Gonadotropin-releasing hormone neurons in the preoptic-hypothalamic region of the rat contain lamprey gonadotropin-releasing hormone III, mammalian luteinizing hormone-releasing hormone, or both peptides

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This study utilized a newly developed antiserum, specific for lamprey gonadotropin-releasing hormone III (l-GnRH-III), to determine the following: in which regions of the rat hypothalamus the neuronal perikarya producing l-GnRH-III are localized; and whether this peptide, known to selectively induce follicle-stimulating hormone release, is coexpressed in neurons containing mammalian luteinizing hormone-releasing hormone (m-LHRH). Double-label immunocytochemistry was performed by using an l-GnRH-III polyclonal antiserum and an LHRH monoclonal antiserum. Immunopositive neurons for l-GnRH-III, m-LHRH, or neurons coexpressing both peptides were detected within the organum vasculosum lamina terminalis (OVLT) region of the preoptic area (POA). Caudal to the OVLT, l-GnRH-III-positive neurons were also observed dorsomedially, above the third ventricle in the medial POA. The m-LHRH neurons were not observed in this area. The lateral POA region contained neurons positive for both peptides along with single-labeled neurons for each peptide. Importantly, neurons that expressed l-GnRH-III, m-LHRH, or both peptides were also detected in the ventral regions of the rostral hypothalamus, dorsolateral to the borders of the supraoptic nuclei. In both of these latter areas, neurons containing l-GnRH-III were slightly dorsal to neurons containing only m-LHRH. The l-GnRH-III perikarya and fibers were eliminated by absorption of the primary antiserum with l-GnRH-III, but not by l-GnRH-I, chicken-GnRH-II, or m-LHRH. These results indicate that, unlike other isoforms of GnRH found in the mammalian brain, l-GnRH-III neurons not only are observed in regions that control follicle-stimulating hormone release but also are colocalized with m-LHRH neurons in areas primarily controlling LH release. These findings suggest an interrelationship between these two peptides in the control of gonadotropin secretion.

immunocytochemistry | l-GnRH-III | m-LHRH | c-GnRH-II antisera | FSH and LH control

Gonadotropin-releasing hormones (GnRHs) have been detected in all vertebrate species studied across the phylogenetic scale from tunicates (1, 2) to humans (2, 3). In mammals, the prevailing view is that luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release is primarily controlled by mammalian luteinizing hormone-releasing hormone (m-LHRH). However, recent studies have uncovered other isoforms of the GnRH peptide besides m-LHRH in the mammalian brain (3–8). Chicken-GnRH-II (c-GnRH-II) (4–7) and lamprey-GnRH (l-GnRH; refs. 3 and 8) have been detected in the mammalian hypothalamus. Specifically, *in situ* hybridization experiments localized c-GnRH-II mRNA in the supraoptic, paraventricular, and suprachiasmatic nuclei as well as in the medial basal hypothalamus, but this mRNA was not detected within neurons that were immunopositive for m-LHRH (5). With regard to l-GnRH, an antiserum to l-GnRH-I bound to cells and nerve fibers projecting through the arcuate nucleus and terminating in the median eminence (ME) in the human brain (3). Additionally, our recent study using an antibody that recognized both l-GnRH-I and l-GnRH-III allowed us to visualize a l-GnRH system in the preoptic-hypothalamic areas of the rat brain that control FSH release (8).

All of these GnRH forms have the ability to release LH; however, only l-GnRH-III has been shown to be a potent, selective inducer of FSH release, as shown in the rat (9) and cow (10). In contrast, l-GnRH-I is ineffective (11) and c-GnRH-II has only slight preferential FSH-releasing activity *in vivo* (12), but not *in vitro* (9). Consequently, because our previous study showed the presence of l-GnRH-like neurons in regions of the hypothalamus that also contain an abundance of m-LHRH neurons (8), we used a double-label immunocytochemical technique using a new antiserum specific to l-GnRH-III to determine the relationship between the m-LHRH and l-GnRH-III neuronal populations.

Methods

Tissue Preparation. Adult male Sprague–Dawley rats were purchased from Charles River Breeding Laboratories. Each rat was anesthetized with 2.5% tribromoethanol and implanted with a third ventricular cannula as described previously (13). While still under anesthesia, an injection of colchicine (100 μg/3 μl) was administered into the third ventricular cannula. The following day, animals were anesthetized with 2.5% tribromoethanol, flushed with saline via cardiac perfusion, and fixed with 4% paraformaldehyde. The brains were removed and postfixed overnight in the same fixative, then rinsed in potassium PBS (KPBS, ph 7.4). Forty-micrometer adjacent sections starting rostrally at regions containing the diagonal band of Broca and proceeding caudally to sections containing the anterior hypothalamus and mammillary nuclei were made by using a vibratome. Sections were also used from the mesencephalon containing the mesencephalic aqueduct.

