Pulsatile secretion of gonadotropin-releasing hormone by rat hypothalamic explants of GnRH neurons without cell bodies

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Abstract

Gonadotropin-releasing hormone (GnRH) is typically secreted in a pulsatile manner. It is still unclear whether pulsatility depends on a GnRH pulse generator residing in the GnRH neurons or in other neurons. Since the cell bodies of GnRH neurons are located rostrally to the optic chiasm and the majority of GnRH terminals in the median eminence of the rat hypothalamus, we have compared GnRH secretion from individual preoptic, retrochiasmatic and median eminence explants using a static incubation system. GnRH is released from the three different types of explant in response to depolarization with veratridine or glutamate receptor stimulation using the agonist N-methyl-*D*-aspartate. Only the retrochiasmatic explants, however, show a characteristic pulsatile secretion of GnRH. The mean (± SD) interpulse interval is respectively 37 + 5,25 ± 4 and 12 ± 1 min when the fractions are collected at 7.5-, 5.0- and 2.5-min intervals. The immunocytochemically stained GnRH cell bodies are normally distributed in the preoptic explants (n = 212-420) while only 3 GnRH cell bodies can be visualized in 7 retrochiasmatic explants. Semi-quantitative RT-PCR shows that GnRH mRNA is present in the retrochiasmatic explant in a ratio of about 1:600 relative to the preoptic explant. We conclude that pulsatile GnRH secretion occurs in the virtual absence of GnRH cell bodies but does not occur from GnRH terminals in the isolated median eminence. These data further indicate that a mechanism of GnRH pulsatility is located in the retrochiasmatic hypothalamus and involves neurons different from the GnRH neurons.

Introduction

Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner which is critical for pituitary gonadotropin secretion in different species [1-3]. Knobil [4] has proposed the existence of a hypothalamic pulse generator evidenced through the periodic electrophysiological manifestations preceding luteinizing hormone (LH) secretory pulses. Whether GnRH pulsatility is generated in the GnRH neurons or through a distinct neuronal apparatus remains elusive. In the rat, the mediobasal hypothalamus retains its capacity to secrete GnRH in a pulsatile manner when deafferented in vivo [5, 6] or when studied in vitro [7, 8]. Since such an isolated hypothalamus does presumably not contain GnRH cell bodies which are located in the preoptic area [9], the existence of a pulse-generating mechanism external to the GnRH neurons is suggested. Recently, however, it has been proposed that GnRH neurons have the intrinsic capacity to generate secretory pulses since the immortalized GnRH neurons (GT-1 cell line) can release GnRH in an intermittent manner [10, 11]. Alternatively, some mechanism of GnRH pulsatility could reside in the terminals independently of neuronal cell bodies since pulsatile GnRH secretion has been shown using the isolated median eminence [12]. The aim of this study was to show whether the cell bodies of GnRH neurons are required or whether GnRH terminals suffice to elicit pulsatile secretion of GnRH from hypothalamic explants.

Materials and Methods

ANIMALS AND EXPLANT DISSECTION

Adult male Wistar rats were housed under standardized conditions of temperature (25 °C) and light darkness rhythm (12/12 h) with free access to food and water. The animals were studied at 50 days of age.

After decapitation, the preoptic and retrochiasmatic explants of the hypothalamus were dissected through 3 coronal incisions: 2 mm rostrally to the optic chiasm, at the caudal border of the optic chiasm and at the rostral border of the mamillary bodies. Two lateral incisions along the lateral sulci and a 2 mm deep frontal section were made to obtain the preoptic and retrochiasmatic explants. The median eminence was dissected following a procedure described by Negro-Vilar et al. [13].

