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Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons

(dye-coupling/synapse-like connections/immortalized cells)

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ABSTRACT **Mammalian reproduction is dependent upon intermittent delivery of luteinizing hormone-releasing hormone (LHRH) to the anterior pituitary. This mode of secretion is required to sensitize maximally the gonadotrophs to LHRH stimulation and to regulate gonadotropin gene expression. While LHRH secretion is pulsatile in nature, the origin of the pulse generator is unknown. In this report, we show that this oscillator could be located within the LHRH neuronal network. When immortalized LHRH neurons are placed into a perifusion system, LHRH is secreted into the medium in a pulsatile fashion under basal conditions. LHRH secretion and the number of LHRH pulses are reduced when calcium is removed from the medium. Perifusion also influences pro-LHRH processing, since the molar ratio of its processed products varies dramatically when the cells are transferred from a static system. Several different cellular mechanisms may underlie these changes in secretion and processing. Lucifer yellow experiments reveal that some cells are dye-coupled. Hence, these cells could be electrically coupled through gap junctions such that secretion from individual cells could be coordinated. Secretion could also be synchronized through the observed synapse-like contacts. These contacts could perform a negative-feedback role to regulate not only the amount of LHRH released but also the molecular forms secreted. The organization of LHRH neurons into interconnected clusters could serve to coordinate LHRH secretion from individual cells and, thereby, orchestrate functions *in vivo* as diverse as the onset of puberty, the timing of ovulation, and the duration of lactational infertility.**

Luteinizing hormone (LH)-releasing hormone (LHRH) is a major regulator of reproduction in mammals (1–3). While LHRH neuronal cell bodies are scattered throughout the anterior hypothalamic region, their nerve terminals converge on the median eminence. LHRH is secreted into the hypophysial portal circulation, where it is transported to the anterior pituitary to stimulate the release of LH and follicle-stimulating hormone.

In all mammalian species studied so far, secretion of LH into blood is episodic in nature (1, 4–8). Interestingly, LHRH is secreted into the hypophysial portal blood in a pulsatile manner (9–11), and LH pulses are preceded by LHRH release (1, 10, 11). In addition, LH pulsatile secretion is lost either after passive immunization with LHRH antiserum (12) or with administration of a LHRH antagonist (13). Lesions of either the medial basal hypothalamus or the arcuate nucleus also eradicate LH pulses (1). By comparison, continuous infusion of LHRH, which desensitizes the gonadotropes through down-regulation of the LHRH receptor (14), also abolishes episodic secretion of LH and reduces LH β- and α-subunit mRNA levels (15, 16). While the relationship between LHRH and LH secretion is well established, it is unclear whether the generator for LHRH pulses is located within the LHRH neuronal network itself or within networks of other neighboring neurons or cells that directly affect LHRH secretion.

Recently, Mellon *et al.* (17) developed an immortalized hypothalamic neuronal cell line that expresses the prepro-LHRH mRNA and secretes LHRH into the medium in response to several stimuli. The purpose of our investigation was to determine whether these immortalized neurons possess an inherent ability to secrete LHRH in a pulsatile manner. Since pulsatile release may involve the coordinated response from a number of neurons, we also sought to identify some of the cellular mechanisms that could underlie this mode of secretion.

MATERIALS AND METHODS

Materials. The A772 and MC-2 antisera were used to immunostain and/or quantitate LHRH and gonadotropin-releasing hormone-associated peptide (GAP), respectively. Dulbecco's modified Eagle's medium, fetal calf serum, horse serum, penicillin and streptomycin were purchased from GIBCO. Glass and plastic two-well Lab-Tek slides, Matrigel, and Cytodex beads were bought from Nunc, Collaborative Research, and Pharmacia, respectively. Goat anti-rabbit IgG (heavy and light chains) coupled to 5-nm colloidal gold particles and the IntenSE M silver enhancement kit were obtained from Amersham, while the Epon LX-112 generic resin and Lucifer yellow dye were purchased from Ladd Research Industries (Burlington, VT) and Molecular Probes, respectively. Synthetic LHRH and human GAP were bought from Peninsula Laboratories.

