Synchronization of Ca\(^{2+}\) Oscillations Among Primate LHRH Neurons and Nonneuronal Cells In Vitro

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Richter, T. A., K. L. Keen, and E. Terasawa. Synchronization of Ca\(^{2+}\) oscillations among primate LHRH neurons and nonneuronal cells in vitro. J Neurophysiol 88: 1559–1567, 2002; 10.1152/jn.00805.2001. Periodic release of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus is essential for normal reproductive function. Pulsatile LHRH release appears to result from the synchronous activity of LHRH neurons. However, how the activity of these neurons is synchronized to release LHRH peptide in a pulsatile manner is unclear. Because there is little evidence of physical coupling among LHRH neurons in the hypothalamus, we hypothesized that the activity of LHRH neurons might be coordinated by indirect intercellular communication via intermediary (nonneuronal) cells rather than direct interneuronal coupling. In this study, we used an in vitro preparation of LHRH neurons derived from the olfactory placode of monkey embryos to assess whether nonneuronal cells, play a role in coordinating LHRH neuronal activity. We found that cultured LHRH neurons and nonneuronal cells both exhibit spontaneous oscillations in the concentration of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) at similar frequencies. Moreover, [Ca\(^{2+}\)]\(_i\) oscillations in both types of cell were periodically synchronized. Synchronized [Ca\(^{2+}\)]\(_i\) oscillations spread as intercellular Ca\(^{2+}\) waves across fields of cells that included LHRH neurons and nonneuronal cells, although waves spread at a higher velocity among LHRH neurons. These results suggest that LHRH neurons and nonneuronal cells are functionally integrated and that nonneuronal cells could be involved in synchronizing the activity of the LHRH neurosecretory network.

INTRODUCTION

Luteinizing hormone-releasing hormone (LHRH) peptide is released from a relatively small number of neurons (600–2000 in mammals, depending on the species) (Silverman et al. 1994) in the hypothalamus in episodic bursts (Clarke and Cummins 1982; Knobil 1981), about once per hour in primates (Terasawa 1995). How the activity of cultured LHRH neurons is coordinated to result in discrete LHRH pulses has remained unknown despite decades of research into this phenomenon (reviewed in Levine et al. 1991; Terasawa 2001). The most commonly accepted hypothesis holds that LHRH neurons are physically coupled to other LHRH neurons to form a cohesive network within which the activity of individual LHRH neurons is coordinated. However, unlike oxytocin and vasopressin neurons, LHRH neurons are not organized in discrete nuclei within the hypothalamus but rather are diffusely distributed throughout the rostral-caudal extent of the hypothalamus (Silverman et al. 1994). Moreover, ultrastructural analyses have consistently failed to document the existence of direct LHRH neuron–LHRH neuron coupling as a likely means of integration (Witkin 1999; Witkin et al. 1995).

Several lines of evidence suggest an alternative hypothesis to direct interneuronal coupling, namely that LHRH neuronal activity is coordinated by indirect signaling via nonneuronal cells. For instance, LHRH neurons in vivo are relatively isolated from other LHRH neurons but are intimately associated with glial cells (Durrant and Plant 1999; Witkin et al. 1997). In addition, cells in the vicinity of LHRH neurons in the hypothalamus, but not LHRH neurons per se (Silverman et al. 1982), exhibit intermittent, synchronous increases in electrical activity that are coincident with LH pulses (Knobil 1989; Sano and Kimura 2000). Thus as is the case in several other CNS systems (Cotrina et al. 2000; Nedergaard 1994), it is possible that nonneuronal cells that are associated with LHRH neurons participate in coordinating interneuronal signaling.

To date, substantial insight into the functioning of LHRH neurons has been gained from observations made in a line of immortalized cells, called GT1, which have the distinctive LHRH neuronal phenotype (Liposits et al. 1991; Mellon et al. 1990; Wetsel 1995) and synthesize and secrete LHRH in a pulsatile manner (Costantin and Charles 1999, 2001; Funabashi et al. 2001; Krismanovic et al. 1992; Wetsel et al. 1992). LHRH release from GT1 cells is Ca\(^{2+}\) dependent (Costantin and Charles 1999, 2001; Funabashi et al. 2001; Krismanovic et al. 1992; Wetsel et al. 1992), and cultures of some subcloned GT1 cell lines (e.g., GT1–1) exhibit waves of increased concentrations of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and synchronous firing of action potentials (Charles et al. 1996; Funabashi et al. 2001; Nunemaker et al. 2001), which could serve as a means of coordinating the activity of networks of these cells. However, it is not possible to evaluate in GT1 cells the idea that the activity of LHRH neurons is coordinated by signaling via nonneuronal cells, rather than by direct interneuronal coupling, because GT1 neuronal cell cultures are comprised almost exclusively of LHRH neurons (Mellon et al. 1992).

