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Whole-Cell Recordings from Preoptic/Hypothalamic Slices Reveal Burst Firing in Gonadotropin-Releasing Hormone Neurons Identified with Green Fluorescent Protein in Transgenic Mice*

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ABSTRACT

Central control of reproduction is governed by a neuronal pulse generator that underlies the activity of hypothalamic neuroendocrine cells that secrete GnRH. Bursts and prolonged episodes of repetitive action potentials have been associated with hormone secretion in this and other neuroendocrine systems. To begin to investigate the cellular mechanisms responsible for the GnRH pulse generator, we used transgenic mice in which green fluorescent protein was genetically targeted to GnRH neurons. Whole-cell recordings were obtained from 21 GnRH neurons, visually identified in 200-μm preoptic/hypothalamic slices, to determine whether they exhibit high frequency bursts of action potentials and are electrically coupled at or near the somata. All GnRH neurons fired spontaneous action potentials, and in 15 of 21 GnRH neurons, the action potentials occurred in single bursts or episodes of repetitive bursts of high frequency spikes (9.77 $\pm$ 0.87 Hz) lasting 3–120 sec. Extended periods of quiescence of up to 30 min preceded and followed these periods of repetitive firing. Examination of 92 GnRH neurons (including 32 neurons that were located near another green fluorescent protein-positive neuron) revealed evidence for coupling in only 1 pair of GnRH neurons. The evidence for minimal coupling between these neuroendocrine cells suggests that direct soma to soma transfer of information, through either cytoplasmic bridges or gap junctions, has a minor role in synchronization of GnRH neurons. The pattern of electrical activity observed in single GnRH neurons within slices is temporally consistent with observations of GnRH release and multiple unit electrophysiological correlates of LH release. Episodes of burst firing of individual GnRH neurons may represent a component of the GnRH pulse generator. (*Endocrinology* **141:** 3731–3736, 2000)

THE NEURONS that synthesize and secrete GnRH form the final common pathway for the central regulation of reproduction. GnRH is released in an episodic manner into pituitary portal blood and stimulates the corresponding pulsatile secretion of the pituitary gonadotropins, LH and FSH (1–5). This episodic GnRH signal is obligatory for the function of the reproductive axis (6). Our current understanding of putative mechanisms linking neuronal activity and GnRH release is based primarily upon extracellularly recorded multiple unit electrical activity in the arcuate region of the medial basal hypothalamus (7–9). Multiple unit activity is an electrophysiological correlate of the GnRH pulse generator and is characterized by episodes of spike activity associated with LH release. This episodic multiple unit firing is separated by protracted intervals of relative quiescence corresponding to the interpulse interval of serum LH levels. The intermittent profile of multiple unit activity is also reminiscent of the secretory pattern of GnRH (1–5).

Efforts to understand the mechanisms responsible for episodic GnRH release have been hampered by the diffuse distribution of these neuroendocrine cells in the hypothalamus; this has greatly limited single cell experiments aimed at identifying the electrophysiological mechanisms responsible for the secretory patterns of GnRH neurons. Previous studies of GnRH neurons have primarily relied on either immortalized transformed GnRH cell lines (GT1–7 cells) (10, 11) or cultured embryonic GnRH neurons (12). There is no report of a pattern of activity from these model systems that reflects the intermittent or pulsatile nature of GnRH release in the intact animal. In contrast, a recent study of GnRH neurons identified by expression of green fluorescent protein (GFP) suggests that GnRH neurons in hypothalamic slices are generally silent (13). Previous studies, therefore, have not assessed the pattern of electrical activity of individual GnRH neurons and have not reported the activity patterns that would be expected to underlie episodic hormone release (*e.g.* similar to the multiple unit electrical activity recorded *in vivo* and thought to correspond to the GnRH pulse generator). In the present study we used whole-cell recording of GFP-

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identified GnRH neurons in slices from the preoptic area and hypothalamus to record patterns of electrical activity congruent with the pulsatile secretion of GnRH.

Materials and Methods

Mice

GnRH neurons in preoptic/hypothalamic slices derived from gonadal-intact male and female GnRH-GFP mice were studied between 26–65 days of age. Details regarding these transgenic animals have been described previously (14). The animal care and use committees of the University of Virginia and Colorado State University approved all procedures.