Cell Culture. The immortalized hypothalamic neurons, the GT1-7 cell subclone, were maintained in medium as described (18). For scanning EM and perifusion experiments, cells were grown on Cytodex beads for 4 days. For all other experiments, cells were grown for 1–3 days on glass coverslips or Lab-Tek slides previously coated with Matrigel.

Immunocytochemistry. GT1-7 cells were fixed with a 4% paraformaldehyde solution in phosphate-buffered saline for 1 hr and treated with a 0.2% solution of Triton X-100 for 10 min as described (19). Cells were immunostained with MC-2 antiserum (1:5000 dilution for 24 hr at 4°C), which recognizes both the pro-LHRH and GAP (18, 20). Slides were then

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Abbreviations: LH, luteinizing hormone; LHRH, LH-releasing hormone; GAP, gonadotropin-releasing hormone-associated peptide.

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incubated with goat anti-rabbit IgG coupled to 5-nm gold particles (1:30 dilution for 2–4 hr at room temperature), followed by silver intensification with the IntenSE M kit. Specificity of the immunocytochemical reaction was evaluated by replacing the primary antiserum with normal sheep serum or by preabsorbing the GAP antiserum with synthetic human GAP. The light microscopic preparations were examined and photographed with a Zeiss Axiophot microscope.

Transmission EM. GT1-7 cells were fixed in a solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate-buffered saline for 1 hr as described (19). They were postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon resin. Serial ultrathin sections were cut on a MT 6000-XL Ultratome (Research and Manufacturing, Tucson, AZ), contrasted with uranyl acetate and Reynolds' lead citrate, and examined with a Zeiss-10 CR electron microscope.

Scanning EM. GT1-7 cells grown on Cytodex beads were fixed either immediately or following a 4-hr perifusion. In the first case, samples were fixed to Matrigel-coated plastic coverslips with a 2% glutaraldehyde solution. Perifused samples were processed as described above. All samples were postfixed, dehydrated, dried to the critical point in carbon dioxide, "sputter-coated" with gold, and evaluated with a JEOL JXA-35 scanning electron microscope.

Vital Dye Image Analyses. Just prior to dye microinjection, Dulbecco's modified Eagle's medium was replaced with Hepes-buffered saline, and cells were maintained at room temperature. GT1-7 cells were individually injected by pressure with micropipettes loaded with potassium or lithium salts of Lucifer yellow in distilled water. Frame-averaged images were obtained with a Perceptics 9200 imaging system equipped with a Dage-MTI SIT 66 camera.

Perifusion of Immortalized LHRH Neurons. After culture on Cytodex beads for 4 days, ≈0.2 ml of beads–cells (see Fig. 1 *c–e*) were loaded into plastic 1-ml syringes. A rubber stopper was positioned directly on top of the beads–cells to minimize dead volume. Cells were perifused at 0.1 ml/min with Krebs–Ringer bicarbonate glucose buffer and maintained at 37°C under 95% O_2/5% CO_2 (21) in the presence or absence of calcium. Eight syringes were perifused simultaneously, and 5-min fractions were collected. Samples were stored at −80°C until assayed.

Quantitation of LHRH and GAP Secretion. LHRH and GAP secretion were quantitated by RIA using A772 and MC-2 antisera (20, 22–24), respectively. Minimal detectable doses for the LHRH and GAP assays were 0.69 pg and 7.8 pg, respectively. All samples from a given experiment were run in a single assay. The intraassay variability was 7.1% and 9.2% for the respective LHRH and GAP RIAs.