The recent development of an in vitro preparation of primary LHRH neurons derived from the monkey embryo has enabled us to assess the hypothesis that the activity of nonneuronal cells that...
are associated with LHRH neurons participate in intercellular signaling among LHRH neurons. In our culture system, LHRH is released in a pulsatile manner at a frequency similar to in vivo LHRH pulses (Terasawa et al. 1993, 1999a) and, as in GT1 cells, neurosecretion of LHRH is Ca\textsuperscript{2+} dependent (Terasawa et al. 1999a). In the present study, we used dynamic video imaging to measure spontaneous changes in [Ca\textsuperscript{2+}]\textsubscript{i} in individual LHRH neurons and nonneuronal cells cultured together.

**METHODS**

**Tissue culture**

We obtained rhesus monkey (Macaca mulatta) embryos by Caesarian section at 35–37 days of gestation (E35–37). All experimental procedures were carried out under the standards established by the Animal Welfare Act in a protocol that was approved by the Animal Care and Use Committee of the University of Wisconsin.

The methods used to establish and maintain cultures of embryonic tissue have been described in detail elsewhere (Terasawa et al. 1993, 1999a,b). Briefly, the olfactory placodes and ventral LHRH neuron migratory pathway (terminal nerve region) were dissected out, divided into small (<1 mm\textsuperscript{3}) pieces and plated on glass coverslips (n = 20–25 individual culture coverslips per embryo). Cultures were grown in growth medium (Medium 199, Life Technologies, Rockville, MD) supplemented with 10% FBS (Hyclone), 0.6% glucose, and 75 μg/ml gentamycin (Roche, Indianapolis, IN) that was replaced every 1–4 days and incubated at 37°C (1.5% CO\textsubscript{2}, 98.5% O\textsubscript{2}) for ≥2 wk until used for imaging studies (3–5 wk).

For this study, we used only those cultures that contained tissue from the terminal nerve region because this tissue contains a relatively large number of LHRH neurons that are migrating from the olfactory pit to the forebrain (Terasawa et al. 1993). In addition to maximizing the number of LHRH neurons obtained, excluding cultures derived from olfactory placode also enabled us to avoid including immature LHRH neurons in our analyses.

**Cell imaging**

The method used to measure [Ca\textsuperscript{2+}]\textsubscript{i} was the same as that described previously (Terasawa et al. 1999b). [Ca\textsuperscript{2+}]\textsubscript{i}, was measured by calculating the ratio of the fluorescence intensity (ΔF/ΔF\textsubscript{0}) of the Ca\textsuperscript{2+} indicator dye, fura-2 AM (Texas Fluorescence Labs, Austin, TX; cells loaded with 18 μM for 30 min at 37°C) in cells excited at 340 and 380 nm (133-ms delay). Light emitted at 510 nm was captured by a vidicon camera (Hamamatsu Photonics, Hamamatsu, Japan) at 10-s intervals. Cultures were perfused continuously with oxygenated medium (Medium 199, Sigma; 50 μl/min) at ~37°C for 100–200 min. A culture was viewed through a ×20 microscope objective, and a 750 × 750 μm recording field that contained the appearance of LHRH neurons (Terasawa et al. 1993) was selected for data capture. Measurements of fluorescence in individual cells were made by delimiting the borders of the cell body on a video image and measuring pixel intensity within the borders of the digitized fluorescence image. Data for individual cells were normalized to baseline (ΔF/ΔF\textsubscript{0} relative to the mean of the lowest 10 ΔF/ΔF\textsubscript{0} values recorded for a particular cell within an experiment. Fluorescence data were captured and analyzed using commercially available software (Metafluor, Universal Imaging, West Chester, PA).

