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Pulsatile Release of Gonadotropin-Releasing Hormone (GnRH) from the Rat Hypothalamus *in Vitro*: Calcium and Glucose Dependency and Inhibition by Superactive GnRH Analogs*

JEAN-PIERRE BOURGUIGNON, ARLETTE GERARD, GEORGETTE DEBOUGNOUX, JOAN ROSE, AND PAUL FRANCHIMONT

Pediatric Clinic (J.-P.B.), University of Liege, CHU, Sart-Tilman, B4000 Liège, Belgium; and Radioimmunoassay Laboratory (J.-P.B., A.G., G.D., J.R., P.F.), Institute of Pathology, CHU, Sart-Tilman, B-4000 Liège, Belgium

ABSTRACT. We have shown previously that the rat hypothalamus retains *in vitro* its capacity of generating pulsatile release of GnRH. The present study evaluated if pulsatile release of GnRH *in vitro* was influenced by metabolic conditions (calcium and glucose availability) and the possible self-regulatory role of GnRH in its pulsatile secretion. In the presence of a calcium-chelating agent (EGTA, 20 mM) or a calcium-channel blocker (D-600, 0.1 mM), the release of GnRH induced by a depolarization (veratridine, 50 μM) was markedly and reversibly decreased. In addition, frequency and amplitude of GnRH secretory pulses were significantly reduced ($P<0.05$). When glucose use was inhibited using 2-deoxyglucose (5.6 mM) the release of GnRH induced by veratridine and the frequency of GnRH pulses were also blunted ($P<0.05$). Superactive agonists of GnRH (Buserelin and D-TRP^6-PRO^9-*N*-ET, 10 nM) caused a prompt decrease of GnRH release in basal conditions and in the presence of veratridine. A significant inhibition ($P<0.05$) was observed using buserelin concentrations greater than 0.01 nM, whereas two GnRH analogs without biopotency (Leu^8-GnRH, Des-gly^{10}-*N* picolylamide GnRH, 100 nM) did not affect GnRH release. The two agonists of GnRH reduced by 43% to 66% ($P<0.05$) the occurrence of significant GnRH pulses. We conclude that, *in vitro*, the hypothalamic neuronal circuitry resulting in GnRH pulsatile secretion is dependent on calcium and glucose availability and is sensitive to an ultrashort-loop inhibitory feedback mechanism. (*Endocrinology* **121:** 993–999, 1987)

THE DEVELOPMENT of *in vitro* perifusion systems has made allowance for studying extensively the release of neuropeptides from the hypothalamus (1–10). GnRH brought on a special interest since, in the rat like in other species, indirect evidence was obtained for the hypothalamic localization of the machinery determining the pulsatile nature of LH secretion (11, 12). In 1981 we reported that the release of GnRH from the hypothalamus of male rats occurred episodically *in vitro* (13). Subsequently we observed that the frequency of pulsatile GnRH release increased between 2 and 4 weeks of age (4). More recently, the release of GnRH from the hypothalamus of female rat (14, 15) and guinea pig (16) was also shown to be pulsatile *in vitro*. The primary mechanism activating GnRH release intermittently has not been elucidated. It could involve a local inhibitory action of GnRH on its own secretion through an ultrashort-loop feedback control, as proposed 25 yr ago (17) and supported by very recent *in vivo* (18, 19) and *in vitro* (19, 20) observations using superagonists of GnRH. The aim of this study was to evaluate if pulses in GnRH release observed *in vitro* result from an active biological process depending on the metabolic conditions in the neural tissue fragment and if GnRH agonists used as a bioactive, nonimmunoreactive GnRH-like material affect the pulsatile nature of GnRH release *in vitro*.

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Address correspondence and requests for reprints to: Jean-Pierre Bourguignon, M.D., Department of Pediatrics, Centre Hospitalier Universitaire du Sart-Tilman, 34000 Liège, Belgium.