Abbreviations: GnRH, gonadotropin-releasing hormone; c-GnRH-II, chicken-GnRH-II; l-GnRH, lamprey gonadotropin-releasing hormone; ME, median eminence; LH, luteinizing hormone; FSH, follicle-stimulating hormone; m-LHRH, mammalian luteinizing hormone-releasing hormone; DAB, 3,3′-diaminobenzidine tetrahydrochloride; POA, preoptic area; OVLT, organum vasculosum lamina terminalis.

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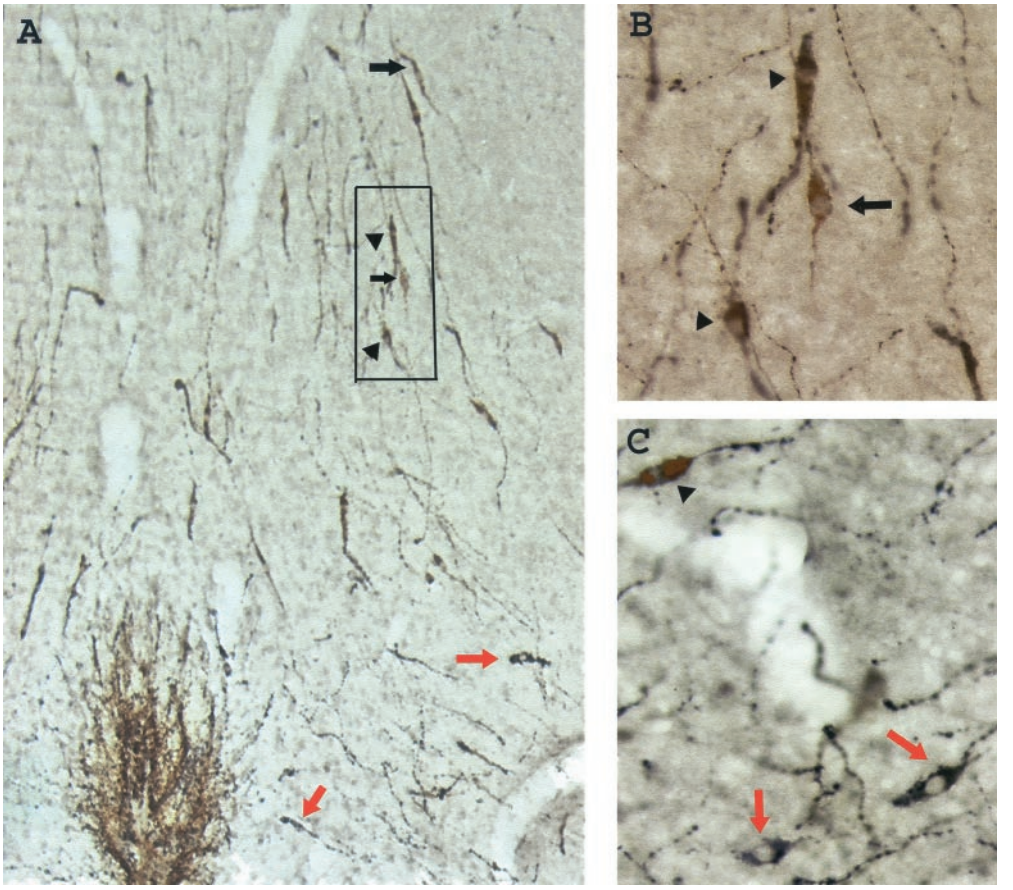

Fig. 1. Localization of l-GnRH-III and m-LHRH in the rostral region of the POA. (*A*) Distribution of l-GnRH-III and m-LHRH nerve fibers and perikarya in the lamina terminalis. Note that l-GnRH-III neurons (black; red arrows) are interspersed among the m-LHRH neurons (brown; black arrows) and those that express both peptides (black/brown; arrowheads). Fiber tracts for l-GnRH-III (black) and m-LHRH (brown) are shown within the OVLT. (*B*) An increased magnification of three neurons shown in the box in *A*. Two of the neurons are immunopositive for both l-GnRH-III and m-LHRH (black/brown; arrowheads), and one neuron is immunopositive only for m-LHRH (brown; black arrow). (*C*) The morphology of two l-GnRH-III positive neurons (black; red arrows) and one neuron containing both l-GnRH-III and m-LHRH (black/brown; arrowhead). (*A*, ×144; *B*, ×305; *C*, ×322.)

