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# Colocalization of galanin and luteinizing hormone-releasing hormone in a subset of preoptic hypothalamic neurons: Anatomical and functional correlates

(coexpression of neuropeptides/neuromodulation/double-labeling/immunocytochemistry/reproductive endocrinology)

ISTVAN MERCHENTHALER*†, FRANCISCO J. LOPEZ‡, AND ANDRES NEGRO-VILAR‡

*Functional Morphology and ‡Reproductive Neuroendocrinology Sections, Laboratory of Molecular and Integrative Neuroscience, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

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**ABSTRACT** **Colocalization of neurotransmitters, including neuropeptides and amines, in the same neuron of certain areas or well-defined nuclei of the central and peripheral nervous systems appears to be the rule rather than the exception. The coexistent neurotransmitters can be coreleased and interact at pre- and postsynaptic levels in a synergistic or antagonistic manner. Galanin is a recently isolated and characterized "gut–brain" peptide. It is colocalized with many neurotransmitters in both the central and the peripheral nervous systems. Among other regions in the central nervous system, galanin is present in neuronal perikarya of the septum and the hypothalamus. The dense accumulation of nerve terminals in the external zone of the median eminence suggests that galanin is an important peptide regulating neuroendocrine functions. Although most galanin and luteinizing hormone-releasing hormone (LHRH) neurons have a distinctly different morphology, a subset of galanin-immunoreactive perikarya in the diagonal band of Broca and the medial preoptic area, near the organum vasculosum of the lamina terminalis, have morphological features similar to those of LHRH neurons. By using double-labeling immunocytochemistry, we have found that in the preoptic region of the male rat brain ≈15–20% of these "LHRH-like" galanin-immunoreactive neurons are also immunopositive for LHRH. Moreover, in the medial preoptic area and the diagonal band of Broca, some of the single-labeled LHRH cells are surrounded with galanin-immunoreactive nerve terminals, suggesting that LHRH perikarya have synaptic contacts with galanin-immunoreactive terminals. Additional studies indicated that galanin can readily enhance *in vitro* release of LHRH from nerve terminals in the median eminence. The observations that (*i*) galanin is coexpressed with LHRH, (*ii*) galanin seems to innervate LHRH-producing neurons, and (*iii*) galanin acts as a putative neurotransmitter to enhance the release of LHRH suggest that galanin should be considered an important regulator of LHRH-containing neurons and, therefore, of reproductive functions.**

Galanin is a 29-amino acid peptide originally isolated from porcine intestine (1). It is widely distributed in the central and peripheral nervous systems (2–5), the gastrointestinal tract (2, 6, 7), the urogenital tract (8), and the adrenal medulla (9). Moreover, a widespread distribution of galanin binding sites in the nervous system has also been described (10). Immunocytochemical mapping of galanin-immunoreactive structures in the brain indicated that high galanin concentrations occur in the median eminence (ME); in the paraventricular, the supraoptic, the arcuate, and the dorsomedial nuclei of the hypothalamus; in the locus ceruleus; in the spinal trigeminal nucleus; and in the nucleus of the solitary tract (1–4).

Although the precise physiological role of galanin has not yet been established, the presence of a dense accumulation of galanin-immunoreactive nerve terminals in the external zone of the ME suggests that galanin is an important hypophysiotropic factor. This particular location and the observation that galanin may affect the release of prolactin, growth hormone (11–13), and luteinizing hormone (LH) (14) further strengthen this possibility.

