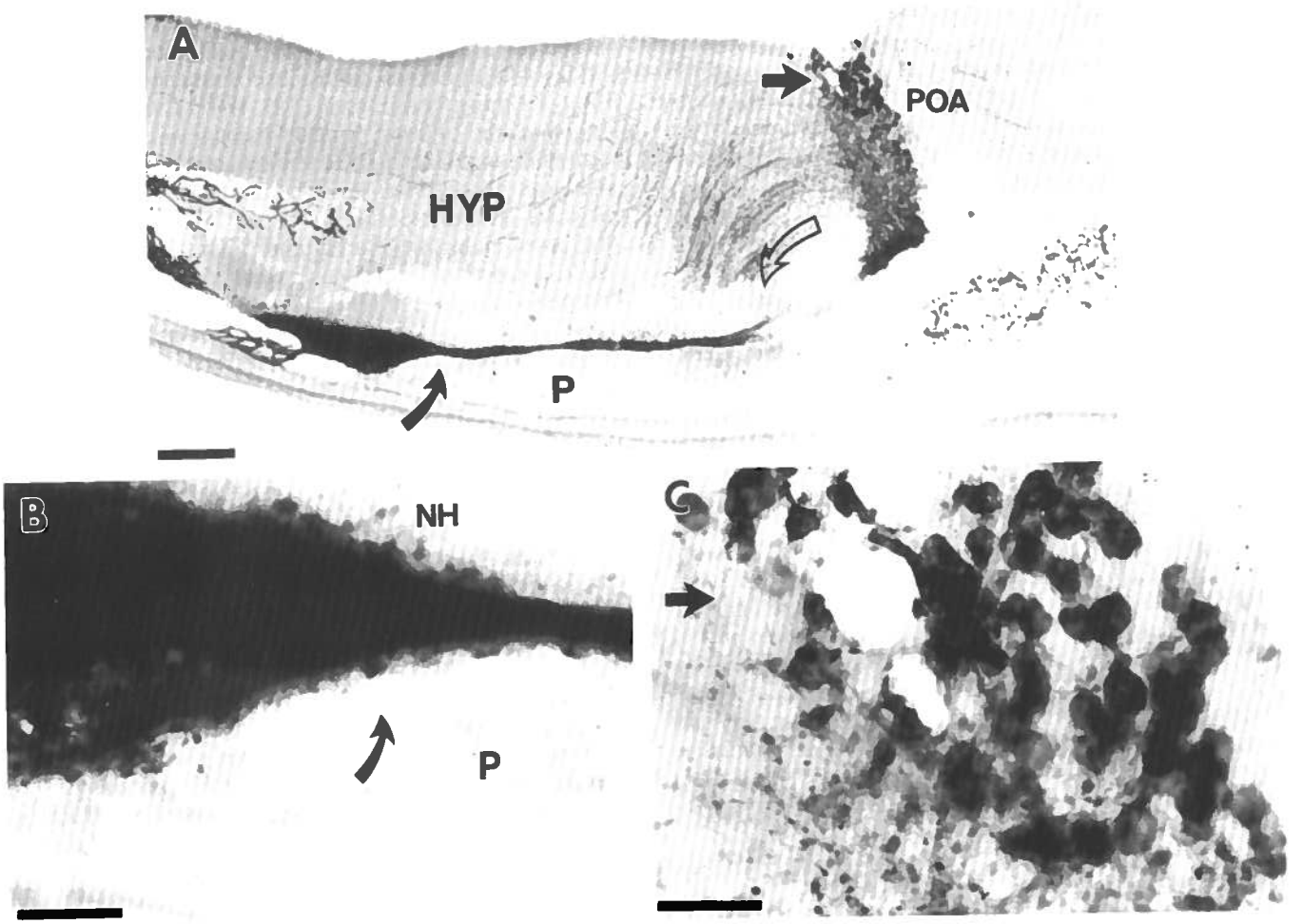

Fig. 5A–C. Photomicrographs from a parasagittal section from a young ammocoete reacted with antiserum 21–134 illustrating the strong immunoreactivity of this antiserum with rostral cell bodies and putative caudal terminals. The regions shown at higher magnification in **B** and **C** are taken from the section shown in **A** and are indicated by the *solid arrows*. The *curved open arrow* in **A** indicates the direction of fibers from the arc of cells in the preoptic area (*POA*) towards the neurohypophysis (*NH*). In contrast to the other antisera, fibers directed caudal to the hypothalamus towards the brainstem were infrequent using 21–134. *HYP* Hypothalamus; *P* pars distalis. *Scale bar*: **A** 100 μm. *Scale bars*: **B** and **C** 20 μm

dially and caudally to reach the neurohypophysis (Figs. 3 and 4). Fibers containing irGnRH collected into fascicles in the rostral neurohypophysis, and appeared to completely fill the caudal neurohypophysis (Figs. 1–4).

Two additional subgroups of irGnRH-containing neurons were recognized on the basis of their location and cellular appearance. One group of immunoreactive cells was located in the midline in the caudal hypothalamus (Fig. 2A, B). The other additional group began immediately caudal to the optic chiasm and was characterized by neurons with wider inter-neuronal spacing than in the arc of cells in the rostral preoptic area (Fig. 2A, C). In ammocoetes less than 10 cm long, cells in both these additional groups contained low levels of immunoreaction product and were difficult to locate. During metamorphosis, the cells in the group immediately caudal to the optic chiasm underwent a striking change in their immunoreactive appearance. These cells became even more elongated, sometimes triangular, and a major process was often visible oriented away from the third ventricle in coronal sections (Fig. 4B, C).

In addition to profuse projections towards the neurohypophysis there were also numerous projections to extrahypothalamic sites. Fibers filled the caudal two-thirds of the olfactory bulbs, appearing as if they had originated in the preoptic area (Fig. 1A, B). There was no evidence of fibers associated with the olfactory epithelium or the olfactory nerve. Fiber projections to sites caudal to the hypothalamus arose from similar sites in