l-GnRH-III Immunocytochemistry. Sections were incubated in 1% H_2O_2 for 60 min. Immunocytochemistry for l-GnRH-III was performed as previously described (8), except for the primary antiserum. The antiserum to l-GnRH-III was generated in rabbits by S.A.S. (Code No. 39-82-78-3). The specificity of the antiserum for l-GnRH-III was tested by immunocytochemistry, and showed no cross-reactivity with l-GnRH-I in the lamprey. Preabsorption of this antiserum with l-GnRH-I also had no affect on the antiserum binding to l-GnRH-III in the lamprey (S.A.S., unpublished observations). Anti-BSA activity was eliminated from this antibody 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 1:1000 with KPBS B (KPBS with 1.5% normal goat serum and 0.5% Triton X-100), then incubated with tissues 48 h at 4 C. Tissues were rinsed and then incubated for 1 h and 15 min in biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories). After washing, the tissues were incubated in avidin biotin complex (ABC, Vector Laboratories) for 30 min at room temperature. After washes in KPBS, the signal was amplified by using a biotin amplification procedure (14) by incubating for 20 min in 0.01% H_2O_2 and biotinylated tyramine (BT, 10 μl/ml). Tissues were rinsed again in KPBS and then incubated in ABC for 60 min at 37 C, rinsed in KPBS, and then washed with sodium acetate buffer (0.175 M; 3 times for 10 min). The sections were exposed to sodium acetate buffer containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_20_2 and 1.25% nickel sulfate (Ni-DAB) for 2–5 min and then rinsed in sodium acetate buffer followed by KPBS buffer. The sections were mounted on gelatin coated slides, dehydrated in graded alcohols, cleared in Histoclear (VWR Scientific), coverslipped, and viewed on a Leitz microscope.

Specificity of the l-GnRH-III antiserum was tested by preabsorbing the serum with synthetic l-GnRH-III peptide synthesized by M. L. Yuban (Louisiana State University, Baton Rouge, LA), l-GnRH-I (Peninsula Laboratories), m-LHRH (human LHRH, Sigma), c-GnRH-II (Bachem) at concentrations ranging from 1–10 μg/0.1 ml of l-GnRH antiserum (1:100 dilution) for 24 h. A final dilution of 1:1000 was used for all assessments.

Double Immunocytochemistry Technique. Colocalization of l-GnRH-III and m-LHRH was performed by using double immunocytochemistry procedure by using Ni-DAB chromagen (black/blue stain) followed by the DAB chromagen (brown stain). Sections were processed as above to detect l-GnRH-III neurons and fibers by using the Ni-DAB chromagen. After the tissues were exposed to the Ni-DAB chromagen, they were washed in KPBS buffer and then incubated overnight at room temperature with a monoclonal LHRH antiserum (1:500, HU11B, provided by H. Urbanski, Oregon Regional Primate Center, Beaverton, OR). The sections were rinsed in KPBS B buffer (1.5% normal horse serum, 0.05% Triton X-100) and then incubated at room temperature with biotinylated anti-mouse