The dynamics of changes in [Ca\textsuperscript{2+}]\textsubscript{i}, in fields of cells were visualized in movies created from fluorescence images using National Institutes of Health Image software (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Stacks of individual digitized fluorescence images captured at 10-s intervals were used to compose streaming movies.

Our cultures are composed of primarily LHRH neurons and numerous epithelial cells, fibroblasts, and other types of unidentified cells. Occasionally, some cultures also contain a small proportion of non-LHRH neurons. To identify LHRH neurons, we followed the procedure detailed in Terasawa et al. (1999b) with minor modifications. Briefly, once a recording experiment was complete, the imaged area was photographed and a coverslip grid reference was obtained to facilitate locating the cells after immunostaining. LHRH neurons were identified by using standard immunohistochemical techniques with a antisera cocktail GF-6 and LR-1 (gifts from Dr. N. M. Sherwood, University of British Victoria, Victoria, Canada; 1:9,000 dilution and Dr. R. A. Benoit, University of Montreal, Montreal, Canada; 1:15000, respectively) and 3,3’-diaminobenzadine as the chromagen as described in Methods.

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were acquired prior to immunostaining. Among nonneuronal cells, epithelial cells were distinguishable during Ca\(^{2+}\) imaging because they showed the appearance of uniform round cells. To confirm the Ca\(^{2+}\) imaging from epithelial cells, selected cultures were immunostained for epithelial cell marker protein (Sigma, St. Louis, MO). Neurons that were not LHRH immunopositive, fibroblasts, and cells that were not clearly distinguishable on the basis of their morphology as either neurons or nonneuronal were excluded from analyses. Nonetheless, we retain the term “nonneural cells” used here because 1) fibroblasts were generally distinguishable because of their shape and size, but they could disguise their appearance and 2) we did not confirm the cell type in all cultures.

**Data analysis**

To detect peaks in [Ca\(^{2+}\)], oscillations, we designed an algorithm that was based on commercially available pulse detection software. A peak in a [Ca\(^{2+}\)] oscillation was identified if an individual \(\Delta F/F_0\) value was preceded and followed by three or more lower values; preceded by a series of at least three sequentially increasing values and followed by a series of at least three sequentially decreasing values; and greater than the mean \(\Delta F/F_0 + 2\) SD for the whole recording period (Fig. 1). The algorithm was used to calculate the number of [Ca\(^{2+}\)] peaks for individual cells during a recording period (equivalent to the oscillation frequency, expressed as number peaks/minute), the amplitude of each [Ca\(^{2+}\)] peak, and the interpeak interval (IPI). To confirm that the algorithm detected true oscillation peaks and not noise (random, transient increases in [Ca\(^{2+}\)]), we generated a control model from 20 randomly selected cells for each culture. Data for each cell were divided into time bins equivalent to half the mean oscillation frequency of that culture and reconstituted as a novel, random series. This was repeated five times for each cell. These randomized data series were then analyzed with the pulse detection algorithm. On average, <1\% of the number of peaks that were detected in the untransformed data were detected in the randomized series.

**Fig. 2.** Individual luteinizing hormone-releasing hormone (LHRH) neurons and nonneuronal cells exhibit spontaneous [Ca\(^{2+}\)] oscillations. A: changes in [Ca\(^{2+}\)], in 3 representative LHRH neurons (a–c) and nonneuronal cells (d–f) with high (a and d–f), medium (b and e), or low (c and f)–frequency [Ca\(^{2+}\)] oscillations. The oscillation frequency for each cell, expressed as interpeak interval (IPI in min), is indicated in parentheses. \(\triangledown\), peaks in [Ca\(^{2+}\)]. \(\triangledown\), a double [Ca\(^{2+}\)] peak. All cells were from the same culture. B: mean \pm SE IPI of [Ca\(^{2+}\)] peaks in LHRH neurons ( ), nonneuronal cells ( ), and random series generated from the same data (see methods for details). Numbers in parentheses indicate the number of individual cells. C: autocorrelation of changes in [Ca\(^{2+}\)] in a representative LHRH neuron and nonneuronal cell from the same culture. Autocorrelation plots are commonly used for examining randomness in a data set. Randomness in data sets can be assessed by computing autocorrelations for data values at varying time lags. If random, such autocorrelations should be near 0 for any and all time-lag separations. The narrow peak at time lag 0 in both cases indicates that the time series is not random but rather has a high degree of autocorrelation between adjacent and near-adjacent observations, i.e., oscillations in [Ca\(^{2+}\)], in the individual cells are periodic.
series, confirming that the algorithm successfully detected genuine oscillation peaks rather than noise (Fig. 1).