LH-releasing hormone (LHRH)- and galanin-containing neurons have a distinctly different distribution and morphology (15–18). In initial studies (18), a subset of galanin-immunoreactive perikarya near the organum vasculosum of the lamina terminalis (OVLT) was observed to have morphological features resembling those of LHRH-containing perikarya. This observation suggests that galanin and LHRH might be colocalized in a subset of neurons in the areas close to the OVLT. In several areas of the brain, galanin has been shown to be colocalized with other peptides or amines—e.g., with vasopressin and dynorphin in the paraventricular nucleus (19), with tyrosine hydroxylase and $\gamma$-aminobutyric acid (GABA) in the arcuate nucleus (5, 20), with norepinephrine in the locus ceruleus, with serotonin in the medullary raphe nuclei (20, 21), and with choline acetyltransferase in the medial septum and the diagonal band of Broca (DBB) (22). LHRH, on the other hand, has not been colocalized so far in the brain with any other known neurotransmitters. Recently, antibodies raised against $\delta$ sleep-inducing peptide, a factor originally isolated from blood, were shown to costain LHRH cells in rabbit brain (23). The presence of galanin-containing cells with an LHRH-like morphology provided the impetus to conduct a systematic analysis to determine if galanin and LHRH are colocalized in the same neurons of the rat brain. This study provides clear evidence for the colocalization of these peptides and additional evidence for the role of galanin in regulating LHRH secretion from nerve terminals *in vitro*.

## MATERIALS AND METHODS

**Materials.** Antisera directed toward LHRH were raised in two different species. Antiserum H-16 was raised in a rabbit (24), and antiserum S772 was raised in a sheep (25). The antisera against rat galanin (FJL #7-3) and thyrotropin-releasing hormone (4/6) were raised in rabbits (26, 27). The immunocytochemical characterization of these antibodies has been described elsewhere (17, 24–27). Sheep anti-rabbit IgG, rabbit anti-sheep IgG, and their respective peroxidase-antiperoxidase (PAP) complexes were obtained from Arnel

Abbreviations: LH, luteinizing hormone; LHRH, LH-releasing hormone; DBB, diagonal band of Broca; MPOA, medial preoptic area; ME, median eminence; OVLT, organum vasculosum of the lamina terminalis; PAP, peroxidase-antiperoxidase.
†To whom reprint requests should be addressed.

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Products (New York). Fluorescent isothyocyanate (FITC)-labeled donkey anti-rabbit IgG and rhodamine-labeled donkey anti-sheep IgG were purchased from Chemicon. 3,3'-Diaminobenzidine (DAB) and 4-chloro-1-naphthol were obtained from Aldrich Chemical. Tri-X 400, Tmax 3200 black-and-white and Ektachrome P 800/1600 films were purchased from Eastman Kodak. Synthetic rat galanin and LHRH were obtained from Peninsula Laboratories.

**Animals.** Male Sprague–Dawley rats (Charles River Breeding Laboratories) weighing 250–300 g were used. The animals were housed in a temperature- and light-controlled facility with a 12-hr light/12-hr dark schedule (lights on at 0800) and a temperature of 22°C. For control studies, brain tissues were also obtained from homozygous hypogonadal mice (The Jackson Laboratory).

For the morphological studies, animals were anesthetized with 2.5% tribromoethanol (1.0 ml/100 g of body weight), and colchicine was injected into the lateral ventricle as described before (28). Twenty-four hours later, the animals were deeply anesthetized and perfused through the ascending aorta with 30 ml of 1% phosphate-buffered paraformaldehyde (pH 7.4), followed by 200 ml of Zamboni's fixative (29). Thirty minutes after the perfusion, the brains were carefully removed from the skull, cut into smaller pieces, and immersed in the same fixative overnight at 4°C. Tissue blocks containing the posterior portion of the DBB and the entire hypothalamus were either embedded into paraffin or cut at 30 $\mu$m with a Vibratome. Paraffin-embedded tissues were serially sectioned at 4–5 $\mu$m.

***In Vitro* Incubations.** For the *in vitro* studies, animals were killed by decapitation, the brain was rapidly removed from the skull, and arcuate nuclei–ME fragments were dissected under a stereomicroscope with a fine pair of scissors (30). The procedure used for the incubation has been described in detail (31). Briefly, dissected tissue fragments were preincubated for 30 min and then exposed for an additional 30 min to galanin at different concentrations, and the changes in the secretion of LHRH during this period were evaluated.

**Immunocytochemical Procedures.** Four different immunocytochemical techniques were used to examine galanin and LHRH colocalization: (*i*) staining of adjacent Vibratome sections for either LHRH or galanin with the PAP technique as described (28), (*ii*) staining of adjacent paraffin sections for either LHRH or galanin with the double PAP technique (32), (*iii*) staining of the same Vibratome section with primary antisera raised in the same species by using contrasting chromogens (33), and (*iv*) staining of the same paraffin sections with primary antisera raised in different species by using contrasting fluorochromes (34, 35). The advantages and disadvantages of the different techniques have been discussed in detail by Hokfelt *et al.* (36).

