

Neuronal systems immunoreactive with antiserum to lamprey gonadotropin-releasing hormone in the brain of *Petromyzon marinus*

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Summary. The role of gonadotropin-releasing hormone (GnRH) in mammalian reproduction has been studied extensively; however, the role of a structurally different, but related, decapeptide is not well characterized in the most primitive class of vertebrates, Agnatha. Utilizing an antiserum directed to the recently characterized lamprey GnRH, we examined immunoreactive neuronal perikarya and nerve fibers in sections from the brain of the sea lamprey, *Petromyzon marinus*, using the unlabeled peroxidase-antiperoxidase method. Neuronal perikarya and fibers were immunopositive with antisera generated to lamprey GnRH and also to certain antisera generated to mammalian GnRH. Immunopositive neuronal perikarya were detected in an arch-shaped population extending from ventral to dorsal pre-optic areas. Fibers from these cells projected to the neurohypophysis via the preoptico-hypophyseal tract, but in addition also protruded into the third ventricle. Additionally, some fibers coursed along the external surface of the brain, and may also release GnRH into meningeal compartments. The presence of fully processed, mature decapeptide is indicated within neuronal perikarya, as well as in projecting nerve fibers and terminals. No reaction product was detected in sections incubated with an antiserum to the interior amino acid sequences of mammalian LHRH. This finding supports the structure reported for lamprey GnRH by Sherwood et al. (1986).

Key words: GnRH, LHRH – Reproduction – Neurohypophysis – Hypothalamus – Lamprey, *Petromyzon marinus*

The role of the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH of fishes, or luteinizing hormone-releasing hormone – LHRH – of mammals) in reproduction and ovulation is well established in mammals and many non-mammalian vertebrates. In these animals, GnRH or LHRH released from neurovascular terminals of the median eminence reaches target anterior pituitary gonadotrophs via a vascular portal system. However, both hagfish and lampreys, the only living representatives of the most ancient class of vertebrates, Agnatha, lack this vascular network (Gorbman 1965; Tsuneki and Gorbman 1975; Tsukahara et al. 1986). Only lampreys have been reported to lack

GnRH terminals in the posterior pituitary (Ball 1981). Thus, mechanisms of delivery of hypothalamic releasing hormones to target cells are unknown in these animals. One purpose of this study was to identify potential release sites of GnRH in the lamprey, by specifying sites of termination of neuronal fibers immunopositive with an antiserum directed to lamprey GnRH. These investigations were performed using adult lampreys in their final reproductive stages prior to the single spawning that occurs in their lifetime.

Several characteristics of the GnRH decapeptide have been conserved among all vertebrate species, e.g., the length of the peptide, the amino acid residues at amino and carboxy terminals as well as modifications of these residues, amidation of the carboxy terminal, and cyclized pyro-Glu at the amino terminal. Differences within the molecule occur primarily in amino acids 7 and 8. Sherwood and colleagues (1986) have demonstrated that the gonadotropin-releasing hormone of lampreys also has additional differences in amino acids in positions 3, 5 and 6, retaining only the pGlu-His in positions 1 and 2, Ser in 4, and pro-Gly NH₂ in positions 9 and 10. Sower and colleagues (1987) have demonstrated that the lamprey GnRH is biologically active in stimulating the pituitary-gonadal axis of lampreys, as evidenced by elevations in plasma steroid levels and advanced ovulation in response to administration of lamprey GnRH. A second goal of this study was to identify neuronal elements immunoreactive with antibodies raised to synthetic lamprey GnRH using immunocytochemistry, and to compare these immunoreactive elements with those detected using selected antisera to mammalian LHRH.

Materials and methods

Adult landlocked sea lampreys, *Petromyzon marinus*, were captured on their spawning migration in the Chobogan River, Michigan, transported to New Hampshire and maintained at the freshwater facility of the University of New Hampshire in 2.6 m cylindrical tanks supplied with flowing reservoir water. The animals, 13 males and 2 females, were sacrificed by decapitation. The brains were quickly removed and immersed in 10% acrolein in 0.1M Sorensen's phosphate buffer, pH 7.2, for 1 h, and washed in Sorensen's buffer. Some brains were kept in a cryoprotectant mixture (Watson et al. 1986). Sections were cut on a Vibratome at thicknesses of 50 and 100 µm. Alternate sections were placed in containers to be treated with different antisera.