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Fig. 4A–D. Photomicrographs from coronal sections from a metamorphic ammocoete reacted with antiserum 3952 illustrating the relationship of the position of the rostral cells, the dense innervation of the neurohypophysis, and the appearance of the ventral hypothalamic cells. The *solid straight arrow* in **B** indicates the general position of the ventral hypothalamic cell group and directly points to a good example of their triangular shape with process directed away from the ventricle. This region is shown at higher magnification in **C**. The *solid curved arrow* in **B** indicates cells from the dorsal caudal portion of the arching cell group that originates in the rostral preoptic area shown in **A**, above the optic chiasm (*OC*). Fiber density in the neurohypophysis (*NH*) is too dense to distinguish individual fibers. *P* Pars distalis. *Scale bar*: 100 μm

the preoptic area that gave rise to the neurohypophyseal projection (Figs. 1A, B and 2A). Caudally directed fibers, however, were not bundled as tightly as those fibers directed toward the neurohypophysis. We did not determine the targets of either the fibers directed caudally beyond the hypothalamus, or of the irGnRH cells that were in the caudal hypothalamus.

irGnRH elements using antisera 1467 or 21–134

Neurons immunoreactive with antiserum 1467 were located in the rostral midline, both as part of the arc of cells in the preoptic area (Fig. 1c–f) and the ventral cells caudal to the optic chiasm. The distribution of irGnRH cells using antiserum 1467 was virtually indistinguishable from lamprey irGnRH-III cells (including after preabsorption with lamprey GnRH-III decapeptide; Fig. 1d). Additionally, the 1467ir cells were also of the same shape and size as lamprey irGnRH-III cells. By contrast, the group of cells immunoreactive with 3951 and 3952, located in the caudal hypothalamus, was not apparent using antisera 1467 or 21–134. The neurohypophysis was completely filled with immunoreaction product using antiserum 1467. Using antiserum 21–134, immunoreaction product was less striking in fibers but similarly dense in rostral cell bodies, and caudally near putative terminals in the neurohypophysis, compared to all other antisera (Fig. 5).