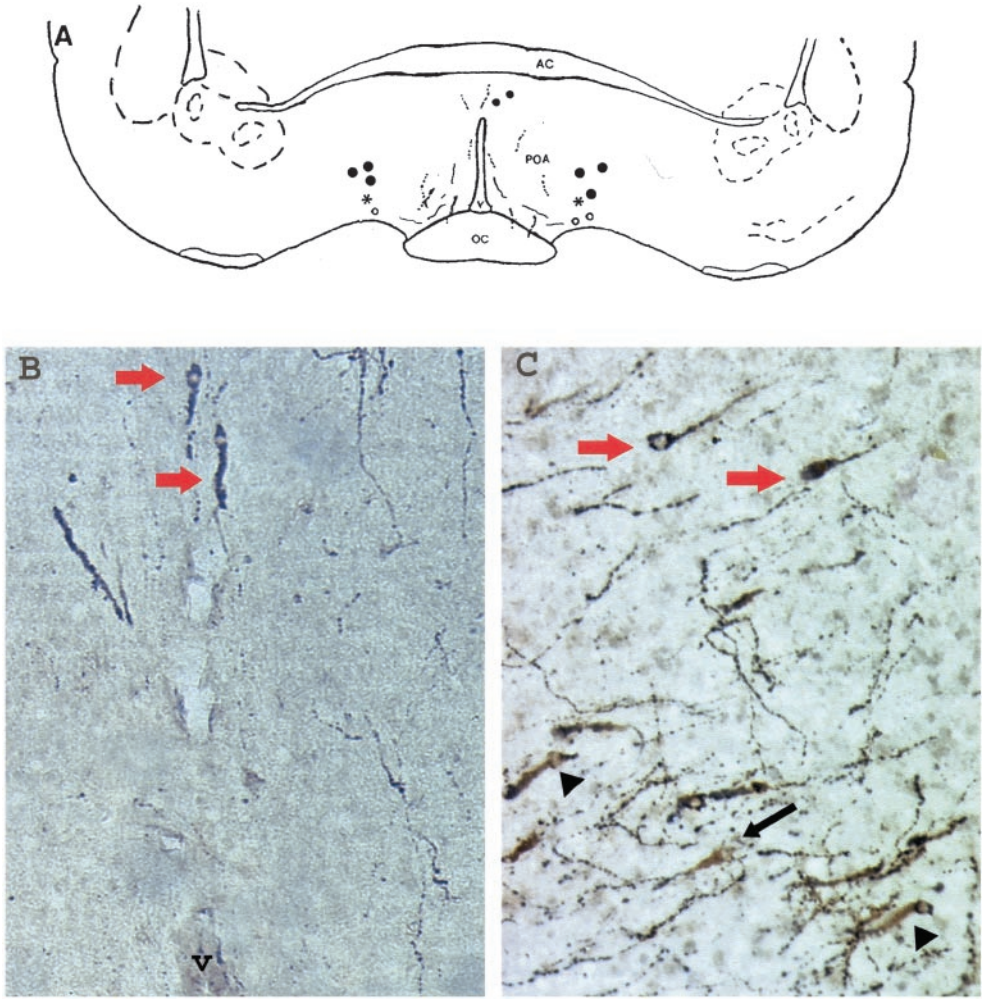

Fig. 2. Localization of l-GnRH-III and m-LHRH in the caudal region of the POA. (*A*) Frontal section showing the distribution of immunoreactive l-GnRH-III neurons (black dots), m-LHRH neurons (open circles), neurons that coexpress both peptides (asterisks), and fibers of both peptides (dotted lines). Note that l-GnRH-III neurons are observed in the dorsomedial and lateral POA. In the lateral region of the POA, the l-GnRH-III are observed dorsal to the neurons that are immunopositive for m-LHRH or both peptides. (*B*) L-GnRH-III neurons observed in the dorsomedial region of the POA above the third ventricle (V). (*C*) Representative neurons immunopositive for l-GnRH-III (red arrow), m-LHRH (black arrow), and both peptides (arrowheads) located in the lateral POA. (*B*, ×242; *C*, ×289.)

IgG (1:500 dilution, Vector Laboratories) for 1 h. Sections were rinsed in KPBS then incubated with ABC complex for 1.5 h. The sections were rinsed and then incubated with DAB (10 mg DAB/50 mls KPBS/0.03% H_20_2) for 5 min. Sections were mounted on gelatin coated slides, dehydrated in graded alcohols, cleared in Histoclear, coverslipped, and viewed microscopically.

Microscopy was performed with a Zeiss Axioplan2 microscope fitted with a Hamamatsu chilled 3CCD color camera (Hamamatsu, Bridgewater, NJ). Digital images were captured by using Adobe Photoshop 4.0 (Adobe Systems, Seattle, WA) and MacIntosh PowerMac G3 computer (Apple Computer, Cupertino, CA). Prints were electronically printed by using a Kodak DS8650 color printer.

Results

Double-labeled immunocytochemistry was used to compare the localization of l-GnRH-III peptide with that of m-LHRH in the rat hypothalamus. L-GnRH-III was detected by using the nickel-intensified DAB stain, which shows a black reaction product, and m-LHRH was detected by using the DAB without nickel, which shows a brown reaction product. Fig. 1 *A–C* illustrates the localization of l-GnRH-III and m-LHRH nerve fibers and perikarya in the lamina terminalis region of the preoptic area (POA). Neurons containing both black and brown chromagen are immunopositive for both peptides, whereas neurons containing only black chromagen are immunopositive for l-GnRH-III and neurons containing only brown chromagen are immunopositive for m-LHRH. L-GnRH-III perikarya are similar in morphology to the m-LHRH perikarya in that they are bipolar or unipolar, ovoid to fusiform in shape, with a centrally located nucleus. In those neurons containing only l-GnRH-III, the reaction product was observed in the cytoplasm of the perikarya and within their extending processes. In some of the neurons coexpressing both peptides, the l-GnRH-III reaction product was mainly observed in the processes of the neuron, whereas the LHRH appeared to be localized to the cytoplasm of the perikarya. However, we also visualized neurons that did not show that distinction, in that both colors were found throughout the cytoplasm and processes.

Caudal to the organum vasculosum lamina terminalis (OVLT) region, a discrete population of l-GnRH-III cells was observed in an area between the dorsal border of the third ventricle and the ventral borders of the anterior commissure (Fig. 2 *A* and *B*). Perikarya containing m-LHRH were not detected in this region.

In the lateral POA of this same region (Fig. 2*C*), neurons were detected that were immunopositive for l-GnRH-III and m-LHRH, and an occasional neuron coexpressed both peptides. The l-GnRH-III neurons were found to be dorsal to those neurons containing either m-LHRH only or both peptides.

