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# Time-Related Neuroendocrine Manifestations of Puberty: A Combined Clinical and Experimental Approach Extracted from the 4th Belgian Endocrine Society Lecture

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**Key Words.** Neuroendocrinology · Puberty · Gonadotrophin-releasing hormone · Pulsatility

**Abstract.** The neuroendocrine manifestations of puberty converge on changes in GnRH secretion. Their appraisal through the assay of GnRH-like material in 24-hour urine extracts shows an increased excretion of this material in the late prepubertal period. The most striking pubertal changes in GnRH secretion occur on a circadian and ultradian basis. In man, they can be evaluated only indirectly. The circadian variations in LH and FSH secretion characteristic of puberty may be observed in timed fractions of 24-hour urine with some delay when compared to the variations of plasma levels. Studies on the frequency of pulsatile LH secretion and during chronic intermittent administration of GnRH support the existence of an increased frequency of GnRH secretory episodes at puberty. LH response to synthetic GnRH is directly related to the frequency of stimulation by endogenous GnRH pulses and provides a very useful index of neuroendocrine maturation in patients with delayed or precocious puberty. A direct evaluation of pulsatile GnRH secretion is possible using the rat hypothalamus in vitro. In these experimental conditions, the frequency of pulsatile GnRH release increases during very early stages of sexual maturation in the male rat. GnRH itself and beta-endorphin are inhibitory regulators of GnRH secretion in vitro and may participate in the mechanisms restraining the pulse-generating machinery in the hypothalamus before puberty.

## Introduction

Pediatricians and endocrinologists are frequently consulted about early or delayed occurrence of pubertal development. This has stimulated clinical and experimental research in order to clarify the biological mechanisms controlling the initiation of puberty. In 1929, indirect evidence of the role of pituitary gonadotrophins in puberty was obtained by Kallas [46]. Using parabiotic immature animals, he reported about occurrence of precocious puberty following castration. In 1952, the role of the hypothalamus in the control of sexual maturation was introduced by Harris and Jacobsohn [43]. These authors showed that fertility of hypophysectomized adult female rats was restored using pituitary grafts from immature rats. In 1971, gonadotrophin-releasing hormone (GnRH) was identified by the groups of Schally and Guillemin [26, 58] as the hypothalamic decapeptide controlling the secretion of pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH). More recently, a most elegant demonstration of the GnRH role in sexual maturation was provided by studying genetically hypogonadal mice. Implantation of fetal preoptic area (wherefrom GnRH secretory neurons originate) into the third ventricle [53] or introduction of an intact GnRH gene into the genome of these mutant mice [57] resulted in development of sex characteristics and fertility.

Since 1974, we have been interested in the evaluation of the hypothalamic control of sexual maturation and the understanding of its mechanisms. This research was primarily clinically oriented though it became soon obvious that a complementary experimental approach was required to evaluate directly some aspects of the hypothalamic control of pituitary gonadotrophins. The aim of this paper is to review some of our contributions to the understanding of the neuroendocrine manifestations of puberty with special emphasis on time-related mechanisms.



## GnRH-Like Material Excretion in 24-Hour Urine

A logical approach to evaluate the role of the hypothalamus in the regulation of pituitary-gonadal function is the development of appropriate techniques for the measurement of GnRH in biological fluids. We [11] and others [1, 62, 65] have developed highly sensitive and specific radioimmunoassays for GnRH. While GnRH is measurable in peripheral blood after administration of the synthetic decapeptide [1, 65], endogenous GnRH is undetectable in peripheral plasma [62]. Although extraction procedures have been used to work out the low peripheral levels of GnRH and its degradation, rapid fluctuations due to the short half-life of GnRH [65] and its episodic pattern of secretion [28] make single peripheral measurements useless. Additional difficulties arise from the dilution effect occurring between the hypothalamo-pituitary system and peripheral venous blood and from the existence of possible extrahypothalamic sources of GnRH.

