

## Release of Luteinizing Hormone-Releasing Hormone from Enzymatically Dispersed Rat Hypothalamic Explants Is Pulsatile<sup>1</sup>

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### ABSTRACT

This study was conducted to investigate 1) the utility of a cell perfusion system to examine questions dealing with the regulation of pulsatile LHRH release and 2) the necessity of cell-cell connections for communication between LHRH neurons and for coordination of LHRH release. To this end, cell perfusion of both hemihypothalamic tissue and enzymatically dispersed hypothalamic tissue isolated from adult male rats was performed. Periodic perfusate samples were collected and assayed to measure LHRH release. LHRH release from both hemihypothalamic and dispersed hypothalamic tissue was clearly pulsatile, with comparable pulse frequencies and amplitudes. These results were interpreted to support the hypothesis that coordination of pulsatile LHRH release can be maintained in the absence of most cell-cell connections. This suggests a paracrine rather than a neural mechanism for the coordination of LHRH secretory events leading to the distinct signals we observe as pulses of LHRH *in situ*.

### INTRODUCTION

LHRH is a decapeptide that controls the release of LH and FSH in mammals, ultimately controlling reproductive function [1, 2]. The cell bodies of LHRH neurons are anatomically dispersed throughout much of the hypothalamus, and LHRH neurons comprise only 800–1600 of the millions of neurons found in the rat hypothalamus [3, 4]. The dispersed anatomical distribution of LHRH cell bodies that lack a clearly defined neuronal network connecting them synaptically suggests that regulation of the timing of LHRH neurosecretory events (pulses) might occur at the neuroterminals of the LHRH neurons concentrated in the stalk-median eminence (S-ME) [5, 6]. The release of LHRH at the S-ME is episodic or pulsatile in all mammalian species studied, including rat [7, 8], sheep [9], and monkey [10]. In order for episodic release of LHRH to occur, LHRH neurons must coordinate and synchronize the release events.

A number of model systems have been used to dissect the complex neuroendocrine interactions in the hypothalamus that are responsible for coordinated hypothalamic LHRH release. *In vivo* models used to measure episodic LHRH release have included frequent sampling of pituitary portal blood [11–14], push-pull perfusion [15–17], and microdialysis [18] of the S-ME and anterior pituitary. These models yield the most physiologically relevant results, but include a complex neuronal network that is difficult to dissect pharmacologically. A pioneering effort that developed an *in vitro* model used a tissue perfusion system to measure episodic LHRH release from hypothalamic explants

[19]. Others have subsequently used hypothalamic tissue perfusion [20] and culture of disrupted hypothalamic tissue [21] to evaluate facets of the mechanism for coordinated LHRH release. The present study used both intact hemihypothalamus explants and enzymatically dispersed hypothalamic neurons in a perfusion culture system to determine whether coordinated hypothalamic LHRH release is dependent on cell-cell connections *in vitro*.

### MATERIALS AND METHODS

#### Animals

Seven adult male Sprague-Dawley rats from Harlan Sprague-Dawley (Madison, WI) weighing 250–320 g were used in these experiments. The rats were housed for 1–4 wk in individual cages under controlled temperature and lighting conditions and were given free access to food and water. The rats were killed by decapitation at 0730–0830 h, and the hypothalami were removed. These experiments were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals.

#### Cell and Tissue Preparation

The mediobasal hypothalamus—delimited laterally by the hypothalamic fissures, anteriorly by a cut 2 mm anterior to the anterior aspect of the optic chiasm, and posteriorly by the rostral portion of the mamillary bodies—was surgically removed by a horizontal cut of approximately 2 mm in depth and divided in half longitudinally down the midline. Each hemihypothalamus was placed in medium, a modified Krebs-Ringer bicarbonate buffer containing 2.2 mM CaCl<sub>2</sub>, 154 mM NaCl, 5.6 mM KCl, 1.0 mM MgCl, 6.0 mM NaHCO<sub>3</sub>, 10 mM glucose, 2 mM HEPES, 0.1% BSA, and 0.006% bacitracin, pH = 7.4. One hemihypothalamus was placed in medium while the corresponding hemihypothalamus was enzymatically dispersed. Each dispersed half was diced, using scalpels, into pieces of approximately 1 mm<sup>3</sup>. These pieces were suspended in medium containing 0.05% trypsin and were incubated at 37°C for 15 min. The suspension was triturated ten times with a sterile 5-ml pipette every 5 min. Each cell preparation sat for 1 min to allow large pieces to precipitate, and the cell suspension was poured into another sterile 15-ml tube and centrifuged for 5 min at 800 rpm to pellet the dispersed hypothalamic cells. The cells and intact tissue were loaded into their respective chambers in the perfusion system, and 5- $\mu$ m membrane filters were added to the afferent and efferent ports of the chambers. Exclusion of methylene blue dye by dispersed cells was determined as an index of viability. Approximately 95–99% of the cells were single or paired cells at the time of dispersion. Four to 8 h later, > 95% of the cells remained dispersed on the basis of microscopic examination.