To determine whether \([\text{Ca}^{2+}]_{\text{i}}\) peaks in individual cells were synchronized with \([\text{Ca}^{2+}]_{\text{i}}\) peaks in other cells in a culture, we calculated and compared the precise times at which \([\text{Ca}^{2+}]_{\text{i}}\) peaks occurred in all cells. The degree of synchronization was defined as the percentage of the population of cells in which an \([\text{Ca}^{2+}]_{\text{i}}\) peak occurred within a window of 30 s. To assess whether synchronization detected among cells was simply due to chance, data for each culture were compared with a model comprising an equivalent number of cells in which the same number of peaks was randomly distributed over a period of time equivalent to that in the real cells. These random models exhibited a maximum synchronization of \(\sim 20\%\) of cells (data not shown), in contrast to 100% synchronization that could be detected in the cell cultures. This confirmed that synchronization of \(>20\%\) of cells in our cultures was not a chance phenomenon and allowed us to define synchronization of oscillations in \([\text{Ca}^{2+}]_{\text{i}}\) among cells as either unsynchronized (\(<20\%\) of cells synchronized), moderately synchronous (21–80% of cells synchronized) or highly synchronous (\(>80\%\) of cells synchronized).

Changes in \([\text{Ca}^{2+}]_{\text{i}}\), during periods in which \([\text{Ca}^{2+}]_{\text{i}}\) peaks were highly synchronous (\(>80\%\) of cells) were analyzed to determine whether synchronization of \([\text{Ca}^{2+}]_{\text{i}}\) peaks arose in cells independent of the activity of other cells or whether synchronization among cells reflects some form of intercellular coupling, which would be manifested as a spatiotemporal \([\text{Ca}^{2+}]_{\text{i}}\) wave. For this analysis, 20 cells of each type were randomly selected from each culture. The distance between each pair of cells (20² combinations) and the time difference with respect to the \([\text{Ca}^{2+}]_{\text{i}}\) peaks in cell pairs were calculated. These data were used to calculate the strength of the correlation between distance between cells and \([\text{Ca}^{2+}]_{\text{i}}\) peak times using standard least-squares regression. The slope of the regression was used to derive the speed (\(\mu\text{m/s}\)) at which intercellular \([\text{Ca}^{2+}]_{\text{i}}\) waves were propagated.

**FIG. 3.** Changes in \([\text{Ca}^{2+}]_{\text{i}}\) are very similar in LHRH neurons and nonneuronal cells within the same culture. All data in this figure are from a single representative culture. A: mean \(\pm\) SE \([\text{Ca}^{2+}]_{\text{i}}\) in LHRH neurons (n = 19) and nonneuronal cells (n = 25) at 10-s intervals. B: representative cross-correlogram for a randomly selected LHRH neuron and nonneuronal cell from the same culture. The narrow peak at time lag 0 is indicative of correlation of oscillations in \([\text{Ca}^{2+}]_{\text{i}}\) between the 2 cells. C: mean \([\text{Ca}^{2+}]_{\text{i}}\) in LHRH neurons (n = 19) plotted against mean \([\text{Ca}^{2+}]_{\text{i}}\) in nonneuronal cells (n = 25) in a single representative culture at each of the 786 time points depicted in A. The slope of the linear regression is unity (Y = 1.0002X – 0.0003, \(r^2 = 0.85\), \(P < 0.001\)), indicating that changes in \([\text{Ca}^{2+}]_{\text{i}}\) are correlated between LHRH neurons and nonneuronal cells within the same culture. D: a random model generated from the data in C (see METHODS for details of how the model was generated). The absence of any correlation between \([\text{Ca}^{2+}]_{\text{i}}\) in LHRH neurons and nonneuronal cells indicates that the correlation observed in C is not due to chance.
Statistics

Standard least-squares regression analysis was used to assess correlations. Student’s t-test was used for between-group comparisons of \([\text{Ca}^{2+}]_i\) oscillation frequencies and \([\text{Ca}^{2+}]_i\) wave speeds between LHRH neurons and nonneuronal cells. ANOVA with Fisher’s protected least square difference (PLSD) post hoc test was used to analyze \([\text{Ca}^{2+}]_i\) peak amplitudes and IPIs. Data are presented as means ± SE. Statistical significance was established at \(P < 0.05\).

