# Maturation of the Hypothalamic Control of Pulsatile Gonadotropin-Releasing Hormone Secretion at Onset of Puberty: II. Reduced Potency of an Inhibitory Autofeedback*

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**ABSTRACT.** At the time of onset of puberty in the male rat, between 15 and 25 days of age, we have reported that pulsatile GnRH secretion by hypothalamic explants showed an increased frequency as indicated by the reduction of the mean interpulse interval from 66 to 40 min ($P < 0.05$). This study aimed to evaluate whether these changes in GnRH secretion involved a self-regulatory mechanism. A 7.5-min exposure of explants obtained at 50 days to 0.1 $\mu$M superagonist D-TRP$^6$-PRO$^9$-$N$-Et-GnRH (GnRH-A) resulted in a delay of the next GnRH secretory pulse so that the mean interpulse interval increased from 35 to 67 min ($P < 0.001$). In addition, after a 7.5-min exposure to GnRH-A, there was a 15-min period with absent or reduced release of GnRH in response to 50 $\mu$M veratridine, a depolarizing agent. A similar refractory period of 15 min was observed using explants obtained at 25 and 50 days whereas, at 15 days, the period of refractoriness lasted for 52.5 min. The inhibitory effect of GnRH-A on the subsequent response to veratridine occurred at similar concentrations of GnRH-A at the three studied ages and the inhibition was prevented using an antagonist of GnRH

together with GnRH-A. The involvement of GnRH itself in an autofeedback mechanism was evaluated by studying the period of refractoriness separating two GnRH pulses elicited by 7.5-min exposures to veratridine. The initial responsiveness to veratridine was recovered after a refractory period of 52.5, 22.5, and 15 min when studied at 15, 25, and 50 days, respectively. While refractoriness occurred during repeated depolarization with K$^+$ or veratridine, such an effect was not observed using $N$-methyl-D-aspartate (NMDA). During exposure to GnRH-A, the NMDA-induced release of GnRH was only reduced by 38% whereas veratridine-induced secretion showed a 94% reduction. Thus, exogenous activation of NMDA-sensitive receptors could bypass the inhibitory autofeedback. We conclude that: 1) pulsatile GnRH secretion is controlled by an inhibitory autofeedback involving NMDA sensitive receptors, 2) the increased frequency of pulsatile GnRH secretion at onset of puberty may be related to a reduced sensitivity of the hypothalamic pulse generator to an inhibitory autofeedback. (*Endocrinology* **127**: 2884–2890, 1990)

R ECENTLY, we and others provided evidence of an ultrashort inhibitory feedback of GnRH on its own secretion (1–5). The functional significance of that hypothalamic autofeedback was suggested by the inhibition of pulsatile secretion of LH or GnRH *in vivo* after intracerebroventricular administration of GnRH or an agonistic analog of the decapeptide (4, 5). However, no role of GnRH autofeedback in a physiological process was documented yet.

In the male Wistar rat, puberty takes place between 25 and 50 days of age as shown by the age-related increase in testis weight and the concomitant increase in the relative proportion of elongated spermatids. Such mature forms of germ cells were first seen in rat testicular cells

at 25 days and attained an adult level by 50 days of age (6). Using hypothalamic explants obtained at those ages, we have shown that the frequency of pulsatile GnRH secretion *in vitro* was slow at 15 days of age whereas a nearly 2-fold increased frequency was seen at 25 days (6, 7). By the end of puberty, at 50 days, the explants released GnRH at a frequency similar to that observed at 25 days. We hypothesized that the secretory pulses of GnRH could exert an autofeedback control on the hypothalamic pulse generator. Accordingly, the reduced interval between GnRH secretory pulses seen at onset of puberty might involve a reduced sensitivity to an autoregulatory control of GnRH. To test this hypothesis, we studied the inhibitory effects of GnRH on its own secretion using hypothalamic explants at different stages of sexual maturation.