The combined use of several different double-labeling techniques is a more reliable approach to examine the coexistence of galanin and LHRH than the use of any single technique. Agreement of all four methods provides conclusive evidence for the colocalization of two or more substances in the same neuron.

All sections were examined with an Axiophot photomicroscope equipped with the necessary excitation and emission filters for immunofluorescence microscopy.

**Immunocytochemical Controls.** Staining specificity was tested by several criteria. The specificity of the technique was tested by a series of increasing dilutions of the primary antisera, resulting in a gradual decrease and eventual disappearance of the immunostaining. The specificity of the primary antisera was also verified: Galanin- and LHRH-immunoreactivities were abolished by absorption of the primary antiserum with as little as 1 $\mu$g/ml of the peptide to which the antiserum was raised. In addition, immunoreactivity was unaffected by absorption of the antisera with as much as 20 $\mu$g/ml of the other peptide. Double-staining performed on both vibratome or paraffin-embedded tissue sections from homozygous hypogonadal mice, which do not express the LHRH prohormone because of a deletional mutation in the distal half of the LHRH gene (37), showed only a single chromogen, indicating the presence of galanin in perikarya of the medial preoptic area (MPOA), and no

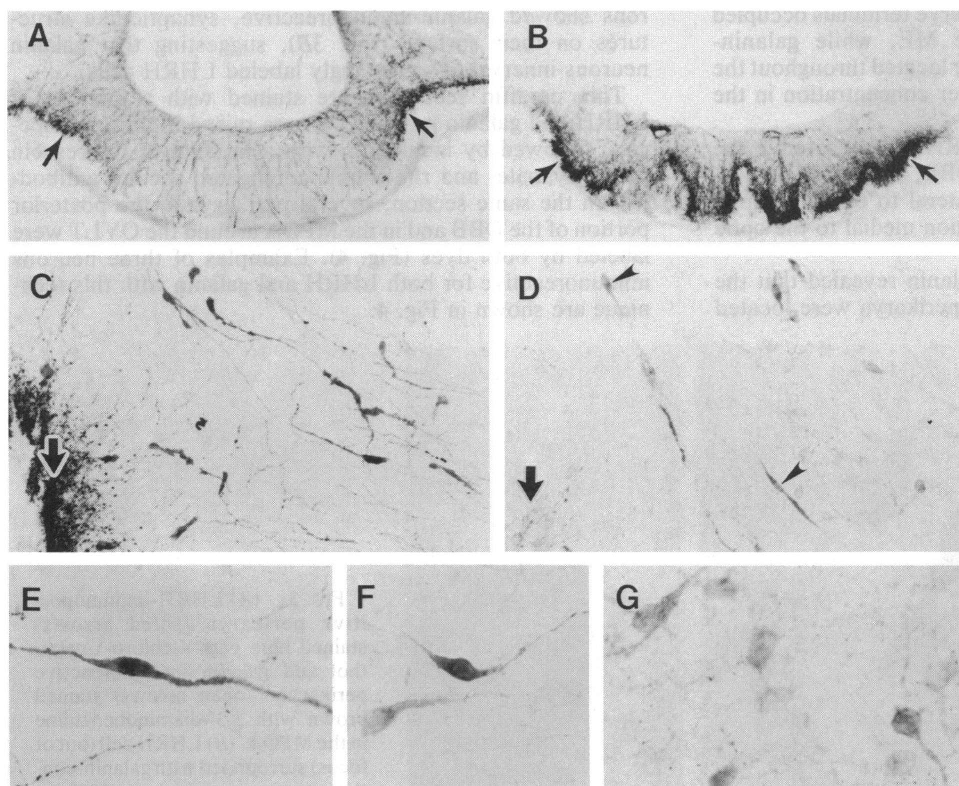


FIG. 1. LHRH (*A*) and galanin (*B*) nerve terminals in the ME. While most of the LHRH nerve terminals occupy the lateral edge of the external zone of the ME (arrows), galanin is evenly distributed in the entire width of the ME with a more prominent localization in the lateral edge (arrows). LHRH (*C*)- and galanin (*D*)-immunoreactive perikarya in the MPOA of the hypothalamus at the level of the OVLT (arrow). (*C*) All LHRH cells are elongated fusiform neurons. (*D*) Similar elongated LHRH-like galanin-immunoreactive perikarya (arrowheads). Higher magnifications are of a typical LHRH neuron (*E*), of galanin-immunopositive neurons resembling the shape of LHRH neurons (*F*), and of typical round, multipolar galanin-containing neurons in the basal preoptic area (*G*). (*A–D*, ×95; *E–G*, ×250.)

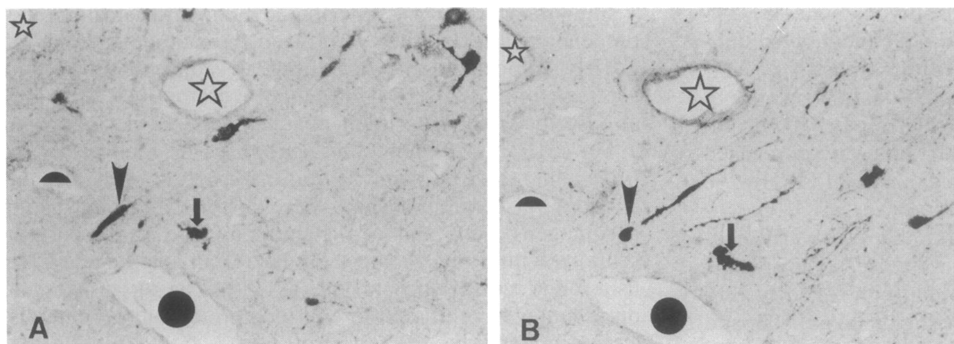