RESULTS

Intracellular \(\text{Ca}^{2+}\) oscillations in individual cells

Spontaneous oscillations in \([\text{Ca}^{2+}]_i\) were observed in individual LHRH neurons and nonneuronal cells in the same cultures. Examples of LHRH neurons and nonneuronal cells with a high, medium, and low \([\text{Ca}^{2+}]_i\) oscillation frequency are illustrated in Fig. 2A. The dynamics of \([\text{Ca}^{2+}]_i\) oscillations in individual LHRH neurons and nonneuronal cells were very similar, and each oscillation was usually associated with a single distinct \([\text{Ca}^{2+}]_i\) peak, although two or three peaks per oscillation were occasionally observed (Fig. 2A). The range of IPIs in individual cells was similar in LHRH neurons and nonneuronal cells (1.4–83.4 min), and the average IPI was 13.9 and 11.9 min for individual LHRH neurons (\(n = 241\)) and nonneuronal cells (\(n = 389\)), respectively, from seven cultures (Fig. 2B). Autocorrelograms of changes in \([\text{Ca}^{2+}]_i\) in individual cells had a narrow peak at lag time zero (Fig. 2C). Autocorrelograms are commonly used for examining randomness in data by computing autocorrelations for data values at varying time lags; if random, such autocorrelations should be near zero for any and all time-lag separations. Thus the peak in Fig. 2C indicates that the oscillatory dynamics of \([\text{Ca}^{2+}]_i\) within individual cells were periodic.

Temporal changes in mean \([\text{Ca}^{2+}]_i\) in cell populations were also very similar in LHRH neurons and nonneuronal cells within the same culture. In Fig. 3A, mean ± SE \([\text{Ca}^{2+}]_i\) in LHRH neurons and nonneuronal cells from a representative culture are shown. It can be seen that changes in \([\text{Ca}^{2+}]_i\) in both types of cell appeared to be strongly correlated but not identical. The temporal correlation of changes in \([\text{Ca}^{2+}]_i\) is further exemplified by cross-correlational analyses of \([\text{Ca}^{2+}]_i\) in individual LHRH neurons and nonneuronal cells from the same culture. As illustrated in Fig. 3B, cross-correlograms for a single randomly selected LHRH neuron and a nonneuronal cell from the same culture were characterized by a narrow peak at time lag zero, indicating a strong correlation of changes in \([\text{Ca}^{2+}]_i\) in both types of cell. The correlation of changes in \([\text{Ca}^{2+}]_i\) between LHRH neurons and nonneuronal cells is fur-
Synchronization of intracellular Ca$^{2+}$ oscillations

Despite individual cells exhibiting a range of different [Ca$^{2+}$], oscillation frequencies (Fig. 2A), the correlation of [Ca$^{2+}$] in individual LHRH neurons and nonneuronal cells in the same culture (Fig. 3) suggested that changes in [Ca$^{2+}$] might be synchronized among some cells in the population. Indeed, when we analyzed the time at which peaks in [Ca$^{2+}$], oscillations occurred in the population of cells within each culture, we found that [Ca$^{2+}$] peaks in individual cells were periodically synchronized across the population. Such synchronization could be readily identified as clusters of [Ca$^{2+}$] peaks when [Ca$^{2+}$] in each cell in a population was plotted on the same time axis (Fig. 4A). Synchronization was indicated by the occurrence of a single [Ca$^{2+}$], peak in several cells within a narrow time window (20 s), as illustrated in Fig. 4, B and C. Synchronization of [Ca$^{2+}$] peaks among different cells included both LHRH neurons and nonneuronal cells. Synchronization of oscillations in [Ca$^{2+}$], was observed over a wide range of proportions of the total cell population.