## Materials and Methods

*Animals, incubation of hypothalamic explants, and RIA of GnRH*

The retrochiasmatic hypothalamic explants obtained in male Wistar rats aged 15, 25, and 50 days were studied *in vitro* after

a procedure described in detail previously (1, 8). Briefly, the explants were dissected and transferred to the incubator within 30 sec to 1 min after decapitation. During each experiment, 12 explants were immersed in 12 individual chambers containing 0.5 ml Eagle's Minimum Essential Medium (Flow, Mc Lean, VA) enriched with glycine (10 nM), magnesium (1 mM), and glucose (25 mM). This was a static perifusion system, the total volume of incubation medium being sampled and renewed every 7.5 min. GnRH was measured in the collected fractions using the highly specific RR-5 antiserum kindly provided by Dr. A. Root (St. Petersburg, FL). This RIA procedure has been described previously (1, 7). The limit of detection was 5 pg/7.5-min fraction which was the value assigned to data below that limit. The coefficient of variation for both the sampling procedure in the incubator and the RIA was 14%.

### Study protocols

*Effect of GnRH-A on pulsatile GnRH secretion at 50 days.* The pulsatile secretion of GnRH from eight explants obtained at 50 days was evaluated during a 3.75-h period. After 2 h of experiment, culture medium enriched with 0.1 $\mu$M D-TRP$^6$-PRO$^9$-$N$-Ethylamide GnRH (GnRH-A), a GnRH superagonist (Dr. J. Rivier, Salk Institute, San Diego, CA) was used for only one interval of 7.5 min. Subsequently, the standard culture medium was again used until the end of the experiment. GnRH was measured in the collected 7.5-min fractions. We showed previously that a 0.1 $\mu$M concentration of GnRH-A did not result in any significant cross-reactivity in the RIA of GnRH (1). The occurrence of significant GnRH secretory pulses was determined using the PULSAR program (9, 10) as described previously (8).

*Effects of GnRH-A on the veratridine-induced secretion of GnRH: specificity, importance of age, and GnRH-A concentration.* Hypothalamic explants (n = 12) of 50-day-old rats were exposed to different peptides for two consecutive periods of 7.5 min. During the second 7.5-min period, 50 $\mu$M veratridine (Sigma, St. Louis, MO), a depolarizing agent, was added to the medium and the increment of GnRH secretion over the pretreatment level was calculated. The response to veratridine was studied in control conditions and in the presence of the following peptides: 1 $\mu$M 1-44 human GRF (Sigma), 1 $\mu$M SRIF (Labaz-Sanofi, Brussels, Belgium), 10 $\mu$M TRH (UCB, Brussels, Belgium), 10 $\mu$M arginine-vasopressin (UCB, Brussels, Belgium), 1 $\mu$M oxytocin (UCB, Brussels, Belgium), 0.01 $\mu$M D-SER (TBU)$^6$-PRO$^9$-$N$-Ethylamide, a GnRH agonist (Buserelin, Hoechst AG, Frankfurt, FRG) and 0.01 $\mu$M GnRH-A without or together with 0.01 $\mu$M Ac-D-$\beta$NAL$^1$-D-$p$-Cl-PHE$^2$-D-PHE$^3$-SER$^4$-TYR$^5$-D-ARG$^6$-PHE$^7$-ARG$^8$-PRO$^9$-D-ALA$^{10}$, a GnRH antagonist (BIM 21009, IPSEN International, Paris, France). At the concentrations used, none of those peptides resulted in a significant inhibition of labeled GnRH binding to the antiserum.

Using 12 explants obtained at 15, 25, and 50 days, the effects of different concentrations (0.0001–0.1 $\mu$M) of GnRH-A on the veratridine-induced secretion of GnRH was studied.