Some investigators have shown that the kidney is an important site for GnRH metabolism, significant amounts of GnRH-like material being excreted in urine [65]. In addition, 24-hour urine collection could provide a means of integrating the rapid fluctuations of GnRH secretion. Thus, we have studied the nature of urinary GnRH-like material and found its immunoreactive and physicochemical properties consistent with a small proportion of intact decapeptide [12] whereas the 2–10 nonapeptide of GnRH is a major constituant of urinary GnRH-like immunoreactivity [13]. This material has been extracted and measured in 24-hour urine of normal subjects at different stages of life [13]. A 2-fold increase of GnRH-like material excretion is observed during the late prepubertal period, the values seen at 9 or 10 years being significantly higher ($p < 0.05$) than at 6 years. Comparing prepubertal and pubertal subjects, Rettig et al. [66] have reported a similar increase in immunoreactive GnRH excretion in urine. However, despite our efforts to characterize and measure urinary GnRH-like material, we have to conclude that the modest increase observed at puberty provides some, but limited insight into the hypothalamic mechanisms of puberty.

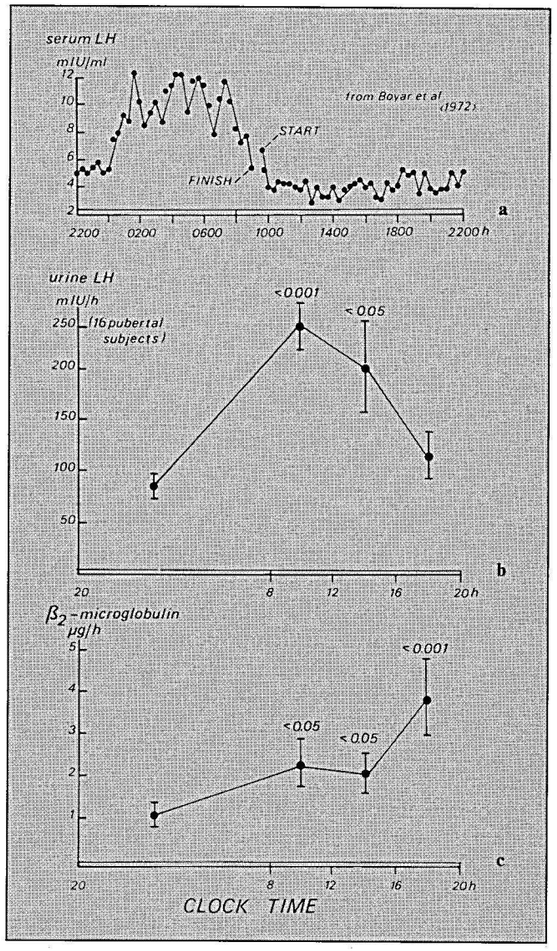


**Fig. 1. a** Variations of serum LH concentrations determined every 20 min throughout 24 h in an early pubertal boy [adapted from 25, with permission]. **b, c** Mean (± SEM) urinary excretion (calculated on an hourly basis) of LH and $\beta_2$-microglobulin in timed fractions of 24-hour urine collected from 20 to 8, 8 to 12, 12 to 16 and 16 to 20 h in 16 subjects at stage 2 to 4 of puberty according to Tanner [71].

## Gonadotrophins in Timed Fractions of 24-Hour Urine

Since the measurement of GnRH does not provide an optimal way of assessing directly the neuroendocrine manifestations of human puberty, an indirect approach has been considered through the study of pituitary gonadotrophins. In some instances, variations in plasma levels of FSH and LH may reflect the hypothalamic control of their secretion. A striking example is the sleep-related increase of LH and FSH secretion demonstrated by Boyar et al. [25]. This circadian pattern is particularly obvious for LH and exclusively observable throughout puberty. As shown in figure 1a representing data from an

early pubertal boy studied by Boyar et al. [25] in 1972, LH secretory pulses are superimposed over the increased level of release seen at night.