Materials and Methods

Rat hypothalamus

Male Wistar rats aged 50 days at the time of the experiment were purchased from Janssens (Beerse, Belgium). They were housed, six per cage, under constant temperature and a light-dark schedule (lights on 0600–2000 h) with free access to food and water, until the time of the experiment. For each experiment, a group of 12 rats were killed around 1300 h. Immediately after decapitation, the retrochiasmatic hypothalamus was dis-

sected and transferred to the incubator. As previously described (4), these fragments were shown to contain the median eminence, the arcuate and ventromedial nuclei, whereas the mammillary bodies, the anterior and the suprachiasmatic nuclei were only partly included.

Incubation procedure

The *in vitro* system has been previously described, as well as the incubation conditions (4). Briefly, 12 retrochiasmatic hypothalami were studied, 1 per chamber, in a water-saturated atmosphere of 95% O_2-5% CO_2 (vol/vol), at 37.5 C. Culture medium [Dulbecco's Modified Eagle's Medium (DMEM), Flow, Mac Lean, VA; 0.5 ml/chamber] contained 1.25 mM calcium and 25 mM glucose in control conditions. It was renewed every 7.5-min period; the sampled medium being collected on ice in tubes containing bacitracin (Sigma, St. Louis, MO; 20 μM final concentration) was frozen until assayed. After incubation of the hypothalamic fragments, their GnRH content was extracted and assayed (21). A static incubation system was preferred because attempts to use a perifusion system resulted in a lower recovery of GnRH due to increased adsorption and degradation.

RIA of GnRH and pulse analysis

Using the RR-5 anti-GnRH antibody generously supplied by Dr. A. Root (St. Petersburg, FL), the RIA of GnRH was performed according to a method described previously (4, 21). The sensitivity varied between 0.5 and 1 pg/tube. From the 0.5-ml samples collected, 0.1 ml was assayed in duplicate; the detection limit of GnRH release was 5 pg/7.5 min and data below this limit were assigned this value. The interassay coefficient of variation of the RIA was 18%. The intraassay coefficient of variation of both tissue incubation and RIA procedures was calculated by measuring in 15 successive fractions the recovery of 4 different concentrations of synthetic GnRH (25–175 pg/0.5 ml) introduced in the incubation chambers and sampled after 7.5 min. The mean (±1 SD) recovery was 94±7% and the mean coefficient of variation was 14±3%. Significance of GnRH secretory pulses was determined according to two different methods. One was derived from Santen and Bardin (22), the original calculation based on a 20% increment from nadir to peak being modified in order to use 4 times the 14% coefficient of variation as the criterion of pulse significance. The appropriate computer program for this calculation was a courtesy of R. A. Steiner (Seattle, WA). The other method used was the PULSAR program (23), version 1.3 B modified for the IBM-PC computer by J. F. Gitzen and V. D. Ramirez (Urbana, IL). The 14% coefficient of variation described above was used as B coefficient to determine the assay SD. The cutoff criteria (G) for peak identification were calculated empirically, G being 2.5 for the single data point peaks, which were the most frequent observation. Comparing both methods, we found that the Santen and Bardin procedure overestimated the frequency of significant pulses by 59% when compared to the frequency calculated by the PULSAR method, or visually. This may result from the absence of smoothing calculation of baseline and noise variations in the method of Santen and Bardin. Therefore, the PULSAR method was chosen to analyze our data.

Study protocol

The release of GnRH was studied basally and under stimulation by 50 μM veratridine (Sigma, St. Louis, MO), a depolarizing agent (24), added to culture medium for two or three consecutive periods of 7.5 min. In the presence of veratridine, the release of GnRH induced by the depolarization was calculated as the maximal increment over the pretreatment level. When compared to control conditions (DMEM, 1.25 mM calcium, 25 mM glucose), the release of GnRH basally and during veratridine stimulation was studied in the presence of 20 mM EGTA as a calcium chelating agent, 0.1 mM D-600 (Methoxy Verapamil, Knoll AG, Ludwigshafen FRG) as a calcium-channel blocker (25), and 5.6 mM 2-deoxyglucose as an inhibitor of glucose use (26). In this latter condition, 2-deoxyglucose was used at a concentration equimolar to glucose. Then, control medium consisted of DMEM with 5.6 mM glucose, 5 g/liter standard monocomponent porcine insulin preparation (Actrapid MC, Novo, Copenhagen), and 5.6 mM D-galactose were added to keep the same osmolarity in control medium as in that containing 2-deoxyglucose.