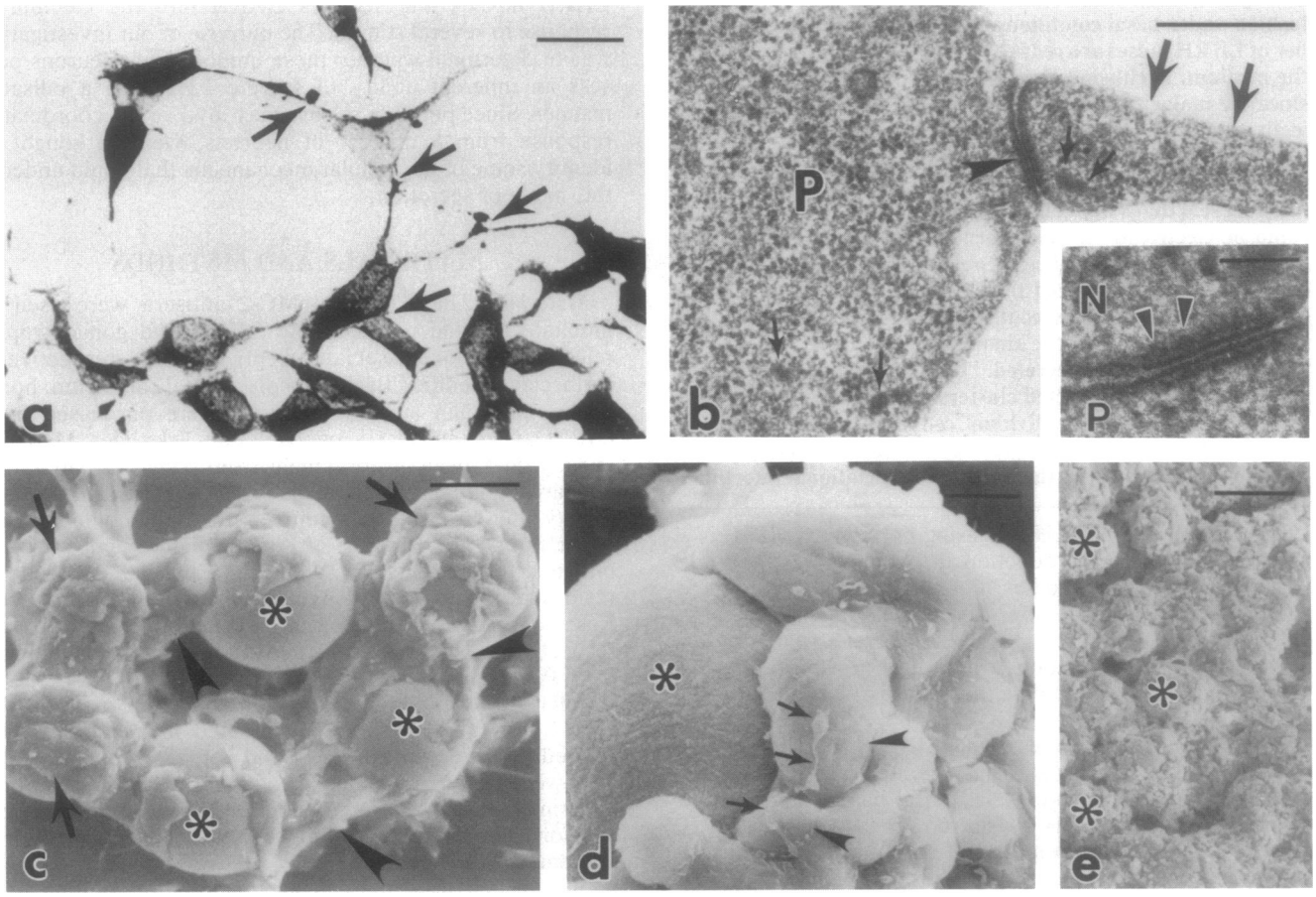

FIG. 1. Morphological evidence for connections among the GT1-7 cells. (*a*) Cultured GT1-7 neurons establish connections (arrows) with each other, and all cells are immunopositive for GAP. (*b*) Transmission electron micrograph demonstrates synapse-like formation (arrowhead) of a neuronal process (large arrows) with the cell body (P) of an immortalized neuron. Small arrows point to secretory granules. (*b Inset*) Membrane specialization at higher power. Two secretory vesicles (arrowheads) are inserted into the presynaptic dense material. N, neurite; P, perikaryon. (*c*) Scanning electron micrograph of GT1-7 neurons grown on Cytodex beads (asterisks). Some of the beads (arrows) are almost completely covered by cells. Note the cell bundles (arrowheads) interconnecting the individual beads. (*d*) Medium power scanning electron micrograph of GT1-7 cells attached to a Cytodex bead (asterisk). A varicose neuronal process (arrows) contacts two of the cells (arrowheads). (*e*) Scanning electron micrograph of the GT1-7 cells coupled to Cytodex beads (asterisks) taken 4 hr after superfusion. Note the aggregation of the beads and continuity of the cells.

Pulse Analysis and Statistics. The data were evaluated for the presence of LHRH pulses by two different methods. First, data were evaluated by setting the threshold value for pulse detection at 3 times the coefficient of variation of the assay for the concentration of that particular sample (see ref. 25). Patterns of pulsatile LHRH secretion were also evaluated by using the algorithm DETECT (26) as described (27). When the results from the two different pulse-detection systems were compared, similar results were obtained. Therefore, in this report the results are depicted from the DETECT algorithm.

RESULTS

