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Early Prepubertal Ontogeny of Pulsatile Gonadotropin-Releasing Hormone (GnRH) Secretion: I. Inhibitory Autofeedback Control through Prolyl Endopeptidase Degradation of GnRH*

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ABSTRACT

$GnRH_{[1-5]}$, a subproduct resulting from degradation of GnRH by prolyl endopeptidase (PEP) and endopeptidase 24.15 (EP24.15) was known to account for an inhibitory autofeedback of GnRH secretion through an effect at the *N*-methyl-D-aspartate (NMDA) receptors. This study aimed at determining the possible role of such a mechanism in the early developmental changes in frequency of pulsatile GnRH secretion. Using retrochiasmatic explants from fetal male rats (day 20–21 of gestation), no GnRH pulses could be observed *in vitro*, whereas pulses occurred at a mean interval of 86 min from the day of birth onwards. This interval decreased steadily until day 25 (39 min), during the period preceding the onset of puberty. Based on $GnRH_{[1-10]}$ or $GnRH_{[1-9]}$ degradation and $GnRH_{[1-5]}$ generation after incubation with hypothalamic extracts, EP24.15 activity did not change with age, whereas PEP activity was maximal at days 5–10 and decreased subsequently until day 50. These changes were consistent with the ontogenetic variations in PEP messenger RNAs (mRNAs) quantitated using RT-PCR. Using fetal explants, the NMDA-evoked release of GnRH was potentiated in a dose-dependent manner by bacitracin, a competitive PEP inhibitor and the desensitization to the NMDA effect was prevented using 2 mM of bacitracin. At day 5, a higher bacitracin concentration of 20 mM was required for a similar effect. Pulsatile GnRH secretion from fetal explants was not caused to occur using bacitracin or Fmoc-Prolyl-Pyrrolidine-2-nitrile (Fmoc-Pro-PyrrCN), a noncompetitive PEP inhibitor. At postnatal days 5 and 15, a significant acceleration of pulsatility was obtained using 1 μM of Fmoc-Pro-PyrrCN or 2 mM of bacitracin. At 25 and 50 days, a lower bacitracin concentration of 20 μM was effective as well in increasing the frequency of GnRH pulsatility. We conclude that the GnRH inhibitory autofeedback resulting from degradation of the peptide is operational in the fetal hypothalamus but does not explain the absence of pulsatile GnRH secretion at that early age. After birth, PEP activity is high and may account for the low frequency of pulsatility. The potency of that effect decreases before the onset of puberty and may contribute to the acceleration of GnRH pulsatility. (*Endocrinology* **140:** 4609–4615, 1999)

AFTER BEING SECRETED, GnRH was shown to be degraded in the hypothalamus and the anterior pituitary gland by two endopeptidases acting in a stepwise manner (1). Prolyl endopeptidase (PEP) resulted in cleavage of the C-terminal glycinamide residue and generated $GnRH_{[1-9]}$, which was in turn cleaved at the tyr^5-gly^6 bond by the metalloendopeptidase EC 3.4.24.15 (EP24.15), thus generating $GnRH_{[1-5]}$ (1). $GnRH_{[1-9]}$ was the preferred substrate of EP24.15 because it was 11 times more potent than $GnRH_{[1-10]}$ in generating $GnRH_{[1-5]}$ (2).

The physiological role of pituitary endopeptidases in limiting GnRH stimulation of the pituitary gonadotropes was supported by the increase in gonadotropin secretion seen after systemic administration of EP24.15 inhibitors in the rat (3, 4). A possible role for brain endopeptidases in the regulation of GnRH effects was suggested by the effect of intracerebroventricular (ICV) administration of EP24.15 inhibitors, which resulted in increased gonadotropin secretion (3) as well as increased recovery of ICV-administered GnRH (5). In ovariectomized estrogen-primed rats, progesterone administration caused a reduction in GnRH degrading activity in the median eminence, which was associated with an increase of the GnRH content (6). In the female monkey, pulsatile GnRH secretion in the stalk-median eminence area was increased by local immunoneutralization of EP24.15, whereas it was suppressed by recombinant EP24.15 (7). In the ovariectomized ewe, however, pulsatile LH secretion was not affected by ICV administration of PEP or EP24.15 inhibitors (8).