The release of GnRH was also studied in the presence of several GnRH analogs either with a highly increased biopotency or without biological activity. Inactive analogs were Des(ARG)-LEU^8-decapeptide and des(GLY)-picolylamide^{10}-nonapeptide (Hoechst AG, Frankfurt, West Germany). The superactive agonistic nonapeptides of GnRH studied were D-TRP^6-PRO^9-*N*-ethylamide (Dr. J. Rivier, the Salk Institute, San Diego, CA), D-SER(TBU)^6-PRO^9-*N*-ethylamide (Buserelin, Hoechst AG, Frankfurt, FRG), and 5-oxo-L-PRO^1-D-LEU^6-PRO^9-*N*-ethylamide (Leuprolide, Abbott, North Chicago, IL). At concentrations up to 100 nM, none of these analogs resulted in a significant cross-reaction in the RIA of GnRH, except buserelin resulting in 0.001% cross-reactivity with synthetic GnRH on a molar basis. However, no significant inhibition of radiolabeled GnRH binding to RR-5 antiserum occurred in the presence of buserelin concentrations less than or equal to 10 nM, used in our experiments.

Statistical analysis

The significance of differences in GnRH release between control conditions and in the presence of several compounds was calculated using paired and unpaired Student's *t* test, respectively, when results were obtained studying the same or different hypothalami.

Results

As shown in Table 1, incubation of rat hypothalami in the presence of EGTA or D-600 did not significantly affect the hypothalamic GnRH content. In contrast, the increase in GnRH release induced by veratridine was markedly reduced during suppression of calcium availability. The inhibitory effect of 20 mM EGTA upon GnRH release induced by veratridine (from 17.3±2.4 to 2.7±1.3 pg/7.5 min, mean ± SEM, n=9) was shown to be reversible: after using control medium for 15 min after EGTA, the response to veratridine was not only recovered (32.1± 8.1 pg/7.5 min) but significantly higher ($P<0.05$) than the pretreatment response. As shown in Fig. 1, EGTA and D-600 resulted in a lowered level of GnRH release, most values being undetectable. In addition, the pulsatile pattern of GnRH release seen in control conditions was inhibited in the presence of EGTA and D-600 (Fig. 1), both frequency and amplitude of GnRH pulses being significantly decreased (Table 1).

Using control medium containing 25 mM glucose, GnRH release induced by veratridine was 21.1±2.2 pg/7.5 min (mean ± SEM, n=35). Using a reduced glucose concentration of 5.6 mM, the response to veratridine was significantly decreased to 10.6±1.7 pg/7.5 min (n=16, $P<0.05$). After addition of 5 g/liter insulin to the medium with 5.6 mM glucose the response to veratridine was restored (20.6±4.8 pg/7.5 min, n=9) to a level similar to that obtained in control conditions using 25 mM glucose. As shown in Table 1, mean GnRH release induced by veratridine (23.4 pg/7.5 min) and mean GnRH pulse frequency (2.4 pulses/120 min) observed using 5.6 mM glucose, 5 g/liter insulin and 5.6 mM galactose were similar to the data obtained in control conditions (25 mM glucose). The replacement of galactose, a nonusable carbohydrate, by 5.6 mM 2-deoxyglucose, an inhibitor of

TABLE 1. Effects of calcium suppression, inhibition of glucose use, and GnRH superagonists upon the hypothalamic content and the release of GnRH *in vitro*

