

Localization of immunoreactive lamprey gonadotropin-releasing hormone in the rat brain[☆]

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Abstract

A highly specific antiserum against lamprey gonadotropin-releasing hormone (GnRH) was used to localize l-GnRH in areas of the rat brain associated with reproductive function. Immunoreactive l-GnRH-like neurons were observed in the ventromedial preoptic area (POA), the region of the diagonal band of Broca and the organum vasculosum lamina terminalis, with fiber projections to the rostral wall of the third ventricle and the organum vasculosum lamina terminalis. Another population of l-GnRH-like neurons was localized in the dorsomedial and lateral POA, with nerve fibers projecting caudally and ventrally to terminate in the external layer of the median eminence. Other fibers apparently projected caudally and circumventrically to terminate around the cerebral aqueduct in the mid-brain central gray. By using a highly specific antiserum directed against mammalian luteinizing hormone-releasing hormone (m-LHRH), the localization of the LHRH neuronal system was compared to that of the l-GnRH system. There were no LHRH neurons in the dorsomedial or the lateral region of the POA that contained the l-GnRH neurons. As expected, there was a large population of LHRH neurons in the ventromedial POA associated with the diagonal band of Broca and organum vasculosum lamina terminalis. In both of these regions, there were many more LHRH neurons than l-GnRH neurons and the LHRH neurons extended more dorsally and laterally than the l-GnRH neurons. The LHRH neurons seemed to project to the median eminence in the same areas as those that were innervated by the l-GnRH neurons. Absorption studies indicated that l-GnRH cell bodies were eliminated by adding 1 μ g of either l-GnRH-I or l-GnRH-III, but not m-LHRH to the antiserum before use. Fibers were largely eliminated by the addition of 1 μ g l-GnRH-III to the antiserum. No chicken GnRH-II neurons or nerve fibers could be visualized by immunostaining. Because the antiserum recognized GnRH-I and GnRH-III equally, we have visualized an l-GnRH system in rat brain. The results are consistent with the presence of either one or both of these peptides within the rat hypothalamus. Because l-GnRH-I has only weak nonselective gonadotropin-releasing activity, whereas l-GnRH-III is a highly selective releaser of follicle-stimulating hormone, and because l-GnRH neurons are located in areas known to control follicle-stimulating hormone release selectively, our results support the hypothesis that l-GnRH-III, or a closely related peptide, may be mammalian follicle-stimulating hormone-releasing factor.

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1. Introduction

Gonadotropin-releasing hormones (GnRHs) have been isolated and sequenced from vertebrate species [15] across the phylogenetic scale including tunicates, primitive protochordates [10]. In several species, more than one form of GnRH exists [10]. An antiserum directed against lamprey

(l)-GnRH-I visualized nerve fibers projecting through the arcuate nucleus and terminating in the median eminence (ME) in human brains [12]. This antiserum was subsequently shown to crossreact with l-GnRH-III, suggesting that this peptide might exist in neurons projecting to the ME in humans. l-GnRH-III, in contrast to l-GnRH-I and many of the other GnRHs isolated from lower forms has preferential follicle-stimulating hormone (FSH)-releasing activity in the rat, with a minimal effective dose of 10 pmol in vivo and 10^{-9} M in vitro [16]. l-GnRH-III released LH in vivo and in vitro only at much higher doses [16]. Therefore, we hypothesized that l-GnRH-III might be a FSH-releasing

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factor (FSHRF). By using a highly specific antiserum directed against l-GnRH, we report the distribution of l-GnRH immunoreactive neuronal perikarya and fibers in regions of the preoptic-hypothalamic areas that control FSH release. The results support the hypothesis that l-GnRH-III, or a closely related peptide, may be mammalian FSHRF.

2. Methods

Male Sprague-Dawley rats received a third ventricular injection of colchicine (100 $\mu\text{g}/3 \mu\text{l}$) and the next day were anesthetized with 2.5% tribromoethanol, flushed via cardiac perfusion with saline, and fixed with 4% paraformaldehyde. The brains were removed, postfixed in the same fixative overnight, and then rinsed with potassium phosphate-buffered saline (PBS; 0.02 M KPBS; pH 7.4). Tissue sections (40 μm) for immunocytochemistry (ICC) were made by using a vibratome and were incubated in 1% hydrogen peroxide for 60 min. Sections were rinsed with KPBS, then washed three times for 10 min each with KPBS A (KPBS with 0.05% bovine serum albumin (BSA) and 0.5% Triton-X-100). This treatment was followed by washes as above with KPBS B (KPBS with 1.5% normal goat serum and 0.5% triton X-100). Highly specific anti-l-GnRH serum generated by Stacia Sower in rabbits (Code No. 3952) was characterized by radioimmunoassay; it cross-reacted 100% with l-GnRH-III and I, 0.1% with m-LHRH, and did not crossreact with c-GnRH-II. This l-GnRH antiserum first eliminated any anti-BSA activity by the addition of 500 μg of BSA to a 1:100 dilution of the antiserum 24 h before use. Subsequently, the antiserum was diluted to 1:1000 with KPBS B, then incubated with tissues for 48 h at 4°C, then 90 min at 37°C. Adjacent sections were incubated as above with a 1:1000 dilution of anti-m-LHRH serum (HU4H); kindly provided by Dr Henryk Urbanski (Oregon Regional Primate Research Center, Beaverton, OR). The m-GnRH antiserum was a monoclonal antiserum highly specific against m-LHRH, which showed of course the characteristic distribution of LHRH perikarya and terminals in the rat hypothalamus and preoptic area. Other alternate sections were incubated with a 1:1000 dilution of anti-c-GnRH-II serum produced by Els O'Hondt, Zoological Institute of the KU Leuven, Leuven, Belgium, and kindly provided by Dr Luc Berghman, Department of Poultry Science, Texas A&M University. Tissues were rinsed with KPBS A and B (three times with each buffer for a total of 30 min), then incubated for 1 h and 15 min at room temperature in either biotinylated goat anti-rabbit IgG (after the primary polyclonal antiserum to l-GnRH and c-GnRH) or horse anti-mouse IgG (after the primary monoclonal antiserum to m-LHRH). Both of the secondary antisera were produced from Vector Labs (Burlingame, CA, USA), and were used at a dilution of 1:500. After washing in KPBS A (6–8 times for a total of 45 min) tissues were incubated in avidin biotin complex (ABC, Vector Labs) for 30 min at room tempera-