EXPLANT INCUBATION AND GNRH RADIOIMMUNOASSAY

The explants were studied using a static incubation procedure which was described in detail previously [7, 8]. In a first experiment, 10 individual retrochiasmatic explants and the corresponding preoptic explants were studied. In a second experiment, 5 retrochiasmatic explants

and 5 median eminences were studied. They were incubated individually in 0.5 ml Eagle's Minimum Essential Medium (Flow, McLean, Va., USA) supplemented with glycine, magnesium, glucose and bacitracin to achieve concentrations of 10 nM, 1 mM, 25 mM and 20 µM, respectively. The medium was collected and renewed every 7.5 min during 4 h. At the end of the experiment, the release of GnRH was evoked by incubation for 7.5 min using 50 µM of veratridine, a Na$^+$ channel opener (Sigma) and, 30 min later, 50 mM of N-methyl-D-aspartate (NMDA, Sigma). In a third experiment, retrochiasmatic explants were incubated and the fractions for GnRH assay were collected at different intervals (15, 7.5, 5 and 2.5 min, 10 explants studied for each sampling interval). The collected samples were frozen until assayed.

GnRH was measured in the collected fractions using the specific RR-5 antiserum (provided by Dr. A. Root). This RIA procedure has been described previously [7, 8]. The limit of sensitivity was 5 pg/7.5-min fractions which was the value assigned to samples with undetectable GnRH level. The release of GnRH evoked by NMDA or veratridine was calculated as the difference between the amounts of GnRH secreted immediately prior to and during incubation with the secretagogue.

IMMUNOCYTOCHEMISTRY

Seven rats anesthetized using chloral hydrate received intracardiac perfusion of Zamboni's buffered picric acid-paraformaldehyde fixative (pH 7.4). The brains were dissected and incubated overnight in the fixative at 4°C. After overnight washing in PBS 0.02 M containing 30% sucrose, the brains were embedded in OCT, frozen on dry ice and sectionned at 14 µm on a cryostat. After preincubation with PBS 0.02 M containing 10% normal swine serum and 0.1% Triton X-100 and washing, the sections were incubated during 4 or 5 days at 4°C with the anti-GnRH LR1 antiserum (a generous gift from Dr. R. Benoit, Montreal, Canada), diluted at 1:2,000. This antiserum was previously characterized [14], After washing, the sections were incubated sequentially with biotinylated swine anti-rabbit immunoglobulins (Prosan, Glastrup, Denmark), used overnight at 4°C at 1:200 dilution and StreptAB Complex (Prosan) at room temperature. Staining was obtained using 3,3'-diaminobenzidine (with β-D-(+)glucose and glucose oxydase) and counterstaining using hematoxylin. All the sections were screened for GnRH-immunoreactive neuronal cell bodies, paying attention to avoid double cell counting.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

After decapitation of 50-day-old male rats, the preoptic and retrochiasmatic explants were dissected and rapidly frozen in RNase-free liquid nitrogen. The samples were weighed and stored at -70°C. Total RNAs were extracted according to the RNAzol method [15] and stored at -70°C. The extracted RNAs were quantified by UV-spectroscopic measurement and the absence of degradation was assessed by electrophoresis on formaldehyde agarose denaturating gel. Based on the rat GnRH cDNA sequence [16,17], GnRH primers were synthesized using the following oligonucleotide sequences which were proposed by Kim et al. [18]. Upstream primer: 5' CACTATGGTCACCAGCGGGG 3' (sequence 894-913; exon 1; accession number M 31670) and downstream primer: 5'AGAGCTCCTCGCAGATCCCTAAGA 3' (sequence 3,635-3,658; exon 3;

accession number M 31670). The predicted size of this PCR product is 375 bp [16, 17], As positive and semi-quantitative controls, the upstream and downstream primers of β-actin were used: 5'GATGGTGGGTATGGGTCAGAAGGA 3' and 5'GCTCATTGCCGATAGTGATGACCT 3' [19], The predicted size of this PCR product is 632 bp. All primers were chosen to flank introns so that the amplified GnRH and β-actin cDNAs can be distinguished from an amplification of possible contaminating genomic DNA.