Morphological Organization and Connections Among the GT1-7 Cells. LHRH and GAP are the major metabolic products of the LHRH precursor protein (18, 24). All of the GT1-7 cells showed immunostaining for GAP (Fig. 1*a*) and LHRH (data not shown; see refs. 17 and 19). These cells had mature processes (Fig. 1*a*), and some of them established synapse-like connections with adjacent neurons (Fig. 1*b Inset*). The cytoplasm of the cells was rich in ribosomes and contained numerous secretory vesicles and granules (Fig. 1*b*).

Prior to perifusion, the cells were grown on Cytodex beads. Cells colonized the beads and extended processes to cells on the same or neighboring beads (Fig. 1 *c* and *d*). During perifusion, these cellular interactions and connections became even more evident as the cells acquired a syncytium-like appearance (Fig. 1*e*). Indeed, they often seemed to form continuous sheets where both perikarya-to-perikarya and perikarya-to-process types of connections were observed.

Besides forming synapse-like contacts, at least 15% of the GT1-7 cells tested were also dye-coupled, suggesting the presence of gap junctions between cells. When individual cells were injected with Lucifer yellow, the dye was found to spread instantaneously throughout the extent of the cell (Fig. 2 *a* and *aa*). In some cases after injection of a single cell, the dye was found to spread to neighboring cells. Interestingly, dye coupling was seen most frequently between cells that were interconnected by fine processes (Fig. 2 *b* and *bb*). As a control, the dye did not diffuse through the cell plasma membrane, and extracellular injection of dye was not taken up by adjacent cells. Besides observing dye-coupling, junctional connections were often seen between cells (Fig. 2*c*; see also refs. 17 and 19).

Perifusion Studies. At the onset of perifusion, the molar ratio of GAP to LHRH (the two major pro-LHRH cleavage products) was >16.0 (Fig. 3). This result is consistent with findings in static cultures (18). When the molar ratio was calculated at hourly intervals, it was found to decline during the first 2 hr of perifusion, after which the ratio was maintained at approximately 2.0 for the duration of the experiment (Fig. 3).