In the rostral hypothalamus near the borders of the supraoptic nuclei, neurons were detected that mainly contained m-LHRH only, but occasionally, neurons were observed that colocalized both peptides (Fig. 3*A*). Dorsolateral to these neurons, we observed another small population of neurons containing only l-GnRH-III immunoreactivity (Fig. 3*B*).

Immunopositive l-GnRH-III nerve fibers were observed rostrally within the OVLT (Fig. 1*A*), and caudally within both medial and lateral POA, passing along the third ventricle and ventrally along the optic chiasm (Figs. 2 and 3). Positive fibers were also observed in the intermediate hypothalamus, mostly within the ventromedial and arcuate nuclei and ventrally along the base of the brain above the optic chiasm and extending just lateral to the borders of the supraoptic nucleus. Numerous fibers were observed coursing ventrally into the ME. Fig. 4*A* illustrates the localization of immunoreactive l-GnRH-III material in the ME. Fiber staining was observed throughout the rostro-caudal extent of the ME. Immunoreactive fibers were also observed in the mammillary bodies and into the periaqueductal region of the mesencephalon, although they were few in number. This fiber distribution is essentially the same as that which is known for m-LHRH. We observed that the relative density of immunopositive fibers for l-GnRH-III in the ME was visualized to be less than that of those fibers that were immunopositive for m-LHRH.

Fig. 4*B* shows that immunoabsorption of the l-GnRH-III antiserum with 1 μg of the l-GnRH-III peptide completely eliminated nerve fiber and neuronal staining (see Fig. 4*A* for comparison). As shown in Fig. 4 *C* and *D*, nerve fiber staining was unaltered when the antiserum was absorbed with 1 μg of m-LHRH and l-GnRH-I, respectively. Absorption with l μg of c-GnRH-II decreased staining of fibers by about 60%.

Discussion

In earlier studies, Stopa *et al.* showed a lamprey-like GnRH in human hypothalami and ME by using a combination of immunocytochemistry, HPLC, and RIA (3). The distribution of the immunopositive l-GnRH-like neurons and fibers was similar to that observed for m-LHRH. We have also described previously a population of immunoreactive l-GnRH-like neurons in the rat ventromedial POA with axons projecting to the OVLT (8). Dorsomedial l-GnRH-like perikarya were also located in the caudal preoptic area, with axons projecting caudally and ventrally to the ME. Conversely, by using a monoclonal antiserum against m-LHRH, we determined the localization of m-LHRH neurons in the regions where the 1-GnRH neurons were observed. Adjacent sections of the ventral POA region revealed that both peptides were localized in neurons within this area, but there were no m-LHRH neurons in the dorsomedial or dorsolateral POA where perikarya of l-GnRH were observed (8). Furthermore, immunoabsorption of the primary antiserum demonstrated that the staining of l-GnRH-like neurons was eliminated by the addition of the l-GnRH-III peptide, but not by the addition of either m-LHRH or l-GnRH-I. Nerve fibers in the ME were eliminated by absorption with l-GnRH-III, but only slightly reduced by absorption with m-LHRH. Because the l-GnRH antiserum (no. 3952) recognized l-GnRH-I and l-GnRH-III equally, a specific antiserum against l-GnRH-III without cross-reactivity with l-GnRH-I was needed to show that the l-GnRH-like neurons visualized in the rat brain were indeed l-GnRH-III neurons.

The present study indicates that an antiserum specific for l-GnRH-III (no. 39-82-78-3) binds the same populations of neurons seen with the less specific l-GnRH antiserum (no. 3952).

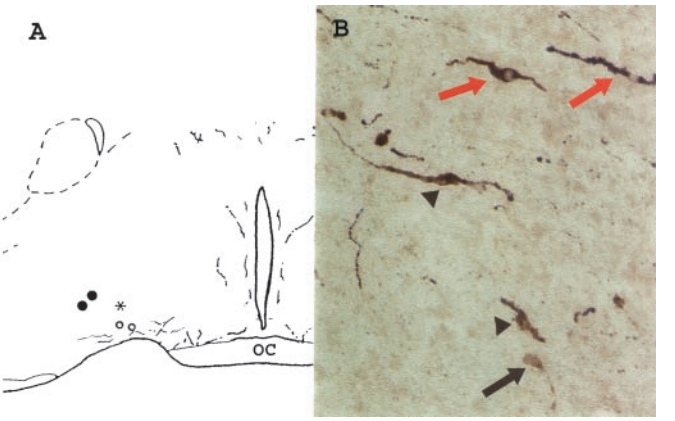

Fig. 3. Distribution of immunoreactive l-GnRH-III and m-LHRH in the rostral hypothalamus. (*A*) Diagram of a frontal section showing the distribution of immunoreactive l-GnRH-III (black dots), m-LHRH (open circles), both peptides (asterisks), and fibers (dotted lines). (*B*) L-GnRH-III neurons (red arrows) dorsolaterally to the borders of the supraoptic nuclei. Neurons containing both peptides (arrowheads) or containing only m-LHRH (black arrow) were detected ventral to l-GnRH-III neurons. (*B*, ×251.)