While the primary aim of endopeptidase degradation was thought to be the limitation of GnRH interaction at pituitary and hypothalamic GnRH receptors, we proposed an additional inhibitory autofeedback role. This was based on the possible inhibition by the $GnRH_{[1-5]}$ subproduct of the secretion of GnRH evoked through stimulation of the *N*-methyl-D-aspartate (NMDA) receptors (9). Because this autofeed-

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back control could affect the frequency of GnRH pulsatility, it appeared relevant to study the role of hypothalamic PEP and EP24.15 in the early ontogeny of pulsatile GnRH secretion. We aimed at elucidation of the endopeptidase contribution in the developmental changes in the hypothalamic GnRH pulse generator leading to the onset of puberty.

Materials and Methods

Hypothalamic explant incubation and RIA of GnRH

In male Wistar rats of different ages between late gestation (fetal day 20–21) and postnatal day 50, the retrochiasmatic hypothalamus was rapidly dissected after decapitation and transferred into a static incubator as described in detail previously (10–12). Noteworthy, the studied retrochiasmatic explants did contain GnRH axons and terminals but virtually no GnRH cell bodies (13). In each experiment, 12 to 15 explants were studied individually for 4 to 6 h through collection and renewal of the incubation medium (0.5 ml) every 7.5 min. GnRH was measured in the collected fractions using a highly sensitive RIA (10, 11). The values below the limit of detection (5 pg/7.5 min) were assigned that value. Two different GnRH antisera were used, generously gifted by Dr. A. W. Root (St. Petersburgh, FL) (14) and by Dr. Y. F. Chen and V. D. Ramirez (Urbana, IL) (15). Both antisera were highly specific of $GnRH_{[1-10]}$ without significant cross-reactivity of neither $GnRH_{[1-9]}$ and $GnRH_{[1-5]}$, nor any of the reagents used in the experiments. [Hyp^9]GnRH resulted in only 5% cross-reactivity in the assay.

Measurement of endopeptidase activity

Retrochiasmatic hypothalamic explants were dissected and immediately homogenized in 10 times as much buffer (20 mM TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethanosulfonic acid), pH 7.4), at 4 C, with a Politron homogenizer. After low speed centrifugation to remove cellular debris, the supernatant was centrifuged at 10,000 × *g*, 4 C for 20 min. The supernatant was kept as a soluble fraction, and the pellet was washed twice by resuspension in the homogenization buffer followed by recentrifugation. The final pellet was resuspended in the homogenization buffer and kept as a membrane fraction. Both fractions were stored at −70 C. Aliquots of the fractions were assayed for protein determination by a dye-binding method using a commercial kit (Bio-Rad Laboratories, Inc., Hercules, CA). Synthetic $GnRH_{[1-10]}$ or $GnRH_{[1-9]}$ (UCB, Brussels, Belgium) was incubated with hypothalamic soluble or membrane fraction (20 to 40 μg of protein) at 37 C in 250 μl of 200 mM TES, pH 7.4. Twenty micrograms of peptide were incubated with the soluble fraction for 30 min and 10 μg with the membrane fraction for 60 min. The reactions were terminated by heating, 100 C for 7 min. After brief centrifugation, 200 μl were injected onto a microBondapak C18 column (3.9 × 300 mm, Waters Corp., Milford, MA). The peptides were eluted from the column by a linear gradient from 10–30% of solvent (0.08% TFA/CH3CN) at a flow rate of 1 ml/min. Chromatography was performed with a Waters HPLC system and monitored by UV detection at 214 nm. The elution peaks of GnRH and degradation fragments were identified by comigration of synthetic $GnRH_{[1-10]}$ and the $GnRH_{[1-9]}$ and $GnRH_{[1-5]}$ subproducts and peak areas were calculated. The $GnRH_{[1-10]}$ or $GnRH_{[1-9]}$ degrading activity was estimated by disappearance of the peptides after incubation. The presence of $GnRH_{[1-5]}$ after incubation was regarded as generation of $GnRH_{[1-5]}$ because this subproduct was relatively stable (16). In the used conditions, the relationship between protein concentration and incubation time was linear.

The specificity of the assays for PEP and EP24.15 activity was assessed using the soluble fraction of hypothalamic extracts prepared from 15-day-old rats. Incubation with 200 μM of bacitracin (Sigma, St. Louis, MO) a competitive PEP inhibitor (17) or 100 nM of Fmoc-Prolyl-Pyrrolidine-2-nitrile (Fmoc-Pro-PyrrCN), a recently proposed noncompetitive PEP inhibitor that is more specific and potent than bacitracin (18), resulted in a profound inhibition of $GnRH_{[1-10]}$ degradation (2–3% of controls), a complete suppression of $GnRH_{[1-9]}$ generation and a reduction of $GnRH_{[1-5]}$ generation (13–14% of controls). This indicated PEP inhibition and residual $GnRH_{[1-5]}$ generation presumably through direct EP24.15 degradation of $GnRH_{[1-10]}$. Incubation with 10 μM of *N*-[1(R,S)carboxy-3-phenylpropyl]Ala-Ala-Tyr-*p*-aminobenzoate (CFP-A-A-Y-*p*AB), a competitive EP24.15 inhibitor (1) resulted in a slight reduction of $GnRH_{[1-10]}$ degradation (77% of controls), an increase of $GnRH_{[1-9]}$ generation (445% of controls), and a profound inhibition of $GnRH_{[1-5]}$ (6% of controls).