*Effects of time after exposure to GnRH-A on the subsequent response to veratridine.* Using explants obtained at 15, 25, and 50 days (12 in each group), the secretion of GnRH induced during a 7.5-min exposure to 50 $\mu$M veratridine was studied after a 7.5-min exposure to 0.1 $\mu$M GnRH-A. This study was repeated after different time intervals between exposures to GnRH-A and veratridine. In an initial experiment using explants obtained at 15, 25, and 50 days, that time interval was progressively reduced from 22.5 to 0 min. In a subsequent experiment, time intervals decreasing from 60 to 0 min were used to further study explants obtained at 15 days.

*Effects of time after a first exposure to veratridine on the response to a second veratridine challenge.* Using explants obtained at 15, 25, and 50 days (12 in each group), the secretion of GnRH induced by veratridine (50 $\mu$M) was studied on two occasions after different time intervals between the two veratridine challenges. In an initial experiment using explants obtained at 15, 25, and 50 days, that time interval was progressively reduced from 30 to 7.5 min. In a subsequent experiment, time intervals decreasing from 60 to 7.5 min were used to further study explants obtained at 15 days. For those experiments with different time intervals, the same explants were used repeatedly.

*Involvement of N-methyl-D-aspartate (NMDA)-sensitive receptors in the autofeedback.* Four explants obtained at 50 days were exposed repetitively (every other 7.5-min period) to 50 mM NMDA (Sigma), 50 mM K$^+$, and 50 $\mu$M veratridine in order to evaluate the refractoriness of GnRH secretion to these different stimuli. In addition, using 12 explants obtained at 50 days, the increase in GnRH secretion induced by 50 mM NMDA or 50 $\mu$M veratridine was studied during simultaneous exposure to 0.1 $\mu$M GnRH-A.

### Statistical analysis

The significance of differences in GnRH interpulse interval was calculated using paired Student's $t$ test. The significance of differences in GnRH response to veratridine was calculated using Scheffé's test for multiple comparison with analysis of variance (11).

## Results
### Effect of GnRH-A on pulsatile GnRH secretion at 50 days

As shown in Fig. 1, the pulsatile secretion of GnRH from 50-day hypothalamic explants was inhibited by a 7.5-min exposure to GnRH-A. Before exposure to the agonist, the mean ($\pm$SD) interval between GnRH pulses was 34.7 $\pm$ 6.0 min. Exposure to GnRH-A for 7.5 min resulted in a 2-fold increase in the interpulse interval (66.6 $\pm$ 10.9 min, $P < 0.001$). GnRH-A worked as a bioactive nonimmunoreactive substitute of a GnRH secretory pulse resulting in a delay of the next endogenous GnRH pulse which occurred 36.6 $\pm$ 7.4 min after exposure to GnRH-A. Subsequently, the normal frequency of GnRH pulsatility was restored (mean interval: 32.5 $\pm$ 6.5 min).

### Effects of GnRH-A on the veratridine-induced secretion of GnRH: specificity, importance of age, and GnRH-A concentration