Reverse transcriptase and PCR were performed in a one-step method, using the RNA PCR Perkin-Elmer kit. Briefly, 10 μg of total RNAs were reverse transcribed into cDNAs with an oligodeoxythymidilate-oligo (dT) as primer, in a volume of 20 μl (10 min at 70°C, 60 min at 42°C and 5 min at 4°C). The reaction mixture contained 25 m*M* MgC1$_2$, 1 m*M* dNTP mix, 1 U/μl RNAse inhibitor, 2.5 μl/ml M-MuLV reverse Transcriptase (Eurogentec, Liege, Belgium) and 2.5 μ*M* oligo (dT)16. The cDNAs obtained were amplified in the presence of DNA polymerase (Goldstar, Eurogentec) and the specific primers. PCR was performed in a Perkin-Elmer PCR automated thermocycler with the following cycles: 1.5 min at 94°C (denaturation), 1.5min at 60°C (primer annealing) and 1.5 min at 72°C (primer extension) followed by a post-PCR incubation during 10 min at 72 ° C. Negative controls (water instead of cDNA) were run in parallel. Ten micrograms preoptic RNAs and 10 μg retrochiasmatic RNAs were used for PCR. After 20 cycles, 5 μl of the preoptic medium were diluted at 1:75, 1:100, 1:150, 1:200 and 1:300 in the PCR mixture. PCR was allowed to run again to cycle 50 for the different dilutions of the preoptic product and for the retrochiasmatic product. The PCR products were studied through electrophoresis on 1.5% agarose gel in TAE buffer, stained with ethidium bromide and photographed under UV illumination. An electronic densitometric scanning of PCR product signals was directly processed from agarose gel under UV transilluminator with a black and white high-sensitive CCD camera connected to an IBM personal computer. Densitometric scanning of the β-actin bands at different PCR cycles (23-27) indicated that the extraction rates were similar in the preoptic and the retrochiasmatic explant. As specificity controls, PCR products from preoptic and retrochiasmatic mRNAS were digested with Eac 1, Hha 1 and Tag 1. This resulted in the following anticipated fragments: 162 and 213 bp, 231 and 144 bp, 321 and 54 bp as predicted by analysis of restriction sites (data not shown).

STATISTICAL ANALYSIS

The occurrence of significant pulses of GnRH secretion was determined using the Pulsar program [20, 21] as described previously [22]. The individual interpulse intervals and the mean ± SD intervals were calculated. The coefficient of variation (CV) of the interpulse interval was also calculated. The significance of difference in mean GnRH release evoked by veratridine or NMDA was calculated using the unpaired Student's t test.

Fig. 1. *Representative profiles of GnRH secretion by 4 individual explants of the retrochiasmatic hypothalamus (A) and the corresponding preoptic hypothalamus (B). The mean (± 1 SD) GnRH release evoked by veratridine (50 µM) and NMDA (50 mM) is studied using 6 individual retrochiasmatic (C) or preoptic (D) explants. The dashed line indicates the limit of sensitivity of the GnRH assay. • = Significant pulse detected by the Pulsar program.*
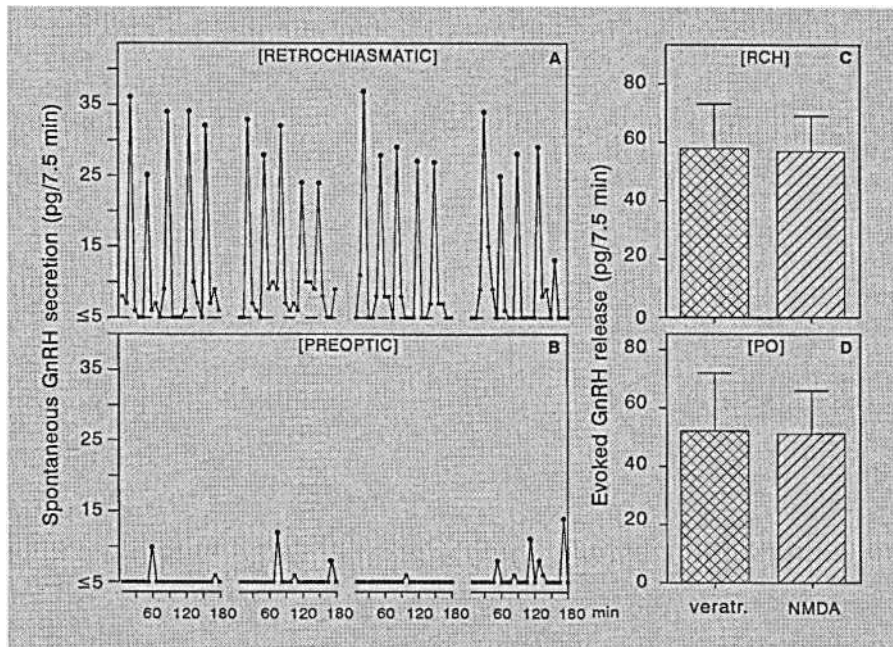