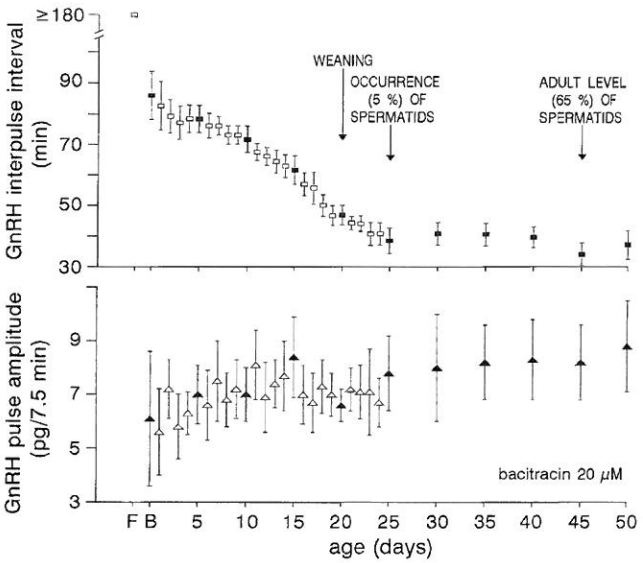

FIG. 1. Developmental changes in frequency and amplitude of GnRH secretion *in vitro* from retrochiasmatic explants (n = 6–36) of the male rat hypothalamus. F, Fetal, days 20–21 of gestation. B, Day of birth. The data are mean ± SD. ANOVA shows a significant reduction in GnRH interpulse interval before onset of puberty and no significant changes in amplitude.

Quantitation of PEP mRNAs

Total RNAs were extracted from retrochiasmatic explants according to the RNAzol method (19). The extracted RNAs were reverse transcribed with Moloney Murine leukaemia virus reverse transcriptase (Expand, Roche Molecular Biochemicals, Mannheim, Germany) using oligo (dT) 12–18 (Eurogentec, Liège, Belgium). The cDNAs obtained were then amplified by PCR (32 cycles at 94 C for 90 sec, 55 C for 90 sec, 72 C for 90 sec) using the 5′-oligonucleotide CATTCTGCAGCTTCACGACC (7–26) and the 3′-oligonucleotide GCGCAAGAAGGACTCTAAGA (91–112) from rat prolyl endopeptidase (accession number H34452) (20). The predicted size of this PCR product was 106 bp. As positive and semiquantitative controls, the 5′-oligonucleotide GTGACTTCACACGCCATAATG (236–256) and the 3′-oligonucleotide CCTTCAAGTCTAGCAGGATTG (677–697) from rat cyclophilin were used (accession number M19533). The predicted size of this PCR product was 453 bp. 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 a UV transilluminator with a black and white highly sensitive camera.

Study protocols

Using hypothalamic explants obtained at 1-day intervals between fetal day 20–21 and postnatal day 25 and at 5-day intervals between days 25 and 50, the frequency and the amplitude of pulsatile GnRH secretion was studied. For consistency with our previous studies (9–12), the present one was performed using 20 μM of bacitracin.

Using the soluble and membrane fractions of hypothalamic explants prepared at 5-day intervals between fetal days 20–21 and postnatal day 50, the activity of PEP and EP24.15 was measured using reversed phase HPLC through the degradation of synthetic $GnRH_{[1-10]}$ and $GnRH_{[1-9]}$ and the generation of $GnRH_{[1-5]}$. Using the total RNAs extracted from the retrochiasmatic hypothalamus on fetal days 20–21 and postnatal