	Hypothalamic GnRH content (ng/hypothalamus)	Release induced by veratridine 50 μM (pg/7.5 min)	Pulse frequency (n/120 min)	Pulse amplitude (pg)
Controls	3.1 ± 0.4 (15)	15.9 ± 1.7 (18)	2.5 ± 0.1 (6)	31.5 ± 4.9 (6)
EGTA (20 mM)	2.5 ± 0.3 (17)	2.5 ± 1.2 (10)	0.5 ± 0.3 (5)[a]	7.9 ± 4.6 (2)[a]
Controls	4.0 ± 0.4 (11)	19.6 ± 2.5 (15)	2.5 ± 0.3 (6)	21.6 ± 3.7 (6)
D-600 (0.1 mM)	3.9 ± 0.4 (11)	4.3 ± 1.1 (11)	1.1 ± 0.4 (5)[a]	7.5 ± 1.9 (4)[a]
Galactose (5.6 mM)	3.1 ± 0.3 (22)	23.4 ± 3.1 (34)	2.4 ± 0.2 (12)	19.4 ± 2.8 (12)
2-Deoxyglucose (5.6 mM)	2.3 ± 0.2 (18)	5.4 ± 1.1 (23)	1.3 ± 0.2 (11)[a]	16.9 ± 3.6 (8)
Controls	2.5 ± 0.2 (10)	16.7 ± 1.7 (21)	2.1 ± 0.5 (10)	10.2 ± 1.3 (9)
Leu^8 (100 nM)			1.7 ± 0.6 (4)	10.9 ± 1.0 (3)
Buserelin (10 nM)	2.7 ± 0.3 (10)	2.6 ± 1.2 (10)	0.7 ± 0.2 (10)[a]	6.2 ± 0.7 (6)[a]
D-TRP^6-PRO^9-*N* ET (10 nM)	2.3 ± 0.2 (7)	9.1 ± 1.1 (12)	1.2 ± 0.4 (8)[a]	6.5 ± 1.8 (5)[a]
Leuprolide (10 nM)	2.9 ± 0.5 (7)	6.5 ± 3.7 (7)		

Values are mean ± SEM (n).
[a] $P < 0.05$ *vs.* controls.

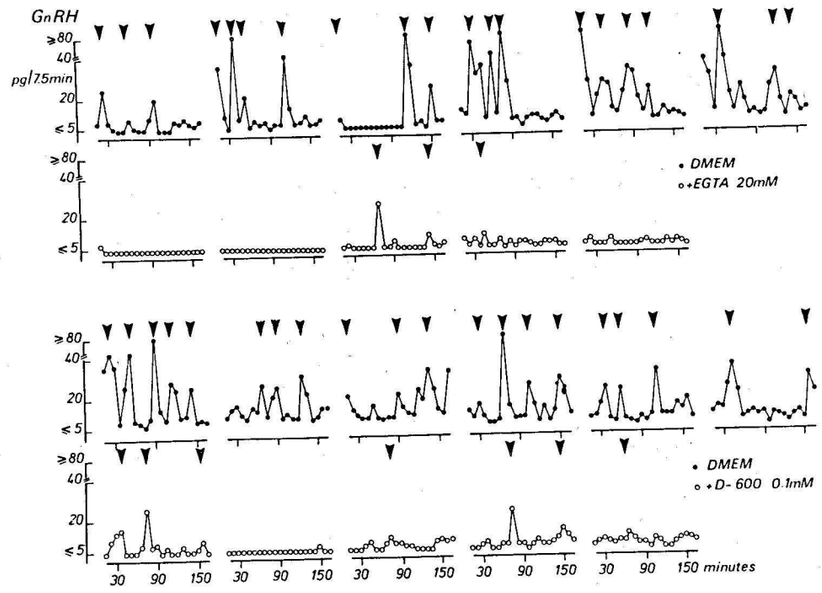

FIG. 1. Time-related release of GnRH *in vitro* from individual hypothalami of 50-day-old male rats incubated in control conditions (DMEM containing 1.25 mM calcium) and in the presence of a calcium-chelating agent (EGTA) or a calcium channel blocker (D-600). The *arrows* denote significant pulses of GnRH release.

glucose use, resulted in a significant reduction of both GnRH response to veratridine and pulse frequency.

