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Maturation of the Hypothalamic Control of Pulsatile Gonadotropin-Releasing Hormone Secretion at Onset of Puberty. I. Increased Activation of *N*-Methyl-D-Aspartate Receptors*

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ABSTRACT. In the male rat the timing of puberty can be estimated by the rapid increase in testicular weight occurring between 25–50 days of age. We found that elongated spermatids, the most mature germ cells identified using flow cytometry, were first seen at 25 days (4% of the testicular cells), while an adult proportion (63%) was attained by 45 days of age. We have shown previously that hypothalamic explants could release GnRH in a pulsatile fashion at a frequency increasing around the age of 25 days, thus consistent with the time of onset of puberty. Since pulsatile GnRH secretion could be suppressed by MK-801, a noncompetitive antagonist of *N*-methyl-D-aspartate (NMDA) receptor activation, we postulated that an increased activation of those receptors could be involved in the neuroendocrine mechanism that activates pulsatile GnRH secretion at the onset of puberty. Such a concept was supported by the NMDA-induced release of GnRH, which was observed using 1 mM NMDA at 25 days, while a dose of 20–50 mM was required at 15 or 50 days of age. MK-801 could provide an index of NMDA receptor activation, since the antagonistic effect of MK-801 is use dependent. This particular property was confirmed by the inability of MK-801 (5 pM) to block the depolarization (veratridine)-induced release of GnRH in the presence of 0.001 mM NMDA, while partial or complete suppression was obtained in the presence of 0.1 and 10 mM NMDA, respectively. Using explants obtained at 5, 10, 15, 20, 25, 30, 35, and 50 days of age, the lowest concentrations of MK-801 that blocked the veratridine-induced release of GnRH were, respectively, 10^7, 10^7, 10^7, 10^3, 10, 10^2, 10^4, and 10^8 pM. In contrast, there was no age-related difference in sensitivity to the inhibitory effect of Mg^{2+}, a noncompetitive NMDA receptor antagonist which is not use dependent. The pulsatile secretion of GnRH occurred at a similar frequency at 25 and 50 days of age (4.7 and 5.4 pulses/3.5 h, respectively) but it was suppressed by a lower MK-801 concentration at 25 days (10^4 pM) than at 50 days (10^8 pM). These data indicate that the NMDA receptors involved in the control of pulsatile GnRH secretion are markedly and transiently activated around the time of onset of puberty in the male rat. (*Endocrinology* **127:** 873–881, 1990)

PUBERTY comprises a sequence of events thought to be initiated in the hypothalamus, where a pulse generator drives the intermittent secretion of GnRH, as evidenced by periodic electrophysiological discharges in the arcuate nucleus correlated with LH secretory episodes (1). In the absence of the preoptic area, where the cell bodies of GnRH neurons are located (2), the pulse generator keeps modulating the secretory activity of GnRH axons, since the pulsatile secretion of GnRH can be observed using retrochiasmatic hypothalamic explants *in vitro* (3, 4).

In several species, including primates, the neuroendocrine mechanism of puberty involves particularly an increased frequency of pulsatile GnRH secretion (3, 5). This process can possibly result from the activation of an excitatory pathway. Such a role has been proposed for the *N*-methyl-D-aspartate (NMDA)-sensitive receptors on the basis of pharmacological experiments using acute (6, 7) or chronic administration of NMDA (8, 9). In addition, we reported recently that MK-801, a noncompetitive antagonist of NMDA receptors, could suppress the pulsatile secretion of GnRH (10). In this study we took advantage of the use-dependent nature of MK-801 action (11) to obtain an index of the endogenous agonistic activity at the NMDA receptors involved in GnRH secretion with the aim of demonstrating a possible increase in that activity at the onset of puberty.

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Materials and Methods

Animals, incubation of hypothalamic explants, and RIA of GnRH

Male Wistar rats (Janssens, Beerse, Belgium) were studied at 5, 10, 15, 20, 25, 30, 35, and 50 days of age. The timing of

puberty was determined using two-parameter flow cytometry (12) and sorting rat testicular cells obtained at different ages between 20–70 days. Eight cell compartments were observed; one was consistent with elongated spermatids, the most mature germ cells identified using this technique. The proportion of those cells relative to the total amount of testicular cells was used as an index of testicular maturation.

The procedure of hypothalamic explant incubation has been described in detail previously (4, 10). Briefly, in each experiment, 12 retrochiasmatic hypothalami were dissected and incubated individually in static chambers containing 0.5 ml Eagle's Minimum Essential Medium (Flow, Maclean, VA), which was renewed every 7.5 min for 4 h. This medium was enriched with glycine, magnesium, and glucose to achieve final concentrations of 10 nM, 1 mM, and 25 mM, respectively. Using the highly specific RR-5 anti-GnRH antiserum generously supplied by Dr. A. Root (St. Petersburg, FL), GnRH was measured in duplicate on 0.1-ml samples from the 0.5-ml fractions collected every 7.5 min. The limit of detection was 5 pg/7.5-min fraction, which was the value assigned to data below that limit. The characteristics of this RIA procedure have been described previously (3, 4).