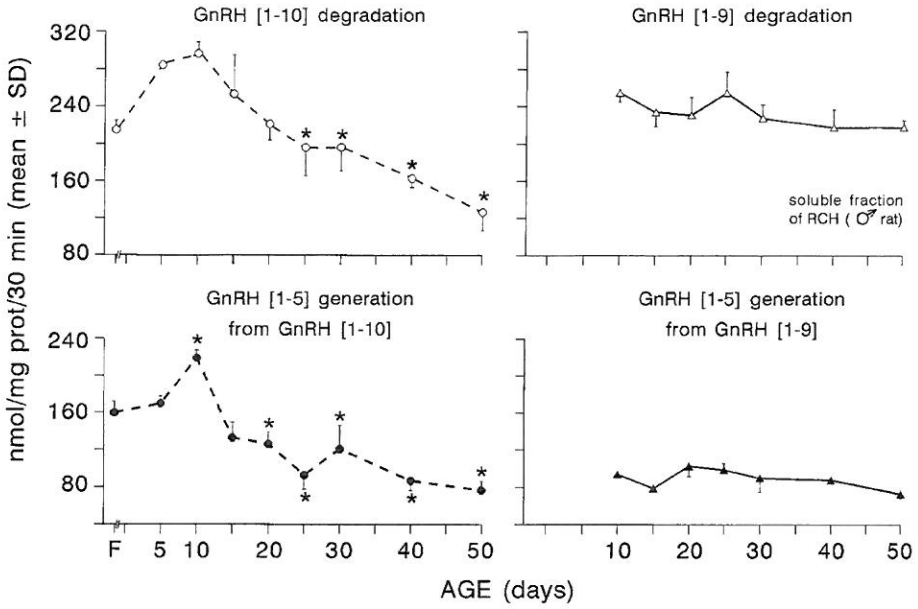

FIG. 2. Developmental changes in endopeptidase degradation of GnRH estimated through HPLC assessment of degradation of $GnRH_{[1-10]}$ or $GnRH_{[1-9]}$ and generation of $GnRH_{[1-5]}$ from those two peptides using soluble fraction extracts of the retrochiasmatic hypothalamus (n = 4). F = Fetal, days 20–21 of gestation. *, $P < 0.05$ *vs.* 5 days.

days 5, 10, 15, 25, and 50, the PEP and cyclophilin gene expression was studied through RT-PCR.

Using explants obtained on fetal days 20–21 and on postnatal day 5, 7.5-min stimulation using 50 mM of NMDA (Sigma, St. Louis, MO) was performed repeatedly at regular intervals of 37.5 min (five fractions) to mimic the frequency of GnRH pulsatility observed from 25 days of age onwards. These experiments were performed in the absence of bacitracin or using different concentrations of the inhibitor (20 μM, 2 mM and 20 mM). The NMDA-evoked release of GnRH was calculated as the difference between GnRH secretion in the fractions collected immediately before and during NMDA stimulation. When no bacitracin was used in the incubation medium, 10 μl of 1 mM bacitracin were added to the collected medium to prevent further degradation of GnRH during the RIA procedure.

Using explants obtained on fetal days 20–21 and postnatal day 5, 15, 25, and 50, pulsatile GnRH secretion *in vitro* was studied in the absence of bacitracin and using 20 μM or 2 mM of the inhibitor. On fetal days 20–21 and postnatal days 5 and 15, similar experiments were performed using Fmoc-Pro-PyrrCN (18).

Statistical analysis

The occurrence of significant pulses of GnRH secretion was determined using the Pulsar program (21, 22) as described previously (23). The individual interpulse interval and pulse amplitude as well as the mean ± SD were calculated. The significance of differences in GnRH pulse amplitude and frequency and NMDA-evoked GnRH release was calculated by ANOVA with correction for repeated measurement and Scheffé's *F* test. The significance of age-differences in GnRH degrading activity and PEP mRNAs was analyzed using one-way ANOVA. The level of significance was $P < 0.05$.

Results

Ontogeny of pulsatile GnRH secretion

The amplitude and the frequency of pulsatile GnRH secretion *in vitro* were studied at different ages between late fetal life and adulthood using 20 μM of bacitracin, which is in conditions similar to our previous studies (9–12). No pulses could be observed using the fetal explants, thus accounting for absence of pulsatility or interpulse interval ≥180 min, which was the minimal study period (Fig. 1). From the day of birth onwards, pulsatility was observed with a mean interpulse interval of 86 ± 8 min. A progressive reduction in interpulse interval was seen between birth and 10 days of age (72 ± 4 min). A 2-fold greater reduction occurred between 10 and 20 days (47 ± 3 min). An adult pattern of frequency was attained by 25 days of age (interval: 39 ± 4 min) when the first evidence of spermatogenetic maturation could be obtained (23). The mean GnRH pulse amplitude was around 6 pg/7.5-min fraction in the early neonatal period and increased progressively but not significantly with age to reach an average of 8 pg/7.5 min after 25 days.