For highly synchronous oscillations in [Ca$^{2+}$], (>80% of cells synchronized), the amplitude of the synchronized [Ca$^{2+}$], peak was significantly greater than [Ca$^{2+}$], peaks that were synchronized among fewer cells ($P < 0.05$, ANOVA with Fisher’s PLSD post hoc test, $n = 496$ cells from 7 cultures; Table 1). In addition, the IPI immediately following a highly synchronous [Ca$^{2+}$], peak was increased (Fig. 5). Separate analysis of data for LHRH neurons and nonneuronal cells revealed that this increase was statistically significant only in LHRH neurons ($P < 0.05$, ANOVA with Fisher’s PLSD post hoc test, $n = 195$ cells from 7 cultures) and not in nonneuronal cells (Fig. 5). The increase in the interval to the next [Ca$^{2+}$], peak following a highly synchronous [Ca$^{2+}$], peak was not related to the peak amplitude (data not shown).

**Intercellular Ca$^{2+}$ waves**

Synchronization of oscillations in [Ca$^{2+}$], was associated with intercellular Ca$^{2+}$ waves that spread across fields of cells.

**TABLE 1. Comparison of the amplitude of synchronized [Ca$^{2+}$], peaks**

<table>
<thead>
<tr>
<th>Percentage of Cells With Synchronized [Ca$^{2+}$], Peaks, %</th>
<th>[Ca$^{2+}$], Peak Amplitude*</th>
</tr>
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<tbody>
<tr>
<td>&lt;20</td>
<td>$0.75 \pm 0.11$†</td>
</tr>
<tr>
<td>20–80</td>
<td>$0.73 \pm 0.12$‡</td>
</tr>
<tr>
<td>&gt;80</td>
<td>$0.90 \pm 0.08$§</td>
</tr>
</tbody>
</table>

* The [Ca$^{2+}$], peak amplitude (mean ± SE) is expressed relative to baseline [Ca$^{2+}$], (that is, ΔF/ΔF$_0$ = 0). † Values are significantly different ($P < 0.05$, ANOVA with Fisher’s PLSD post hoc test, $n = 628$ cells from 7 cultures). ‡ Note that data for luteinizing hormone-release hormone neurons and nonneuronal cells have been combined for this table, because statistical analysis of the separate data produced the same (statistically significant) result.

This is exemplified in movies constructed from digitized fluorescence images, an example of which can be seen at http://www.primate.wisc.edu/people/terasawa/trichter/Moviepage.html. An example of a typical intercellular Ca$^{2+}$ wave is illustrated in Fig. 6, where a Ca$^{2+}$ wave can be seen to spread across a population containing both LHRH neurons and nonneuronal cells. Ca$^{2+}$ waves were observed to originate within the recording field or spread into the recording field from outside. Because the recording field only include <0.5% of the whole culture, we were unable to determine the origin of the Ca$^{2+}$ waves. Nevertheless, we noted that waves originating within the recording field rarely started in the same source on successive occasions.

Interestingly, synchronization of oscillations in [Ca$^{2+}$], and intercellular Ca$^{2+}$ waves were only observed in cultures that contained LHRH neurons (7/7 cultures) and were absent from cultures that did not contain LHRH neurons (13/13 cultures). Moreover, the Ca$^{2+}$ waves of LHRH neurons with nonneuronal cells were observed only when cultures were relatively dense. The average speed at which Ca$^{2+}$ waves were propagated was 12.55 μm/s ($n = 280$ cells representing 35 waves from 7 cultures; data combined). Separate analysis of data for LHRH neurons and nonneuronal cells indicated that intercellular Ca$^{2+}$ waves spread more rapidly ($P < 0.01$, Student’s t-test, data from 7 cultures combined; Fig. 7) between LHRH.
DISCUSSION

Pulsatile neurosecretion of LHRH is crucial for normal reproductive function in many mammalian species, but how activity of LHRH neurons in the hypothalamus generates pulsatile neuropeptide release is not known. Because there is little evidence to suggest that LHRH neurons in vivo are physically coupled to other LHRH neurons, direct communication among LHRH neurons cannot account for pulsatile LHRH release that is thought to result from the synchronization of LHRH neuronal activity. However, the fact that LHRH neurons are intimately associated with numerous glial cells in the hypothalamus (Durrant and Plant 1999; Witkin 1999) suggests an alternative mechanism to direct interneural coupling for coordinating the activity of LHRH neurons. Specifically, if glia and LHRH neurons were coupled, glial cells could mediate indirect interneural signaling among LHRH neurons. We assessed this idea by examining how Ca$^{2+}$ signaling in cultures of primary LHRH neurons is related to Ca$^{2+}$ signaling in the nonneuronal cells with which they are associated. Spontaneous oscillations in [Ca$^{2+}$]$_i$ among cells observed in the present study is remarkably similar to the pattern of electrical activity that emerges in simulated fields of cells containing both LHRH neurons and nonneuronal cells.

neurons (17.6 μm/s) than between nonneuronal cells (11.1 μm/s).