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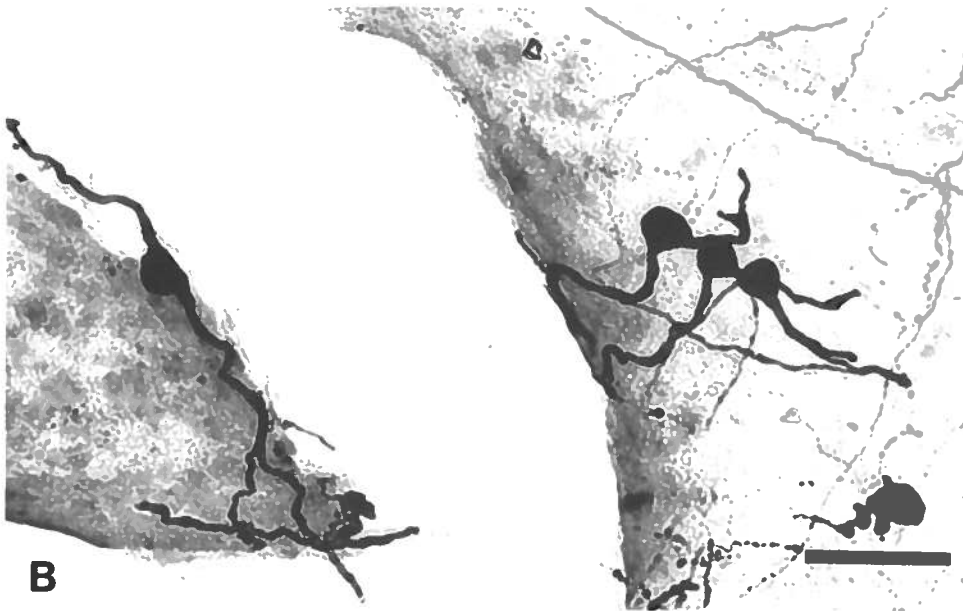
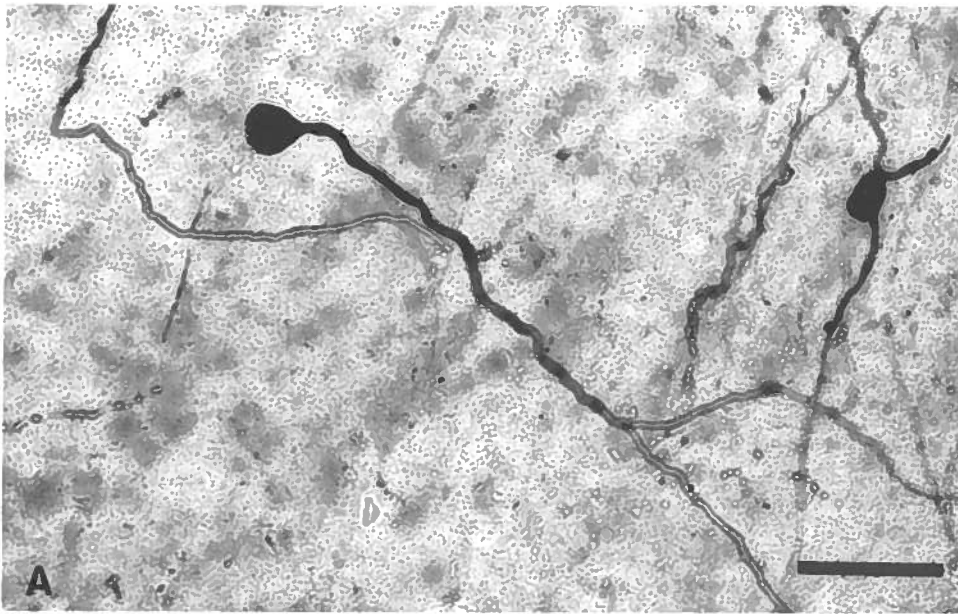


Fig. 1 A, B. Neuronal perikarya immunoreactive with an antiserum directed to the N-terminal p-Glu and C-terminal amidated Gly of mammalian LHRH (AA422) are present in the preoptic area of an adult sea lamprey (A). Immunopositive perikarya cluster in the vicinity of a large blood vessel in the preoptic area (B). $\times 380$. Bar = 50 μ m

Prior to application of antisera, sections were pre-treated with 0.1 M sodium metaperiodate and 1% sodium borohydride to remove residual reactive aldehyde as well as 10% normal goat serum with 1% hydrogen peroxide to remove endogenous peroxidase activity.

An antiserum generated to lamprey GnRH was obtained from Judy A. King and Robert P. Millar (JAK 1467, University of Cape Town); antisera to mammalian LHRH were obtained from R.P. Millar (RM 1076), and Akira Arimura (AA 422, Tulane University); antisera to gonadotropin-associated peptide (GAP) of the human precursor were obtained from Peter Seeburg (PS 39A, Genentech, Inc.) and R.P. Millar (RM 8/5); an antiserum to salmon GnRH was obtained from R.P. Millar (RM 802). Lamprey antiserum demonstrated 0.2% cross reactivity with m-LHRH in radioimmunoassay; therefore, in some cases this antiserum (JAK 1467) was incubated with m-LHRH at a concentration of 2×10^{-6} M for 24 h prior to use to remove any

cross-reacting antibodies. In addition, lamprey, salmon and mammalian antibodies were each incubated with their respective antigens prior to use as controls for non-specific binding of polyclonal antisera.