TABLE 1. Developmental changes in endopeptidase degradation of GnRH estimated through HPLC assessment of $GnRH_{[1-10]}$ degradation and $GnRH_{[1-5]}$ generation using membrane fraction extracts of the retrochiasmatic hypothalamus in male rats

Age (days)	$GnRH_{[1-10]}$ degradation (nmol/mg prot/30 min)	$GnRH_{[1-5]}$ generation from $GnRH_{[1-10]}$ (nmol/mg prot/30 min)
Fetal 21	6.5 ± 5.5[a]	4.2 ± 0.3[a]
Postnatal 5	71.9 ± 8.9	21.9 ± 2.3
10	73.9 ± 2.9	19.3 ± 3.5
15	48.6 ± 8.8	18.2 ± 3.3
20	37.9 ± 6.7	15.9 ± 1.4
25	39.7 ± 11.6	8.0 ± 2.6
30	36.4 ± 8.8[a]	7.4 ± 1.2[a]
35	24.4 ± 8.6[a]	7.7 ± 2.0[a]
40	36.3 ± 2.2[a]	6.3 ± 1.2[a]
45	29.2 ± 7.3[a]	4.2 ± 1.0[a]
50	22.0 ± 13.2[a]	6.5 ± 1.4[a]

Data are mean ± SD (n = 3–10).
[a] $P < 0.05$ *vs.* 5 days.

Ontogeny of hypothalamic GnRH endopeptidase activity

Using the soluble fraction of retrochiasmatic hypothalamic extracts prepared at different ages, the degradation of $GnRH_{[1-10]}$ and the generation of $GnRH_{[1-5]}$ assessed through reversed-phase HPLC showed significant and parallel changes during development (Fig. 2). The $GnRH_{[1-10]}$ degrading activity (PEP and EP24.15) was present in the fetal

hypothalamus, increased to maximal levels at postnatal days 5–10, and decreased subsequently during prepubertal and pubertal periods. In contrast, degradation of $GnRH_{[1-9]}$ and generation of $GnRH_{[1-5]}$ from $GnRH_{[1-9]}$ did not show any significant change between day 10 and day 50 (Fig. 2), indicating that EP24.15 activity did not change throughout development. Using the membrane fraction of retrochiasmatic hypothalamic extracts (Table 1), the endopeptidase activity was lower than in soluble fraction and showed similar developmental variations. $GnRH_{[1-10]}$ degradation and $GnRH_{[1-5]}$ generation were low in the fetal hypothalamus, reached maximal levels at days 5–10, and decreased subsequently during postnatal life. Taken together, these data suggested that the developmental changes in $GnRH_{[1-10]}$ degradation involved changes in PEP activity.

Ontogeny of hypothalamic PEP mRNAs

Based on the ratio between PEP and cyclophilin mRNAs in the retrochiasmatic hypothalamus, the highest PEP mRNA level was found at fetal days 20–21 as well as on postnatal day 5 and 10 (Fig. 3). Significantly lower levels ($P < 0.05$) were observed using hypothalamic extracts from 25- and 50-day-old rats.