FIG. 6. Synchronization of [Ca$^{2+}$], peaks is propagated as intercellular Ca$^{2+}$ waves across fields of cultured LHRH neurons and nonneuronal cells. A: a 340-nm image containing a large number of LHRH neurons, which form a chain and ganglion, seen in the center area of image field. Two LHRH neurons (cells 1 and 3) and nonneuronal cells (cells 2 and 4) have been highlighted to illustrate the progression of a Ca$^{2+}$ wave through LHRH neurons and nonneuronal cells in the same culture. B: time-lapse sequence (0–170 s) of digitized fluorescence images of [Ca$^{2+}$], (340/380 ratio image, see METHODS) captured from the field of cells in A. The time (t) at which an image was captured (relative to the 1st image at t = 0 s) is indicated in the top right corner in each image. Changes in fluorescence intensity (ΔF) are equivalent to changes in [Ca$^{2+}$], and are visualized as pseudo-color changes, according to the scale shown. The Ca$^{2+}$ wave in this case first appeared in the left upper portion of the imaged field (80 s), proceeded to spread diagonally toward the right bottom area, and diffused around the ganglion of LHRH neurons (see at 110 and 120 s). Note that LHRH neuron 1 and nonneuronal cell 2 were activated earlier than LHRH neuron 3 and nonneuronal cell 4. Although individual cells were difficult to identify in the 340/380 ratio images, they were clearly recognized in the 340-nm image. C: tracings of [Ca$^{2+}$] in LHRH neurons (●) and nonneuronal cells (○). Note that the Ca$^{2+}$ wave was initiated from outside of this viewing field, reached neuron 1 and nonneuronal cell 2, and spread to LHRH neuron 3 and nonneuronal cell 4. The sequential progression of the wave through the areas in which cells 1–4 are located is indicated by the shift in peak [Ca$^{2+}$].
networks of coupled cells in which sparse, random activation of individual cells occurs spontaneously to produce intercellular activity waves (Lewis and Rinzel 2000). Because a prerequisite for the development of intercellular waves in such theoretical model systems is some form of coupling among neighboring cells (Lewis and Rinzel 2000), our observation that Ca\textsuperscript{2+} waves were propagated through fields of cells that included LHRH neurons and nonneuronal cells indicates that LHRH neurons are functionally coupled to nonneuronal cells. This suggests that LHRH neurons and nonneuronal cells are functionally integrated and that nonneuronal cells exhibit behavior that is functionally relevant to LHRH neuronal activity. Such integrated signaling among neurons and nonneuronal cells has also been shown to exist in cultures of rat forebrain tissue, where astrocytes are able to transmit Ca\textsuperscript{2+} signals to neurons (Nedergaard 1994).

The mechanisms that underlie the synchronization of LHRH neuronal activity are yet to be fully elucidated, but diffusion of Ca\textsuperscript{2+} among cells is crucial for synchronization (Charles et al. 1996; Terasawa et al. 1999b). Changes in [Ca\textsuperscript{2+}], in individual cells during Ca\textsuperscript{2+} waves are likely to be propagated to neighboring cells by diffusion of Ca\textsuperscript{2+} and/or other ions and molecules, such as K\textsuperscript{+}, inositol 1,4,5-trisphosphate (InsP\textsubscript{3}), ATP, and cAMP (Cotrina et al. 2000; Guthrie et al. 1999; Vitalis et al. 2000). Intercellular diffusion usually occurs through gap junctions. Neurons and glia appear to be coupled by gap junctions in the rat brain (Alvarez-Maubecin et al. 2000) and cultures of cortical astrocytes and neurons (Froes et al. 1999), but it is not known whether LHRH neurons form gap junction associations with glial cells in vivo. Blocking gap junctions abolishes synchronous oscillations in [Ca\textsuperscript{2+}], and intercellular Ca\textsuperscript{2+} waves in cultures of GT1 cells (Charles et al. 1996).