Following pretreatment of sections and pre-absorptions of antisera, sections were incubated with antisera at concentrations from 1/500 to 1/750, free floating in a vial on a shaker at 4° C for 2 to 4 days. Following this incubation with primary antisera, sections were washed and incubated with the secondary antiserum, goat anti-rabbit IgG (Antibodies, Inc., Davis, California), at a concentration of 1/50 for 1–2 h. They were then washed and incubated with the tertiary antiserum, rabbit peroxidase anti-peroxidase (Sternberger-Meyer Immunocytochemicals, Inc., Jarrettsville, Maryland), at a concentration of 1/100 for 1 h. Incubations with secondary and tertiary antisera were carried out at room temperature on a shaker. Following washes, sections were incubated with diaminobenzidine (25 mg%)

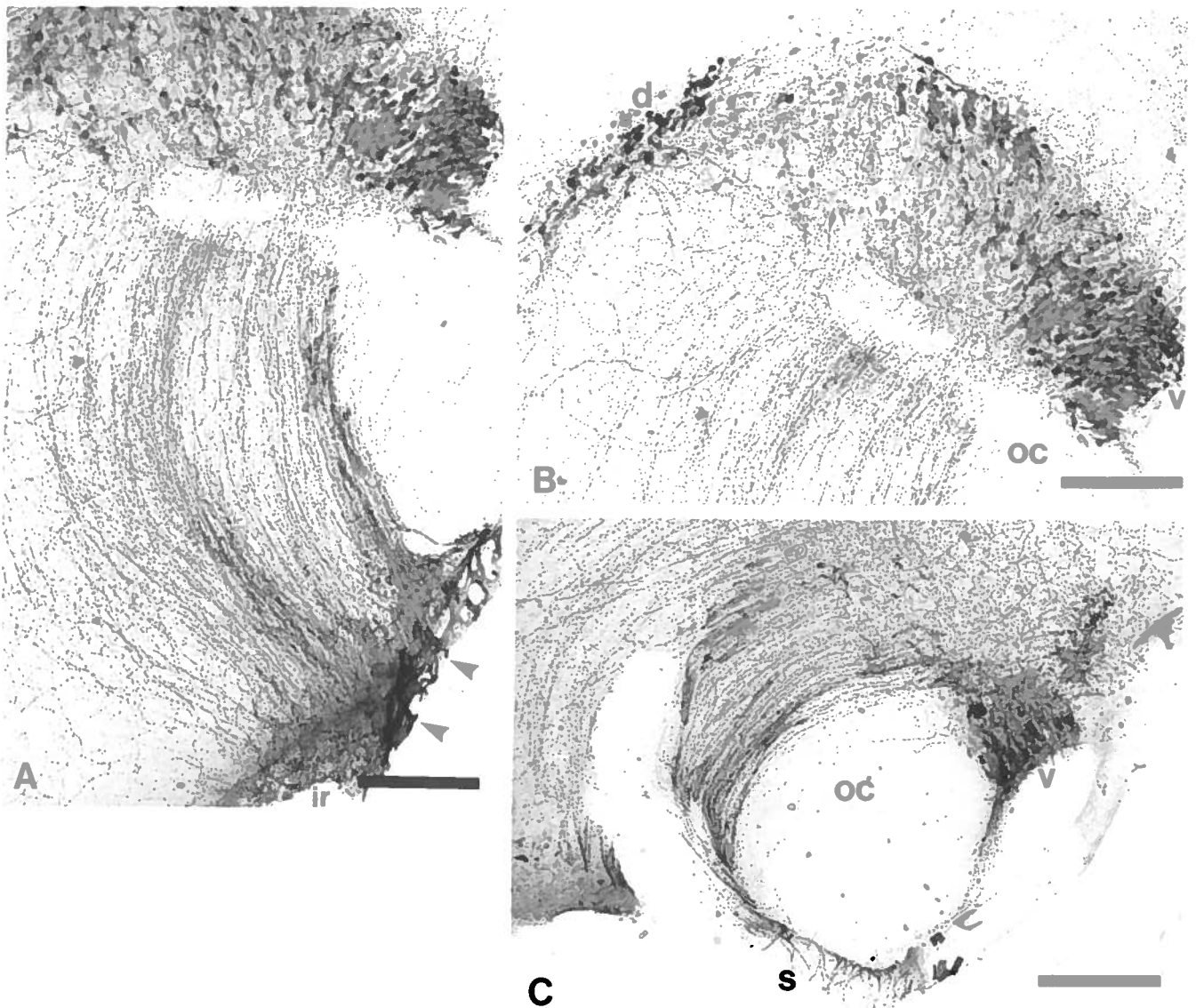


Fig. 2A-C. Neuronal perikarya immunoreactive with an antiserum generated to lamprey GnRH (JAK 1467) form a population of cells spanning ventral (*v*) to dorsal (*d*) preoptic areas; this organizational pattern is particularly obvious in sagittal sections (A-C). The ventral population is rostral to the optic chiasm (*oc*; B, C). Nerve fibers from both these populations stream ventrally toward the base of the brain (*arrowheads*) and into the neurohypophysis, which has been torn away from the section. The infundibular recess (*ir*; A) is present. Other fibers reach the neurohypophysis via the external surface of the brain (*s*; C). $\times 90$. Bar = 200 μ m