Fig. 2. *Representative profiles of GnRH secretion by 5 individual explants of the retrochiasmatic hypothalamus including the median eminence (A) or the median eminence only (B). The mean (± 1 SD) GnRH release evoked by veratridine (50 µM) and NMDA (50 mM) is studied using the retrochiasmatic (C) or median eminence (D) explants. The dashed line indicates the limit of sensitivity of the GnRH assay. • = Significant pulse detected by the Pulsar program.*
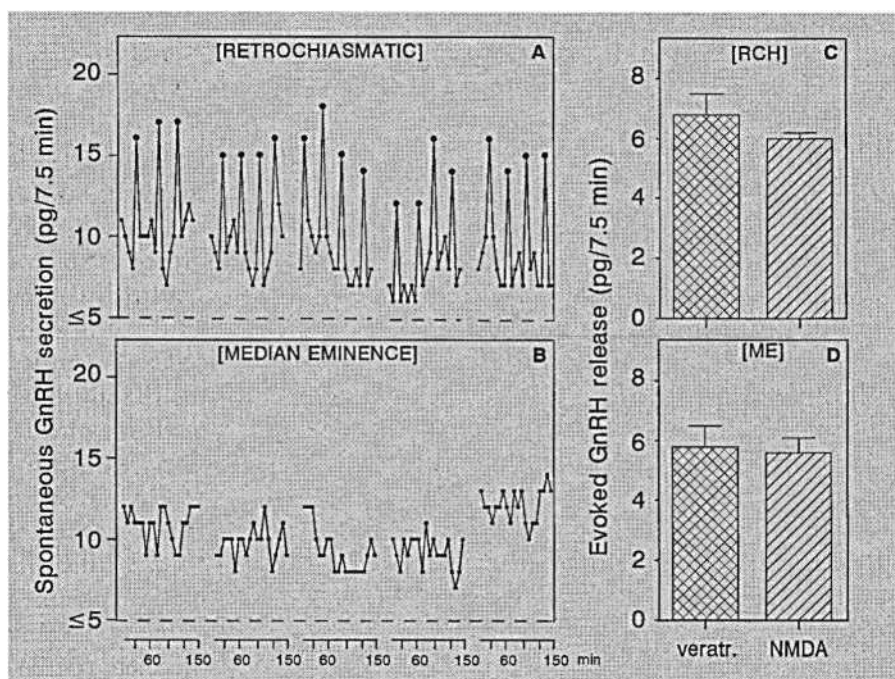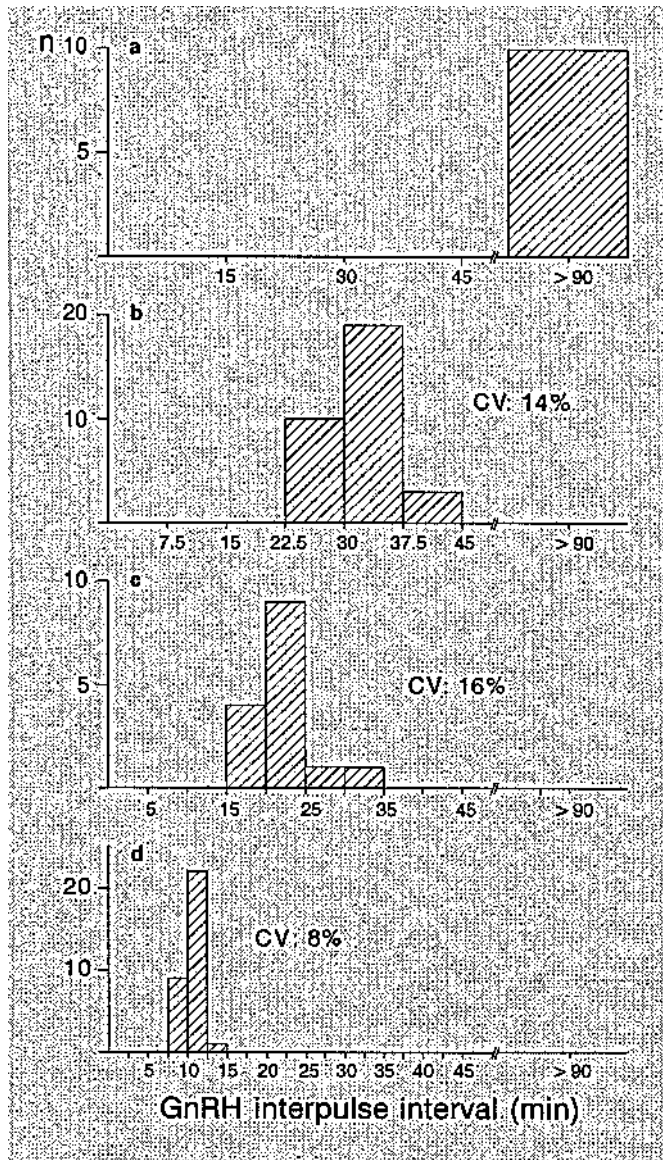
Fig. 3. *Distribution of GnRH interpulse intervals observed during static incubation of individual retrochiasmatic explants from 50- day-old rats and collection of fractions at intervals of 15 (a), 7.5 (b), 5 (c) or 2.5 min (d). The CV of the GnRH interpulse interval is given in each condition.*