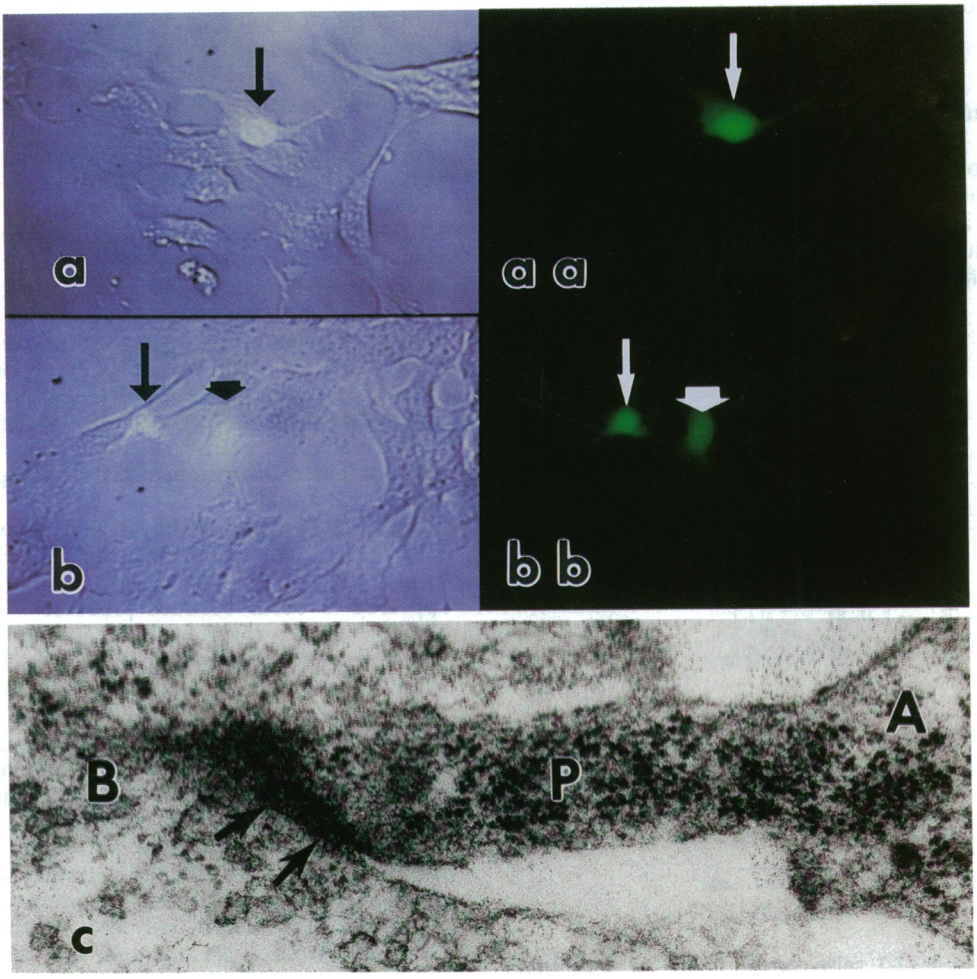

FIG. 2. Presence of dye-coupling between GT1-7 cells. Phase contrast (*a* and *b*) and fluorescent micrographs (*aa* and *bb*) of GT1-7 cells injected with Lucifer yellow. (*a* and *aa*) In the phase-contrast picture (*a*), one cell (arrow) was injected with Lucifer yellow, and only this cell (arrow) was labeled with the dye (*aa*). (*b* and *bb*) In another case, a single cell was injected with Lucifer yellow (arrow in *b* and *bb*), and the dye can be seen to spread to a neighboring cell (arrowhead in *b* and *bb*). (*c*) Transmission electron micrograph shows that a process from cell A establishes contact (arrows) with the perikaryon of cell B.

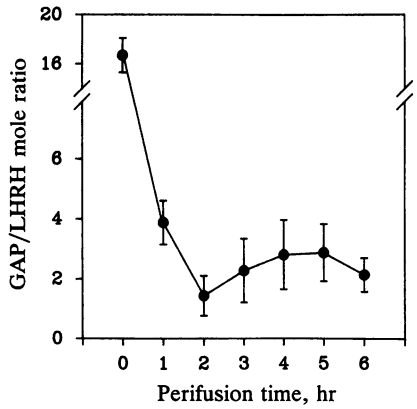

FIG. 3. Molar ratio of GAP to LHRH secreted into media by the GT1-7 cells during perifusion. The concentrations of GAP and LHRH in media were quantitated, and their molar ratios were determined at hourly intervals during a 6-hr perifusion. Secretion from eight different syringes of cells were compared.