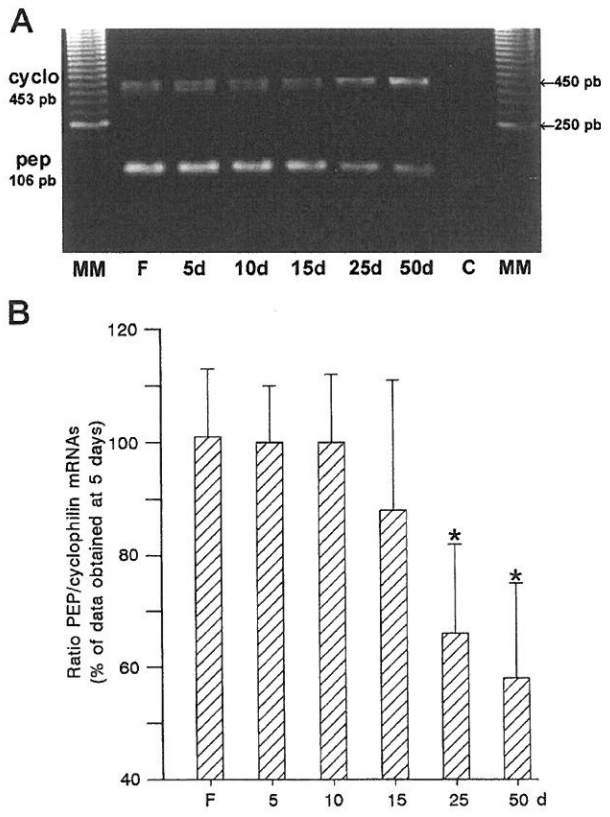

FIG. 3. Developmental changes in PEP mRNAs estimated through RT-PCR of extracts from the retrochiasmatic hypothalamus of male rats (A) and densitometric analysis of the ratio between PEP and cyclophilin mRNAs (B). The data are mean ± SD of six estimates. *, $P < 0.05$ *vs.* data obtained at 5 days. The *upper photograph* shows the electrophoretic pattern of cyclophilin (cyclo) and PEP mRNAs at the different ages of study. F, Fetal; d, days, postnatal age; c, control; MM, molecular markers.

Effects of PEP inhibition on the NMDA-evoked release of GnRH

Using fetal hypothalamic explants incubated without bacitracin, NMDA stimulation repeated every 37.5 min resulted in a progressive extinction of the initial response (Fig. 4). Bacitracin 20 μM and 2 mM caused a dose-related increase in the initial secretory response of GnRH and prevented partially (20 μM) or totally (2 mM) the inhibition of the NMDA-evoked release of GnRH that occurred during repeated stimulation (Fig. 4). Using explants of 5-day-old rats, the GnRH secretory response to repeated NMDA stimulation was reduced only at the fifth and sixth stimulatory episode when 20 μM or 2 mM of bacitracin was used. A 20 mM concentration of bacitracin was necessary to prevent the inhibition of the NMDA-evoked release of GnRH at 5 days.

Effects of PEP inhibition on the frequency of GnRH pulsatility

Using Fmoc-Pro-PyrrCN, a noncompetitive inhibitor of PEP, pulsatile GnRH secretion was not caused to occur from fetal explants. At 5 and 15 days postnatally, the GnRH interpulse interval was significantly reduced by the inhibitor (Fig. 5). Similar observations were made using bacitracin, a competitive antagonist of PEP. At 5 and 15 days, 2 mM of bacitracin were required to obtain a significant reduction of

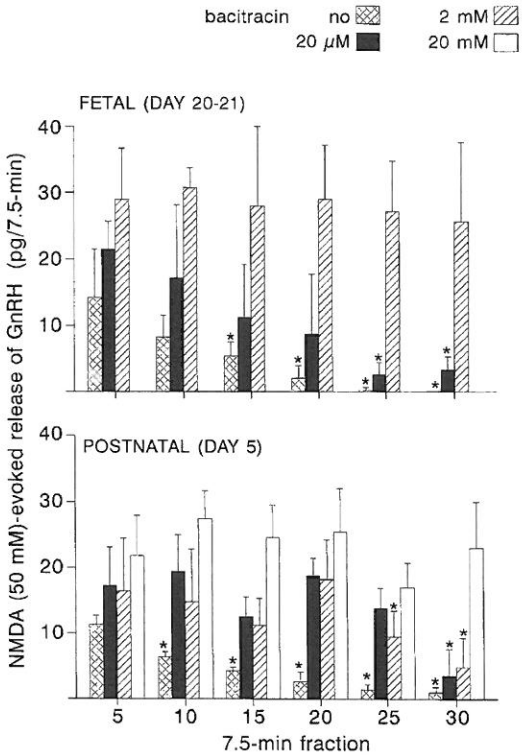

FIG. 4. Mean (± SD) secretory response of GnRH to repeated (every 37.5 min) NMDA stimulation of retrochiasmatic hypothalamic explants from fetal or 5-day-old male rats. Six explants were studied in each age group. The release obtained using medium without bacitracin was compared with that seen using three different bacitracin concentrations. The *asterisks* denote significant differences *vs.* the initial secretory response (fraction 5).