FIG. 2. Colocalization of galanin (*A*) and LHRH (*B*) immunoreactivities. The adjacent 4-$\mu$m paraffin sections are from a colchicine-treated male rat and are stained with the double PAP technique. Identical symbols label perikarya immunoreactive for both antigens. Stars and dots indicate the same blood vessels. (×120.)

staining for LHRH as reported (37). As a further control for spurious colocalization, the same Vibratome section was stained with rabbit antiserum against LHRH and thyrotropin-releasing hormone having contrasting chromogens. Thyrotropin-releasing hormone and LHRH were always localized in completely separate populations of nerve cells, further validating our colocalization methods.

**RIA.** LHRH levels in media samples were measured by RIA with the LHRH antiserum Rice no. 5. A complete characterization of this antiserum has been reported (38, 39). Under equilibrium conditions, the assay has a sensitivity of 0.5–1 pg per tube. To avoid interassay variability, all samples in this study were measured in a single assay. The intraassay variability was approximately 7%. Rat galanin was included in the assay to evaluate any possible interference. None of the concentrations of rat galanin used displaced LHRH.

**Data Analysis.** The levels of LHRH secreted *in vitro* are expressed as means ± SEM. Statistical analysis was performed by using analysis of variance followed by Duncan's multiple range test (40). Differences with a value of $P < 0.05$ were considered to be significant.

## RESULTS

**Immunocytochemical Observations.** Both galanin and LHRH were present in high concentrations in the external zone of the ME. LHRH-containing nerve terminals occupied mostly the lateral aspects of the ME, while galanin-immunoreactive nerve terminals were located throughout the entire width of the ME, with a higher concentration in the lateral regions (Fig. 1 *A* and *B*).

Perikarya immunopositive for LHRH were located in the vertical and horizontal limbs of the DBB, in the MPOA (Fig. 1*C*), in the region just above and lateral to the supraoptic nuclei, and more posteriorly in a region medial to the optic tracts.

Vibratome sections stained for galanin revealed that the majority of galanin-immunoreactive perikarya were located in a region ventral to the DBB, in the MPOA (Fig. 1*D*), and in the periventricular, paraventricular, supraoptic, arcuate, and dorsomedial nuclei.