The increment of GnRH secretion induced by 50 $\mu$M veratridine (mean $\pm$ SD: 11.4 $\pm$ 3.9 pg/7.5 min) was not
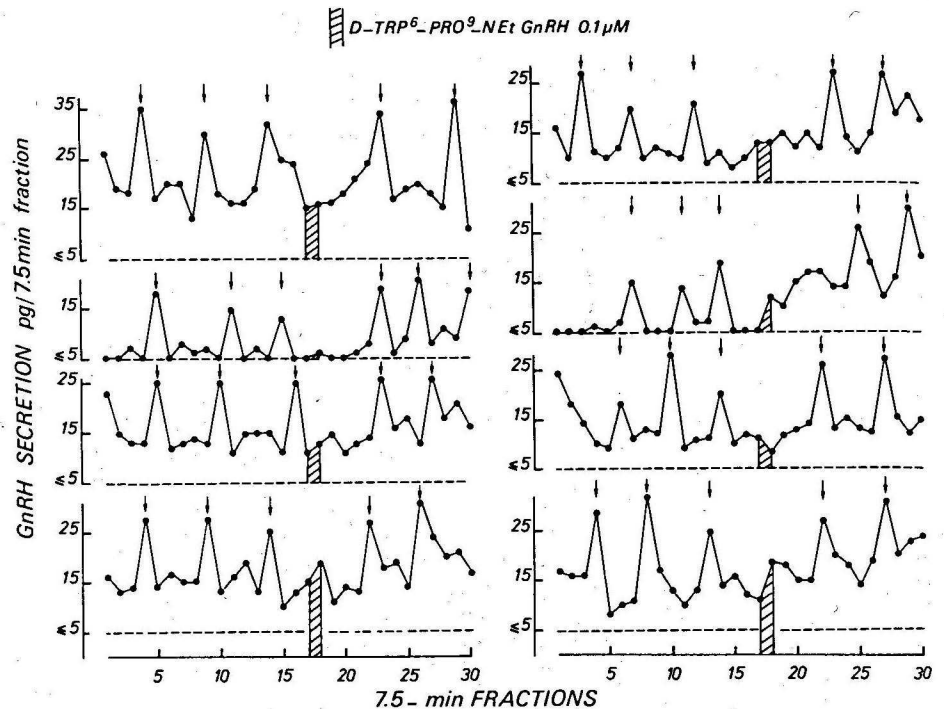
FIG. 1. Individual profiles of pulsatile GnRH release from eight 50-day hypothalamic explants before, during, and after a 7.5-min exposure to 0.1 $\mu$M GnRH-A. Total study period is 225 min. The *arrows* denote significant secretory pulses. The *dashed lines* indicate the detection limit of the assay.

significantly affected in the presence of micromolar concentrations of hGRF, SRIF, TRH, AVP, and OXY (Table 1). In contrast, incubation in the presence of two different superagonists of GnRH (0.01 $\mu$M) resulted in a reduction (83–87%) of the response to veratridine. This inhibitory effect was completely prevented by simultaneous incubation of the agonist with equimolar concentrations of BIM 21009, a GnRH antagonist (Table 1).

As shown in Fig. 2, similar GnRH-A concentrations of

TABLE 1. Effect of hypothalamic hormones and GnRH analogs on the veratridine-induced increment of GnRH secretion from hypothalamic explants at 50 days

| Peptide | Concentration ($\mu$M) | Increment of GnRH secretion (% of controls, mean ± SEM, n = 12) |
|---|---|---|
| Controls | | 100.0 ± 9.9 |
| hGRF | 1 | 94.7 ± 16.0[a] |
| SRIF | 1 | 85.5 ± 13.7[a] |
| TRH | 10 | 112.2 ± 19.8[a] |
| AVP | 10 | 124.4 ± 19.8[a] |
| OXY | 1 | 95.4 ± 15.3[a] |
| Buserelin | 0.01 | 12.7 ± 2.6[b] |
| GnRH-A | 0.01 | 16.8 ± 3.8[b] |
| GnRH-A + | 0.01 | |
| BIM 21009 | 0.01 | 100.0 ± 13.8[a] |

[a] P NS *vs.* controls.
[b] P < 0.001.

0.01 and 0.1 $\mu$M were required at 15, 25, and 50 days to suppress the secretion of GnRH in response to veratridine. There was no significant difference between the data obtained at the three studied ages.

*Effects of time after exposure to GnRH-A on the subsequent response to veratridine*

Immediately after the exposure to GnRH-A or after a time interval of 7.5 min, there was a suppression of the GnRH secretion induced by veratridine (Fig. 3). This effect occurred similarly at 15, 25, and 50 days. At 25 and 50 days, the response to veratridine was partially but not completely recovered when evaluated 15 min after exposure to GnRH-A whereas, after 22.5 min, the response to veratridine was no longer significantly different from that seen before exposure to GnRH-A. In contrast, at 15 days, exposure to GnRH-A was followed by a refractory period of 52.5 min before recovering the pretreatment response to veratridine (Fig. 3).