We have examined the possibility of studying the circadian pattern of gonadotrophin secretion by means of urinary measurements. Early studies by Kulin et al. [54] had concluded to higher concentrations of urinary gonadotrophins excreted at night than during daytime. Using four timed urinary fractions collected over a 24-hour period, we have shown [14] that, during pubertal development, urinary excretion of LH and FSH is the lowest at night while the highest excretion is seen between 8 and 12 in the morning (fig. 1b). This delay between the increase in serum LH levels and the rise in urinary excretion of LH may be related to an increased rate of kidney tubular reabsorption of the protein in resting conditions. Such an explanation is supported by the fact that $\beta_2$-microglobulin, a protein with a molecular weight close to that of FSH and LH and no circadian variations of its serum concentrations, has a lower rate of excretion at night than during daytime (fig. 1c). Thus, the measurement of FSH and LH in timed fractions of 24-hour urine provides a simple means of studying the sleep-related neuroendocrine manifestations of puberty. The circadian variations which are especially obvious at stage 3 of puberty according to Tanner [71], might be relevant for investigating disorders of puberty.

There are some important methodological aspects in the radioimmunoassay of urinary gonadotrophins: the need for a time-consuming extraction has stimulated our efforts to develop a method using unextracted urine [14]. Urinary immunoreactive material, particularly LH [5, 14], is heterogeneous and its nature can change in some pathological conditions, such as the increased secretion of α subunit in treated precocious puberty [55]. This emphasizes the importance of the specificity of the antibodies used. Also, the degradation of urinary immunoreactive material in frozen urine samples has been shown [61].

### Gonadotrophin Response to Synthetic GnRH as an Index of Exposure to Pulsatile GnRH Secretion

While the development of sex characteristics and the increase in gonadal steroid secretion take place over several years [27, 71], the most striking neuroendocrine events related to puberty occur as ultradian variations of LH and FSH secretion. The functional importance of the frequency of pituitary stimulation by GnRH has been well documented by the group of Knobil [52] who have studied castrated monkeys. Following a hypothalamic lesion, these monkeys are deprived of their endogenous hypothalamic generator of GnRH pulses. Therefore, they are dependent on intermittent administration of synthetic GnRH to maintain a proper level of LH and FSH secretion. Optimal frequency of stimulation has been determined according to the frequency of LH pulses seen every hour in castrated monkeys with intact hypothalamus.

Using prepubertal monkeys with lesioned hypothalamus, Wildt et al. [75] showed that puberty could be induced experimentally by long-term pulsatile administration of GnRH on an adult frequency basis of 1 pulse/h. More recently, a similar observation was made in monkeys treated intermittently with N-methyl-*D,L*-aspartate, an agonist of neuroexcitatory amino acids which triggers pulsatile release of GnRH [24a, 64]. In women with anorexia nervosa, pulsatile administration of synthetic GnRH for several days results in the progressive development of pubertal secretory levels of LH and FSH [56].

All those observations raised the hypothesis that an increase in the frequency of pituitary stimulation by GnRH could be a possible neuroendocrine mechanism controlling onset of puberty. This hypothesis is illustrated by some of the data reported by Knobil [52] as shown in figure 2b. When the frequency of GnRH stimulation is increased from a putative prepubertal pattern of 1 pulse/3 h to an adult pattern of 1 pulse/h, there is a slight reduction in plasma FSH while plasma LH increases progressively. These observations are remarkably similar to the variations in LH and FSH responses to synthetic GnRH (fig. 2a) that we reported in normal children at onset of puberty [41]. This indicates that the study of gonadotrophin responses to a bolus administration of synthetic GnRH may provide indirectly information about the neuroendocrine status and especially the frequency-related aspects of endogenous GnRH secretion. Such a concept is consistent with the positive regulation of pituitary GnRH receptors by pulsatile GnRH administration [48].

### GnRH-Gonadotrophin Interactions: Importance in Disorders of Puberty

In our conditions, the study of LH and FSH responses to GnRH has proven to be very useful in patients with delayed sexual maturation. While, after age 10, increased levels of FSH and LH, even when measured in a single blood sample, are helpful in diagnosing primary gonadal deficiency [34], the differential diagnosis between constitutional delay of puberty and gonadotrophin deficiency is usually difficult. Our findings in these patients are based on the fact that neuroendocrine manifestations of puberty precede its physical signs [16]. In boys with constitutional delay of puberty who present with no or only incipient signs of puberty, a pubertal pattern of LH response to GnRH can be observed in contrast with the low LH response seen in patients with hypogonadotrophic hypogonadism (fig. 3). This is of great prognostic value to reassure patients who will subsequently develop puberty or to start sex steroids in those who will need a replacement therapy. A similar approach is possible in hGH-treated patients. At prepubertal bone ages, those who are gonadotrophin deficient already have lower LH (fig. 3) and FSH responses than patients who will subsequently develop a spontaneous puberty [16].