Study protocols

In a first set of experiments the pulsatile secretion of GnRH was evaluated over a 3.5-h period using hypothalamic explants of 15-, 25- and 50-day-old rats. We had previously reported preliminary data from a similar study (3). That experiment was repeated because a different culture medium was used in the present study. Testicular weight was measured to evaluate the time of the onset of sexual development. As stated above, testicular morphology has also been studied between 20–70 days of age.

In a second set of experiments the secretion of GnRH from hypothalamic explants of 15-, 25-, and 50-day-old rats was studied in the presence of increasing concentrations (0.1–50 mM) of NMDA (Sigma, St. Louis, MO). Each NMDA concentration was used for a 7.5-min period, preceded by two 7.5-min periods under control conditions. The results were expressed as the increments in GnRH secretion, which were the differences between the concentrations of GnRH measured in the medium before and during exposure to NMDA.

In a third set of experiments we used (+)-5-methyl-10,11-dihydro- 5H-dibenzo [a,d]-cyclohepten-5,10-imine maleate (MK-801), a noncompetitive NMDA receptor antagonist (13) generously supplied by Merck, Sharp, and Dohme Research Laboratories (Rahway, NJ). Using explants obtained in 50-day-old animals, the effect of MK-801 (5 pM) on the increment in GnRH secretion induced by 50 μM veratridine (Sigma), a depolarizing agent, was studied in the absence or presence of different concentrations of NMDA. The hypothalamic explants were first exposed to NMDA for 7.5 min. Then, during two subsequent 7.5-min periods, MK-801 and veratridine were used together with NMDA. These experiments were carried out to show whether the concept of use dependency was relevant to the effects of MK-801 on GnRH secretion by hypothalamic explants.

In a fourth set of experiments we studied the effects of different concentrations of MK-801 (0.1–10^9 pM) on the secretion of GnRH induced by either 50 mM NMDA (explants obtained at 15, 25, and 50 days) or 50 μM veratridine (explants obtained at 5, 10, 15, 20, 25, 30, 35, and 50 days). By comparison with the use-dependent inhibitory action of MK-801 on the veratridine-induced release of GnRH, the effect of 1–4 mM magnesium, a noncompetitive antagonist of NMDA receptors which is not use dependent, was studied at 25 and 50 days. For each antagonist concentration, 12 explants were studied. Based on the concept of use dependency, these experiments were carried out to evaluate the degree of NMDA receptor activation through the concentrations of MK-801 required for antagonizing the release of GnRH mediated through those receptors.

In a fifth set of experiments the pulsatile pattern of GnRH secretion was studied over a 3.5-h period using hypothalamic explants of 25- and 50-day-old rats. The explants obtained at 25 days were studied under control conditions or in the presence of 10^2, 10^3, and 10^4 pM MK-801. During each experiment, 6 control explants were compared to 6 explants exposed to MK-801. After having discarded the data obtained using explants accidentally shaken or fragmented during the experiment, a total of 28 explants were studied under control conditions, while 6–12 explants were studied using each concentration of MK-801. Using explants obtained at 50 days of age, the effects of 10^6 and 10^7 pM MK-801 on pulsatile GnRH secretion were studied in addition to the data reported previously using MK-801 doses of 10^4 and 10^8 pM (10).

Pulse and statistical analyses

The occurrence of significant GnRH secretory pulses was estimated using an IBM-PC-compatible version (14) of the Pulsar program (15). The B coefficient used to determine the assay SD was 14%, which is the coefficient of variation of both the incubation and RIA procedures. The cut-off criteria for peak identification were 2.5 (G1) and 2.0 (G2), as determined empirically. Pulse frequency was calculated as the number of significant pulses observed over 3.5 h, which was the study period for each explant. The interval between pulses was not used because it could not be calculated in experiments with only one pulse observed during the study period. To avoid biasing the data by the detection limit, GnRH pulse amplitude was calculated only for pulses occurring when the basal secretory rate of GnRH was equal to or above 5 pg/7.5 min. The significance of the differences in NMDA- or veratridine-induced GnRH release or the differences in frequency of pulsatile GnRH secretion within a single experiment or between the different age groups was calculated using Student's *t* test or Scheffe's test for multiple comparisons with analysis of variance, respectively (16).