Discussion

The present study reveals the localization of lamprey GnRH-III peptide in larval lampreys which, based on radioimmunoassay and HPLC experiments (Youson and Sower 1991), are higher than lamprey GnRH-I prior to metamorphosis. Our immunocytochemical results also indicate that lamprey GnRH-III is the primary form of GnRH present in premetamorphic GnRH-containing neurons. A significant component of antiserum 1467 immunoreactive material in larvae likely was due to the presence of lamprey GnRH-III. Only a limited number of cells remained faintly immunoreactive with 1467 after preabsorption with lamprey GnRH-III peptide, and these cells were not detected after additional preabsorption with lamprey GnRH-I peptide. Thus, authentic lamprey GnRH-I peptide may be represented by the immunoreactive elements found after preabsorption with lamprey GnRH-III, and not present after further preabsorption with lamprey GnRH-I. The preabsorption of antiserum 1467 with lamprey GnRH-III in adult animals (M. Nozaki, A. Gorbman and S. Sower, unpublished) only minimally reduced the amount of reaction product, whereas preabsorption with lamprey GnRH-I eliminated most of the reaction product (M. Nozaki, A. Gorbman and S. Sower, unpublished; King et al. 1988). This pattern of preabsorption results can be explained if there is significantly more lamprey GnRH-I peptide in adults compared to the larvae. Indeed, HPLC and RIA analyses of GnRH content have shown a 10-fold increase of lamprey GnRH-I in adults compared to larvae (Fahien and Sower 1990; Youson and Sower 1991; Bolduc and Sower 1992). Additional selective antisera must be generated to fully test this possibility.

All of the antisera used in the current study recognized lamprey GnRH-III to some extent. Based solely on immunocytochemical results, antiserum 3952 was specific for lamprey GnRH-III, while antiserum 1467 was the most selective for lamprey GnRH-I. Antiserum 21–134 is composed of antibodies that recognize both lamprey GnRH-I and GnRH-III equally well. Antiserum 21–134 did not detect as much reaction product in nerve processes as did antisera 1467 or 3952, although reaction product was dense throughout the neurohypophysis. Since lamprey GnRH -I and -III differ in amino acid positions 3, 5 and 6, antiserum 21–134 behaves immunocytochemically as if it is directed towards the N and/or C terminals of the molecule, where the two peptides are identical in positions 1 and 2 and 7–10 (Sower et al. 1993). Interestingly, independent modification of positions 2 or 7 eliminates the reactivity of 21–134 with lamprey GnRH-I decapeptide in RIA (Calvin et al. 1993), indicating that both N and C terminals are both important determinants for immunoreactivity and suggesting that 21–134 might be a conformational dependent antiserum (Copeland et al. 1979).

The distribution of the majority of cell bodies containing lamprey GnRH-I and -III corresponds well to that described in earlier studies for both adult (Crim et al. 1979; King et al. 1988) and developing lamprey (Crim et al. 1979; Wright et al. 1993). Cell bodies were primarily restricted to a small region within the rostral hypothalamus and preoptic area. These cell bodies gave rise to numerous fibers which filled the neurohypophysis with immunoreactive fibers containing GnRH. Our data show that in larvae, the cell bodies and fibers of a GnRH system exist primarily in the form of lamprey GnRH-III and thus could possibly be primed by physiological changes which could lead to the addition of lamprey GnRH-I and to activation. It is likely that in previous immunocytochemical studies which detected less irGnRH in ammocoetes of the same (Wright et al. 1993) or different species (Crim et al. 1979) significant amounts of irGnRH were extracted by processing for paraffin embedding (Goldsmith and Ganong 1975; Clayton et al. 1981). However, the stage-related increases observed in the previous studies (Crim et al. 1979; Wright et al. 1993), similar to changes noted in the current study, clearly indicate that after irGnRH cell bodies and fibers are detectable, they do produce significantly more peptide during metamorphosis. The increased synthesis is further suggested by the changing appearance of the immunoreactive neurons in the current study. Thus, as described previously, irGnRH neurons in adult lamprey were noted as pear shaped and pseudo-unipolar (King et al. 1988) whereas in the ammocoetes of the present study, cells were small and rounded with low levels of reaction product in proximal processes and highly immunoreactive distal processes in the neurohypophysis.