Electrophysiology

Coronal slices from the diagonal band of Broca, preoptic area, and hypothalamus were prepared as described previously (14). Mice were anesthetized with halothane and decapitated; the brain was then rapidly removed and placed in cold (1−2 C) artificial cerebrospinal fluid (14) containing 125 mM NaCl, 24 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2.5 mM KCl, 10 mM glucose, and 1.0 mM $CaCl_2$, pH 7.3–7.4. The region containing the preoptic area and hypothalamus was blocked, and two or three 200-μm coronal slices were prepared. Slices were incubated at 32−35 C in artificial cerebrospinal fluid for at least 2 h before electrophysiological recordings. Whole-cell recordings were obtained with patch pipettes containing 130 mM potassium gluconate, 10 mM HEPES, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 5 mM biocytin, and 2 mM ATP and brought to pH 7.2–7.4 with KOH. Pipette resistances were 2.5–5.0 MΩ. Biocytin was included in the pipette to both facilitate *post-hoc* identification of recorded cells and also to examine coupling between GnRH neurons (15). Biocytin is a low molecular mass molecule (372 Da) that can pass through cytoplasmic bridges and gap junctions (15, 16). After fixation, biocytin was detected using avidin conjugates.

Data collection and analysis

GnRH neurons, both individual neurons and neurons that were closely associated with another GFP-positive neuron, were targeted for recording based on visual identification of GFP expression and accessibility within the slice. Whole cell patch-clamp recordings were obtained using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), with filtering at 5 kHz. Data were digitized on-line with a Neurocorder (DR-484, Neurodata, Inc., New York, NY) and were stored on videocassettes for off-line analysis with Axotape and pClamp software (version 6.0, Axon Instruments). Current-clamp recordings were used to assess endogenous action potential firing. Cells with 15 min of current-clamp recording were assessed for stability by the following criteria before inclusion in the analysis: initial resting membrane potential was more negative than −50 mV, input resistance at initiation and termination of the recording was more than 1 GΩ, and action potential amplitude was more than 60 mV initially and was maintained within 10 mV of the initial amplitude throughout the recording. Frequency histograms were used to analyze the frequency and duration of activity of the 21 cells passing these criteria. GnRH neurons fired high frequency, repetitive action potentials that were grouped in episodes. As illustrated in Fig. 2, the duration of an episode was measured from the onset of 4 Hz or more firing to the end of the last burst of 4 Hz or more firing. An episode included both the bursts (*e.g.* 11 bursting episodes in Fig. 2) and the intervening periods of quiescence (<1 min). The assessment of possible electronic coupling was made in voltage-clamp recordings from 1 member of a pair of closely apposed GnRH neurons (9 of the 32 pairs anatomically examined). Resting membrane potential was compared using Student's t test, with significance accepted at $P < 0.05$.

Immunofluorescence

After recording, sections were fixed in 4% paraformaldehyde. Biocytin was detected with strepavidin-Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or avidin-rhodamine (Vector Laboratories, Inc., Burlingame, CA), and GnRH was detected with LR1 primary antibody and Cy5-conjugated secondary (Jackson ImmunoResearch Laboratories, Inc.) (14). As previously reported (14), GnRH peptide content and GFP expression in the slice were 100% congruent.

Results

Pattern and characteristics of spontaneous action potential firing

Stable whole cell recordings of 15 min or more were obtained from 21 GFP-identified GnRH neurons in slices derived from 17 animals. GnRH neurons exhibited spontaneous action potentials with slow oscillations in membrane potential of 10–30 mV. Spontaneous high frequency bursts of action potentials were observed in 15 of 21 GnRH neurons (Fig. 1). A burst of action potentials was defined as a period during which firing frequency was 4 Hz or more (Fig. 2, *dashed line*). The initial resting potential of neurons that gen-