Accepted April 23, 1998.

Received February 24, 1998.

<sup>1</sup>This work was supported by a University of Wisconsin-Whitewater (UW-W) Faculty Research Grant and a UW-W Undergraduate Research Award.

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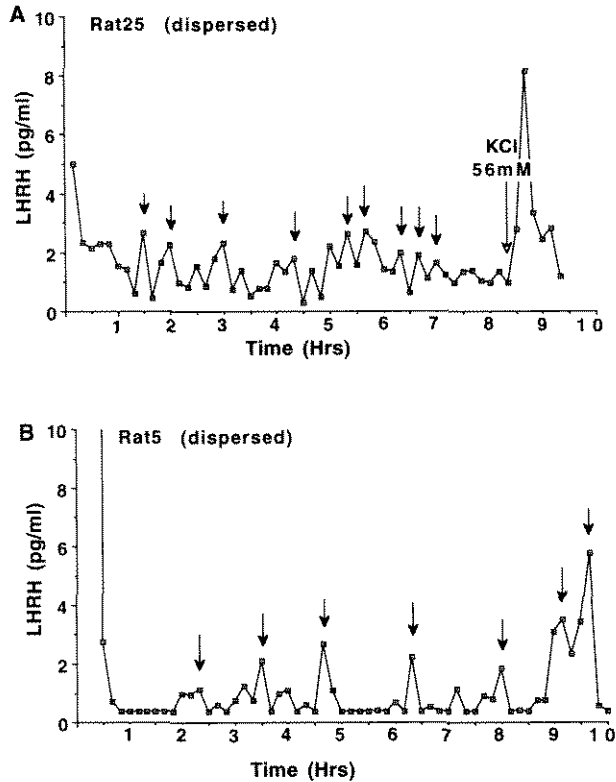


FIG. 1. LHRH release from enzymatically dispersed hypothalamic neurons isolated from two rats (A and B). Release events determined to be significant pulses by the PULSAR algorithm are indicated by arrows. Each data point represents the average LHRH concentration for a 10-min fraction. A 56-mM KCl challenge is indicated for 10 min during the final hour for rat #25 (A).

#### Perfusion Procedures

An Endotronics Accusyst S cell perfusion system (Cellex Biosciences Inc., Minneapolis, MN) was used during these experiments. The previously described medium was pumped into chambers containing either hemihypothalamus (intact tissue) or dispersed cells at a flow rate of 100  $\mu$ l/min. Carbon dioxide flowed into the chamber area to maintain the pH of the medium, having access through gas-permeable afferent lines into the chamber. The system was run for  $\sim$ 10 h/experiment, and 1-ml fractions were collected at 10-min intervals using a refrigerated Gilson (Middleton, WI) FC205 fraction collector. Some of the tissues were challenged with 56 mM KCl 1 h before the completion of the experiment. Samples were frozen at  $-70^{\circ}\text{C}$  until RIA.

#### LHRH RIA

The LHRH RIA used has been described previously [15, 22]. LHRH concentration in perfusates was estimated using anti-LHRH provided by Dr. T. Nett (Colorado State University, Fort Collins, CO) (R1245). Synthetic LHRH used as trace and standard was purchased from Richelieu Laboratories, Inc. (Montreal, PQ, Canada). Sensitivity of the assay was 0.1 pg/tube at 95% binding. The intraassay and interassay coefficient of variation were 6.8% and 9.4%, respectively.

#### Pulse Detection

Pulsatile release of LHRH by this preparation was determined by the PULSAR computer algorithm [23]. The

TABLE 1. Characteristics of LHRH release from enzymatically dispersed vs. intact hypothalami in vitro ( $n = 7$ ).

Parameter	Dispersed*	Intact*
Pulse amplitude (pg/ml/pulse)	$2.25 \pm 0.44$	$3.99 \pm 0.71$
Pulse frequency (pulses/hr)	$0.87 \pm 0.15$	$0.99 \pm 0.06$
Interpulse interval (in min)	$69.0 \pm 11.9$	$60.6 \pm 3.7$

\* Means  $\pm$  SE.

parameters used for LHRH pulse detection were similar to those previously reported for LHRH release in vivo [15, 22]. The cut-off criteria for G1, G2, G3, G4, and G5 were, respectively, 3.8, 2.6, 1.9, 1.5, and 1.2 standard deviations. The intraassay coefficient of variation for LHRH was described by the formula  $y = 3.38X + 3.14/100$ .