ture. This was followed by washes with KPBS (6–8 times for a total of 45 min), then incubating for 20 min in 0.01% H_2O_2 and biotinylated tyramine (BT, 10 $\mu\text{l}/\text{ml}$) to amplify the signal [1]. Tissues were rinsed again in KPBS for 45 min, then incubated in ABC for 60 min at 37°C. Tissues were rinsed in KPBS (3 \times 10 min) then washed with 0.05M Tris-buffer (TB, pH 7.4, 2 \times 10 min). Tissues were exposed to TB containing 0.02% diaminobenzidine and 0.03% H_2O_2 for 2–5 min, then rinsed with TB. Sections were mounted on gelatin coated slides, dehydrated in graded alcohols, cleared in Histoclear (VWR, Philadelphia, PA, USA), coverslipped, and viewed on a Leitz microscope.

The l-GnRH antiserum was tested for specificity of staining by preabsorbing the serum with synthetic l-GnRH-III peptide synthesized by Martha L Yuban (LSU), l-GnRH-I (Peninsula Labs, Belmont, CA, USA), mammalian (m) LHRH (human LHRH; Sigma Chemical, St. Louis, MO, USA), or chicken (c) GnRH-II (Peninsula Labs, Belmont, CA, USA) at concentrations ranging from 1 to 100 $\mu\text{g}/0.4 \text{ ml}$ of 1:100 dilution for 24 h. A final dilution of 1:1000 was used for all assessments.

3. Results

3.1. l-GnRH-like perikarya

Immunoreactive cell bodies were confined to three discrete regions of the preoptic area (POA). All of the cells localized were bipolar, ovoid to fusiform in shape with a centrally located nucleus. Reaction product was confined to the cytoplasm of the cell bodies and to their extending processes (Fig. 1A–C). A few l-GnRH-like perikarya were observed in the midline within the region of the diagonal band of Broca (Fig. 1A), but the majority of the l-GnRH-like cells were found in the rostral preoptic area, at the level of the organum vasculosum of the lamina terminalis (OVLT) (Fig. 1B). Fig. 1A and B also depict the localization of these cells in relation to the distribution of m-LHRH in these specific regions of the POA. m-LHRH cell bodies were found in both of these areas that contained l-GnRH perikarya, but in the ventral POA, at the level of the OVLT, the region containing the m-LHRH neurons extended further laterally and dorsally than that containing l-GnRH-like cell bodies.

In the area just caudal to the OVLT, a diffuse population of l-GnRH-like cells was observed in an area between the dorsal border of the third ventricle and just ventral to the medial borders of the anterior commissure (Fig. 1C). Similarly, just caudal to this area, at the level where the anterior commissure crosses medially, this population of cells continues to be present above the dorsal border of the third ventricle and additionally, another discrete population of l-GnRH-like cells are aggregated in the lateral POA (Fig. 2A). Caudally, throughout the hypothalamus, no other populations of l-GnRH-III like cells were observed, even in the