While LHRH-immunoreactivity was located in smaller, elongated, fusiform (either smooth or "spiny") nerve cells (Fig. 1*E*), galanin immunoreactivity was primarily present in larger, round perikarya (Fig. 1*G*). However, some galanin-immunopositive perikarya were similar in appearance to typical LHRH neurons (Fig. 1*F*). The staining for galanin and LHRH in adjacent sections revealed that a small number of cells near the OVLT were immunoreactive for both peptides (Fig. 2). When the same 30-$\mu$m Vibratome sections were stained consecutively for LHRH and galanin by the technique of Joseph and Sternberger (33), the smaller elongated perikarya were labeled by brown precipitate, indicating the presence of LHRH, and larger perikarya were labeled with blue reaction product, indicating the presence of galanin (Fig. 3*A*). More important, some of the smaller perikarya near the OVLT contained a mixture of brown and blue precipitates, suggesting the presence of both peptides in the same cell. Because of the difficulty in reproducing the true color in a photomicrograph, these data are not shown. By counting singly and doubly labeled LHRH cells in brains from 12 animals, we have estimated that about 15–20% of LHRH perikarya around the OVLT were also immunoreactive for galanin. Furthermore, some LHRH-immunoreactive neurons showed galanin-immunoreactive, synaptic-like structures on their surface (Fig. 3*B*), suggesting that galanin neurons innervated some singly labeled LHRH cells.

Thin paraffin sections were stained with a mixture of LHRH and galanin primary antisera raised in different species, followed by incubation with a mixture of fluorescein isothiocyanate- and rhodamine-conjugated second antibodies. In the same section, several perikarya in the posterior portion of the DBB and in the MPOA around the OVLT were labeled by both dyes (Fig. 4). Examples of three neurons immunoreactive for both LHRH and galanin with this technique are shown in Fig. 4.

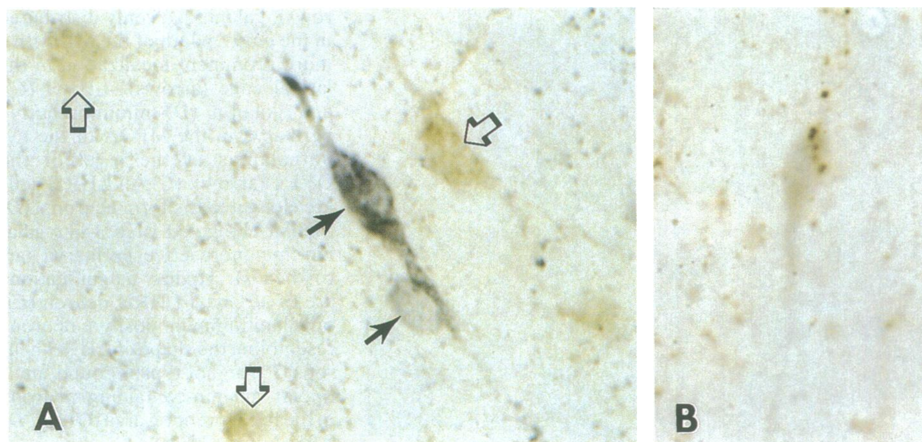


FIG. 3. (*A*) LHRH-immunopositive perikarya (filled arrows) stained blue with 4-chloro-1-naphthol and galanin immunoreactive perikaryon (open arrows) stained brown with 3,3′-diaminobenzidine in the MPOA. (*B*) LHRH cell (out of focus) surrounded with galanin-containing nerve terminals. (×1000.)