Results

Puberty-related changes in pulsatile GnRH release

As shown in Fig. 1, the frequency of pulsatile GnRH release increased significantly ($P < 0.001$) between 15 and 25 days of age from 1.8 ± 0.6 pulses/3.5 h [mean ± 1 SD; n (of explants) = 12] to 4.7 ± 0.8 pulses/3.5 h (n = 28). This increase in GnRH pulse frequency was concomitant with the time when testicular weight started to increase rapidly (Fig. 1). In addition, the age of 25 days was concomitant with the primary observation of elongated spermatids in the testis, while at 50 days of age, an adult proportion of those cells was observed (Table 1). At 50 days, GnRH pulse frequency (5.4 ± 1.2 pulses/3.5 h; n = 33) was not significantly different from that seen at 25 days. Using the pulse data obtained when the basal secretory rate of GnRH was detectable, the mean amplitude of GnRH secretory pulses increased significantly ($P < 0.05$) between 15 days (8.4 ± 2.5 pg; n = 14) and 25 days (10.6 ± 2.9; n = 95) as well as between 25 days and 50 days (12.4 ± 6.4; n = 112).

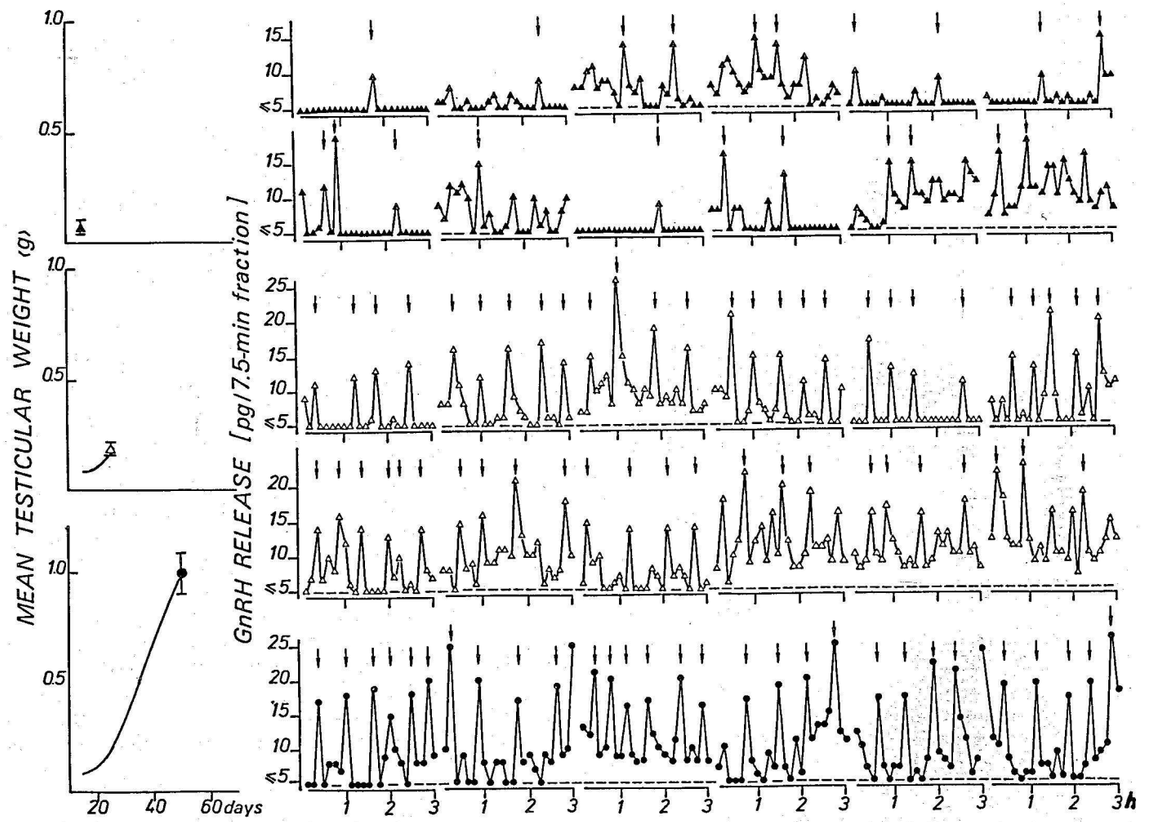

FIG. 1. *Right panels*, GnRH release in 7.5-min fractions collected for 3.0 h during incubation of individual hypothalamic explants obtained at 15 days (▲), 25 days (△), and 50 days (●) of age. The *arrows* denote significant secretory pulses. The *dashed lines* indicate the detection limit of the assay. *Left panels*, Testicular weight (mean ± 1 SD) increase in relation to age from 15–50 days.

Age-related changes in sensitivity of GnRH secretion to NMDA

As shown in Fig. 2, very high concentrations of NMDA (20–50 mM) were required to obtain a significant incre-

TABLE 1. Age-related changes in the relative proportion of elongated spermatids in rat testicular cells analyzed using two-parameter flow cytometry

	Age (days)								
	20	25	30	35	40	45	50	60	70
Elongated spermatids (% of testicular cells)	0	4 ± 2	21 ± 5	23 ± 4	44 ± 4	63 ± 3	66 ± 5	67 ± 2	69 ± 3

Values are the mean ± SD; n = 5.