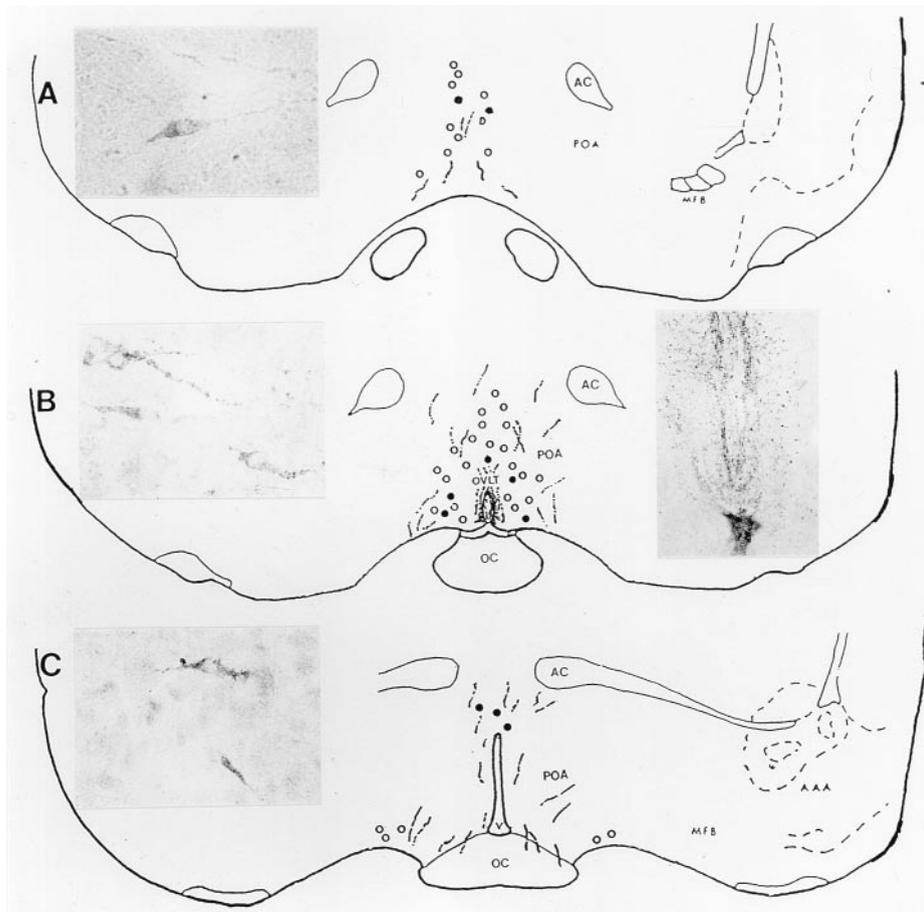


Fig. 1. Frontal sections through the rostral and middle regions of the POA showing the distribution of immunoreactive 1-GnRH neuronal cell bodies (black circles) and fibers (small dotted lines), as well as m-LHRH neuronal cell bodies (open circles). (A), the brain at the level of the diagonal band of Broca, which is the most rostral region of the POA where 1-GnRH neurons are localized; (B), the POA at the level of the lamina terminalis; (C), the POA at the level of anterior commissure before it crosses the midline. Insets on the left and right of the diagram illustrate cell bodies of 1-GnRH neurons (magnification, $\times 500$) and terminals (magnification $\times 140$), respectively. *Abbreviations:* AC, anterior commissure; POA, preoptic area; OVL, organum vasculosum lamina terminalis; MFB, medial forebrain bundle; OC, optic chiasm; AAA, anterior amygdaloid area; GP, globus paladis.

more ventral regions just lateral to the borders of the optic chiasm, where m-LHRH cells are often observed (Fig. 2A and B). These discrete localizations seem to be specific for the 1-GnRH-like cells, as m-LHRH cells were not observed in those regions in the present experiments and to our knowledge have not been reported in those regions.

3.2. 1-GnRH-like nerve fibers

Immunopositive nerve fibers were observed as thin, beaded varicosities from the POA rostrally, into the mammillary region of the hypothalamus caudally. Specifically, in the more rostral POA, a few fibers were observed projecting dorso-ventrally within the more medial aspects of the diagonal band of Broca. A similar fiber projection was also seen at the level of the OVL in which the fibers appeared to concentrate and converge ventrally in a terminal field at the midline either in the OVL or rostral tip of the 3V (Fig. 1B). Caudal to the OVL, beaded fibers continued to be observed mainly in a dorsoventral arrangement through-

out the medial POA (Figs. 1C and 2A and B). In these areas, numerous fibers were also observed along the entire dorsal border of the optic chiasm.

Within the rostral and intermediate regions of the hypothalamus, dorso-ventrally oriented fiber projections could be seen in the periventricular nucleus and dispersed throughout the rostral hypothalamic area, the lateral hypothalamic area, the dorsal and ventromedial nuclei, and the arcuate nucleus. Fig. 3 illustrates the immunoreactive 1-GnRH-like material throughout the ME. The most rostral region of the ME contained the most concentrated staining, mainly in the external layer adjacent to the hypophyseal portal system, with less, but still intense, immunoreactivity within the internal layer of the organ (Fig. 3A). Within the intermediate region of the ME, in the area of the infundibular recess, the more lateral accumulation of the peptide in the external layer became more apparent, with also appreciable staining in the internal layer (Fig. 3B). More caudally, the greatest accumulation of the peptide seen was observed medially within the ME (Fig. 3C).