and hydrogen peroxide (0.05%) in a chamber (Paull and King 1985) on a shaker at room temperature for 30 min. Selected sections containing immunoreactive perikarya in the periventricular preoptic area were subsequently processed for electron microscopy as previously described by Anthony and King (1986). Remaining sections were washed, mounted, air dried, viewed and photographed using a Zeiss Photophot. Details of the immunocytochemical procedure have been described previously (King et al. 1983; Anthony and King 1986).

Results

Neuronal perikarya immunopositive with antisera to lamprey GnRH (JAK 1467) and mammalian antisera (AA 422) were simple pseudo-unipolar or bipolar neurons with few collaterals (Fig. 1); occasionally, tripolar neurons were also

observed. The pear-shaped perikarya measured approximately 13–18 by 13–18 μ m. No reaction product was observed in sections incubated with antisera to lamprey GnRH pre-absorbed with synthetic lamprey GnRH or with antisera to mammalian LHRH pre-absorbed with synthetic mammalian LHRH. GnRH-immunopositive neurons were observed in two cell groups of the basal forebrain, one in the most ventral aspect of the preoptic area, rostral to and abutting the optic chiasm, and a second group more dorsally positioned in the periventricular preoptic area (Figs. 2, 3). These two groups, which appeared distinct in some sagittal and horizontal sections, formed a continuous arc-shaped population, when viewed in a mid-sagittal plane (Fig. 2B).

GnRH-immunoreactive nerve fibers projecting from cells in the dorsal preoptic area arched ventrally (Fig. 2A) toward the base of the brain, condensing in the ventral

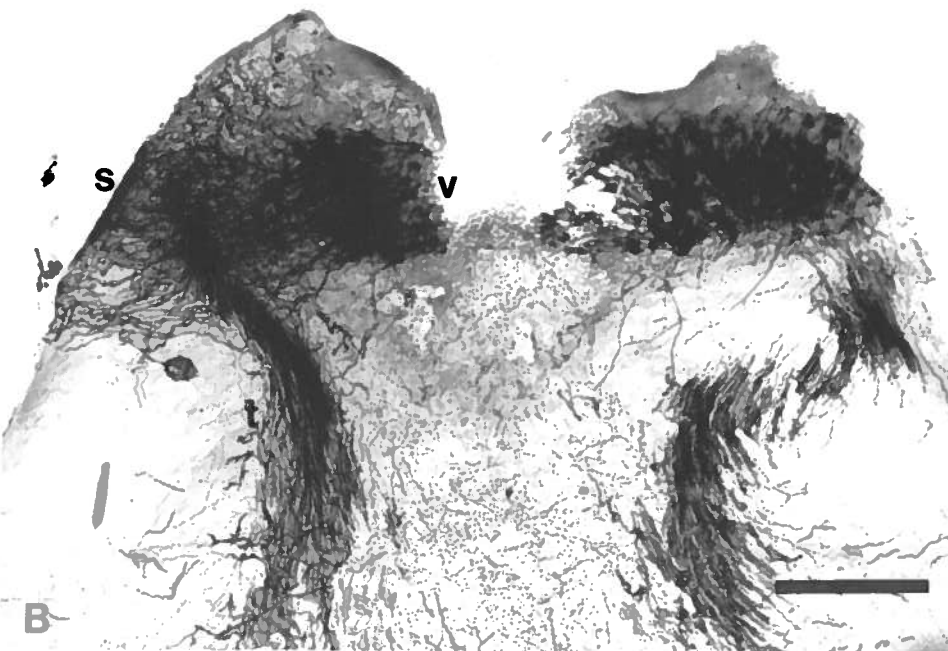
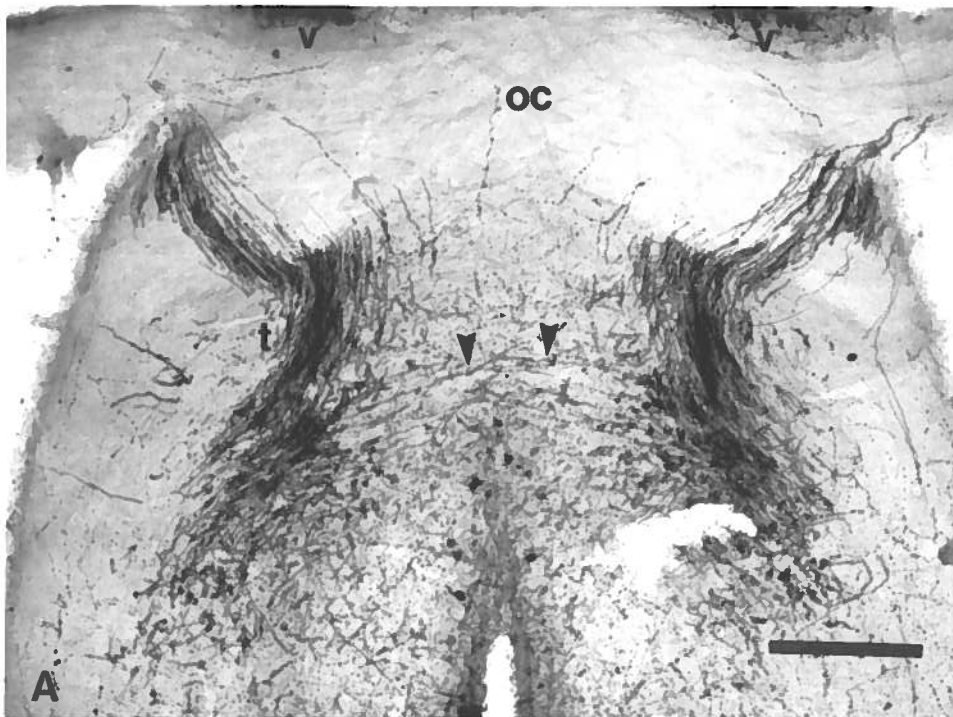