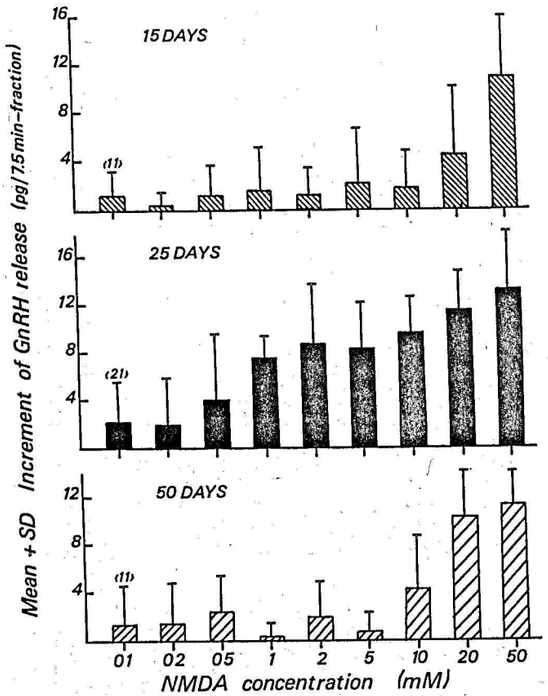

FIG. 2. Mean (±1 SD) increment of GnRH release induced by increasing NMDA concentrations from hypothalamic explants of 15-, 25-, and 50-day-old male rats. The number of explants studied in each group is indicated in *parentheses*.

ment of GnRH release at 15 and 50 days. At 25 days, the greater sensitivity to NMDA ($P < 0.001$) was illustrated by the significant GnRH release induced by lower NMDA concentrations (≥1 mM).

Use-dependent nature of MK-801 inhibitory effects on GnRH secretion

As shown in Fig. 3, using hypothalamic explants of 50-day-old rats, the release of GnRH induced by veratridine was not significantly affected by MK-801 (5 pM) when studied in the absence of NMDA or in the presence of a low NMDA concentration (0.001 mM). In the presence of a higher NMDA concentration (0.1 mM), a partial suppression by MK-801 of the veratridine-induced release of GnRH was seen ($P < 0.05$). A further increase in NMDA concentration (10 mM) resulted in an abolition by MK-801 of the GnRH release induced by veratridine ($P < 0.001$).

Age-related differences in inhibitory effects of MK-801 and Mg^{2+} on the veratridine-induced release of GnRH

As shown in Fig. 4, very low concentrations of MK-801 (1–5 pM) could block the release of GnRH induced by 50 mM NMDA. Using this high agonist concentration, the explants obtained at 25 days were 5- to 10-fold more sensitive to the inhibitory effects of MK-801 than the explants of 15- and 50-day-old rats ($P < 0.005$).

Using veratridine instead of NMDA to elicit the release of GnRH, it was possible to study the effect of the endogenous substrates activating the NMDA receptors on the sensitivity to MK-801 (Fig. 5). At 5, 10, and 15 days of age, the veratridine-induced release of GnRH was blocked by 10^7 pM MK-801. At 20 days, there was a marked increase in sensitivity to MK-801 ($P < 0.001$), since a 10^3-pM concentration could block the release of GnRH induced by veratridine. The sensitivity was maximal at the age of 25 days ($P < 0.001$ *vs.* 20 days), since a 10-pM concentration of MK-801 resulted in a suppression of GnRH release. At 30 days, the sensitivity to MK-801 decreased significantly ($P < 0.05$ *vs.* 25 days), with a further reduction at 35 days. At 50 days, the dose-related inhibitory effect of MK-801 was similar to that seen at 15 days. Thus, the explants obtained at 25 days were 1,000,000 times more sensitive to the inhibitory effects of MK-801 than the explants of 15- and 50-day-old rats.

Using magnesium as a nonuse-dependent NMDA receptor antagonist, a similar concentration of 2.5 mM resulted in a suppression of the veratridine-induced release of GnRH at 25 and 50 days of age (Table 2). This indicated that use dependency accounted for the age-related differences in the sensitivity of GnRH secretion to MK-801.

Age-related differences in MK-801 inhibitory effects on pulsatile secretion of GnRH

As shown in Fig. 6, using hypothalamic explants obtained at 25 days, the pulsatile secretion of GnRH was similar under control conditions and in the presence of 10^2 pM MK-801. A reduction of GnRH pulsatility ($P < 0.001$) was observed using a dose of 10^3 pM, while an almost complete suppression was seen in the presence of 10^4 pM MK-801. To obtain a similar suppression of pulsatile GnRH secretion from explants obtained at 50 days, a MK-801 concentration 10,000 times greater than that used at 25 days of age was required (Table 3).