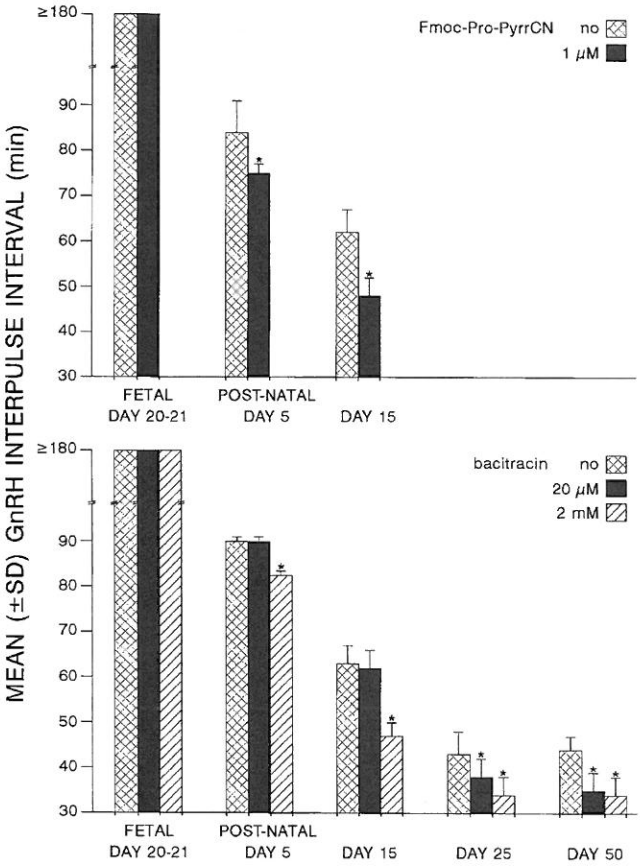

FIG. 5. Developmental changes in the effects of noncompetitive PEP inhibition using Fmoc-Pro-PyrrCN (*upper panel*) or competitive PEP inhibition using bacitracin (*lower panel*) on the frequency of pulsatile GnRH secretion from retrochiasmatic hypothalamic explants of male rats. Five to 10 explants were studied in each age group. The *asterisks* denote significant differences *vs.* the data obtained without inhibitor.

the interpulse interval, whereas a 20 μM concentration was already effective in causing a reduction at 25 and 50 days.

Discussion

To assess the activity of the hypothalamic endopeptidases involved in GnRH degradation, we have combined a bioassay using synthetic $GnRH_{[1-10]}$ and $_{[1-9]}$, an assessment of the PEP transcripts and a study of the effects of bacitracin, a competitive PEP inhibitor, on GnRH secretion. It was crucial to study directly the GnRH degrading activity because the information provided indirectly by bacitracin effects on the NMDA-evoked secretion of GnRH involved not only the inhibitory interaction of $GnRH_{[1-5]}$ but also possible developmental changes in activity of glutamatergic neurons and expression of NMDA receptors. Such developmental changes were suggested by our previous studies showing age-related changes in sensitivity of GnRH secretion to NMDA as well as to MK-801, a NMDA-receptor antagonist (12, 23). Because bacitracin was not a highly specific PEP inhibitor, we also studied the effects of Fmoc-Pro-PyrrCN, a highly specific inhibitor recently made available (18). All together, the HPLC estimation of $GnRH_{[1-5]}$ generation, the RT-PCR estimation of PEP transcripts and the concentrations of bacitracin required to prevent GnRH inhibitory autofeedback concur to demonstrate developmental changes in hypothalamic PEP activity, which is maximal by 5–10 days of age postnatally. Very few data were available on the early ontogeny of PEP and EP24.15 in the brain. Whole brain PEP was found to increase between days 4 and 15 when maximal levels were reached in the rat (24). In a study starting at 15 days of age, EP24.15 activity in the arcuate nucleus-median eminence area did not change throughout development (25). These data are consistent with the absence of developmental changes in EP24.15 activity, as found in the present study. The data reported using whole brain extracts (24) are of little significance because regional differences may be seen such as between preoptic area and mediobasal hypothalamus (25). We elected to study the retrochiasmatic hypothalamus because the inhibitory autofeedback of GnRH was shown to occur in this explant (9). The occurrence of GnRH degradation in the vicinity of GnRH terminals in the median eminence area is supported by the presence of EP24.15 in the perivascular spaces of the median eminence (4). GnRH degrading activity in the median eminence was also found to increase during anestrus and proestrus in the female rat (26). We studied both the soluble and membrane-associated forms because it is unclear which fraction was physiologically relevant. If the membrane-associated form truly reflects the extracellular endopeptidase activity, it is of note that the two-step degradation of GnRH was found to occur in the extracellular milieu of median eminence endothelial cells (27). While the observed changes in PEP activity are thought to influence the GnRH pulse generator through $GnRH_{[1-5]}$ inhibitory effects, we cannot exclude an interaction of other peptides such as TRH or substance P, which involve PEP in their metabolism as well and could thus be affected by the PEP inhibitors.