*Effects of time after a first exposure to veratridine on the response to a second veratridine challenge*

As shown in Fig. 4, when explants obtained at 15, 25, and 50 days were exposed to veratridine on two occasions separated by a 7.5-min interval of time, there was a suppression of the secretion of GnRH in response to the second veratridine challenge. At 25 and 50 days, the pretreatment response to veratridine was recovered after a period of 30 and 22.5 min, respectively. In contrast, at
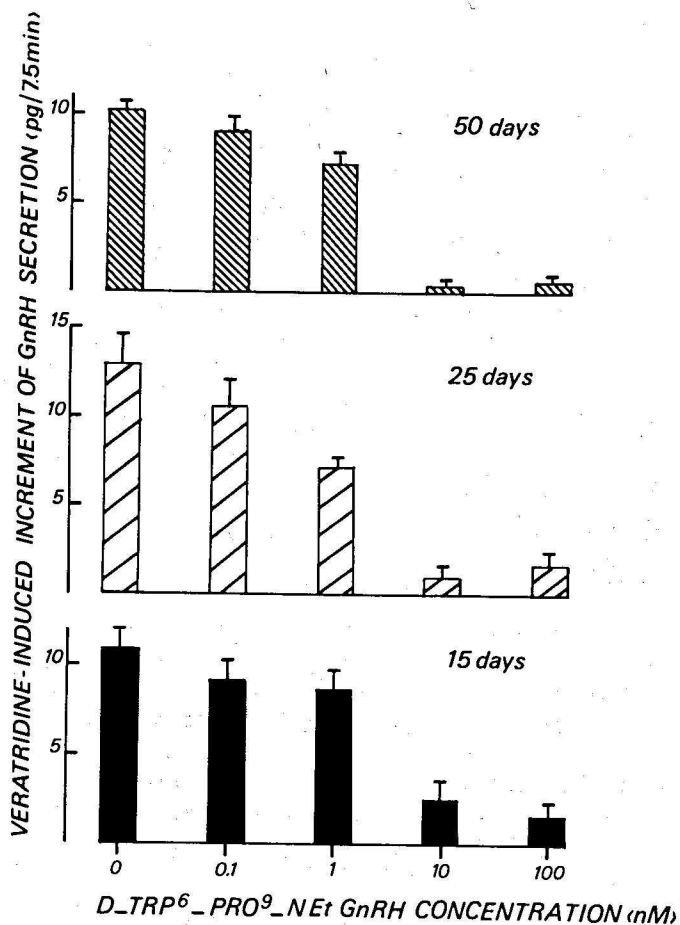
FIG. 2. Using 12 hypothalamic explants obtained at 15, 25, or 50 days of age, the effect of different concentrations of GnRH-A is studied on the increment of GnRH secretion (mean ± SEM, n = 12) induced by a 7.5-min exposure to veratridine (50 μM). In this experiment, veratridine and the GnRH-A were used together at the same time.

15 days, there was a 52.5-min period of refractoriness before recovering a response to veratridine not different from that seen initially.

*Involvement of NMDA-sensitive receptors in the autofeedback*

As shown in Fig. 5, repeated exposure of hypothalamic explants to 50 mM NMDA every other 7.5-min period resulted in an increase in GnRH secretion which did not change significantly with time. In contrast, using 50 mM K⁺ or 50 μM veratridine, the increase in GnRH secretion seen in response to the initial stimulation was followed by a suppression ($P < 0.001$) of the response to the subsequent challenges. During exposure to GnRH-A, the increment in GnRH secretion induced by 50 mM NMDA was partially reduced (62 ± 4% of controls, mean ± SEM) but significantly less impaired ($P < 0.001$) than the response to veratridine which was totally suppressed (6 ± 3% of controls).