Despite the importance of gap junctions to intercellular signaling, direct physical coupling of cells via gap junctions is not necessarily required for transmission of Ca\textsuperscript{2+} signals. Instead, diffusion of signaling molecules into the intercellular medium (“volume” transmission) (Agnati et al. 1995) could induce changes in other cells that do not rely on direct physical coupling via gap junctions. For instance, ATP released from glial cells into the extracellular medium can transmit Ca\textsuperscript{2+} waves to nearby cells without any physical contact (Cotrina et al. 2000; Guthrie et al. 1999; Newman 2001). Similarly, astrocytes can activate other cells from which they are physically separated by releasing glutamate (Parpura et al. 1994). Nevertheless, intercellular Ca\textsuperscript{2+} signaling is more efficient in the presence of functional gap junctions (Rouach et al. 2000), and it is likely that a combination of direct (gap junctions) and indirect (volume transmission) coupling regulates intercellular signaling that underlies Ca\textsuperscript{2+} waves. Finally, another potential means by which intercellular communication might occur is via chemical synapses. It has been reported that intercellular Ca\textsuperscript{2+} wave propagation in response to mechanical stimulation in cultured astrocytes is greatly enhanced in the presence of neurons (Rouach et al. 2000); in the present study, intercellular Ca\textsuperscript{2+} waves spread more rapidly among LHRH neurons than among nonneuronal cells, which might reflect synaptic coupling among the LHRH neurons.

How synchronization of [Ca\textsuperscript{2+}], oscillations is related to neurosecretion of LHRH is unclear at present. Because LHRH neurosecretion requires an increase in [Ca\textsuperscript{2+}], the increase in [Ca\textsuperscript{2+}] that occurs during a highly synchronous event could produce a suprathreshold stimulus for neurosecretion. This hypothetical mechanism is similar to that which is thought to underlie the rhythmic generation of Ca\textsuperscript{2+} waves in other tissues, such as in cardiac muscle (Izu et al. 2001). The finding in the present study that the amplitude of [Ca\textsuperscript{2+}], peaks of synchronized oscillations was significantly larger than nonsynchronized peaks supports the hypothesis that the increase in [Ca\textsuperscript{2+}], associated with highly synchronized [Ca\textsuperscript{2+}], oscillations provides a stimulus for neurosecretion. Specifically, the larger peak amplitude could reflect an increase in [Ca\textsuperscript{2+}], that is sufficient (i.e., a suprathreshold stimulus) to stimulate Ca\textsuperscript{2+}-dependent neurosecretion and would cause concomitant changes in [Ca\textsuperscript{2+}], in neighboring cells, thereby transmitting a neurosecretion-inducing stimulus to other neurons.

An alternative to the above-mentioned hypothesis is that neurosecretion does not only occur as a result of highly synchronized [Ca\textsuperscript{2+}] peaks but that neurosecretion occurs with every increase in [Ca\textsuperscript{2+}], when cells are not synchronized, levels of LHRH peptide would be undetectably low, whereas synchronization of neurosecretory activity among many cells would produce a discrete LHRH “pulse.” This hypothesis implies that synchronized [Ca\textsuperscript{2+}], peaks are not different from nonsynchronized peaks, which is contrary to our finding that synchronized [Ca\textsuperscript{2+}], peaks are larger than nonsynchronized peaks. Assessment of the aforementioned hypotheses will require the simultaneous measurement of [Ca\textsuperscript{2+}], and neurosecretion from single cells.

In summary, we found that cultures of primary LHRH neurons and the nonneuronal cells with which they are associated have remarkably similar oscillatory spontaneous [Ca\textsuperscript{2+}], dynamics and that both types of cell exhibit highly synchronized increases in [Ca\textsuperscript{2+}], that are propagated as intercellular Ca\textsuperscript{2+} waves. Thus nonneuronal cells would appear to be functionally integrated with LHRH neurons. This raises the possibility that nonneuronal cells, such as glia, might be a crucial component of the in vivo LHRH neurosecretory system, providing an indirect coupling mechanism to facilitate the syn-
chronization of isolated LHRH neurons. Future studies of GnRH neurons in situ, such as slice preparations, are required to evaluate this hypothesis.

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