As shown in Fig. 2, the mean level of GnRH release was significantly reduced after 15-min incubation in the presence of three different superagonists of GnRH (10 nM) whereas two analogs of GnRH without biological activity (100 nM) did not affect the release of GnRH. Using several concentrations of a superagonist, buserelin, it was shown that a significant inhibitory effect was obtained at concentrations greater than or equal to 0.01 nM, up to 10 nM. The three superagonists of GnRH caused a significant decrease in the GnRH release induced by veratridine (Table 1). The inhibition of the response to veratridine in the presence of buserelin, 10 nM (from 17.8±2.0 to 6.8±0.8 pg/7.5 min, mean ± SEM, n=5), was shown to be reversible from the first 7.5-min period control medium was used. Then, the response to veratridine (36.3±5.1 pg/7.5 min) was significantly higher than the pretreatment value ($P<0.05$). The *upper panel* of Fig. 3 illustrates the occurrence of pulses in GnRH release from hypothalami incubated in control conditions or in the presence of Leu^8-GnRH, an analog without biopotency. In the presence of buserelin or D-TRP^6-PRO^9-*N* Et-GnRH, two superactive agonists, the frequency of GnRH pulses was significantly reduced by 67% and 43%, respectively (Fig. 3, *lower panel*). A significant reduction in GnRH pulse amplitude was also observed (Table 1).

Discussion

In this paper we show that the pulsatile release of GnRH from the rat hypothalamus *in vitro* is dependent on calcium and glucose availability. In addition, our data provide evidence of negative ultrashort-loop feedback regulation of GnRH secretion resulting in a reduction of both frequency and amplitude of GnRH pulses.

The observation of pulsatile secretion of GnRH from the rat hypothalamus *in vitro* is consistent with the presence of a hypothalamic pulse generator conceptualized from periodic electrophysiological manifestations (12) and periodic activation of LH release (11). When compared with *in vivo* pulsatility of LH in the intact adult male rat (27), the frequency of GnRH pulses *in vitro* is clearly greater. It is possible that, *in vivo*, the hypothalamus generates more GnRH pulses than expected according to LH pulse frequency, some GnRH pulses being ineffective in eliciting LH response at the pituitary level (28). *In vitro*, an increased frequency of GnRH pulsatile release could also result from the disconnection of the hypothalamus from other brain areas and the subsequent suppression of possible afferent inhibitory pathways (29, 30). Alternatively, some pulses observed *in vitro* could result from a passive peptide leakage from axons and terminals rather than from an active neurosecretory process. In order to rule out this hypothesis the role of calcium was studied since its transfer through the axonal membrane permits depolarization (25) and the subsequent neurosecretory activity, particularly documented for GnRH release (7, 8, 10, 31, 32). Our data indicate that spontaneous pulses in GnRH release are calcium-dependent after a channel blocked by D-600. However, some pulses were detected by the computer analysis in the presence of D-600, although the basal level of GnRH release was often below the detection limit and could account for an underestimation of pulse occurrence. It is possible that some pathways activating GnRH release are not sensitive to D-600 or that distribution of D-600 to the different areas in the hypothalamic fragment was not sustained at a high enough concentration.