Discussion

In this paper we show that hypothalamic maturation at the onset of puberty results in an increased frequency of pulsatile GnRH secretion involving a transient increase in NMDA receptor activation. The experimental model used is based on the concept that in the hypothalamus, a neuronal circuitry distinct from the GnRH neurons works as a pacemaker, modulating the secretory activity of GnRH axons presynaptically (4, 10). This

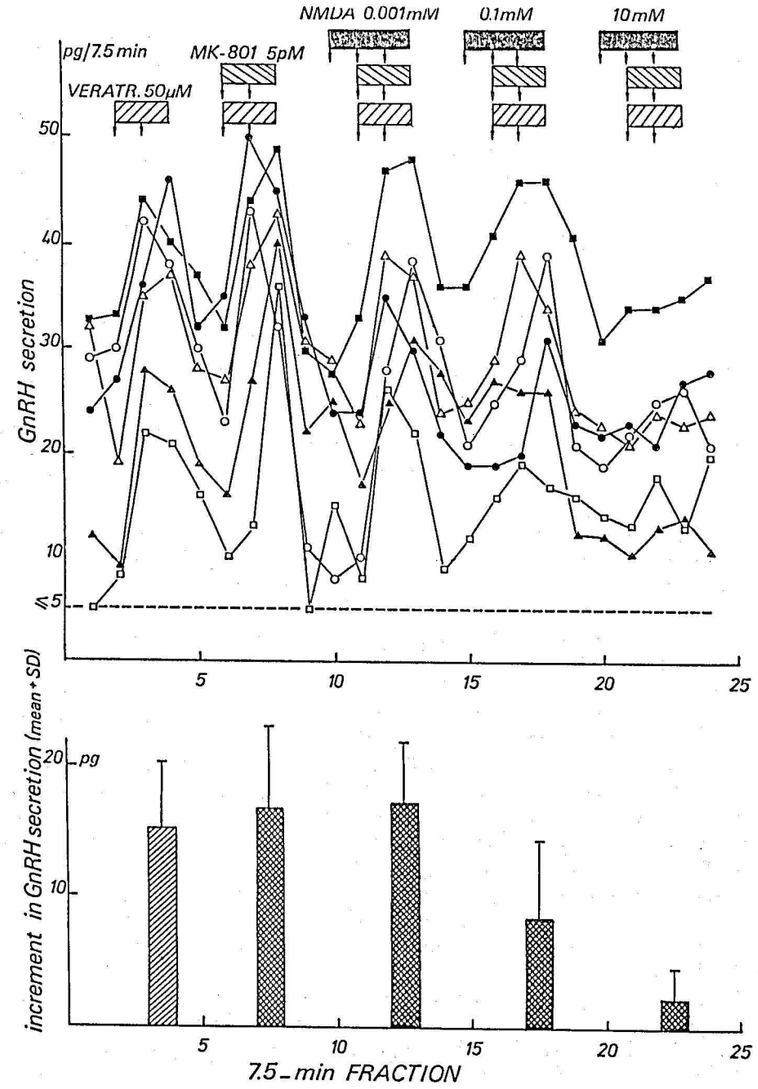

FIG. 3. Variations in GnRH release during a 15-min depolarization using veratridine in the absence or presence of MK-801 (5 pM). The inhibitory effect of MK-801 was studied in the absence of NMDA or in the presence of increasing concentrations of NMDA applied for 22.5 min. The *dashed line* indicates the detection limit of the assay. *Upper panel*, Individual profiles of 6 hypothalamic explants of 50-day-old male rats; *lower panel*, mean increment observed during exposure of 12 explants to veratridine.

pulse generator or oscillator has a dramatic functional importance, since the secretory level of the pituitary gonadotropins and, consequently, their gonadal effects depend on the frequency of pulsatile GnRH secretion (5, 17, 18). This is particularly obvious at the onset of puberty, as indicated by the increased frequency of LH or GnRH secretory episodes in different species (19–22). In the male rat our data indicate that the onset of puberty takes place around 25 days of age, as evidenced by the rapid acceleration of testicular weight increase and the occurrence of mature germ cells in the testis. The time relationship between those signs of testicular maturation and the increase in frequency of pulsatile GnRH secretion between 15–25 days of age suggests that the latter phenomenon is an important neuroendocrine manifestation of puberty. Compared to our previous observations (3), the present findings provide a more obvious illustration of that phenomenon. This may relate to the different nature of the culture medium, particularly the concentration of glycine, which was decreased from 400 to 0.01 μM in order to achieve optimal conditions for activation of the NMDA receptors (23). Also, the use of an appro-

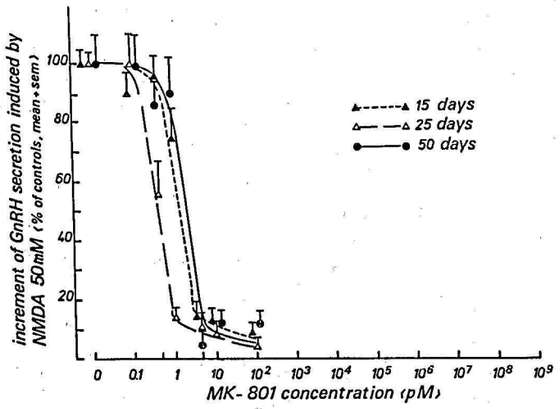

FIG. 4. Mean (±SEM) increment of GnRH release induced by NMDA from 12 hypothalamic explants of 15-, 25-, and 50-day-old male rats in the absence (controls) or presence of increasing concentrations of MK-801.

priate method for the detection of significant GnRH pulses may have played some role. In agreement with our previous data (3), the acceleration of GnRH pulse frequency is a very early manifestation of puberty, while no further changes have been observed throughout sexual development between 25–50 days of age.