While the development of a pubertal pattern of LH secretion seems to be related to an increase in the frequency of GnRH pulses, there is an optimal frequency required to maintain a normal adult level of gonadotrophin secretion. Further increase of the stimulation beyond that frequency may paradoxically result in a reduction of LH secretion through a desensitization process. This is consistent with data obtained in vitro using



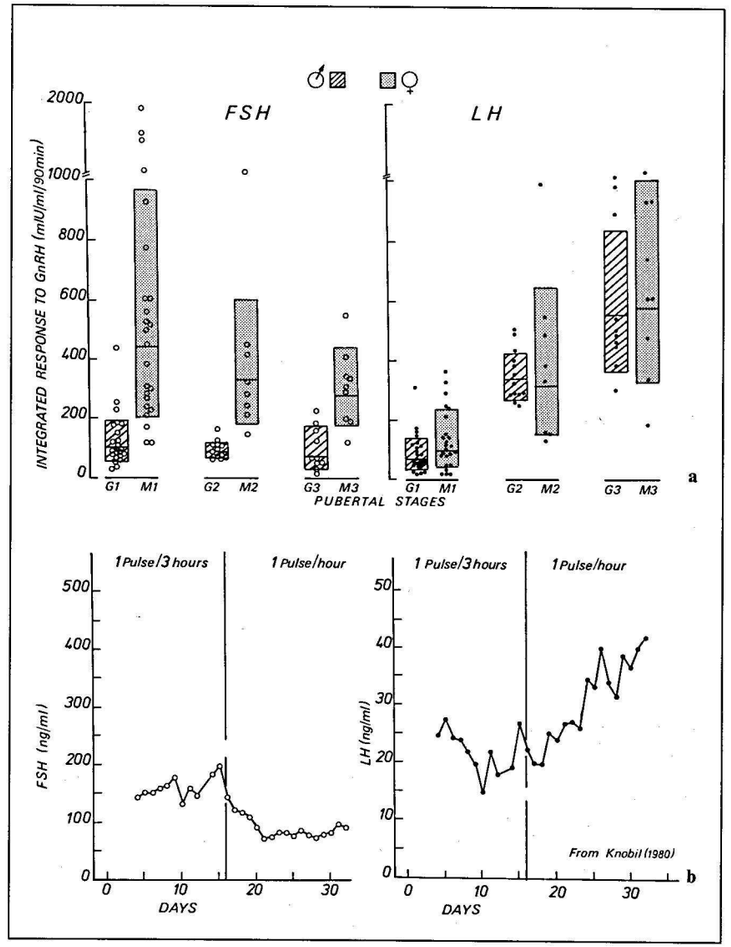


**Fig. 2. a** Individual values of FSH and LH integrated responses to intravenous bolus injection of GnRH 25 μg/m² in normal children of both sexes before puberty (genital score G1 or breast development stage M1 according to Tanner) or during early puberty (stage 2 and 3 of Tanner). Bars represent geometric means ± 1 SD. **b** Plasma levels of FSH and LH in a female ovariectomized monkey with lesioned hypothalamus receiving intermittent injections of synthetic GnRH at the frequency of 3 pulses/h for 17 days followed by 1 pulse/h during 15 days [adapted from 52, with permission].