Results

The retrochiasmatic explants exhibit a spontaneous pulsatile pattern of GnRH secretion (fig. 1A, 2A). The mean interpulse interval is 34.7 ± 3.9 min (fig. 1A) or 37.9 ± 4.5 min (fig. 2A). Using the preoptic explants, few irregular pulses of low amplitude are seen (fig. 1B). Using the median eminences, no GnRH secretory pulses are observed (fig. 2B). The release of GnRH evoked by veratridine or NMDA is similar using the retrochiasmatic (fig. 1C) and preoptic explants (fig. 1D). Likewise, a similar GnRH release is evoked by veratridine or NMDA using the retrochiasmatic (fig. 2C) and median eminence explants (fig. 2D). Using retrochiasmatic explants, the GnRH interpulse

interval is a function of the sampling interval (fig. 3). Using 15- min fractions, no more than one single pulse was secreted by each explant during a study period of 180 min. Therefore, the interpulse interval could not be calculated (>90 min). Using 7.5-, 5- and 2.5- min fractions, the interpulse intervals show a respective reduction: 37 ± 5, 25 ± 4 and 12 ± 1 min. In those conditions, the intervals between GnRH pulses are distributed in a relatively narrow range with a similar pattern peaking at 37.5, 25 and 12.5 min (fig. 3).

Immunocytochemical study of the preoptic explant shows the normal distribution of GnRH cell bodies in that region (fig. 4). The total number of cell bodies found in 7 preoptic explants varies between 212 and 420. Among the 7 retrochiasmatic explants studied thoroughly, only one GnRH cell body can be observed in three of them. These cell bodies are located at the border of the optic chiasm, in the vicinity of the section separating the 2 explants. In the median eminence area, the convergent network of GnRH fibers is observed.

Using RNAs extracted from preoptic explants, the RT- PCR reproducibly generates a (Lactin band of 632 bp and a GnRH band of 375 bp. Using RNAs from retrochiasmatic explants, the RT-PCR generates a (Lactin band and a slight GnRH band. Gel electrophoresis of the RT-PCR products (fig. 5) shows that the intensity of the GnRH band from retrochiasmatic extracts is similar to that obtained from the 1:200 diluted preoptic extract. Taking into account the difference in tissue weight and RNAs obtained from preoptic and retrochiasmatic explants, it can be estimated that GnRH mRNA is distributed in retrochiasmatic and preoptic explants in a ratio of about 1:600.