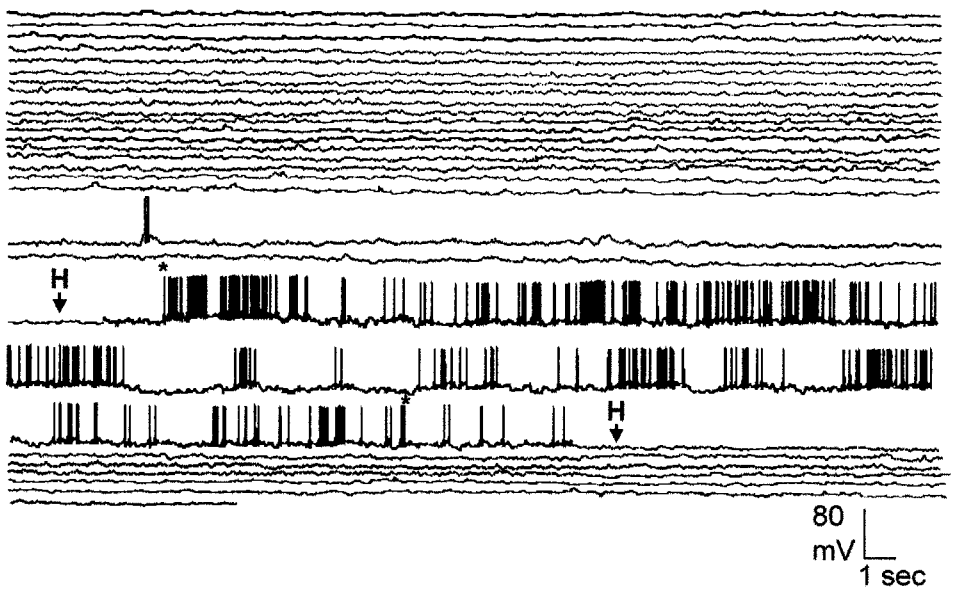

FIG. 1. Representative patterns of electrical activity over 15 min of continuous recording in a single GnRH neuron from a 55-day-old female. Each line represents about 34 sec. Resting potential during the quiescent intervals ranged from −69 to −52 mV. Firing was initiated at approximately −42 mV. *Asterisks* indicate the onset and termination of the firing episode, as defined in *Materials and Methods*. The letter H marks the start and end of the data included in the frequency histogram for Fig. 2.

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erated bursts was −59.8 ± 1.8 mV (mean ± SEM) *vs.* −68.2 ± 2.6 mV for neurons without bursts ($P < 0.05$). Bursting and nonbursting GnRH neurons were present in the same animal, and in one instance in the same slice.

GnRH neurons often fired in clusters or episodes of repetitive bursts. An episode was defined as a period with two or more bursts of action potentials, independent of the nature of intervening activity. Importantly, the onset of episodes was sufficiently abrupt that the criteria chosen to define them did not affect their detection or our interpretation of these data. Before these episodes of repetitive firing, GnRH neurons were either quiescent (8 of 15) or fired infrequently (7 of 15; ≥0.01–0.02 Hz). The mean duration of the quiescent period before the episodes of activity was 13.5 ± 1.4 min (range, 0.5–30.6 min), which presumably represents a minimum. The onset of burst activity was manifest as an abrupt shift to high frequency firing that ranged from 4–13 Hz (see *Materials and Methods* for definition of bursts). Figure 3 illustrates the episodes of increased activity, without the prior or subsequent quiescent periods, from 3 representative GnRH neurons from different animals. The duration of the episodes of firing averaged 42.6 ± 11.3 sec (range, 3–120 sec). During these episodes, high frequency bursts (9.8 ± 0.9 Hz; range, 5.9–12.1 Hz) comprised approximately half of the overall duration of activity (mean, 21.3 ± 5.8 sec; range, 3–84 sec), but periods of infrequent firing and short-term quiescence (<1 min) were also observed. GnRH neurons with bursts were from animals that were 51.1 ± 3.1 days of age, and those without bursts were from animals that were 47.7 ± 5.5 days; 30% of recordings from both groups were females.