When a rapid perifusion paradigm (5-min samples) was utilized, LHRH was secreted in a pulsatile manner (Fig. 4 *Upper* and *Middle*). Detailed characterization of this secretory activity can be found in Table 1. Omission of calcium from the buffer (Fig. 4 *Lower*) depressed LHRH secretion and reduced the number of pulses relative to that observed in controls (data not shown).

DISCUSSION

In the present study we show that immortalized hypothalamic neurons secrete LHRH into the medium in a pulsatile fashion. The synchrony of these cells needed to elicit a secretory event implies that there must be a high degree of coordination among the individual GT1-7 neurons. This synchronization may be the result of the numerous connections that are present among the GT1-7 cells. In this regard, we found that

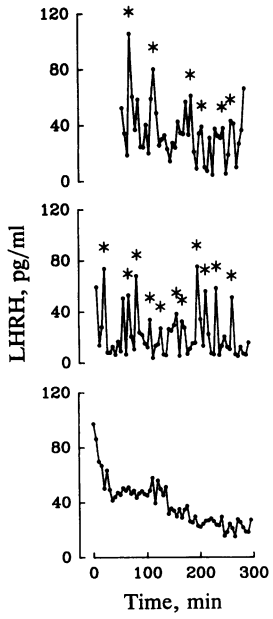

FIG. 4. Perifused GT1-7 cells secrete LHRH in a pulsatile fashion. (*Upper* and *Middle*) Profiles from two chambers of basal LHRH secretion over a 5-hr perifusion. Samples were collected at 5-min intervals. Asterisks signify a pulse of LHRH secretion as determined by the algorithm DETECT (25). (*Lower*) LHRH secretory profile from a single chamber of cells perifused with buffer lacking calcium.

Table 1. Pulsatility parameters for perifused GT1-7 cells

Parameter	Mean ± SEM*	
Frequency, pulses per hr	1.28 ± 0.18	(10)
Duration, min	20.00 ± 1.96	(48)
Interval, min	36.03 ± 3.00	(39)
Peak values, pg/frac.	69.48 ± 10.01	(48)
Trough values, pg/frac.	21.42 ± 2.94	(48)
Amplitude, pg/frac.	48.06 ± 7.64	(48)
Area, pg/frac. × time	1130.46 ± 308.26	(48)

pg/frac., pg per fraction.
*Numbers within parentheses indicate either the number of chambers perifused (frequency) or the number of interpulse intervals or pulses evaluated (the rest of the parameters).

the GT1-7 cells are dye-coupled and that they establish synapse-like connections among themselves. These connections may provide the cellular foundation necessary to support the pulsatile release of LHRH from these neurons.

Pulsatile secretion of LHRH is evident, at least, over the 5 hr of perifusion. It is intriguing that the interval between pulses of LHRH from these cells is similar to the interpulse interval reported for LH *in vivo* (28, 29). These data indicate that the generator for LHRH pulses may reside within the LHRH neuronal network and that inputs from other neurons are not required to maintain pulsatile activity. This characteristic would be expected if these cells were true oscillators. The neuronal circuitry superimposed from multiple neurotransmitter and neuromodulator systems known to innervate LHRH neurons may form additional neural networks that can modify basal LHRH episodic secretory activity.

Pulsatile secretion implies a coordinated release. When calcium is removed from the medium, pulsatile LHRH secretion was practically abolished. These results indicate that our pulsatility data are not spurious and that this mode of secretion is regulated. For synchronous release to occur and for a LHRH secretory episode to be detected, clusters of cells would have to undergo secretion at the same time. In this regard, the GT1 cells establish numerous connections among themselves in static cultures (17, 19). Contacts are made between perikarya, cell bodies and processes, and neurites. When the GT1-7 cells are grown on Cytodex beads, similar types of connections are seen. Frequently, connections are not only established between cells on the same bead but also between adjacent beads. The interactions within this neural network could be maintained by both gap junctions and synaptic contacts.