Fig. 4. *Rostrocaudal distribution of cell bodies of GnRH neurons in the hypothalamus of a 50-day-old male rat. Each bar represents the data from 3 adjacent coronal sections of 14 µm. The level of dissection between the preoptic and retrochiasmatic explants is indicated.*
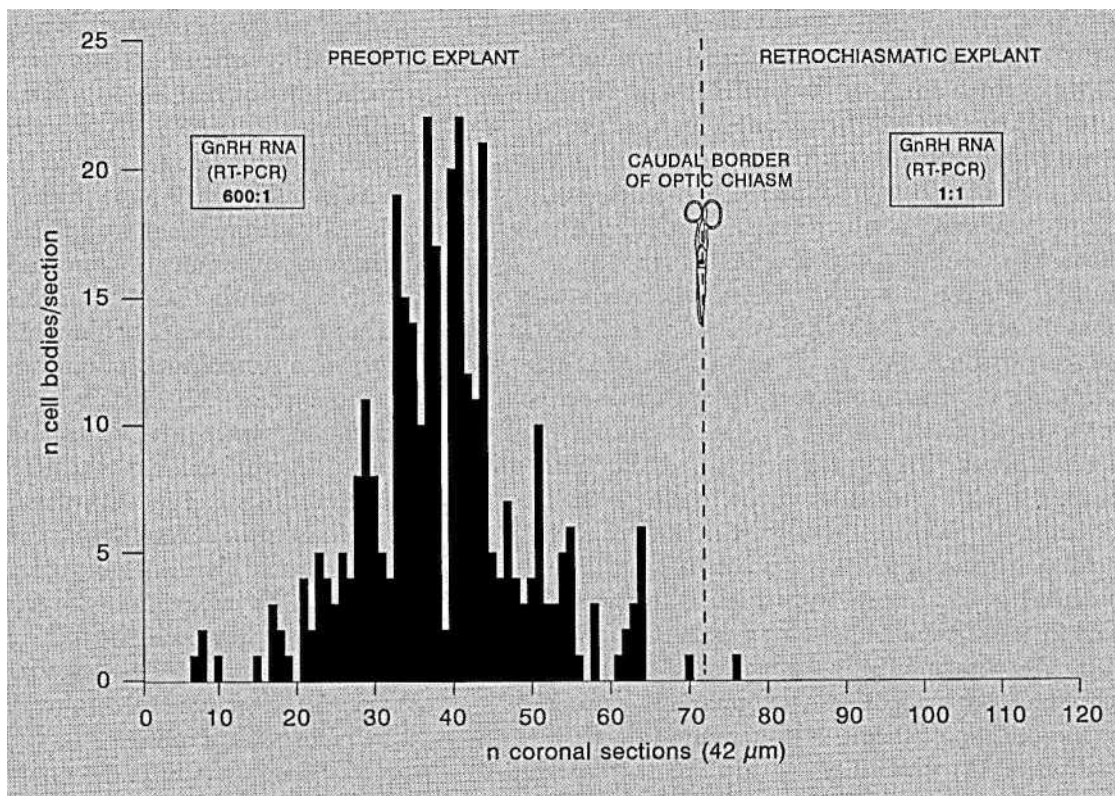
Fig. 5. *Electrophoresis of RT-PCR products (10 µl) from RNAs extracted from the retrochiasmatic (RCH) and preoptic (PO) parts of the rat hypothalamus. PM = 1-kb DNA ladder. For each band (A-G), the RNA origin, the number of PCR cycles and the studied concentration are shown. Densitometric analysis indicates that the amount of retrochiasmatic PCR products (B) is comparable to the 1:200 dilution of preoptic PCR products (F).*

	PM	A	B	C	D	E	F	G
origin		PO	RCH	PO	PO	PO	PO	PO
n cycles		45	50	50	50	50	50	50
concentr.		1/1	1/1	1/75	1/100	1/150	1/200	1/300