The most notable developmental change in the distribution of GnRH cells in the current study was in a ven-

tral hypothalamic cell group caudal to the optic chiasm. This cell group was barely detectable in ammocoetes, but during metamorphosis filled with irGnRH, particularly lamprey GnRH-III. These cells were also significantly more elongated than the rostral preoptic cells which are noted for their early cuboidal appearance and their later pear-shaped appearance. Cells in this grouping differed further from those in the densely packed main preoptic area by their relatively wider spacing. An earlier analysis of adult lampreys had mapped these cells, but considered them part of the arc-shaped preoptic area group (King et al. 1988). Their appearance in the larvae and metamorphic animals of the present study suggests that they are not a simple continuation of the arc-shaped preoptic area group. Even though the cells in this group were located close to the rostral pars distalis and neurohypophysis they maintained major processes directed away from the third ventricle. The striking increase in irGnRH in these cells in larger ammocoetes and during metamorphosis, coupled with their location and the orientation of their processes, lead us to suggest that they may play a unique role during metamorphosis.

The small number of caudal irGnRH neurons which contained only lamprey irGnRH-III, or the elongated cells caudal to the optic chiasm, may constitute homogenous functional subgroups within the population of GnRH neurons (Hiatt et al. 1992; King and Rubin 1992). Even though the irGnRH cell distribution in the lamprey brain is unique in its relative lack of dispersion, there may be physiologically relevant regulation that is related to cell position, or selective expression of lamprey GnRH-III. For instance, among GnRH neurons in mammals, subgroups have been defined by the expression of specific cell surface carbohydrates (Tobet et al. 1993), the synthesis of galanin (Merchenthaler et al. 1991), and the expression of Fos protein at particular times during the estrous cycle (Lee et al. 1990).

In other species, in which two forms of GnRH have been found there have been significant differences in their distribution. In the non-mammalian vertebrates studied, chicken GnRH-II has been the most common alternate form of GnRH reported (Muske and Moore 1990; Amano et al. 1991; Millam et al. 1993; van Gils et al. 1993; Millam et al. 1993; Lepretre et al. 1993). With one exception (Conlon et al. 1993), the localization of chicken GnRH-II has been generally in regions not typically associated with pituitary function. There are two reports of multiple forms of GnRH in mammals; one in musk shrews (*Suncus murinus*), in which a distinct caudal cell group was found containing immunoreactive chicken GnRH-II (Delovade et al. 1993). The other report provides an apparent exception to the "rule" that alternate forms of GnRH are usually found in separate neurons, and rather than chicken GnRH-II, involves lamprey GnRH-I. In humans, lamprey GnRH (ostensibly lamprey GnRH-I, but potentially lamprey GnRH-III) was found in the same sites as mammalian GnRH (Stopa et al. 1988). In the current study, two forms of GnRH were found in the lamprey brain. Interestingly, the distribution of two forms of GnRH in lamprey brain was more similar to humans than other vertebrates with a substantial portion of the cell populations apparently containing both lamprey GnRH-I and -III.

In the current study, irGnRH neurons in the brains of larval lamprey were not detected in positions suggestive of migration from the olfactory placode into the brain (Muske and Moore 1988; Schwanzel-Fukuda and Pfaff 1989; Wray et al. 1989; Daikoku-Ishido et al. 1990; Ronnekleiv and Resko 1990; Norgren and Lehman 1991; Tobet et al. 1993). All irGnRH neurons were detected in the preoptic area or caudal to it. Fibers that contained irGnRH reached into the olfactory bulb, but appeared as if they had originated from cells located caudal to the olfactory bulbs. To determine the developmental origin, and migratory pathway of GnRH cells, embryos and younger larvae will need to be examined. The sensitivity of the methods used for lamprey GnRH-III in the current study will be important for determining when and where GnRH neurons first appear during lamprey development.

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