Additionally, we now describe another population of l-GnRH-III neurons in the rostral hypothalamus, dorsolateral to the borders of the supraoptic nuclei. Also, the staining of neurons and fibers with this new antiserum could be eliminated by preabsorption with the l-GnRH-III peptide, but was not affected by preabsorption with l-GnRH-I or m-LHRH. Although preabsorption with c-GnRH-II reduced staining somewhat, probably because this peptide has 80% homology with l-GnRH-III, there are virtually no c-GnRH-II neuronal fibers in the ME. Importantly, this study shows a close relationship between two different forms of GnRH in the mammal, in that l-GnRH-III and m-LHRH are colocalized in neurons in the ventral POA and the rostral hypothalamus. Interestingly, the OVLT area of the POA has been shown to contain FSH-releasing activity by bioassay (15). We have shown here that this region contains an abundance of LHRH neurons, as well as cells that express both peptides. Lesions in this region eliminate the preovulatory type of LH but not FSH release (15). This type of LH release has been thought to be due to LHRH release; however, the presence of l-GnRH-III neurons in the area and neurons that express both peptides suggests an interaction of these two peptides to either amplify or reduce the preovulatory surge of FSH and LH. Further experiments are needed to clarify this interrelationship. In addition to the neurons that contained both peptides, neurons that contain only l-GnRH-III or m-LHRH were also observed in these same areas.

These isolated l-GnRH-III neuronal populations suggest that these cells may play a separate role in the control of FSH release. First, the region encompassed by the dorsomedial and dorsolateral neurons includes or is just rostral to the region that, when stimulated, induced selective FSH release (16) and, when destroyed, interfered with pulsatile FSH release (17, 18). Additionally, of the GnRH isoforms tested *in vivo*, l-GnRH-III is the only one that can selectively release FSH with a high degree of potency (9), an action that was not shared by other GnRH forms, except for c-GnRH-II, which had only a slight preferential FSH-releasing activity (12). Recently, FSH-releasing activity was eluted from Sephadex columns in a fraction that contained immunoassayable l-GnRH (19). The FSH-releasing activity of this fraction was abolished by the same antiserum used for immunoassay and immunocytochemistry in our previous study (8). This antiserum crossreacts with both l-GnRH-I and -III; however, l-GnRH-I has little selective gonadotropin-releasing