←375 bp
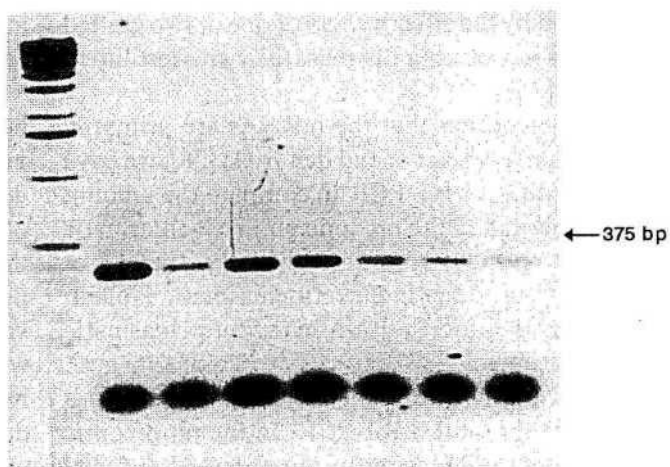
Discussion

In this paper, we show that retrochiasmatic explants of the rat hypothalamus secrete GnRH in a pulsatile manner in the absence of GnRH cell bodies. In contrast, only few irregular pulses are observed using explants of the preoptic hypothalamus where GnRH cell bodies are located and no pulses are seen using explants of the median eminence. The absence of GnRH cell bodies in the retrochiasmatic area of the rat hypothalamus is consistent with previous observations [9, 23-25]. We found a single GnRH cell body in some of the studied retrochiasmatic explants. This raises the question as to whether a single retrochiasmatic GnRH neuron may be sufficient to act as pulse generator. Pulsatile LH secretion can be restored in the hpg mouse by third-ventricular graft of fetal preoptic tissue containing as few as one single GnRH neuron [26]. In such experiments, however, it is possible that GnRH synthesized by a single neuron is stored in the median eminence in a sufficient amount for pulsatile secretion driven by a pulse generator in the arcuate nucleus-median eminence area. This hypothesis is supported by experiments in rats with hypothalamic deafferentation which show restoration of LH pulsatility by third ventricular graft of mediobasal hypothalamic tissue containing no GnRH neurons [27], The observation of GnRH pulsatility in the absence of GnRH cell bodies also raises the question as to whether synchronization or integrated activation of different GnRH neurons is a critical component of pulsatile GnRH secretion.

Synchronization may in fact occur at the axon-terminal level of GnRH neurons if the pulse generator involves the input of external neurons in the retrochiasmatic hypothalamus. Evidence against communication between GnRH cell bodies as a prerequisite to pulsatility is provided by the effectiveness of one or two grafted GnRH neurons in restoring LH pulsatility and fertility in the hpg mouse [26].

Based on the concept that the mRNAs are primarily located in neuronal perikarya and dendrites [28], we have used semi-quantitative RT-PCR to compare the amount of GnRH transcripts in the preoptic and retrochiasmatic explants. These results are consistent with the presence of very few GnRH cell bodies in the retrochiasmatic explant. The low amount of GnRH mRNA in the retrochiasmatic explant is in agreement with the observations of Kim et al. [18] who have used the mediobasal hypothalamus as a negative control for GnRH RT-PCR. In the rat preoptic area, hundreds of GnRH neurons are observed and distributed as described previously [9,24,25,27,29]. In this respect, the rat hypothalamus provides an original model to separate the GnRH cell bodies from their terminals since in other species such as man, the monkey and guinea pig, the GnRH neurons migrate more caudally in the retrochiasmatic area [30-32], The similar amplitudes of GnRH release evoked by veratridine and NMDA using the preoptic and retrochiasmatic explants attest that GnRH axon-terminals and NMDA receptors are present in these two regions and are still functional at the end of the in vitro experiment. This observation is consistent with the existence of GnRH axon projection to the median eminence in the retrochiasmatic explant as well as to the organum vasculosum of the lamina terminalis in the preoptic explant [33-35]. Thus, the absence of GnRH pulsatility using the preoptic explant cannot be explained by the absence of functional GnRH terminals since neurosecretion of GnRH can be evoked from this fragment.