Fig. 3A, B. Nerve fibers that condense to form the preoptico-hypophyseal tract (*t*) are particularly apparent in horizontal sections through the ventral preoptic area. In the most ventral section (**A**), which contains the crossing fibers of the optic chiasm (*oc*), a very small portion of the ventral group of cells (*v*) is present rostral to the chiasm. The preoptico-hypophyseal tract is composed of fibers originating from both the ventral group and fibers that have descended from the dorsal group, which is not seen at this horizontal level, but is present in Fig. 4B. Note that some fibers appear to decussate (*arrowheads*) from one tract to the other. The ventral group of cells (*v*) is more fully represented in a section that is slightly more dorsal (**B**) than that in **A**. In addition to the fibers of the preoptico-hypophyseal tract (*t*), fibers projecting laterally to the external surface of the brain (*s*) are also present. Antiserum = AA422. $\times 100$. Bar = 200 μm

hypothalamus to form a dense bundle, consisting of large numbers of nerve fibers organized into well-formed preoptico-hypophyseal tracts (Fig. 3). Fibers from the ventral cells joined with those from the dorsal preoptic cells in the preoptico-hypophyseal tract to project caudally to the neurohypophysis (Figs. 2A, 3). These tracts were bilateral, but decussating fibers were often apparent just caudal to the optic chiasm (Fig. 3).

Two other hypothalamic projection routes were equally represented. One of these additional routes was composed of nerve fibers that approached the neurohypophysis by

way of the external surface of the brain, in association with the meninges (Fig. 4A). These were apparent along the lateral margins of the brain (Fig. 3B) as well as along the base of the brain ventral to the optic chiasm (Fig. 2). The second route was represented by fibers projecting to, and directly contacting, the third ventricle. The ventricular projections appeared as bulbous protrusions emanating from sub-ependymal periventricular dorsal preoptic cells (Fig. 4B). Electron microscopy confirmed that these bulbous terminals directly contact the third ventricle (Fig. 5). The cell groups and pathways are diagrammed in Fig. 6.