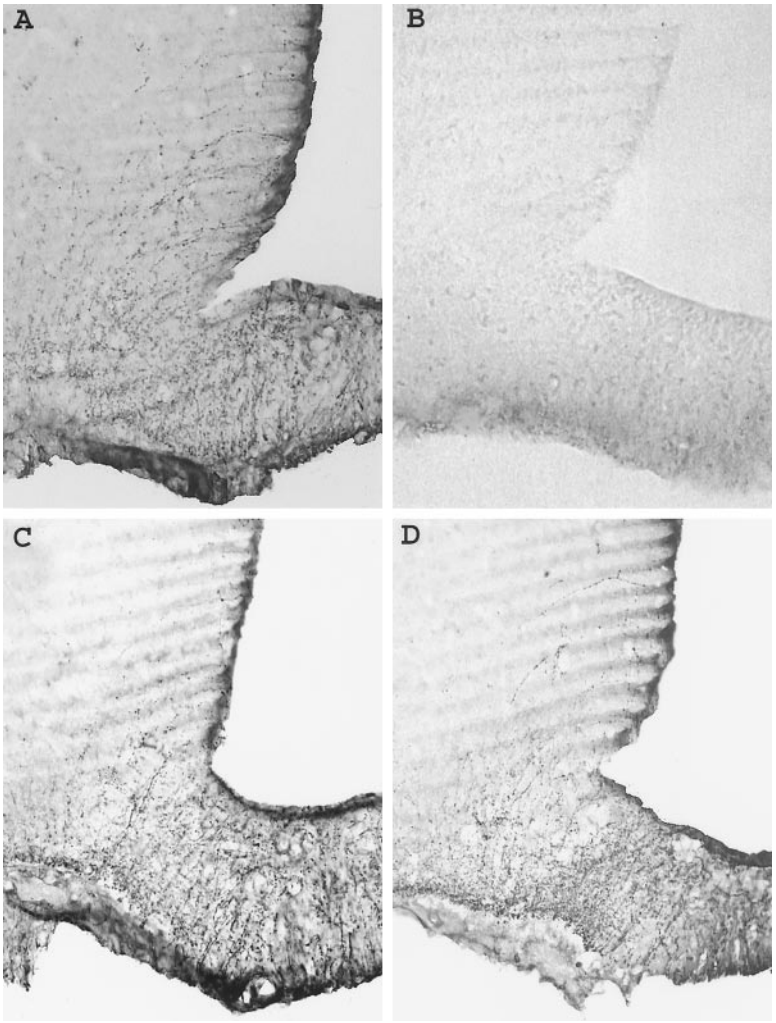

Fig. 4. Frontal sections through the middle region of the ME showing the efficiency of immunoabsorption of the l-GnRH-III antiserum. (*A*) Positive staining after tissue incubation with unabsorbed primary antiserum. (*B*) The ability of 1 μg of l-GnRH-III to eliminate fiber staining; *C* and *D* demonstrate clearly that preabsorption with 1 μg of m-LHRH (*C*) or 1 μg of l-GnRH-1 (*D*) does not diminish the immunoreactivity when compared with the l-GnRH-III unabsorbed antiserum (*A*). (*A–D*, $\times$141.)

action. Therefore, we believe that the FSH-releasing ability of this fraction was caused by l-GnRH-III. Final proof awaits isolation and determination of the structure of follicle-stimulating hormone releasing factor.

Further evidence of a separate control of FSH release has been demonstrated in studies by using drugs, such as alcohol (20, 21) and delta-9-tetrahydrocannibanol (22), that eliminate pulsatile LH release without affecting FSH release. Cytokines, such as interleukin-1, have also been shown to act centrally to selectively inhibit LH release without affecting FSH secretion (23). Thus, the neuronal populations that contain only l-GnRH-III may control the selective release of FSH, whereas those that contain both peptides work in concert to control the simultaneous pulses of FSH and LH.

Another GnRH isoform that is localized in the mammalian hypothalamus and midbrain is c-GnRH-II. Previously, we used a c-GnRH-II antiserum, which has been shown to detect this peptide in chicken brain (24) and found that there was no c-GnRH-II neuronal or fiber staining in the hypothalamus of the rat (8). Recently, after using an amplification technique, we were able to detect scant immunopositive c-GnRH-II fibers, but again no perikarya, in the POA, as well as the hypothalamic/ME region of the rat (J.K.H. and W.L.D., unpublished observation). We did, however, detect a few immunopositive c-GnRH-II cells in the mesencephalon. This result was similar to the staining observed in the musk shrew (6), although, in our hands we did not detect fibers in the mammillary region and only a few fibers in the mesencephalon, whereas abundant fibers of c-GnRH-II were described in these regions of the shrew. Importantly, others (7) have not observed immunopositive c-GnRH-II neurons in the hypothalamus of the normal mouse. Conversely, in the hypogonadal mouse lacking m-LHRH, c-GnRH-II cells have been detected in the arcuate nucleus. Since the c-GnRH-II antiserum was conjugated to BSA (25) and because the study does not indicate that the anti-BSA activity was absorbed (26), these putative c-GnRH-II cells found in the arcuate nucleus may represent BSA stained cells by the anti-BSA antibodies within the antiserum and not c-GnRH-II neurons. Similar false-positive results for the localization of GnRH have been previously demonstrated by using an antiserum generated against GnRH-BSA hapten-conjugates (27).

Additionally, the morphology and distribution of c-GnRH-II neurons and fibers is different from that of m-LHRH and l-GnRH-III. C-GnRH-II cells are described as round in shape and smaller in size than m-GnRH neurons (4, 6, 7), whereas l-GnRH-III neurons have a similar morphology as that of

m-LHRH neurons. Both types of these neurons are oval to diffuse in shape and are similar in size (8).

The distribution of c-GnRH-II cells has been shown in several studies to differ from the distribution of m-LHRH cells. In the musk shrew and mouse, it has been demonstrated, by using immunocytochemistry, that there is no overlap in the distribution of neurons containing c-GnRH-II and m-LHRH (6, 7). In the primate, by using *in situ* hybridization, Latimer *et al.* detected c-GnRH-II mRNA in the hypothalamus, but determined it was not colocalized with m-LHRH (5). Furthermore, immunocytochemical localization of c-GnRH-II perikarya and fibers was not reported, raising the possibility that the mRNA was not translated into the peptide (5). In an earlier study, using fetal primate brains, c-GnRH-II was detected in only a few neurons and fibers in the caudal hypothalamus, suggesting that this peptide may not reach the pituitary via the portal vessels (4), and may not play a role in gonadotropin release.

In summary, we report the colocalization of l-GnRH-III with m-LHRH, together with localized separate populations of l-GnRH-III and m-LHRH neurons in the ventromedial preoptic area, a region known to control the preovulatory release of gonadotropins (15). These observations suggest that both peptides are cooperating to control the preovulatory surge of LH and to a lesser extent FSH.