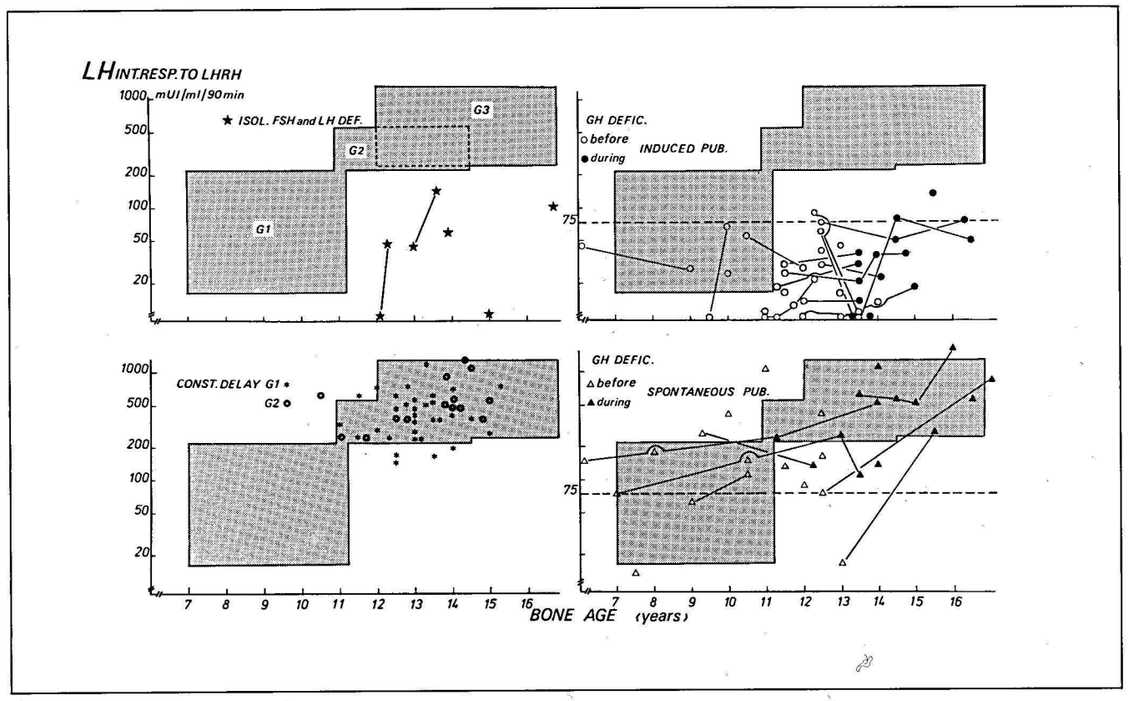


**Fig. 3.** LH integrated responses to GnRH 25 μg/m² (log. scale) in relation to age. Shaded rectangles represent mean ± 2 SD of the LH responses seen in control boys with prepubertal genital score (G1) or at stages 2 and 3 of puberty according to Tanner. Individual values are plotted in relation to bone age in 4 different groups of male patient: (1) isolated gonadotrophin deficiency, (2) constitutional delay of puberty before any physical evidence of puberty (G1) or with a testicular volume ≥ 4 ml as the only sign of puberty, (3) hGH-treated boys with associated gonadotrophin deficiency, before and during therapy with testosterone enanthate 100 mg/month, and (4) hGH-treated boys with normal gonadotrophin function, before and during spontaneous puberty. Symbols connected by lines represent patients retested longitudinally.

pituitary cell cultures [47] and in vivo in the monkey [6]. A fascinating application of this phenomenon is the use of superactive agonist analogs of GnRH in the treatment of central precocious puberty, which was initially proposed by Crowley et al. [35]. These patients are characterized by an early central maturation obvious from their pubertal pattern of LH response to GnRH. Intranasal or subcutaneous administration of a GnRH agonist results in a complete suppression of the pituitary response to a synthetic GnRH challenge [20, 35]. The pituitary gland is extremely sensitive to this effect since we found that LH response to GnRH was abolished during GnRH agonist therapy even when other biological and clinical parameters indicated only partial inhibition of the pubertal process [21]. An interesting finding is the increased serum levels of α subunit in patients treated with a GnRH agonist [55]. This may be related to the recent demonstration that expression of α subunit and β LH messenger RNA is regulated by the frequency of GnRH stimulation [42].

### Frequency of LH Secretory Episodes as an Index of Pulsatile GnRH Secretion

The pulsatile nature of GnRH secretion determines the pulsatile pattern of LH secretion as shown by LH measurements in peripheral plasma concomitant with GnRH measurements in sheep portal blood [31] and monkey cerebrospinal fluid [73]. Since the assay of GnRH in peripheral biological fluids does not provide direct information on pulsatile GnRH secretion, an indirect approach has been attempted through the study of pulsatility in LH secretion. In prepubertal children, only few data are available on account of the low peripheral levels of LH and FSH measured by radioimmunoassay and the requirement of several blood samples. More accurate data may be expected in the future using immunoradiometric assays which are more sensitive.