Since we found previously that activation of NMDA receptors elicited the release of GnRH (23), whereas antagonists of NMDA receptor activation could block the pulsatile secretion of GnRH (10), we attempted to delineate the possible involvement of NMDA receptors in the maturation of the hypothalamic GnRH pulse generator at the onset of puberty. Such a hypothesis was consistent with the findings of Cicero *et al.* (7), who showed an increased responsiveness of LH to NMDA administration in the male rat between 20–35 days of age, whereas younger or older animals were less sensitive to NMDA. In agreement with those data, we showed that the sensitivity of GnRH secretion to the stimulatory effect of NMDA was greater at 25 days than at 15 or 50 days. This age-related difference may have accounted for the very high concentrations of NMDA required in our previous experiments using hypothalamic explants from 50-day-old rats (10, 23).

The increased LH responsiveness to NMDA at the onset of puberty suggested an increased synthesis of endogenous excitatory amino acids activating the NMDA receptors. We are still ignorant about the nature of the substrate or the mechanism specifically activating the NMDA receptors involved in the GnRH pulse generator. We hypothesized that MK-801 could provide a unique means for indirectly evaluating the degree of NMDA receptor activation, on account of the use dependency that characterizes its effects (11). At the NMDA-receptor complex, MK-801 is a noncompetitive antagonist because it binds at a site distinct from that of glutamate or its agonist NMDA (12). This binding site is closely related to the NMDA receptor-associated ion channel (24). While the concept of noncompetitive antagonism usually requires that the effect be independent of the agonist, MK-801 action appears to be modulated by the agonist. In fact, L-glutamate or other NMDA receptor agonists markedly stimulate the binding of tritiated MK-801 to the receptor complex (25). This potentiation of the antagonistic effect by the agonist is defined as use dependency. The mechanism of use dependency resides on a binding site with two components showing distinct affinities; the high affinity component is expressed only when the receptor is activated by the agonist (26). Consequently, the antagonistic capacity of MK-801 will be inversely related to activation of the NMDA receptor, thus providing an indirect estimate of the agonistic activity. Our data on the veratridine-induced release of GnRH, showing that MK-801 inhibitory effects developed in the presence of increasing NMDA concentrations, indicated that use dependency was operating in our experimental conditions. Using magnesium, a noncompetitive antagonist of NMDA receptors which does not exhibit use dependency (27), it was possible to confirm that the age-related differences in reactivity to MK-801 resulted from use dependency and reflected differences in activation of the NMDA receptors.

The endogenous level of NMDA receptor activation was studied through the release of GnRH induced by depolarization using veratridine. We have shown previously that antagonists of NMDA receptors, such as MK-801, could suppress the release of GnRH induced by veratridine (10). This suppression implies that veratridine effects occur proximally on the GnRH axons and that the circuitry involving the NMDA receptors keeps the depolarizing stimulus under a permissive control at a presynaptic site or is directly depolarized by veratridine. Therefore, variations in the responsiveness to veratridine can be related to differences in endogenous activation of the NMDA receptors. Thus, the age-related differences in sensitivity to the inhibitory effects of MK-801 on the response to veratridine reflect very important differences in NMDA receptor activation, since at 25 days the sensitivity to MK-801 is 1,000,000 times greater than that at 15 or 50 days.

The differences in NMDA receptor activation with age were also shown by evaluating the concentrations of MK-801 required for blocking the pulsatile secretion of GnRH. This more physiological approach was not used before 25 days of age, because the lower frequency of pulsatile GnRH secretion made difficult any evaluation of a reduction of that frequency over a 3- or 4-h experiment. Between 25–50 days, there was a 10,000 times