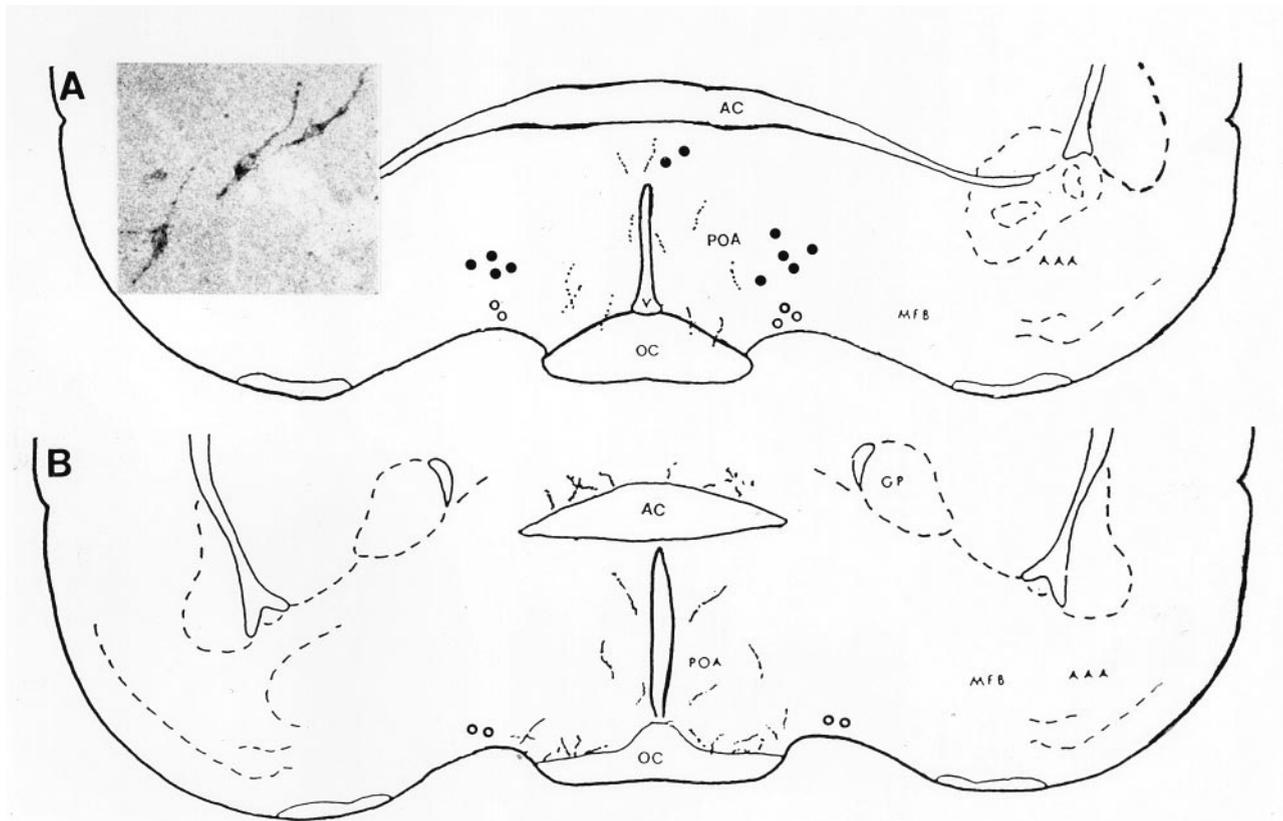


Fig. 2. Frontal section through the more caudal regions of the POA showing the distribution of immunoreactive 1-GnRH neuronal cell bodies (●) and fibers (small dotted lines), as well as m-LHRH neuronal cell bodies (○). (A), 1-GnRH cell bodies in the lateral POA dorsal to where m-LHRH cells have been localized. The insert illustrates three 1-GnRH cell bodies and their processes (magnification, 500 ×); (B), represents the caudal most region of the POA which is devoid of immunoreactive 1-GnRH cell bodies but does contain 1-GnRH positive nerve fibers and m-LHRH cell bodies. Abbreviations are the same as depicted in Fig. 1.

Staining was quite intense along the ventral walls of the ventricle with fibers apparently projecting to the ventricular ependyma (Fig. 3). In the most caudal regions of the hypothalamus, beaded fibers were observed in the periventricular nucleus dorsally, and within the supramammillary and mammillary nuclei ventrally (Fig. 4). Immunostained fibers were seen near the ventricle that projected caudally, surrounding the ventricle to the region of the aqueduct and the mid brain central gray (Fig. 5).

No differences in fiber distribution or relative content of m-LHRH or 1-GnRH were detected throughout the hypothalamus, including the ME. With regard to c-GnRH-II, no positive immunostaining for this peptide was observed throughout the hypothalamus.

3.3. Immunoabsorption studies

Immunoabsorption of the anti-1-GnRH serum was conducted to assess specificity of staining. Neuronal staining in the POA was abolished when the antiserum was absorbed with as little as 1 μ g of 1-GnRH-I or -III, but was unaffected by 1 μ g of m-LHRH. Nerve fibers in the more concentrated terminal field areas of the OVLT and ME were still present,

even though substantially reduced after immunoabsorption with 1–10 μ g of 1-GnRH-III. Fig. 6A and B, respectively, depict staining with unabsorbed antiserum and the modest amount of staining left in the ME after absorption with 1 μ g 1-GnRH-III. Also, sections of ME stained with the antiserum absorbed with 1 μ g of m-LHRH showed markedly more immunoreactive fibers in the ME (Fig. 6C) than those sections shown above (Fig. 6B) stained with the antiserum absorbed with 1 μ g 1-GnRH-III.

Quantitation of fiber staining in the ME was conducted by comparing unabsorbed 1-GnRH antiserum with the antiserum immunoabsorbed with 1 μ g of 1-GnRH or m-LHRH. The tissue labels on each slide were covered, and three stained tissues from each of the three groups were rated for their relative fiber density by six individuals in the laboratory. A total of 18 observations were made for each group, and each tissue section was ranked from 1 to 4, with 1 being greatest and 4 being the least staining. The unabsorbed antiserum produced dense fiber staining and received a mean (\pm SEM) rating score of 1.1 ± 0.07 . The antiserum when absorbed with m-LHRH and 1-GnRH-III produced mean (\pm) scores of 1.9 ± 0.07 and 3.9 ± 0.07 , respectively, showing that the 1-GnRH was very efficient at di-