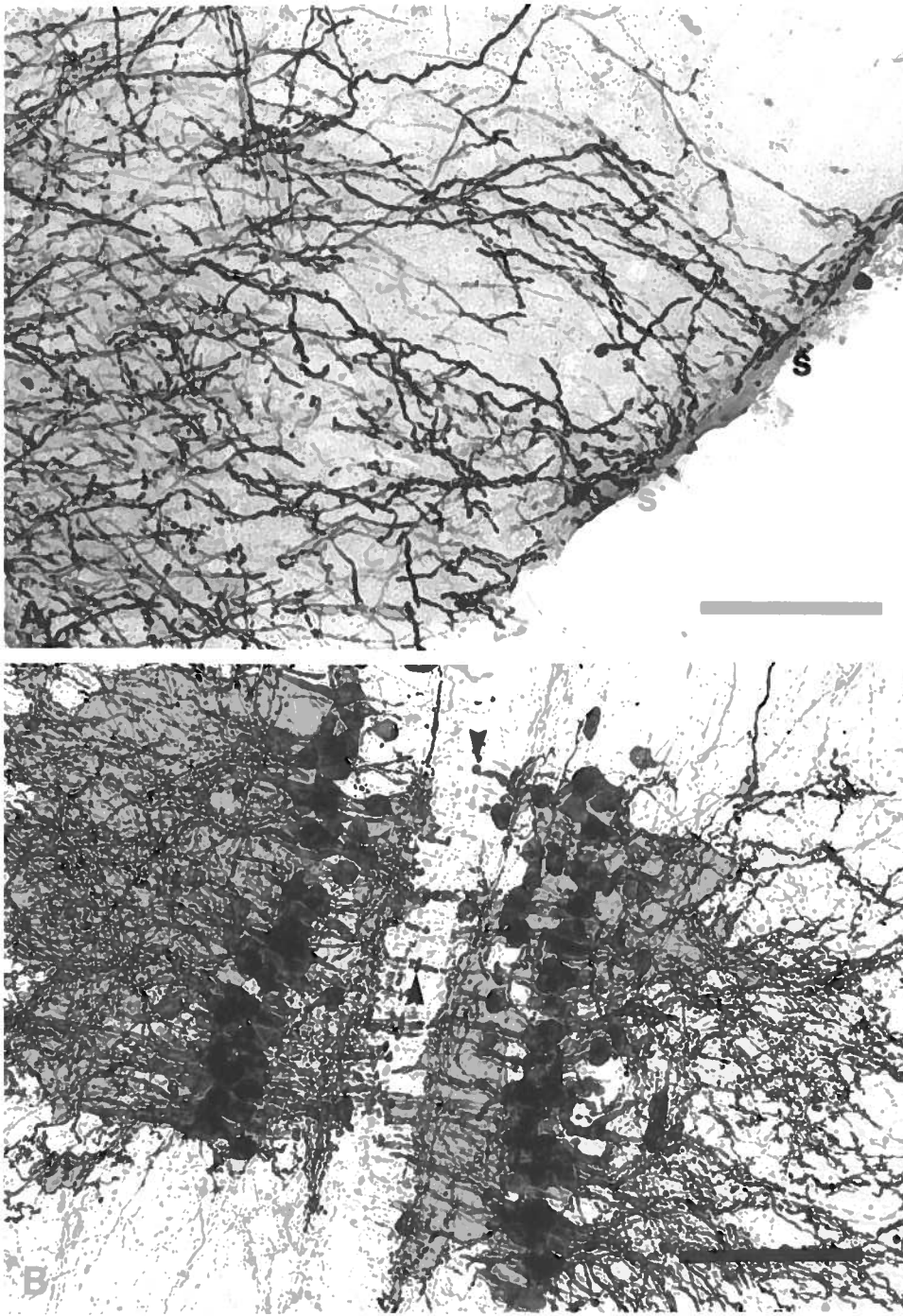


Fig. 4. In horizontal sections at the level of the dorsal preoptic area, two projections of periventricular GnRH-immunoreactive cells are apparent: lateral projections to the external surface of the brain (*s*; **A**), and ventricular projections into the third ventricle (*arrowheads*; **B**). Antiserum = AA422. $\times 250$. Bar = 100 μm

In addition to these two well-organized hypothalamic projection systems directed to the neurohypophysis and the third ventricle, extrahypothalamic projections were numerous, but were more diffuse and not organized into discrete bundles. Fibers were apparent in the stria medullaris projecting to the habenula, in the olfactory stria, and in descending tracts of the brainstem.

While immunoreactive with the lamprey antiserum (JAK 1467) and a mammalian antiserum directed to the N- and C-terminals of the mammalian decapeptide (AA 422), neuronal elements in lamprey brain were not im-

munopositive with a mammalian antiserum directed to the interior sequence of amino acids (RM 1076). Little or no reaction product was detected following treatment with two antisera directed to different amino acid sequences in the gonadotropin-associated peptide (GAP) portion of the human precursor (RM 8/5; PS 39A). Neuronal elements in sections of lamprey brain were immunoreactive with an antiserum generated against salmon GnRH (RM 802). Some cells in both groups (e.g., dorsal and ventral preoptic areas) were detected with this antiserum as well as nerve fibers projecting to the neurohypophysis and the third ventricle.