The dorsomedial caudal POA is known to control the selective release of FSH, based on lesion and stimulation studies (16–18). This area contains only perikarya of l-GnRH-III neurons. The axons of both m-LHRH and l-GnRH-III neurons project to the ME, releasing the peptides into the capillaries of the hypophysial portal vessels, which transport them to the anterior pituitary where l-GnRH-III preferentially releases FSH, and m-LHRH preferentially releases LHRH.

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1. Powell, J. F. F., Reska-Skinner, S. M., Prakash, M. O., Fischer, W. H., Park, M., Rivier, J. E., Craig, A. G., Mackie, G. O. & Sherwood, N. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10461–10464.
2. Fernald, R. D. & White, R.B. (1999) *Front. Neuroendocrinol.* **20,** 224–240.
3. Stopa, E. G., Sower, S. A., Svendsen, C. N. & King, J. C. (1988) *Peptides* **9,** 419–423.
4. Lescheid, D. W., Terasawa, E., Abler, L. A., Urbanski, H. F., Warby, C. M., Millar, R. P. & Sherwood, N. M. (1997) *Endocrinology* **138,** 5618–5629.
5. Latimer, V. S., Rodrigues, S. M., Garyfallou, V. T., Kohama, S. G., White, R. B., Fernald, R. D. & Urbanski, H. F. (2000) *Mol. Brain Res.* **75,** 287–292.
6. Rissman, E. F., Alones, V. E., Craig-Veit, C. B. & Millam, J. R. (1995) *J. Comp. Neurol.* **357,** 524–531.
7. Gestin, E. D., White, R. B. & Fernald, R. D. (1999) *FEBS Lett.* **448,** 289–291.
8. Dees, W. L., Hiney, J. K., Sower, S. A., Yu, W. H. & McCann, S. M. (1999) *Peptides* **20,** 1503–1511.
9. Yu, W. H., Karanth, S., Walczewska, A., Sower, S. A. & McCann, S. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9499–9503.
10. Dees, W. L., Dearth, R. K., Hooper, R. N., Brinsko, S. P., Romano, J. E., Rahe, C. H., Yu, W. H. & McCann, S. M. (2001) *Domest. Anim. Endocrinol.* **20,** 279–288.
11. Yu, W. H., Millar, R. P., Milton, S. C. F., Del Milton, R. C. & McCann, S. M. (1990) *Brain Res. Bull.* **25,** 867–873.
12. Lumpkin, M. D., Moltz, J. H., Yu, W. H., Samson, W. K. & McCann, S. M. (1987) *Brain Res. Bull.* **18,** 175–178.
13. Antunes-Rodigues, J. & McCann, S. M. (1970) *Proc. Soc. Exp. Biol. Med.* **133,** 1464–1469.
14. Adams, J. C. (1992) *Histochem. Cytochem.* **40,** 1456–1463.
15. Samson, W. K., Snyder, G., Fawcett, C. P. & McCann, S. M. (1980) *Peptides* **1,** 97–102.
16. Ojeda, S. R., Jameson, H. E. & McCann, S. M. (1977) *Endocrinology* **100,** 595–1603.
17. Lumpkin, M. D. & McCann, S. M. (1984) *Endocrinology* **115,** 2473–2480.
18. Lumpkin, M. D., McDonald, J. K., Samson, W. K. & McCann, S. M. (1989) *Neuroendocrinology* **50,** 229–235.
19. Yu, W. H., Karanth, S., Sower, S. A., Parlow A. F. & McCann, S. M. (2000) *Proc. Soc. Exp. Biol. Med.* **224,** 87–92.
20. Dees, W. L. & Kozlowski, G. P. (1984) *Alcohol* **1,** 429–433.
21. Dees, W. L., Rettori, V., Koziowski, J. G. & McCann, S. M. (1985) *Alcohol* **2,** 641–646.
22. Wenger, T., Rettori, V., Snyder, G. P., Dalterio, S. & McCann, S. M. (1987) *Neuroendocrinology* **46,** 488–493.
23. Rettori, V., Gimeno, M. F., Karara, A., Gonzales, M. C. & McCann, S. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2763–2767.
24. van Gils, J., Absil, P., Grauwels, L., Moons, L., Vandesande, F. & Balthazart, J. J. (1993) *Comp. Neurol.* **334,** 304–323.
25. Okusawa K., Amano M., Kobayashi, M., Aida, K., Hanyu, I., Hasegawa, Y. & Miyamoto, K. (1990) *Gen. Comp. Endocrinol.* **80,** 116–126.
26. Chen, A., Yahalom, D., Ben-Aroya, N., Kaganovsky, E., Okon, E. & Koch, Y. (1998) *FEBS Lett.* **435,** 199–203.
27. Kozlowski, G. P. & Dees, W. L. (1984) *J. Histochem. Cytochem.* **32,** 83–91.