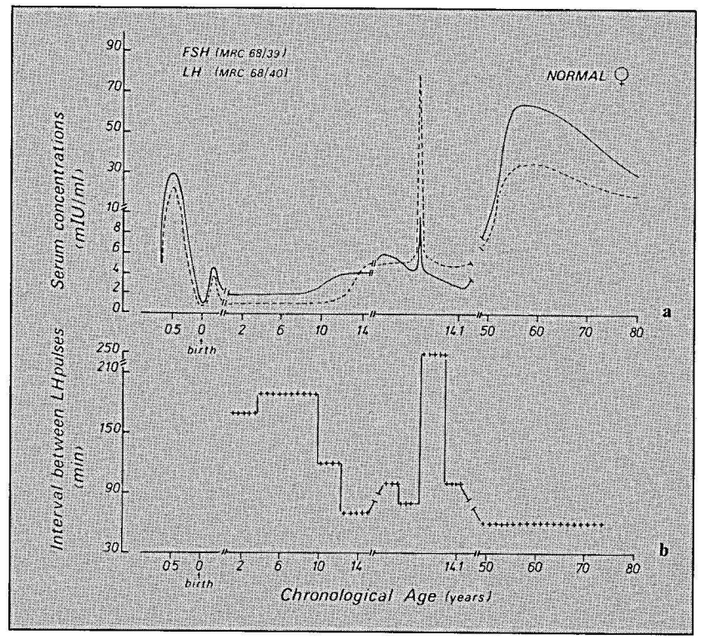


**Fig. 4.** Serum concentrations of LH and FSH (**a**) and interval between LH pulses (**b**) in normal females at different periods of life.

In figure 4, the age-related changes in serum concentrations of FSH and LH from fetal life to menopause are schematically illustrated [24]. The frequency of LH secretory episodes is also shown. Although the secretion of both gonadotrophins is pulsatile, the periodicity is more apparent for LH than for FSH on account of their different half-lives. This is the reason why LH has been preferred to FSH in analyzing pulsatility. In very young infants [74] and in prepubertal children after 2 years of age [45], the mean interval between LH pulses is around 180 min. However, a higher frequency has been reported in the former group with premature thelarche [4]. From 10 years onwards, LH pulses can be observed every 120 min [45] whereas, during puberty, this interval decreases to 70 min [3, 25, 63]. During the menstrual cycle, LH pulse frequency is the highest during late follicular phase and the lowest during early luteal phase [2, 77].

In the absence of gonadal steroids as a result of physiological situations such as menopause [77] or pathological conditions like gonadal dysgenesis [67], LH pulse frequency is increased. In agonadal patients [67] or castrated monkeys [7], the frequency of LH pulsatility which is already increased at prepubertal ages, does not further rise at the age of normal puberty while gonadotrophin levels increase at that time. These studies suggest that an increased amplitude of GnRH secretion may also contribute to the central mechanism of puberty. While the frequency of LH may be regarded as an index of GnRH neurosecretion, the amplitude of LH pulses is influenced by the amplitude of GnRH secretory pulses [76] and by direct interactions of gonadal steroids at the pituitary level as well [37].

### Pulsatile GnRH Secretion: Experimental Approach

From the studies on pulsatile LH secretion and LH response to administration of synthetic GnRH, the concept of an increased frequency of GnRH secretion at puberty has been put forward. In order to go thoroughly into that hypothesis, experimental models are required. We have elicited to use the male rat. This rodent shows a rapid increase in testicular weight starting by the age of 3 weeks while an adult testicular weight is achieved by 2 months of age [17].