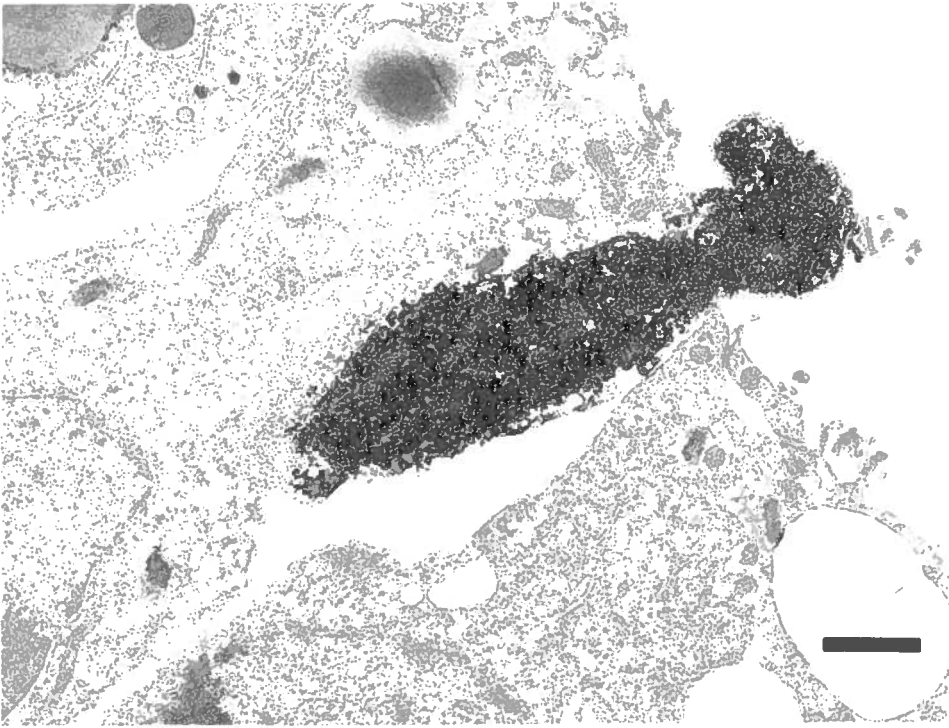


Fig. 5. An electron micrograph illustrates the projection of an GnRH-immunopositive process interdigitating between two ependymal cells to reach the third ventricle. Antiserum = AA422. Bar = 1 μ m. \times 12700.