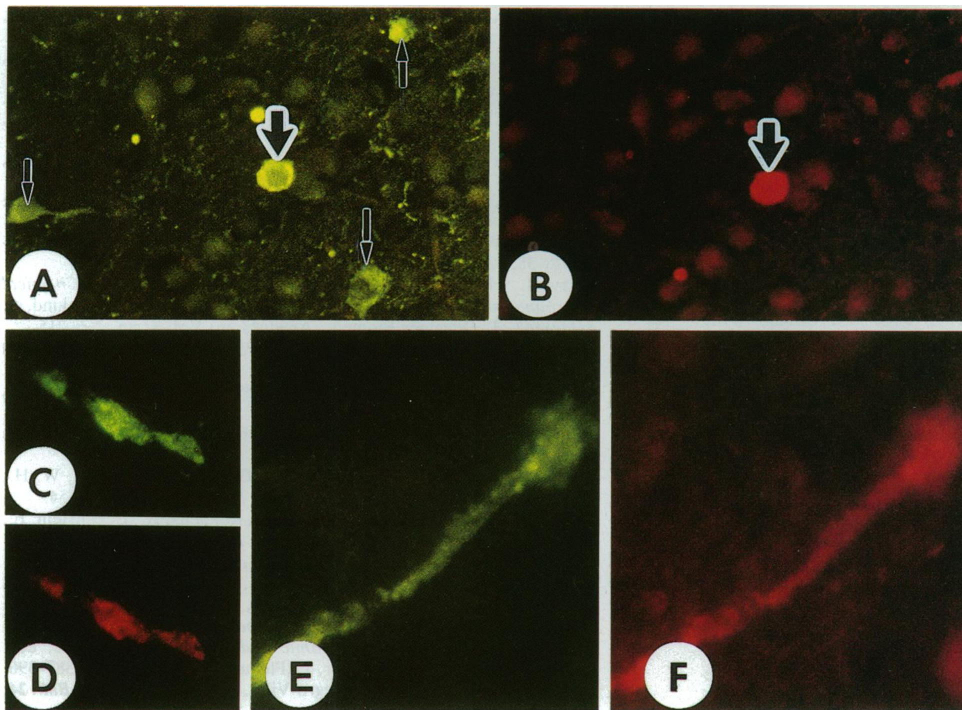


FIG. 4. Colocalization of LHRH and galanin in the same neuron by staining the same thin paraffin sections with primary antisera raised in different species with contrasting fluorochromes. Three pairs of sections (*A* and *B*; *C* and *D*; and *E* and *F*) immunostained for galanin (*A, C,* and *E*) and LHRH (*B, D,* and *F*) are shown. Yellow fluorescein isothiocyanate labels galanin-immunoreactive perikarya, and red rhodamine labels LHRH-immunoreactive perikarya. Identical symbols label identical neurons. Note that on *A*, four galanin-containing cell bodies are indicated (different arrows), and only one of them (large arrow) is immunoreactive for LHRH (large arrow on *B*). (*A–D*, ×300; *E* and *F*, ×1100.)

**Effects of Galanin on LHRH Secretion *in Vitro*.** The observation that most of the LHRH-containing perikarya are surrounded by galanin-containing nerve terminals prompted us to evaluate whether galanin could affect LHRH release from arcuate nuclei-ME fragments *in vitro*. The data depicted in Fig. 5 indicate that galanin stimulates LHRH release in a dose-dependent fashion. A 2-fold increase in LHRH release was observed when 100 nM galanin was added to the medium. This effect was replicated in several independent experiments and suggests that galanin is a potent stimulator of LHRH release from nerve terminals in the ME.

## DISCUSSION

The immunocytochemical evidence presented indicates that perikarya immunoreactive for both LHRH and galanin constitute a distinct population of neurons near the OVLT in the MPOA of the male rat brain. Moreover, the data also suggest that neurons immunopositive only for LHRH are innervated by synaptic button-like structures that are immunoreactive for galanin. Pharmacological studies indicate that galanin can

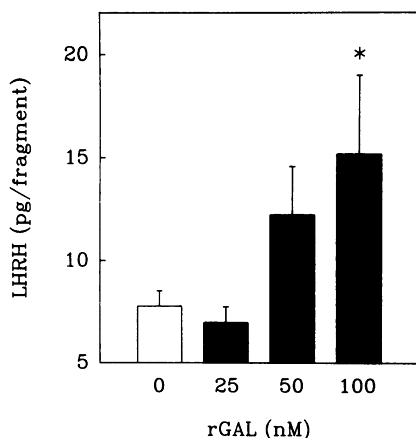


FIG. 5. Effects of rat galanin (rGAL) on LHRH release from arcuate nucleus-ME fragments *in vitro*. *, $P < 0.05$ vs. control group (analysis of variance followed by Duncan's multiple range test).

enhance secretion of LHRH *in vitro* from nerve terminals in the ME. Taken together, the observations that galanin is coexpressed with LHRH, that it appears to innervate LHRH neurons, and that it acts as a putative neurotransmitter to enhance release of LHRH suggest that galanin should be considered an important regulator of LHRH-containing neurons and, therefore, of reproductive functions.