The GT1-7 cells were found to be dye-coupled. This finding suggests that some of the GT1-7 cells may be interconnected by gap junctions. Interestingly, most of the dye-coupling appeared to occur between processes. The proclivity for junctions to be present in this region of the LHRH neuron may have important implications *in vivo*. In rodents, LHRH neurons are scattered along the base of the forebrain while their axons converge on the median eminence (30). This region of the hypothalamus is very densely innervated with LHRH nerve terminals, and it contains the highest concentrations of LHRH in the entire LHRH neuronal system (31). Such an arrangement could lend itself most easily to stimulus-secretion coupling at this terminal region. Since most of the described dye-coupled neurons in brain and retina are also electrically coupled (32), it is tempting to suggest that the dye-coupled GT1-7 cells may also be electrically coupled. There may be some precedent for this coupling *in vivo*. The incidence of gap junction formation in the arcuate nucleus is enhanced by estradiol treatment (33). Since this region of the hypothalamus is involved in the regulation of gonadotropin release, increased gap junction formation could be associated with enhanced electrotonic coupling. In this regard, pulsatile LH secretion is preceded by bursts of electrical activity from

the medial basal hypothalamus (34). These findings suggest that gap junctions could play an important role in the synchronous release of LHRH from the hypothalamus.

Besides forming possible gap junctions, synapse-like contacts are also established among GT1-7 cells. In this regard, the GT1-7 cells have been shown to express the mRNA for several synaptosomal proteins (17). While synaptic connections could serve to coordinate secretion from clusters of GT1-7 cells, these contacts could also perform an additional role. For instance, LHRH has been shown to depress its own secretion both *in vivo* and *in vitro* (35, 36). Since the GT1-7 cells express the mRNA for LHRH and not for a number of other neurotransmitters (17), the synapse-like connections observed among the GT1-7 cells may represent areas of the neuron where secretory vesicles congregate in close proximity to receptors. Thus, the putative synapses on the GT1-7 cells could affect pulsatile LHRH secretion during periods of basal activity through negative-feedback effects. In addition, this feedback system could also play an important role *in vivo* by coordinating volleys of LHRH secretion as during the preovulatory surge.

Feedback between cells may also affect processing of the LHRH prohormone. When the GT1-7 cells are grown on tissue culture plates for 24 hr, the molar ratio of GAP to LHRH was often variable and it ranged from 16.0 to >27.0 (18). By comparison within the first 2 hr of perifusion, the molar ratio declined to a value of <2.0 (which is closer to the expected value of 1.0; see ref. 23), and it remained at this level for the duration of the experiment. Since our GAP antiserum recognizes both pro-LHRH and GAP (18, 20, 24), whereas the LHRH antiserum binds primarily LHRH (18, 22, 24), these data indicate that pro-LHRH processing may be more complete under perifusion than under static culture conditions. Indeed, perifusion serves to remove products (e.g., LHRH and GAP) from the cell that could feedback to inhibit some of the processing enzymes. For this reason, the perifusion system may provide a better physiological model to study LHRH secretion and processing than the conditions imposed under the static culture conditions.

The physiological regulation of pulsatile LH and LHRH secretion has been extensively studied for many years. During this time the pulse generator was found to reside within the hypothalamus, but its cellular origin was unknown. Using the GT1-7 cells, we have shown that this oscillator may reside, at least in part, within the LHRH neuronal network. In addition, detailed light and electron microscopic studies have revealed several cellular mechanisms which may subserve this generator. The use of these immortalized LHRH cells should help to provide additional insights into the cellular and molecular mechanisms that underlie the physiology of pulsatile gonadotropin secretion.

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