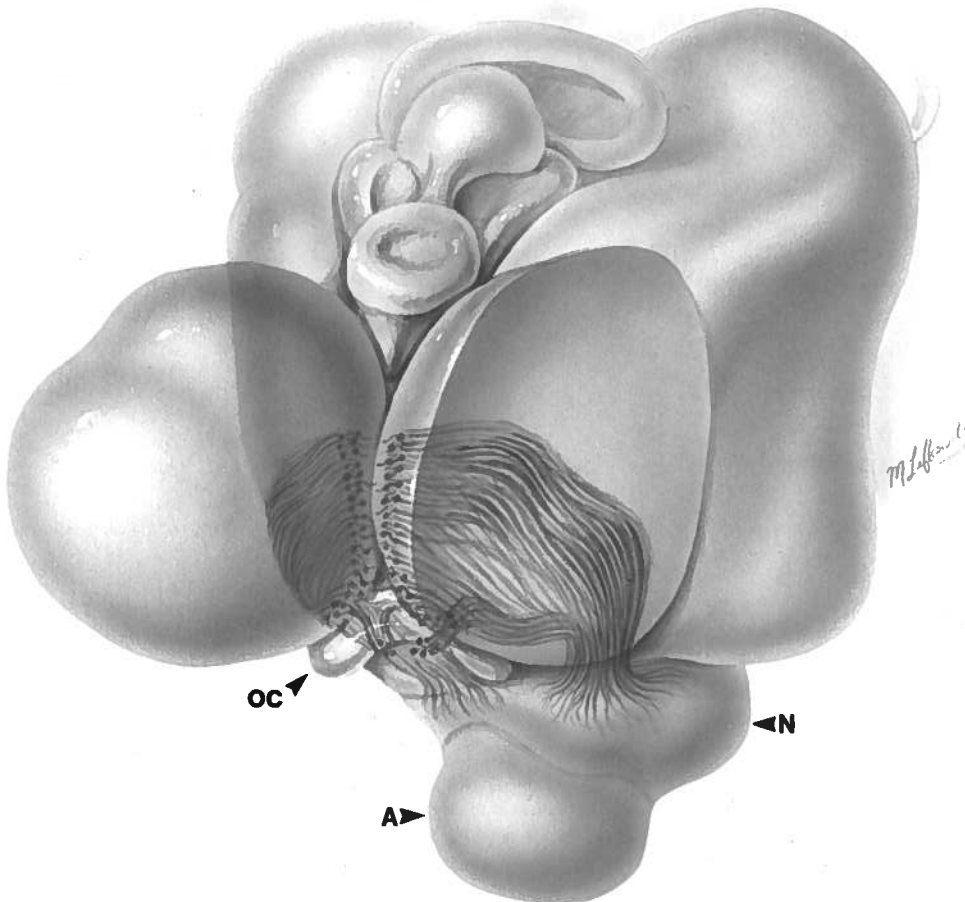


Fig. 6. Diagrammatic representation of the system of GnRH cells and their projections in the brain of the lamprey. The brain is viewed from the front so that the telencephalon appears as large rounded masses, the optic chiasm (*oc*) is seen on the ventral surface rostral to the adeno- (*A*) and neurohypophysis (*N*). Projections of ventral preoptic cells join those of the dorsal preoptic cells to form the preoptico-hypophyseal tract; also, both these cell groups project along the external surface of the brain to the neurohypophysis. Finally, processes of both cell groups directly contact the midline third ventricle.

However, extrahypothalamic fibers appeared less numerous with the salmon-directed antiserum than with antiserum AA 422, directed to the N- and C-terminals of the mammalian decapeptide.

In summary, two groups of perikarya located in the ventral and dorsal preoptic area, immunoreactive with lamprey GnRH antisera and selected mammalian LHRH antisera, projected to the neurohypophysis, the third ventricle and along the surface of the brain. The molecular species of GnRH present within these cells and fibers were immunopositive with antisera generated against lamprey LHRH and N- and C-terminals of mammalian LHRH; fewer were immunopositive with antiserum to salmon GnRH. No reactivity was observed with antisera directed to internal amino-acid sequences of mLHRH or to human precursor GAP sequences. Thus, while the predominant molecular form present in lamprey neurons appears to be lamprey GnRH, immunoreactivity with salmon GnRH suggests that other different, but related forms of GnRH may also be present in the lamprey.

Discussion

Release of GnRH from the neurohypophysis of lampreys, suggested by dense projections of GnRH neurons via the preoptico-hypophyseal tract to the neurohypophysis, agrees with projections of GnRH preoptic neurons to the external layer of the neurohypophysis described in another species of lampreys, *Lampetra tridentatus*, by Nozaki et al. (1984). Putative release into cerebrospinal fluid is suggested by projections of GnRH processes into the third ventricle. In support of this interpretation is the finding by Crim (1981) of extensions of LHRH-immunoreactive processes to the ventricular surface with blebs protruding into the ventricle. Furthermore, "CSF (liquor)-contacting" neurons have been described by Vigh and Vigh-Teichman (1973) as periventricular hypothalamic neurons with projections interdigitating between ependymal cells to contact the third ventricle. In addition, supraependymal neurons have been shown to contact intraventricular processes (Chiba et al. 1981). We propose that, in addition to the neurohypophysis, GnRH neurons project to the ventricular surface and release decapeptide into the third ventricle.

LHRH fibers projecting along the external surface of the brain to reach the median eminence have been described in rats (King et al. 1982; Jennes and Stumpf 1984; Hoffman and Gibbs 1982). Whether the externally projecting surface fibers in the lamprey release GnRH into the meningeal compartments, and whether substances released into meningeal compartments reach the anterior pituitary is not known. Whereas Tsukahara et al. (1986) have shown that substances injected into the neurohypophyseal ventricular space are able to reach secretory cells in the anterior pituitary of the hagfish by simple diffusion, this relationship has not been demonstrated in lampreys. Secretion into meningeal compartments surrounding the brain would require that these external fibers transverse the external limiting glial membrane to contact meningeal compartments.

In this study we have also demonstrated that in lampreys, preoptic neuronal perikarya and projections were immunopositive with antisera generated to synthetic lamprey GnRH and with an antiserum requiring the cyclicized Glu N-terminal and amidated Gly C-terminal of mammalian LHRH. These findings are consistent with the elucidation

of the structure of lamprey GnRH by Sherwood et al. (1986) who demonstrated that the releasing hormone in lamprey differs from mammalian LHRH by substitutions in the interior amino-acid sequence, while no changes are apparent in the N- and C-terminals of the decapeptide. The molecular form of GnRH detected in preoptic neurons was the fully processed and mature decapeptide. Whether lamprey GnRH is initially synthesized as a precursor similar to mammalian LHRH is not known (Adelman et al. 1986; Millar et al. 1986). Data from this study suggest that precursors, if present, differ in the region of C-terminal peptide from mammalian precursor.

The primary mechanism of GnRH transport to the pituitary that has been suggested in lampreys is simple diffusion from neuronal terminals in the neurohypophysis to the adenohypophysis. We propose that an additional route is via secretion into the third ventricle and transport by tanycytes to the adenohypophysis.

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