In this paper, we show that, in the explanted male rat hypothalamus, the GnRH pulse generator is silent before birth though GnRH is already releasable by episodic stimulation using NMDA. These findings suggest that GnRH synthesis and storage as well as NMDA receptor synthesis are not the ultimate factors that control the expression of the GnRH pulse generator. We showed previously that the $GnRH_{[1-5]}$ subproduct of GnRH degradation was involved in an inhibitory autofeedback effect on pulsatility (9), and here we have obtained evidence that GnRH degrading activity is present in the fetal hypothalamus. PEP inhibition results in facilitation of GnRH secretion in response to exogenous NMDA challenges that can mimic an adult pattern of GnRH secretion from fetal explants. The inhibitors, however, do not cause pulsatility to occur. Thus, endopeptidase degradation of GnRH is effective in the fetal hypothalamus but does not represent the key factor that restrains the pulse generator. Increased activity of other inhibitory mechanisms such as GABAergic inputs or insufficient activity of stimulatory mechanisms such as glutamatergic inputs deserve further studies to elucidate why the GnRH pulse generator is silent in the fetal explant. Quite remarkably, pulsatile GnRH secretion *in vitro* can be observed from the first postnatal day onwards. This may suggest that some inhibitory factors are related to the intrauterine conditions. Recent studies have

shown that GnRH existed in the hypothalamus in a particular [Hyp^9] GnRH form with hydroxyproline instead of proline as the ninth residue (28). This form accounts for the majority of hypothalamic GnRH immunoreactivity in the fetus and decreases progressively throughout postnatal life. Because [Hyp^9] GnRH is less sensitive than $GnRH_{[1-10]}$ to PEP activity, this peptide could account for less inhibitory autofeedback, thus adding to our conclusion that such a mechanism does not explain absent pulsatility in the fetus. An alternative hypothesis was that [Hyp^9] GnRH was secreted but less detected than $GnRH_{[1-10]}$ in the GnRH RIA. It is, however, unlikely that the secretion of [Hyp^9] GnRH, which is almost not detected in our RIA, could account for undetected pulsatility because GnRH secretion would then be undetectable in response to secretagogues such as NMDA. This is in contrast to our observation that GnRH pulsatility evoked by NMDA is well observable. Also, the sudden occurrence of pulsatility after birth is not consistent with the progressive postnatal changes in proportion of [Hyp^9] GnRH (30–40%) found in the immunoreactive material released by hypothalamic explants between birth and 6 weeks of age (29).

The detailed study of the ontogeny of pulsatile GnRH secretion from the explanted male rat hypothalamus shows a remarkably progressive increase in pulse frequency during the 2 weeks preceding the onset of puberty. If relevant to *in vivo* physiology, this finding emphasizes that the neuroendocrine changes leading to onset of puberty take place relatively early in life before the age of 3 weeks, whereas no subsequent changes are seen throughout puberty. Unfortunately, due to the size of the rat and the limited sensitivity of LH assay, pulsatile LH could not be studied *in vivo* so far, and no correlation between *in vitro* and *in vivo* data were possible. Our data indicate that the reduction in potency of GnRH inhibitory autofeedback, possibly resulting from a reduced PEP activity with age, can contribute to the acceleration of the GnRH pulse generator that occurs before the onset of puberty, in agreement with our earlier findings (30). In the presence of PEP inhibitors, a developmental reduction of the GnRH interpulse interval is still observed, indicating that other factors such as the GABA-receptor mediated inhibition play an important role as well (31). It is unclear whether the inhibitory autofeedback effects of GnRH are mediated only through the interaction of $GnRH_{[1-5]}$ at NMDA receptors, as we suggested, or whether GnRH receptors are involved as well. Reduced degradation of GnRH could account for increased activity of the GnRH pulse generator through a stimulatory effect of GnRH at its own receptors as long as these receptors mediate a positive autofeedback. Indeed, central microinjection of GnRH in the arcuate nucleus-median eminence area of the female rat causes premature electrophysiological manifestation of the pulse generator (32) and the preovulatory LH surge is delayed by central microinjection of a GnRH antagonist that does not generate any $GnRH_{[1-5]}$ (33). Using hypothalamic explants from male rats incubated with GnRH antagonists or GnRH receptor antisense oligodeoxynucleotides, we also obtained evidence of a positive autofeedback of GnRH (34). Importantly, such observations may not apply to other species. In the ram or the ewe, GnRH antagonists cause an acceleration of LH or GnRH pulsatility, thus pointing to an inhibition mediated via the GnRH receptors (35–37). GnRH antagonists do not appear to affect the electrophysiological correlates of the pulse generator in the monkey (38) nor the frequency of pulsatile LH secretion in man (39). Based on these studies, the mechanism of GnRH positive autofeedback warrants further delineation and integration with the inhibitory autofeedback mediated through $GnRH_{[1-5]}$.

Acknowledgments

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