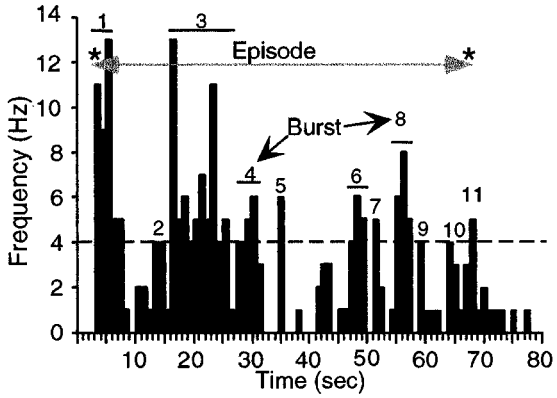

FIG. 2. Frequency histogram illustrating designations of the firing patterns (*i.e.* bursts and episodes of action potentials). The average frequency for each 1-sec interval is plotted *vs.* time. *Asterisks* indicate the beginning and end of the firing episode (*double-headed arrow*). A firing frequency of 4 Hz or more (*i.e.* above the dashed line) represents burst firing within this episode, and individual bursts are numbered (*e.g. arrows* to bursts 4 and 8). The record for this cell, including the two periods of quiescence, is shown in Fig. 1.

GnRH neuron coupling

The incidence of possible coupling of GnRH neurons was examined both anatomically and electrophysiologically. All cells were filled with biocytin during recording. We examined coupling in 32 GFP-identified GnRH neurons that had been selected in recorded slices for closely apposed or overlapping GFP-positive cell bodies and in which a biocytin-filled cell was located after fixation. Additionally, 60 other GFP-labeled cells were examined. We observed no evidence that GnRH neurons were coupled to non-GnRH neurons. Further, possible tracer coupling of biocytin was observed in only 1 GFP-positive neuron, and that was with a GFP-positive neuron that was near the recorded neuron. Voltage-clamp recording of the cell that had possibly transferred

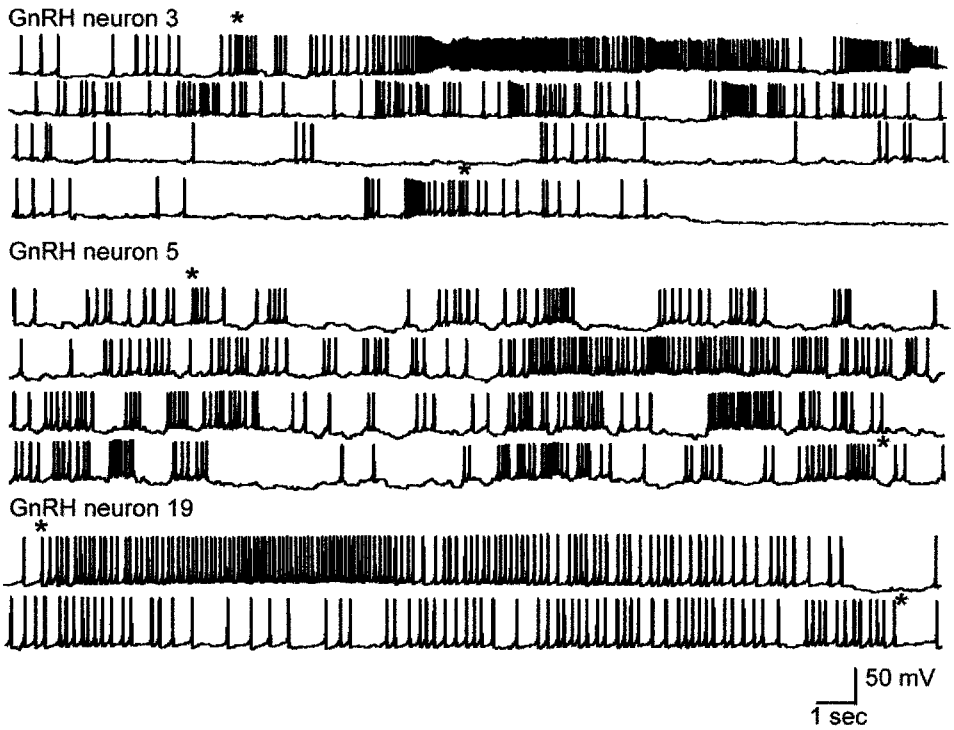

FIG. 3. Firing episodes of three representative GnRH neurons. Cells 3 (*top*), 5 (*middle*), and 19 (*bottom*) were quiescent for 5.2, 1.6, and 13.9 min before the illustrated activity, respectively. Slices were from mice aged 38, 42, and 65 days, respectively.

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biocytin to a neighboring GnRH neuron revealed putative coupling currents (Fig. 4A). The initial resting potential in this cell was −62 mV. During the voltage-clamp recording, the cell was held at −71 mV to prevent action currents from originating in the recorded cell. The observed action currents had a shorter time course and larger amplitude (Fig. 4B) than excitatory postsynaptic currents (Fig. 4, B and C) detected in the same cell, suggesting that they reflected action potential firing in a coupled cell.