Several different techniques have been used to demonstrate the colocalization of LHRH and galanin. Staining of LHRH and galanin in adjacent thin sections provided clear-cut evidence for the coexistence of these two peptides in the same neuron. Because of the fact that LHRH neurons are small and not densely packed, this approach becomes impractical when trying to evaluate systematically the colocalization of these peptides. For this reason, we used antibodies to LHRH and galanin raised in two different species to stain for both peptides in the same tissue section using either contrasting chromogens or fluorochromes. Using this second approach, we also obtained conclusive evidence that both peptides are colocalized in a certain number of LHRH-containing neurons and were able to analyze a large number of sections. Staining of thick Vibratome sections with antisera against galanin and LHRH raised in rabbits also provided confirmation for coexistence of these two peptides. Therefore, the demonstration of the colocalization of these peptides is reinforced by the perfect agreement found with the three different technical approaches employed. The studies using hypogonadal mice brains also provided an important negative control, indicating positive immunostaining for galanin in perikarya in the MPOA and no immunostaining for LHRH neurons. The absence of immunopositive LHRH cells in these animals has been documented (37).

Colocalization of neurotransmitters, including peptides and amines, within the same neurons in a certain area or a well-defined nucleus of the central and peripheral nervous systems appears now to be the rule rather than the exception (36). Galanin, in particular, has been shown to be colocalized with several neurotransmitters in different areas of the brain (19, 22, 41). The physiological significance of the coexistence of multiple messengers in the nervous system is not clearly understood. Studies in the peripheral nervous system indicate that classical neurotransmitters and peptides are core-

leased and interact in either a synergistic or antagonistic manner (36). With the exception of colocalization and interactions of corticotropin-releasing factor and vasopressin in adrenalectomized rats (42–45), similar observations of physiological interactions within the central nervous system are not readily available. Our studies with the arcuate nucleus-median eminence preparation (30) provide intriguing evidence for an interaction between both peptides in the central nervous system.

These studies clearly demonstrate that a subpopulation of LHRH-containing neurons is also immunoreactive for galanin. Since galanin mRNA distribution (46) correlates well with the location of galanin-immunoreactivity observed by immunocytochemistry, we could assume that neurons containing both LHRH and galanin immunoreactivities actually synthesize both peptides. In addition, the colocalization (and probably synthesis) of both peptides within the same neuron, suggests that LHRH and galanin may be cosecreted from nerve terminals. Recently, we attempted to evaluate this hypothesis by measuring LHRH and galanin in portal plasma and by analyzing the pulsatile secretion of both peptides. As is the case for LHRH, galanin is also present in portal blood and at concentrations several fold higher than those found in the peripheral circulation (26). Additional studies (47) revealed that LHRH and galanin are secreted in a pulsatile fashion. Interestingly, secretory episodes in both series presented a statistically significant number of coincidental pulses. These observations support the idea that both peptides are either coreleased or that they are secreted synchronously. The latter phenomenon would require a common functional link to an endogenous intrinsic oscillator. In either case, a close functional link is established for the control of secretion of these two peptides.

It is well known that the steroid background significantly influences the release of LHRH into the portal circulation in the ME and, consequently, the secretion of LH by the anterior pituitary (48–50). Since the LHRH neurons themselves do not appear to contain estradiol receptors (51), the mechanism through which gonadal steroids affect LHRH neurons is still unknown. Since galanin neurons in the MPOA do contain estrogen receptors (52) and galanin mRNA increases with estrogen therapy (46), the possibility should be considered that the effects of estradiol on LHRH and on the consequent LH release may be mediated, at least in part, through the galanin-containing neurons. If this were true, galanin could represent an important mediator of estrogen-induced changes in LH release.

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