#### RESULTS

The LHRH secretion patterns of enzymatically dispersed LHRH neurons isolated from rat hypothalami in a perfusion culture are shown in Figure 1. LHRH release from this cellular preparation was pulsatile, as determined by analysis of the assay results using the PULSAR algorithm. The LHRH pulse frequency for enzymatically dispersed cells was  $0.87 \pm 0.15$  pulses/h (interpulse interval = 69 min,  $n = 7$ ; Table 1). The LHRH secretion patterns of the two intact hemihypothalami corresponding to the cellular preparations in Figure 1 are shown in Figure 2. The intact hypothalami represent control tissue for the enzymatic dispersion. Analysis of the pulse frequency results using a paired *t*-test revealed no significant difference between the

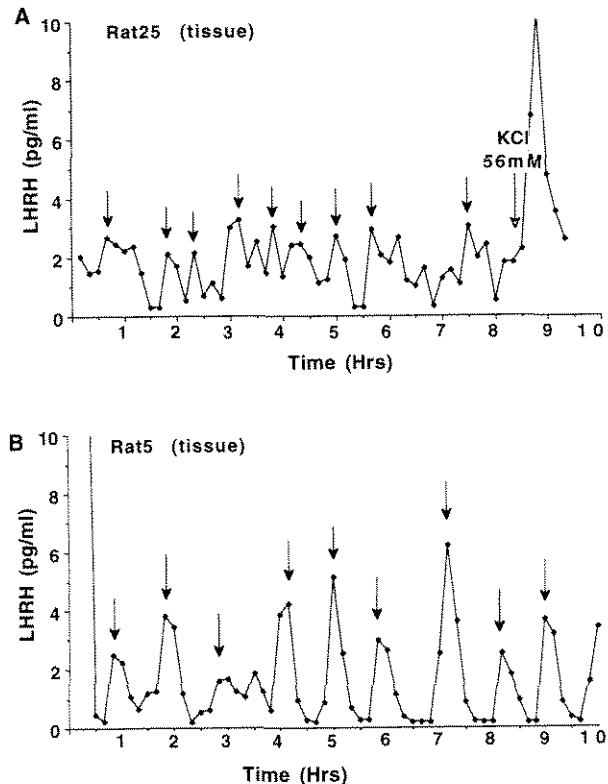


FIG. 2. LHRH release from undispersed hemihypothalami that correspond to the hypothalamic tissue dispersed in the preparations for Figure 1.

dispersed cells and the intact hemihypothalami (Table 1). The pulse amplitude of the intact hemihypothalami was higher, but not significantly different from the pulse amplitude of the dispersed cells (Table 1;  $p < 0.088$ ).

Of interest is the fact that time = 0 on these figures represents a temporal delay of less than 80 min from the time the rats were killed and less than 20 min from the removal of the dispersed cells from the centrifuge. The viability of the dispersed cell preparations was determined during the first 60 min of the perfusion using cells remaining in the centrifuge tube after the perfusion chamber was loaded. Cell viability was checked 8 h later using cells stored in medium during the experiment.

## DISCUSSION

The results of the present study provide compelling evidence in support of the hypothesis that coordinated LHRH release can occur in the absence of endogenous cytoarchitecture. Whether intact hemihypothalami or enzymatically dispersed hypothalamus tissue was perfused, LHRH release was episodic, indicating preservation of adequate cell-cell communication to coordinate LHRH release from synaptically estranged neurons. A classic anatomical manipulation to evaluate the reproductive importance of neural input to the hypothalamus was conducted over 20 years ago by Blake and Sawyer [24]. Rat medial basal hypothalamus was surgically isolated from anterior nerve projections in female rats, and estrous cycles were monitored. Frequent blood sampling and assay for LH demonstrated that pulsatile LH release was preserved. More traditional anatomical approaches have yielded significant advances in our understanding of how LHRH neurons may interact *in vivo*. Most germane to the present study is the work evaluating potential synaptic connections between individual LHRH neurons. Synapses have been reported between LHRH neurons in the rat [25, 26] and monkey [27]. The physiological importance of these observations may be subtle, since careful microscopic analysis of apposed hypothalamic cells immunopositive for LHRH indicates that only ~5% of LHRH neurons in female rats and ~8% of LHRH neurons in the female monkey are connected [5]. This is not to say that either direct synaptic connections between LHRH neurons or indirect connections through an interneuronal network are not potentially important to *in situ* cell-cell communication between these widely dispersed neurons. This finding [5] is consistent with the results of the present study, in which LHRH neurons communicated to elicit coordinated LHRH release in the apparent absence of endogenous extracellular organization.