Discussion

We report here that action potentials in some GFP-identified GnRH neurons in preoptic/hypothalamic slices were organized into bursts and episodes of multiple bursts. The neurons were otherwise either entirely silent or fired at a low frequency most of the time. Evidence for soma to soma coupling was revealed in only one case.

The episodic electrical activity of single GnRH neurons in slices was qualitatively similar to the episodic function of the GnRH pulse generator. Our finding of quiescence in GnRH neurons (in some neurons, up to 30 min) is consistent with the long intervals reported between multiple unit bursts, GnRH pulses, and LH pulses (1–5, 7–9) in intact rodents, ruminants, and primates (*i.e.* often >1 h). In mice, the model employed in the present study, similar LH interpulse intervals have been observed, ranging from 60–80 min in castrates to over 3 h in gonad-intact animals (17–19). Further, the abrupt onset of firing observed in the present study is consistent with the rapid rise in GnRH release during a pulse (1, 20). The duration of episodes of action potential firing of GnRH neurons in the present study (3–120 sec) was of the same order of magnitude as that of other measures of GnRH neuron activity. These include the duration of GnRH release in estradiol-treated ovariectomized ewes (60–300 sec) (20), intracellular calcium spikes in cultured GnRH neurons from embryonic rhesus monkeys (~90 sec) (21), and multiple unit activity in intact rhesus monkeys (132–246 sec) (22). These observations suggest a more prolonged event than the episodes of neuronal firing that we observed in the present study. In this regard, pulses of GnRH could reflect secretion from multiple GnRH neurons that may not fire in complete synchrony. Furthermore, the onset of multiple unit activity, rather than the entire duration, appears to be associated with LH secretion (23). Mathematical extraction of single unit activities from the multiple unit recordings suggests that the episodes result from increased activity of individual neurons rather than activation of additional neurons (24). We observed such an increase in spike frequency in 7 of 15 neurons, whereas episodes of activity in 8 other GnRH neurons were due to activation of previously quiescent cells. In the present study, however, GnRH neurons did not exhibit more than 1 episode of enhanced firing. Given the LH interpulse intervals (*i.e.* often >1 h) (17–19), however, one would not anticipate multiple episodes of activity if these intervals of action potential firing underlie GnRH release.

Although episodic bursts of action potentials have been associated with peptide release in other neuroendocrine systems, GnRH release has not yet been linked directly to any particular pattern of electrical activity. In the magnocellular neuroendocrine systems controlling water balance and milk ejection through the release of vasopressin and oxytocin, the amount of hormone released per action potential is enhanced when spike activity is clustered into bursts (25, 26). Magnocellular neuroendocrine cells alter their firing behavior, shifting between bursting and nonbursting modes in response to physiological or experimental stimuli (27). Similarly, individual GnRH neurons in this study expressed firing patterns that included periods of both quiescence and episodes of repetitive firing. By analogy with other neuroendocrine systems, the temporal shifts in electrical activity of GnRH neurons reported here suggest that burst firing may contribute to pulsatile hormone release. More direct evidence of a link between burst firing and GnRH release would support the