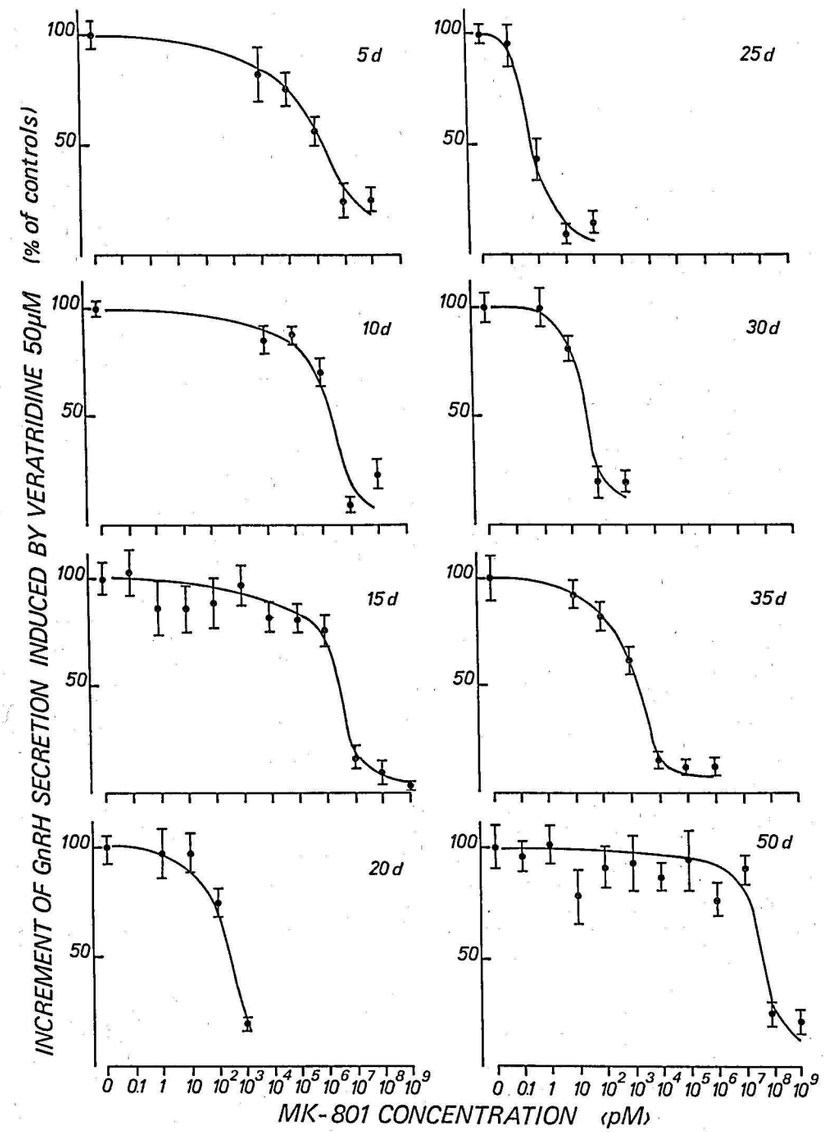

FIG. 5. Mean (±SEM) increments of GnRH secretion induced by veratridine from hypothalamic explants of male rats aged 5–50 days, in the absence (controls) or presence of increasing concentrations of MK-801. Twelve explants were studied in each age group.

TABLE 2. Effect of Mg^{2+} on the increment in GnRH secretion induced by veratridine (50 μM) from hypothalamic explants of 25- and 50-day-old male rats

	Mg^{2+} conc. (mM)							Age (days)
	1.0	1.5	2.0	2.5	3.0	3.5	4.0	
Mean (±1 SD)								
increment in	11.2 ± 3.6	9.3 ± 1.8	9.5 ± 4.0	1.3 ± 1.5	3.7 ± 2.9	1.4 ± 1.9	2.3 ± 2.6	25
GnRH secretion (n = 6)	13.6 ± 5.6	16.3 ± 8.8	12.8 ± 4.3	1.5 ± 2.3	1.8 ± 2.7	1.6 ± 2.1	1.6 ± 1.5	50