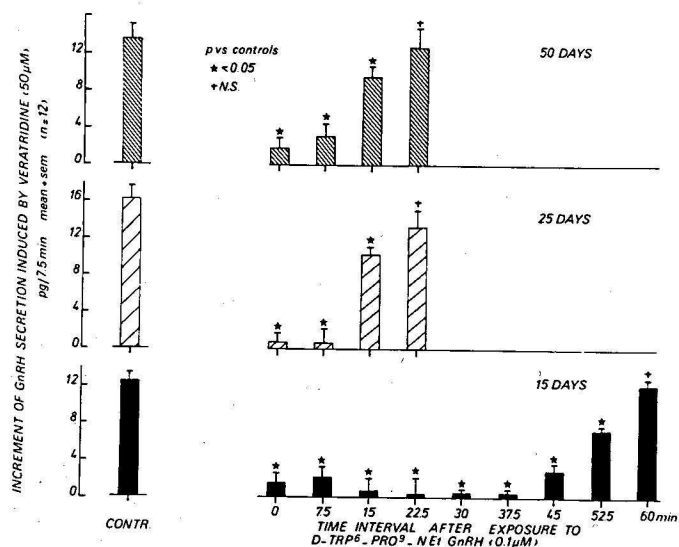FIG. 3. Using 12 hypothalamic explants obtained at 15, 25, and 50 days, the effect of a 7.5-min exposure to 0.1 μM GnRH-A on the subsequent response to 50 μM veratridine is studied after different time intervals.
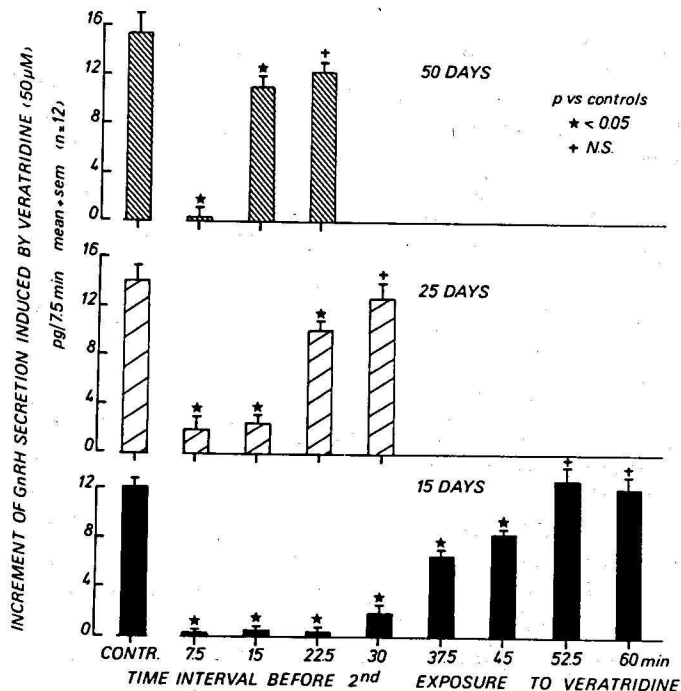


FIG. 4. Using 12 hypothalamic explants obtained at 15, 25, and 50 days, the effect of an initial 7.5-min exposure to 50 μM veratridine on the secretion of GnRH induced by a second exposure to veratridine is studied after different time intervals between the two veratridine challenges.