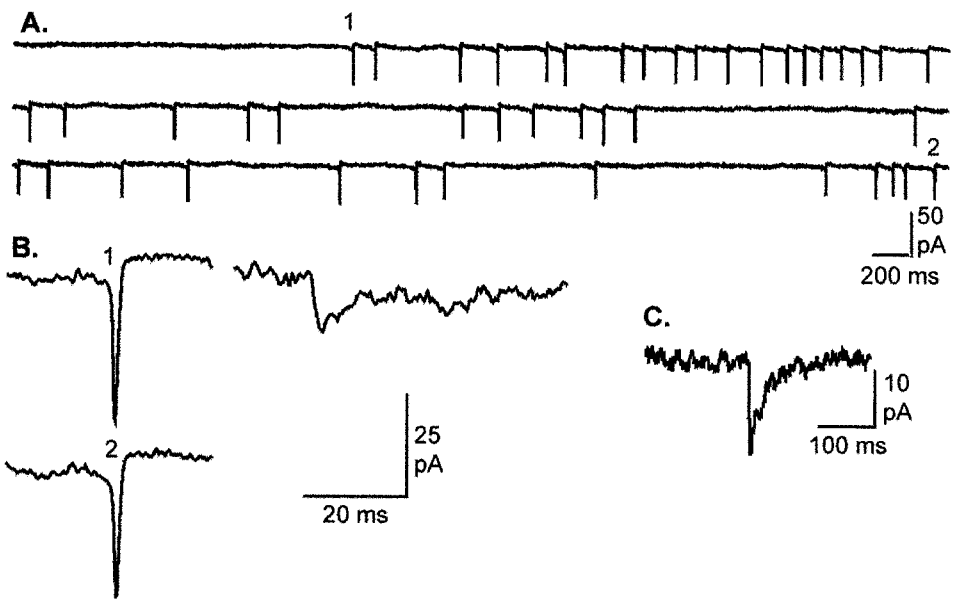

FIG. 4. Evidence of putative coupling currents in the two potentially tracer-coupled GnRH neurons. The resting potential of the clamped cell was −62 mV. During electrophysiological recording, a holding potential of −71 mV was imposed. A, Voltage-clamp recording of putative coupling currents. B, Expanded time scale illustrating time course and stability of the coupling currents. Numbers over expanded currents correspond to those in A. An excitatory postsynaptic current is also shown at the same scale, demonstrating the smaller amplitude and longer duration of these events compared with the putative coupling currents. C, The same excitatory postsynaptic current illustrated in B on a different scale.

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general hypothesis that episodic hormone release depends directly on the activity pattern of neuroendocrine cells.

Episodic GnRH release is presumed to reflect synchronization of the firing patterns of multiple GnRH neurons. Several mechanisms have been proposed to underlie such coordination, including direct soma to soma communication through cytoplasmic bridges (28) or gap junctions (29), synaptic interactions (30), or diffusable second messengers such as nitric oxide (31, 32). In the present study only the possibility of direct coupling was explored. Of 92 GnRH neurons examined for electrical and tracer coupling, we observed evidence for such a link in only 1 instance, suggesting that this is a rare event in this neural system.

The assessment of cell to cell coupling is complicated by limitations of both neuroanatomical and electrophysiological techniques. Although tracer coupling is generally regarded as a valid indication of coupling, artifactual labeling of damaged cells can occur. Additionally, it can be difficult to visualize the precise coupling site in sections of this thickness (200 μm). Hypothetical coupling currents may reflect action potentials originating from unclamped dendrites of the recorded cell, as opposed to action potentials transmitted from a coupled cell. In the sole pair of hypothetically coupled neurons, the amplitude of coupling currents was severalfold smaller than that which would be expected from typical unclamped action potentials, and the waveform of the coupling currents was faster than that of an excitatory postsynaptic current. These data together with weak evidence of possible tracer coupling in the same pair of neurons suggest the GnRH neuron was functionally coupled to its neighboring GnRH neuron.

The low coupling rate observed in the present study (3%) is similar to the prevalence of soma to soma cytoplasmic bridges previously reported between GnRH neurons of rats (28). Our findings are also consistent with those of Kusano *et al.* (12), who demonstrated a lack of direct cell to cell communication in cultured olfactory placode GnRH neurons. In contrast, our observed coupling rate is much lower than that reported for GT1–7 cells, in which 15–30% of cells appear to be dye coupled (33, 34). Our data suggest that any putative synchronization among GnRH neurons that leads to pulsatile release of the hormone probably involves mechanisms other than direct coupling at the level of the cell body. Some other form of local circuit communication in this region of the brain may contribute to synchronization of the GnRH neuronal network

These data suggest that the episodic electrical activity ascribed to the GnRH pulse generator can be observed at the level of the single GnRH neuron in preoptic/hypothalamic slices. The similarity between the action potential firing in GnRH neurons and other biological markers used to monitor activity of the GnRH pulse generator (*i.e.* multiple unit recordings and GnRH release) is striking. Taken together, our data are consistent with the hypothesis that intermittent episodes of burst firing of individual GnRH neurons underlie the episodic secretion of GnRH. The mechanism by which such activity in these neurons might be synchronized to produce coordinated release, however, remains to be further elucidated.

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