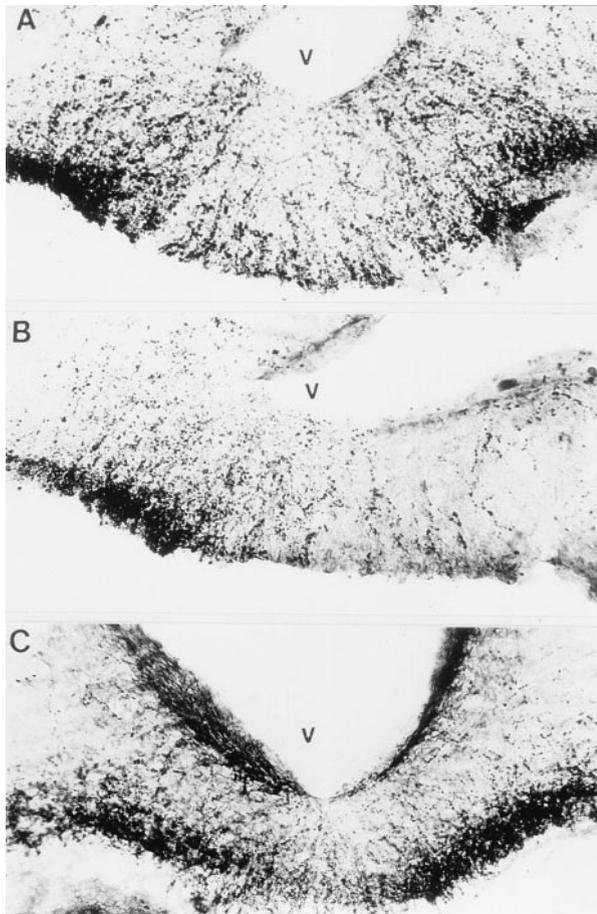


Fig. 3. Distribution of immunoreactive I-GnRH nerve terminals from rostral (A), mid (B), and caudal (C) regions of the median eminence. Magnification, 400 \times ; V = third ventricle.

minishing ($P < 0.01$) immunoreactive fiber staining on comparison with m-LHRH. Almost identical results were obtained by assessing sections stained after absorption with 10 μg of the respective peptides. The addition of 50 μg of I-GnRH-III completely eliminated this immunostaining (Fig. 6D). The fiber immunostaining was also completely eliminated with the addition of 50 μg of either I-GnRH-I or c-GnRH-II. Combining 20 μg each of I-GnRH-I and III also completely eliminated staining. The addition of 100 μg of m-LHRH did, however, eliminate both neuronal and fiber staining.

4. Discussion

The results of our ICC studies indicate that I-GnRH is expressed in two small but distinct neuronal populations. One, in the dorsomedial and lateral POA, the other localized in the ventromedial POA within the diagonal band of Broca and adjacent to the OVLT. The latter neurons are in areas known to contain large numbers of LHRH neurons. However, the former population of neurons is in an area that has not been shown to contain LHRH neurons. On the basis of

the dorso-ventrally oriented fiber projections, we hypothesize that the axons of the I-GnRH neurons located in the dorsomedial and lateral POAs project either ventrally to the OVLT or caudally through the internal layer to the external layer of the ME, where a large number of immunoreactive nerve fibers and terminals can be seen. At the rostral end of the ME, these fibers are distributed throughout the external layer. In the mid-ME, they are located in the lateral wings of the ME, and finally, caudal to the separation of the pituitary stalk, they occur with their greatest density within the medial aspects of the external layer of the ME. The region encompassed by these dorsomedial and lateral neurons includes or is just rostral to the region that, when stimulated, gave selective FSH release [9] and when lesions were placed, interfered with pulsatile FSH release [5]. On the basis of the results from stimulation and lesion experiments, we previously thought that the region containing perikarya of putative FSHRF neurons would be at the level of the paraventricular nucleus; but our immunocytochemical results point to the fact that the region is located more rostrally in the most caudal aspects of the dorsomedial POA. In all probability, the lesions and stimulations, respectively, activated or destroyed these neurons or their axons projecting caudally and ventrally into the ME.

The population of immunopositive I-GnRH neurons located in the ventromedial preoptic area contained as expected numerous LHRH cell bodies that seem to extend more dorsally and somewhat more laterally than the I-GnRH cells. A major projection of these I-GnRH cells appears to be to the rostral tip of the third ventricle and the OVLT, but we cannot rule out possible projections of these neurons caudally to the ME. It was previously shown by bioassay for FSHRF and LHRH, and RIA for LHRH, that extracts of the OVLT possessed much greater FSH-releasing activity than could be accounted for by the content of immunassayable LHRH [11]. The localization of I-GnRH cell bodies and axons in this region supports the results of the earlier study because I-GnRH-III is a potent FSH-releasing peptide [16]. The question is: what is the function of this connection of the I-GnRH neurons to the wall of the 3V and the OVLT? In the case of the OVLT, does this subserves a sensory function by which the steroid environment is monitored in this region where the blood brain barrier is essentially nonexistent, or is the peptide being released into the OVLT, where it subserves an unknown function, and ventricle where it may proceed caudally to have effects on brain function in regions adjacent to the ventricle? Interestingly, considerable staining for I-GnRH was found surrounding the ventral ependymal wall of the 3V above the ME as well.