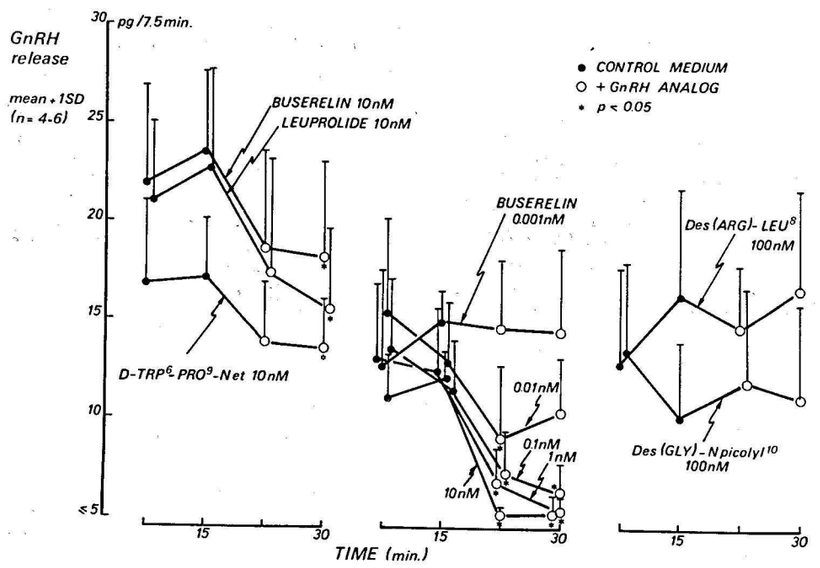

FIG. 2. Release of GnRH *in vitro* from individual hypothalami of 50-day-old male rats studied in control medium (●, DMEM) or in the presence of several analogs of GnRH (○), either with a highly increased biological activity (buserelin, leuprolide, D-TRP^6-PRO^9-*N*-Et GnRH) or without biopotency (Des-(ARG)-LEU^8-GnRH, Des(GLY)-picolylamide^{10}-GnRH).

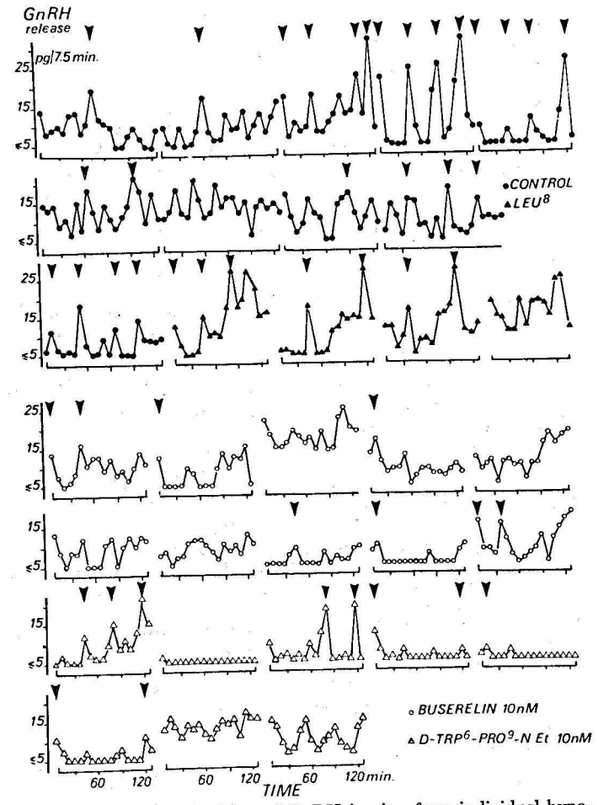

FIG. 3. Time-related release of GnRH *in vitro* from individual hypothalami of 50-day-old male rats studied in control conditions (●: DMEM), in the presence of GnRH analogs without biological activity (▲: Des(ARG)-LEU^8-GnRH), or with a highly increased biopotency (○: Buserelin, △: D-TRP^6-PRO^9-*N* Et-GnRH). The *arrows* denote significant pulses of GnRH release.

The role of glucose in the release of GnRH *in vitro* has not been extensively studied. Most authors have used culture medium with glucose concentrations between 5 and 10 mM (7, 9, 10) whereas we started using a higher concentration of 25 mM glucose. From our data such high concentrations seem to be required unless insulin is added to culture medium. This suggests that insulin may facilitate the use of glucose by the hypothalamus. However, since insulin may still have some unknown interactions with neural tissue (33, 34) we elected working with high glucose concentration and without insulin in culture medium. Using 2-deoxyglucose our data indicate that use of glucose may influence the neurosecretory activity of GnRH neurons *in vitro* whereas others did not support the same view (35). There is no explanation for this discrepancy. Since few authors did study the optimal glucose concentration for *in vitro* incubation of the hypothalamus this issue requires more systematic evaluation.