The localization of a pulse generator mechanism at the GnRH terminal level was suggested by the observation of episodic GnRH release from the isolated rat median eminence as reported by Rasmussen et al. [12], However, using median eminence explants in the same conditions as the retrochiasmatic explants, we did not obtain any evidence of pulsatile GnRH secretion. This cannot be explained by an altered viability of the preparation since GnRH release could be evoked through depolarization or NMDA receptor stimulation in a manner which was similar to that seen using retrochiasmatic explants. Thus, while the median eminence appears to contain the components required for the autofeedback to be operational, pulsatility is not observed in our conditions. We cannot exclude that dissection of the median eminence has resulted in the loss of some anatomical structures involved in a pulse generator. Taken together, our data suggest the existence of a retrochiasmatic GnRH pulse generator mechanism which is external to the GnRH cell bodies and the GnRH terminals.

The question was raised whether methodological aspects such as periodic agitation when the incubation medium was renewed, could account for pulsatility. Indeed, the GnRH interpulse interval changed as a function of the sampling interval. Using a perifusion system where the samples were drawn at constant interval and varying flow rate, flow rate dependency of GnRH interpulse interval was observed as well [36]. Thus, mechanical factors were unlikely to be involved and the effect of sampling interval should not be biased by missing pulses not detected when that

interval was too long. While the frequency of pulsatile GnRH secretion seen in different experiments was reproducible, important variations in GnRH pulse amplitude were seen from one experiment to the other. It was therefore important to compare two types of explants in the same experiment as we did. A useful parameter in the evaluation of pulsatility is regularity of the process. This can be estimated through the distribution of GnRH interpulse intervals. While we found that the interpulse interval was distributed within 3-4 fractions of 7.5 min (coefficient of variation, CV: 8-16%), it was more variable in the study by Rasmussen [12] using the median eminence. From his data, we calculated a CV of 53-56%. That difference in CV is unlikely to be explained by the incubation system since Rasmussen et al. [37] observed a more regular periodicity using perifused human mediobasal hypothalami in vitro (CV: 35%). More recently, other authors also reported pulsatile GnRH secretion showing less variable intervals using the mediobasal hypothalamus than using preoptic explants [38], We tend to conclude that different mechanisms of episodic GnRH secretion may coexist because pulsatility is less regular using the median eminence than using the retrochiasmatic or mediobasal hypothalamus.

The mechanism of pulsatile GnRH secretion from the retrochiasmatic explants of the rat hypothalamus is not fully elucidated. The present data are consistent with an inhibitory autofeedback regulation of GnRH pulsatility. An autoregulatory mechanism of pulsatility is supported by the demonstration of an inhibitory autofeedback of GnRH which can affect the frequency of pulsatile secretion [39], possibly through NMDA receptor antagonism by 1-5 GnRH, a physiological degradation product of the secreted decapeptide [40].

The developmental increase in GnRH pulse frequency which precedes the onset of puberty, is observable using our in vitro paradigm. This indicates that not only a pulse generator but also the neuronal network involved in maturational changes are located in the retrochiasmatic area [7, 22, 39]. In agreement with our data, in vivo electrophysiological experiments suggest that a GnRH pulse generator is located in the rat arcuate nucleus instead of the median eminence or the preoptic area [41] and deafferentation studies show that integrity of the arcuate nucleus is required to maintain a pulsatile LH secretion [5, 6]. Such a concept is also consistent with our recent demonstration that antisense oligodeoxynucleotides for some subunits of NMDA or GABA receptors can affect pulsatile GnRH secretion in the absence of the preoptic area [42, 43]. Finally, it is of interest that mathematical modelization of the GnRH pulse generator has come to the conclusion that a minimal external neuronal unit with a facilitatory neuron and an inhibitory interneuron should be involved [44]. Virtually, every neuron has the capacity to function as a pacemaker. The challenge remains to determine where in the hypothalamus and which type of neurons will superimpose their periodic activity on that of the other neurons to result in the characteristic pulsatility of LH secretion.

Acknowledgments

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