The major part of immunoreactive GnRH extracted from the rat hypothalamus is identical to the synthetic decapeptide [12, 13, 19, 20]. Between 21 and 60 days of age, hypothalamic GnRH content shows a 3-fold increase which is not immediately affected by castration while hypothalamic GnRH content is rapidly depleted following orchidectomy in adults [17]. This suggests that the increase in hypothalamic GnRH content with age reflects a process of central maturation independent of the presence of the gonads. In a pituitary monolayer culture system, GnRH-like bioactivity of hypothalamic extracts is dose-related and the ratio bioactivity/immunoreactivity is similar in extracts from 21-and 50-day-old rats [19]. This indicates that measurement of immunoreactive GnRH is a suitable approach of the activity of GnRH secretory neurons at different ages.

We have examined the possibility of studying pulsatile GnRH secretion in vitro. In rats, the GnRH pulse generator system is localized electrophysiologically in the arcuate nucleus where GnRH axons are projected to the median eminence [49]. In contrast, no periodic electrical activity is detected in the preoptic area where most GnRH perikarya are localized [49]. Complete hypothalamic deafferentiation in vivo does not affect the pulsatile nature of LH release as long as the arcuate nucleus is not included in the section [70].

On that basis, pulsatile GnRH secretion was assumed to be observable in vitro from the isolated retrochiasmatic hypothalamus. Using one single nonfragmented hypothalamic explant per incubation chamber, we have been able to demonstrate that GnRH secretion is intermittent in vitro [15]. This finding has been confirmed later by two other groups [51, 60]. In the presence of EGTA, a calcium-chelating agent or D-600, a blocker of calcium channel, the occurrence of GnRH secretory pulses is markedly reduced [22]. The calcium dependency suggests that an active neurosecretory process is involved in the pulsatile release of GnRH in vitro. In addition, glucose concentration affects the spontaneous release of GnRH and its response to veratridine, a depolarizing agent [22].

The frequency of GnRH pulses observed in vitro (2.5 pulses/2 h at 50 days of age) is greater than that expected according to LH pulse frequency in intact adult male rats [38]. Possible explanations include the occurrence of more GnRH pulses than LH pulses, some GnRH pulses being not effective in inducing pituitary LH release [32]. An alternative explanation is the suppression of some inhibitory pathways by the disconnection of the hypothalamus from the rest of the brain [33].

## Pulsatile GnRH Secretion: Mechanisms and Age-Related Variations

As shown in figure 5, pulsatile release of GnRH in vitro can be observed from hypothalami of male rats at 12 days of age. A similar pattern of secretion is seen at 17 days. Pulse amplitude is significantly increased at 21 days. The most striking change is the increased frequency seen from 23 and 25 days of age (data not shown). At that time and at 27 days, GnRH pulse frequency is similar to that observed at 50 days. This indicates that increased frequency of GnRH secretion may occur very early during sexual maturation in the male rat [18].

Superactive agonist analogs of GnRH show an inhibitory effect on pulsatile release of GnRH in vitro [22], in agreement with the reduction in LH pulse frequency following intracerebroventricular administration of GnRH [36]. While other studies confirm the autoregulatory effect of GnRH on its own secretion [68, 72, 78], our data emphasize the ultrashort loop feedback effect on pulsatility. Thus, GnRH might play a role in the regulation of its pulsatile release. This warrants further research to evaluate if the sensitivity of the hypothalamus to that ultrashort loop feedback decreases with age and may contribute to the increased frequency of GnRH release.

In the hypothalamus, endogenous opiate peptides, particularly β-endorphin, exert an important inhibitory control upon gonadotrophin secretion [29]. Involvement of opiate peptides in the hypothalamic mechanism of puberty is suggested indirectly by the age-related differences in response to naloxone. Opiate antagonists cause minimal or no increase of LH secretion in immature male rats [10, 30, 44] as well as in prepubertal children [40, 59, 69], while naloxone or naltrexone have a marked stimulatory action on LH release in mature animals [10, 30, 44] and pubertal subjects [40, 59, 69]. These findings do not fit with a high activity of endogenous opiate peptides in the hypothalamus before puberty since, in this case, a greater LH response to naloxone should be expected. More consistent with this hypothesis is the decreased inhibitory effect of an enkephalin analog at puberty in the male rat [8]. A possible explanation for the puberty-related increase in responsiveness to naloxone is the facilitatory role played by gonadal steroids [9].