Our data provide further evidence of negative ultrashort loop feedback action of GnRH confirming previous *in vivo* (18, 19) and *in vitro* (19, 20) observations. After intraventricular administration of GnRH, De Paolo *et al.* (19) showed that frequency of LH pulses was reduced *in vivo*. Here we demonstrate that the frequency of GnRH secretory pulses *in vitro* is reduced by GnRH agonists. We did not observe any change in GnRH release *in vitro* in the presence of somatostatin, GRF, TRH, and vasopressin, 100 nM (data not shown). However, others reported that GnRH release *in vitro* was inhibited by somatostatin (36), CRF (37), and oxytocin (38). Somatostatin was also found to have an inhibitory effect upon its release (39–41). All of these observations suggest the existence of complex interactions between neuropeptides within the hypothalamus. Discussing their possible functional significance requires better understanding of their mechanisms and localizations. So far very few studies have evaluated the presence of neuropeptide-binding sites to specific membrane or cytoplasmic receptors or enzymes in the hypothalamus. To our knowledge, GnRH-binding sites have been described in the rat hypothalamus but their significance has not been elucidated (42). The retrograde portal blood flow (43, 44) may account for a wide redistribution of neuropeptides in the basal hypothalamus thereby creating conditions similar to the use of neuropeptides *in vitro*. However, the occurrence of ultrashort-loop feedback *in vivo* is likely requiring vicinity of the sites where the neuropeptide is delivered and those where the autoregulatory effect originates. Therefore, localization of the site of ultrashort-loop feedback is a critical issue. The inhibitory action of CRF and oxytocin on GnRH release was more obvious in median eminence than in mediobasal hypothalamus, suggesting its localization at the terminals of these neurons (37, 38). In contrast, for both somatostatin (41) and GnRH (19), the autoinhibitory effect could not be observed using median eminence but was present within the mediobasal hypothalamus. This indicates that the ultrashort-loop negative feedback was mediated through other neuronal structures than terminals of neurons producing GnRH and somatostatin. Recently, Epelbaum *et al.* (41) have shown, using immunocytochemical techniques, that autosynapses with somatostatin were located on the perikarya or dendrites of somatostatin-containing neurons. For GnRH, a similar hypothesis was proposed by De Paolo *et al.* (19) based on the existence

of GnRH cell bodies within the mediobasal hypothalamus. However, this is a controversial issue and most studies conclude to the predominant or exclusive location of GnRH perikarya in the septal-preoptic-suprachiasmatic area (reviewed in Ref. 45). Accordingly, our data would suggest that, in the absence of GnRH perikarya, a pacemaker system driving periodic stimulations of GnRH axons presumably at a presynaptic level is present in mediobasal hypothalamus. In the monkey hypothalamus, several pulse generating areas were identified (46). Kesner *et al.* (47) did not find any alteration of their electrophysiological manifestations after iv injection of GnRH agonists. Although these data do not necessarily involve exposure of the hypothalamus to a sufficiently high agonist concentration, they do not suggest an inhibition of the pulse generator by GnRH or by LH. In the rat, it is reasonable to assume that the pulse-generating neurons are sensitive to calcium and glucose as well as to GnRH since GnRH pulse frequency was reduced during calcium or glucose deprivation and in the presence of GnRH agonists. In these conditions, the response to veratridine depolarization, which may directly involve GnRH axons, was also depressed suggesting either different localizations for GnRH sensitive sites in the hypothalamus or activation by GnRH agonists of an inhibitory neural pathway ending distally close to the terminals of GnRH axons. The possible role of GnRH ultrashort-loop feedback is unknown. *In vitro*, it might explain why the basal secretion of GnRH has been found in the same range between 4 and 20 pg/7.5 min, using one (14, 19, 36, 37), two to three (8, 9, 34) or six (10) hypothalamic units per chamber. Studies on the effects of GnRH analogs with a biopotency similar to that of the native decapeptide or with antagonistic properties could help in further delineating the mechanisms and the role of GnRH ultrashort-loop feedback.

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