As indicated above, the major projection of the I-GnRH neurons seems to be to the ME. The entire ME is stained and the distribution and intensity is similar to that found for LHRH staining. Based on previous bioassay results that the caudal ME contained more FSH-releasing activity than could be accounted for by the content of LHRH [8], we expected to find a more intense staining of the caudal ME

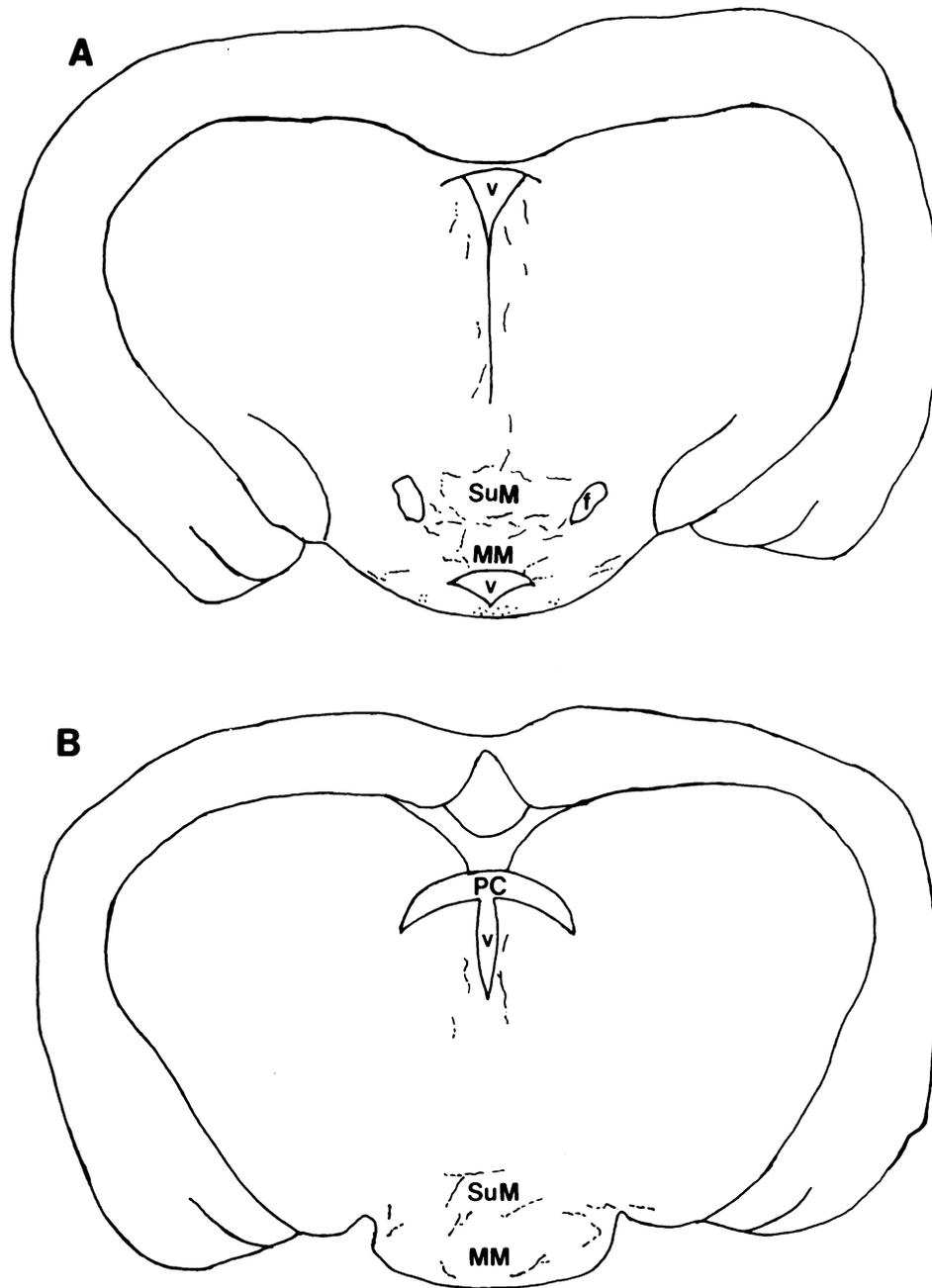


Fig. 4. Distribution of immunoreactive l-GnRH nerve fibers in the caudal hypothalamus (A, rostral mammillary region; B, caudal mammillary region). Abbreviations: SuM, superior mammillary nucleus; MM, medial mammillary nucleus; f, fornix; PC, posterior commissure.

for l-GnRH than for LHRH. The current results do not bear this out, possibly because of the high intensity of staining for both peptides in all regions of the ME.

The antiserum used for these immunocytochemical studies was a polyclonal antibody that cross-reacted only 0.1% with m-LHRH and did not crossreact with c-GnRH-II in radioimmunoassay. There was complete cross-reactivity with l-GnRH-I and III, but no cross reactivity to other known GnRHs tested. Immunoabsorption of the anti-l-GnRH serum was conducted, and neuronal staining was abolished with as

little as 1 μg of the l-GnRH-III peptide. The nerve fibers in the terminal fields of the OVLT and ME were greatly reduced with 1 μg of l-GnRH-III and little affected by m-LHRH, and were completely eliminated with 50 μg of either l-GnRH-I or III, or with 20 μg of each in combination.