Other physiological approaches to analysis of the mechanism of LHRH pulse generation included a number of sophisticated *in vitro* models. Mechanistic studies, by virtue of the pulsatile nature of LHRH release, require periodic sampling to determine baseline and induced effects on the parameters of LHRH release. This inspired the development of an elegant *in vitro* model system by Gallardo and Ramirez 20 years ago using perfusion of hypothalamic explants and repeated sampling [19]. This work laid the foundation for the physiologically meaningful *in vitro* study of LHRH release.

A recent innovative application of the *in vitro* perfusion technique used an immortalized LHRH neuronal cell line developed by Mellon and colleagues [28] in a perfusion culture system. Use of the GT1 cell line has resulted in studies of second messenger systems associated with LHRH release [29, 30] and of cell-cell communication be-

tween immortalized LHRH cells [31, 32]. This model has the obvious experimental advantage over *in situ* model systems in consisting of a relatively pure culture of LHRH neurons. However, the GT1 cell line does have limitations as a physiological model for the study of LHRH release. These limitations, many of which are characteristic of the use of immortal cell lines, are discussed eloquently by Selmanoff in a recent commentary [33]. Of the seven points Selmanoff [33] makes, three are particularly appropriate to this discussion. First, use of a homogenous cell line (GT1 cells) isolates the LHRH neurons from normal *in situ* interactions with other afferent neurons and supporting glia [33]. Second, when normal LHRH cells are stereotaxically placed in the hypothalamus of hypogonadal mice, they innervate the median eminence [34]. When GT1 cells are implanted, the axons rarely project to the median eminence [35]. Third, the GT1 cell preparation generally uses several hundred thousand to millions of GT1 cells in a culture, while rodents typically have only 800–1600 LHRH neurons in the entire hypothalamus [4, 5]. Thus, we need to be extremely conservative when assigning physiological relevance to work conducted using an immortalized cell line.

The primary hypothalamic cell culture perfusion system used in the present study provides a simple but elegant window through which researchers can evaluate the role of complex synaptic networks in the regulation of LHRH release. By comparing LHRH release from intact hemihypothalami with the release from the corresponding hemihypothalami that were enzymatically dispersed into largely single cells, we have a model that can be used to evaluate the importance of interneuronal connections on various modulators and regulators of LHRH release. The results of the present study provide compelling evidence that the coordinated release of LHRH in primary cultured hypothalamic cells from adult rats is pulsatile in the absence of most, if not all, of the endogenous synaptic organization. This observation agrees with the earlier observation by Krsmanovic and colleagues, who reported that enzymatically dispersed fetal hypothalamic cells released LHRH episodically [29]. Previous work reported that GT1 cells in culture grown on either coverslips [36] or beads [37] form interconnections within 36 h. GT1 cells cultured on beads required two days of culture and fetal hypothalamic neurons required three days of culture before pulsatile secretion of LHRH was detectable [29]. This is in stark contrast to the present study, in which episodic LHRH release from enzymatically dispersed hypothalamic neurons from adult rats was detectable within 1 h of culture.

Another study using GT1 cells on coverslips demonstrated that while cell-cell contacts occurred in that system on each coverslip, superfusion using two physically separated coverslips mounted in a Sykes-Moore chamber resulted in synchronized release from the entire preparation [31]. This suggests that a diffusible mediator coordinated the release of the separate neuronal populations from each coverslip. The present study extends this observation, suggesting that the LHRH neurons are capable of coordinating release primarily through a diffusible mediator. This, of course, represents only the minimum level of input necessary for coordinated LHRH release.

Synaptic communication between LHRH neurons, either directly through LHRH-to-LHRH connections or indirectly through an interneuronal network, most likely plays an important role in coordinated LHRH release *in situ*. Regardless, discovery that these cells can apparently communicate to coordinate episodic LHRH release through a diffusible

mediator is an important step in determining the underlying mechanism for the generation of LHRH pulses in vivo. The present results demonstrate that cell perfusion can provide a unique window through which the site of action of various neuromodulators of LHRH release can be observed.

## ACKNOWLEDGMENTS

The authors are indebted to Mr. Fritz Wegner and Dr. David Abbott of the Wisconsin Regional Primate Research Center for use of the RIA facilities and for their generous sharing of expertise and materials. We also wish to thank Ms. Terre Golembiewski for her work maintaining the animal quarters.

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