Studies on LH pulsatility [39] and electrophysiological discharges of the hypothalamic pulse generator [50] indicate indirectly the inhibitory effect of opiate peptides on GnRH pulsatility. Using hypothalami of 50-day-old male rats, we have shown that β-endorphin 1 n*M* resulted in an obvious suppression of the pulsatile pattern of GnRH release [23]. These data are consistent with an intrahypothalamic inhibitory control of GnRH pulsatility by endogenous opiate peptides which may be a neurochemical basis for the hypothalamic 'brake' controlling the onset of puberty. In addition, neuroexcitatory amino acids which stimulates the secretion of GnRH through hypothalamic receptors [24a, 64] may be involved in the hypothalamic mechanism of puberty.



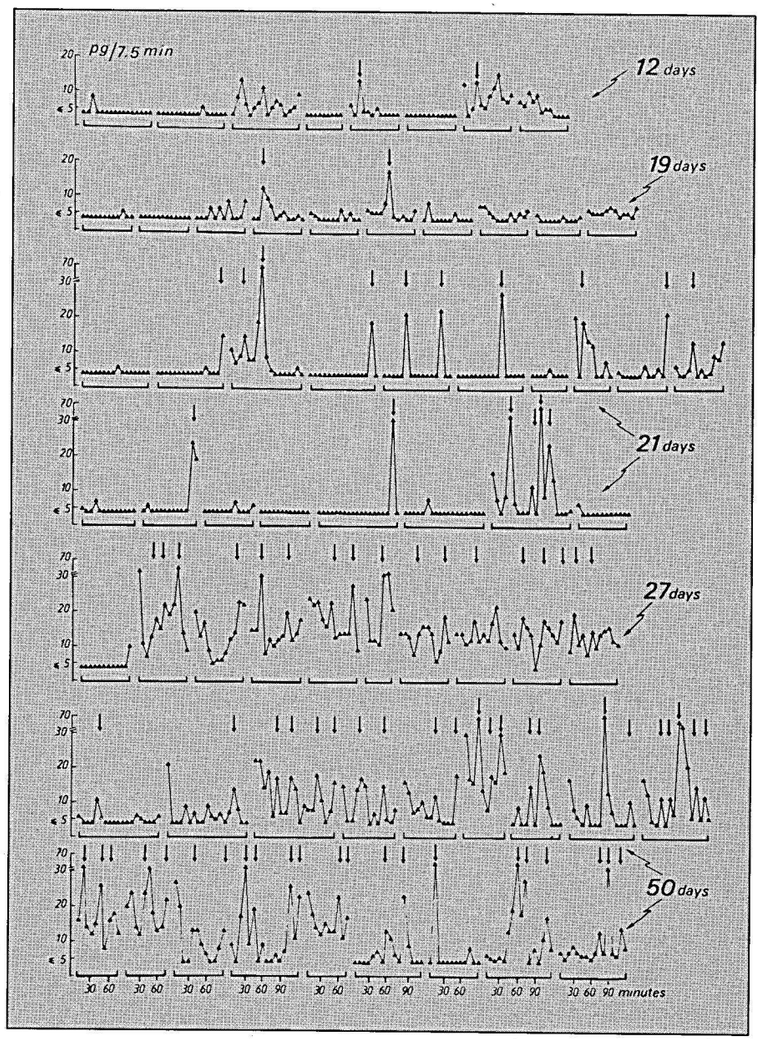


**Fig. 5.** Time-related release of GnRH in vitro from individual hypothalamic explants of male rats at different ages. Horizontal bars represent the study period for one hypothalamus. The arrows denote significant pulses [from 18, with permission].

In summary, time plays a very different role in the central and peripheral manifestations of puberty. The development of sex characteristics and the pubertal growth spurt are relatively time-insensitive processes taking place over years and occurring in several pathologic conditions irrespective of the pattern of exposure to sex steroids [19a, 24b]. In contrast, pubertal changes in pituitary responsiveness to hypothalamic GnRH stimulation are exquisitely sensitive to the frequency of pulsatile GnRH secretion. Therefore, puberty can be regarded as a matter of minutes in the brain and not even a matter of years in the bones.

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