Neuronal staining was not altered with 1 μg of m-LHRH and it was much less effective than l-GnRH-III, requiring 100 μg of the peptide to eliminate fiber staining. We suggest that the removal of fiber staining by m-LHRH was due to the 60% homology between l-GnRH-III and m-LHRH. The results in-

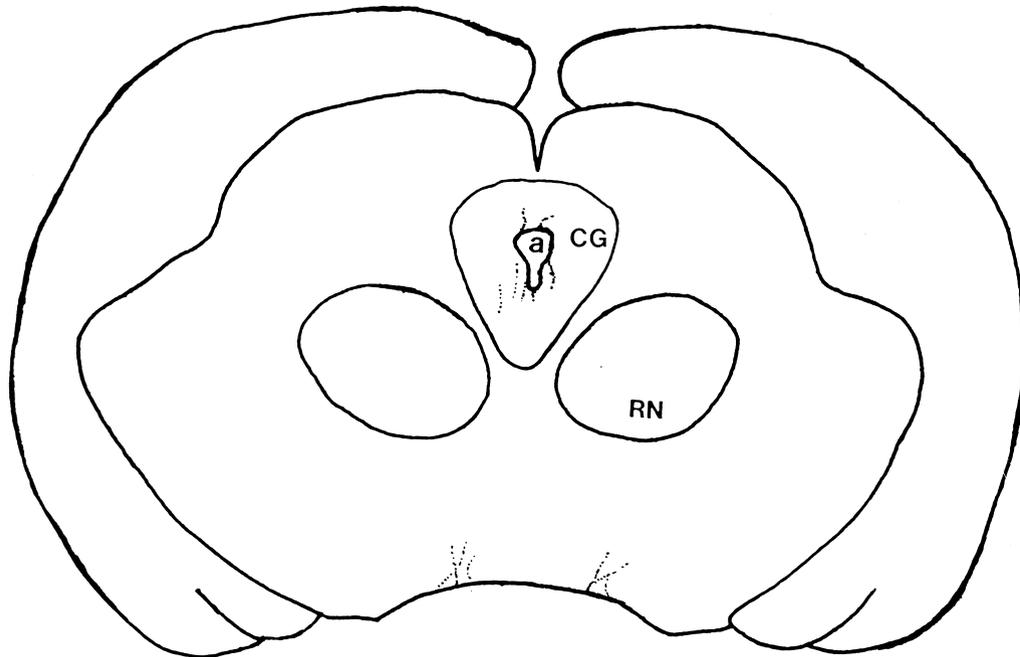


Fig. 5. Distribution of immunoreactive l-GnRH nerve fibers in the mesencephalon. *Abbreviations:* a, aqueduct; CG, central gray; RN, red nucleus.

dicate that the l-GnRH perikarya are not LHRH perikarya and that nearly all of the l-GnRH-like fibers demonstrated contain l-GnRH. Furthermore, LHRH perikarya were not found in the dorsal area containing l-GnRH neuronal perikarya.

Because the antiserum cross-reacted 100% with l-GnRH-I in radioimmunoassay (RIA), it is possible that we immunostained this peptide as well as l-GnRH-III. Because l-GnRH-I nonselectively stimulates both FSH and LH release only at high concentrations (10^{-6} – 10^{-5} M), whereas l-GnRH-III stimulates FSH and LH release at concentrations of 10^{-9} and 10^{-7} M, respectively, we believe that selective control of FSH release is mediated by l-GnRH-III neurons in the immunostained areas; however, we cannot rule out the presence of l-GnRH-I neurons in these areas as well.

The l-GnRH fiber system projected caudally around the ventricle to terminate in the central gray in the region just lateral to the aqueduct of Sylvius. This is an almost identical projection to that of m-LHRH and suggests a role for l-GnRH in mediating sexual reflexes, such as lordosis and penile erection, as has been shown to be the case for LHRH [7]. Whether this role is facilitatory or inhibitory will be determined by future experiments.

In recent years, it has been shown that in vertebrates, at least two different GnRH forms are expressed within the brain of a single species; generally, one GnRH functions as a neurohormone regulating the pituitary in mediating the release of gonadotropins, and the other form may have a neurotransmitter/neuromodulatory function and is generally localized in areas outside the hypothalamus or midbrain regions. However, in mammals, only a limited number of species (monotremes, marsupials, and primitive eutherians)

have been shown to contain more than one form of GnRH using indirect methods of HPLC, RIA, and ICC. In these species, the second form identified was c-GnRH-II. The only direct evidence, by identification of complementary DNA of other GnRHs other than mammalian GnRH, has been characterized in three species: the tree shrew, in which two preproGnRH mRNAs to mammalian and c-GnRH-II [3] were identified; the guinea pig, in which a preproGnRH encodes for a new form of GnRH: guinea pig GnRH [2]; and most recently in the human, in which two genes for mammal GnRH and c-GnRH-II were demonstrated [14]. In earlier studies, Stopa et al. [3] demonstrated a lamprey-like GnRH in human hypothalami and ME using a combination of ICC, HPLC, and RIA. In these same studies, the hypothalamic distribution of immunopositive lamprey-like GnRH neurons was similar to that observed for those containing the m-LHRH. More recently, also using similar indirect methods, Lescheid et al. [4] demonstrated a c-GnRH-II-like form in stump-tail and rhesus monkeys. In these same studies, only a few of the ir-c-GnRH-II like cells were in the posterior basal hypothalamus; most of the immunopositive neurons to c-GnRH-II were shown in the midbrain. White et al. [2] demonstrated that most of the expression of c-GnRH-II was in the caudate nucleus with little expression in the hippocampus and amygdala. These studies did not screen for other forms of GnRH, such as the lamprey GnRH forms, nor do their data suggest that c-GnRH-II is a neurohormone. Thus, there is incomplete and contrasting data on the nature of GnRHs in primates as well as other mammals.