## Discussion

In this paper, we provide a direct evidence of the involvement of an inhibitory autofeedback in the physiological control of GnRH pulsatile secretion. Between 15 and 25 days of age which is before onset of puberty, this inhibitory autofeedback shows a reduced potency
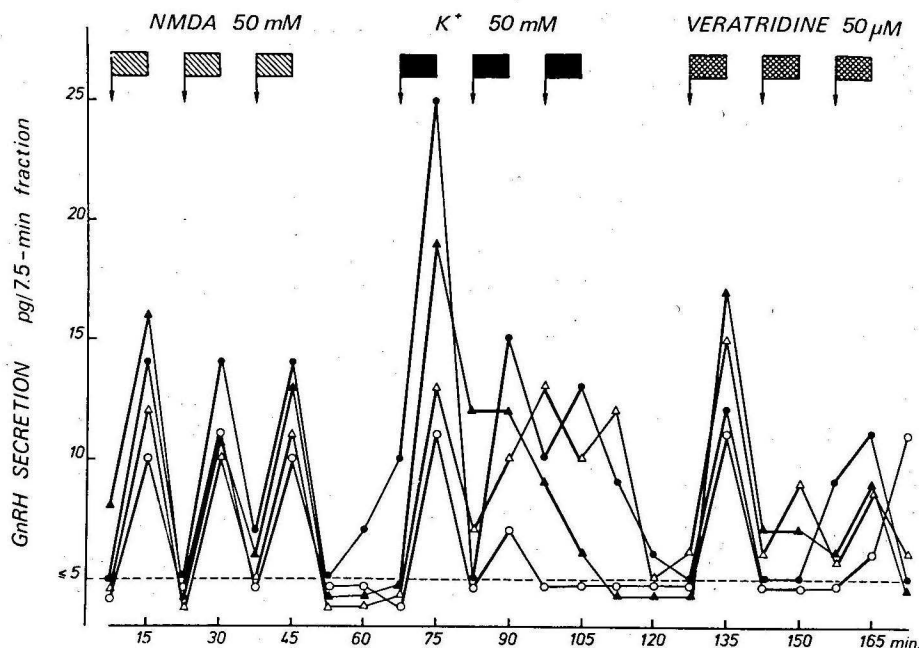
FIG. 5. Effects of repeated exposures to NMDA, K⁺, or veratridine on the secretion of GnRH from four hypothalamic explants obtained at 50 days.

which is concomitant with the reduction of the interval between GnRH secretory pulses. There is a close temporal relationship between the duration of the refractory period determined by the autofeedback and the variations in frequency of pulsatile GnRH secretion at the different ages studied (6). An increase in frequency of pulsatile GnRH secretion at onset of puberty has been observed in different species (3, 6, 12–14). In the male rat, the increase in frequency of pulsatile GnRH secretion and the reduction in sensitivity to GnRH autofeedback take place before onset of puberty, between 15 and 25 days of age whereas no further changes are observed throughout puberty, between 25 and 50 days. This sequence of events suggests that the changes in sensitivity to GnRH autofeedback characterize the neuroendocrine mechanism of onset of puberty. This is in contrast with some parameters such as the hypothalamic GnRH content which shows a steady increase during the first 2–3 months of life in male rats (7, 15, 16).

The superagonists of GnRH offered an interesting opportunity of studying the autofeedback since most of these peptides do not cross-react in the assay of GnRH while they have an increased biopotency. In this study, we confirm the previous demonstrations of an inhibitory action of the agonists upon GnRH secretion (1). As reported here and by others (3, 4), this effect is specific since it is electively blocked by a GnRH antagonist. The threshold concentration of GnRH agonist for an effective autofeedback was found to be not different among the three ages studied. The refractory period resulting from the autofeedback showed a different duration with age, irrespective of the agonist concentration. This suggests that the variations in GnRH autofeedback observed at puberty do not result from changes in intensity of the feedback signal. Such a concept is consistent with the fact that age and puberty only account for small differences in amplitude of spontaneous GnRH pulses or in response to veratridine (6, 7). However, our experiments do not exclude minor differences in sensitivity related to smaller variations in GnRH concentration than those studied in this paper.

Whereas the hypothalamic effect of GnRH agonists may raise some questions with respect to the clinical use of those agents, this pharmacological observation does not imply a physiological role of GnRH autofeedback. This is why we looked for a possible refractory period after the induction of an endogenous GnRH secretory pulse by veratridine. As a matter of fact, the use of veratridine was followed by a period of refractoriness which was similar to that seen after exposure to a GnRH agonist. These data are consistent with the fact that, during a 30-min incubation of explants with veratridine, the initially increased secretion of GnRH returns to the prestimulation level after 15 min despite the continuing presence of veratridine (data not shown). In addition, the autofeedback may account for the rebound increase of GnRH secretion reported after a depolarization using veratridine (17). This observation may reflect the reactivation of the pulse generator after the refractory period caused by the veratridine-induced secretion of GnRH. Thus, our data provide strong evidence of a physiological GnRH autofeedback in the rat. However, such a mechanism may not exist in other species, as suggested by recent data obtained in castrated rams (18).