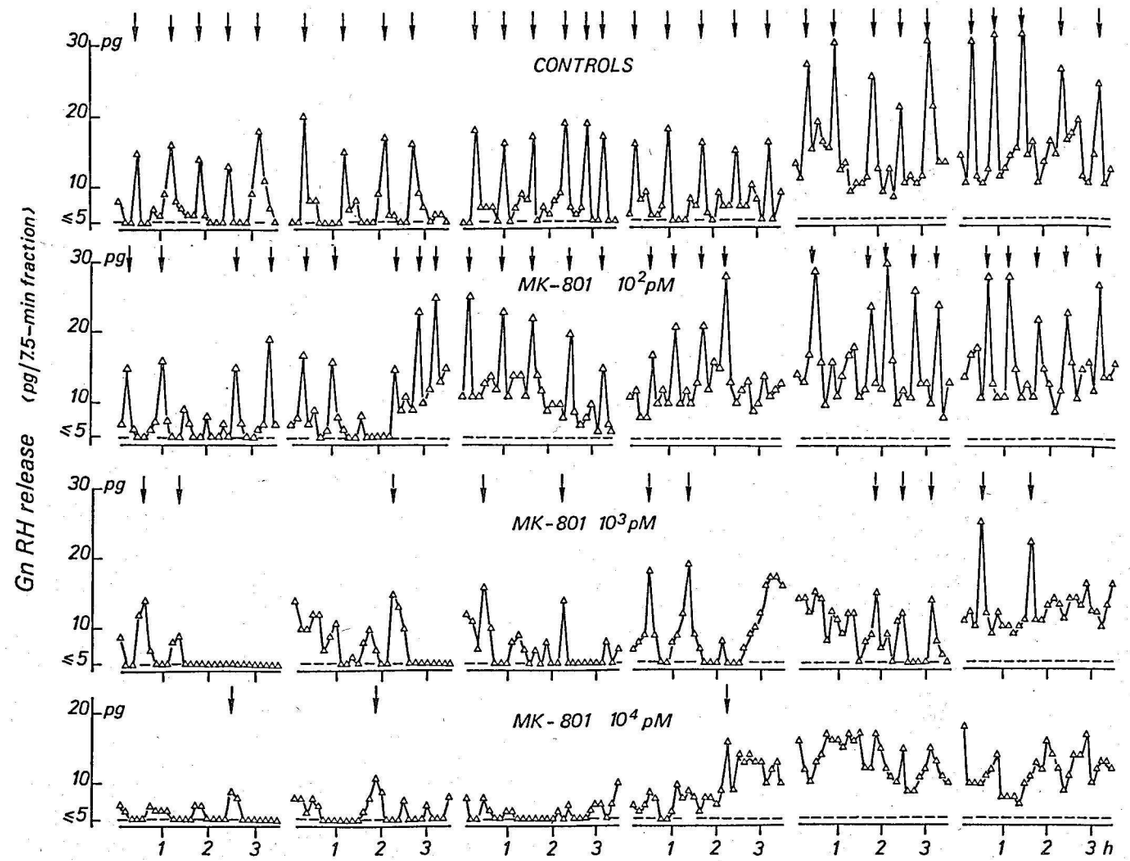

FIG. 6. GnRH release in 7.5-min fractions collected for 3.5 h during incubation of individual hypothalamic explants of 25-day-old male rats in the absence of MK-801 (controls) or in the presence of increasing MK-801 concentrations. The *arrows* denote significant secretory pulses. The *dashed lines* indicate the detection limit of the assay.

TABLE 3. Effect of MK-801 on the frequency of pulsatile GnRH secretion from hypothalamic explants of 25- and 50-day-old male rats

	MK-801 conc. (pM)							Age (days)
	0	10^2	10^3	10^4	10^6	10^7	10^8	
Mean (±1 SD) GnRH pulse frequency (no./3.5 h)	4.7 ± 0.8 (28)[a]	4.7 ± 0.5 (6)[b]	2.3 ± 1.3 (12)[b]	0.7 ± 0.5 (11)[b]				25
	5.4 ± 1.2 (33)			2.3 ± 1.6 (10)[b]	3.3 ± 1.0 (6)[b]	3.8 ± 0.8 (5)[c]	0.8 ± 0.9 (12)[b]	50

[a] The number of explants studied in each group is in parentheses.
[b] $P < 0.001$ *vs.* controls (in the absence of MK-801).
[c] $P < 0.01$.

difference in the concentration of MK-801 required for blocking pulsatile GnRH secretion. While this was qualitatively consistent with the data obtained using veratridine, the quantitative discrepancy may result from the different neuronal pathways involved in a GnRH pulse induced by veratridine or by the hypothalamic pulse generator.

When the secretion of GnRH was induced by adding 50 mM NMDA to the incubation medium of hypothalamic explants, the sensitivity to MK-801 inhibitory effects was very high and relatively similar at the different ages studied. This indicates that regardless of the concentrations of endogenous substrates and receptors, a very high concentration of NMDA can overcome the mechanism controlling the activation of NMDA receptors involved in pulsatile GnRH secretion.

An interesting finding is that the increased activation of NMDA receptors taking place at the onset of puberty only persists for 2–3 weeks, in agreement with the data of Cicero *et al.* on the LH response to NMDA (7). While it was expected to see an increased activation of NMDA receptors timely related to the acceleration of GnRH pulse frequency, it is intriguing to find that in the adult animal the NMDA receptor system returns to the prepubertal level of activation without concomitant regression to a prepubertal pattern of pulsatile GnRH secretion. This is not related to the disappearance of NMDA receptor involvement in pulsatile GnRH secretion, since the antagonists of NMDA receptors can suppress pulsatile GnRH secretion in 50-day-old rats (10). Only hypotheses can be raised based on the known interplay between sex steroids, opiate peptides, and the GnRH pulse generator. It is possible that a maturational reduction of the opiatergic inhibitory control of the GnRH pulse generator (28) would play a facilitatory role, making unnecessary the increased activation of the NMDA receptors seen earlier in life. The increase in plasma levels of testosterone occurring throughout puberty is known to determine the opiatergic control of GnRH secretion (29, 30), and the interaction of sex steroids with NMDA receptor activation warrants further studies.

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