By using an antibody against c-GnRH-II that readily

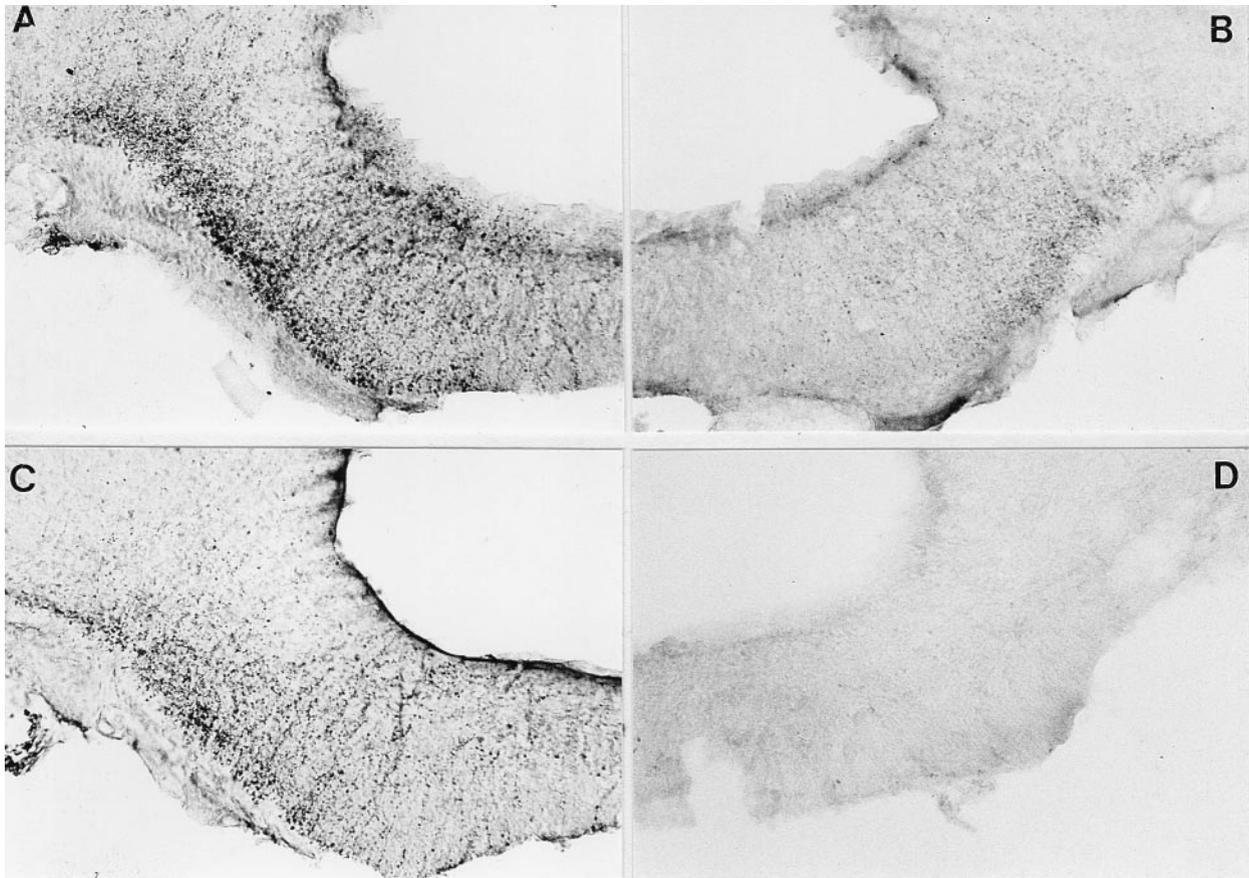


Fig. 6. Frontal sections through the middle region of the ME showing efficiency of immunoabsorption of l-GnRH antiserum. (A) shows intense staining after tissue incubation with unabsorbed primary antiserum; (B) shows marked reduction in fiber staining following preabsorption of the antiserum with 1 μ g of l-GnRH-III; (C) demonstrates clearly that preabsorption with 1 μ g of m-LHRH is much less effective at diminishing immunoreactivity when compared to the l-peptide absorptin shown in (B); (D) depicts the ability of 50 μ g of l-GnRH-III to eliminate fiber staining. Magnification, 400 \times .

demonstrated this peptide in chicken brains by ICC [13], we were also unable to demonstrate any staining in the rat hypothalamus; therefore, we believe that our results are not accounted for by this peptide. Furthermore, in contrast to l-GnRH-III, c-GnRH-II has only slight preferential FSH releasing activity in vivo [6] but not in vitro [16]. It is probable that l-GnRH-III, or a peptide very closely related to it, such as one with only one amino acid substitution in the molecule, is the native mammalian FSHRF. In conclusion, our results provide further evidence that l-GnRH-III may be FSHRF, as it is found in the brain in areas that mediate the selective secretion of FSH in the rat.

Acknowledgments

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