The anatomical localization of the site of GnRH autofeedback is not elucidated. Dendro-dendritic and axo-dendritic contacts between GnRH neurons have been found in the preoptic area and in the arcuate nucleus and the median eminence as well (19–21). GnRH receptors have been identified in the brain though the hypothalamus contains few binding sites (22, 23). Those studies support a central effect of GnRH but they do not help in determining where this effect takes place. Since the hypothalamic explants used in our studies do not contain the preoptic area where the vast majority of GnRH perikarya are located (21), it is very likely that there are at least some more posterior sites involved in the autofeedback. The use of GnRH agonists *in vivo* or *in vitro* results in an inhibition of GnRH secretion from the mediobasal hypothalamus *in vitro* (1, 2) whereas, using explants of median eminence, such an effect is observed only after *in vivo* administration of the agonist (5). These findings suggest that the autofeedback does not take place directly at the terminals in the median eminence. A putative view of the autofeedback system is that some GnRH axons may project to the GnRH pulse generator which controls the secretory activity of GnRH axons at a presynaptic level in the arcuate nucleus. In such a system, it is possible that only GnRH supplied directly by the axons to the site of autofeedback could induce the autoregulatory mechanism. This is consistent with the observation that increased concentrations of GnRH in culture medium resulting from dissection of the explant are not followed by a period of refractoriness to a depolarization (data not shown). In addition, single or pulsatile iv administration of GnRH to the rat *in vivo* does not prevent the occurrence of spontaneous LH pulses (24, 25).

The mechanism of GnRH autofeedback is unknown. Kesner *et al.* (26) reported that a single iv administration of GnRH agonist in the monkey did not alter the electric manifestations of the pulse generator in the arcuate nucleus. In agreement with our previous findings (6, 27), we showed that exposure of rat hypothalamic explants to NMDA every other 7.5 min resulted in repeated pulses of GnRH secretion showing a similar amplitude with time. Thus, in contrast to the refractoriness observed after repeated depolarization using $K^+$ or veratridine, activation of NMDA receptors could bypass the autofeedback mechanism. In addition, the NMDA-induced secretion of GnRH was only partially reduced during exposure to a GnRH agonist. The partial inhibition observed might reflect the reduced secretion of endogenous neuroexcitatory amino acid which may contribute to the response to an exogenous substrate such as NMDA. This concept is supported by our previous studies on the NMDA-induced secretion of GnRH (6). At 25 days, when endogenous activation of NMDA receptors is maximal, the explants are more sensitive to NMDA stimulation than at 15 or 50 days, indicating that the level of endogenous activation of NMDA receptors affects the response to NMDA.

Since the use of exogenous substrates of the NMDA receptors prevented the autofeedback, at least partially, it is likely that endogenous neuroexcitatory amino acids are involved in mediating the autofeedback. We have shown recently that neurons secreting excitatory amino acids and controlling pulsatile GnRH release are transiently activated at the time of onset of puberty (6). Further studies are required to show whether the reduction in sensitivity to the inhibitory autofeedback of GnRH occurring at onset of puberty involves a difference in activation of NMDA receptors.

The electrophysiological basis of maturation of the pulse generator and the role of the autoregulatory feedback in that process have to be elucidated. It is likely that the target of the autofeedback and the primary event of hypothalamic maturation at onset of puberty are closely related. This is why it is critical to further study the autoregulatory feedback which can provide a clue of the central mechanism of puberty.

## Acknowledgments

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