Effect of Stage of Development and Sex on Gonadotropin-Releasing Hormone Secretion in *In Vitro* Hypothalamic Perifusion (44255)

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Abstract. **Marked sexual and ontogenic differences have been described in gonadotropin regulation in the rat. These could arise from events occurring both at the hypothalamic or hypophyseal levels. The present experiments were designed to evaluate the capacity of the hypothalamus in releasing GnRH *in vitro,* basally and in response to depolarization with KCl, during ontogeny in the rat. To that end we chose two well-defined developmental ages that differ markedly in sexual and ontogenic characteristics of gonadotropin regulation, 15 and 30 days. We compared GnRH release from hypothalami of females, neonatal androgenized females and males. Mediobasal hypothalami were perifused *in vitro,* and GnRH measured in the effluent. Basal secretion of the decapeptide increased with age in the three groups with no sexual differences encountered. When studying GnRH release induced by membrane depolarization, no differences within sex or age were encountered. On the other hand FSH serum levels decreased with age in females and increased in males, and in neonatal androgenized females followed a similar pattern to that of females. LH levels were higher in infantile females than in age-matched males or androgenized females. Such patterns of gonadotropin release were therefore not correlated to either basal or K^+-induced GnRH release from the hypothalamus.**

We conclude that sexual and ontogenic differences in gonadotropin secretion in the developing rat are not dependent on the intrinsic capability of the hypothalamus to release GnRH in response to membrane depolarization. The hormonal differences observed during development and between sexes are probably related to differences in the sensitivity of the GnRH neuron to specific secretagogue and neurotransmitter regulation, and/or to differences in hypophyseal GnRH receptors and gonadotrope sensitivity. [P.S.E.B.M. 1998, Vol 217]

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The hypothalamic-pituitary-gonadal system undergoes complex morphological and physiological changes during progression to puberty in mammals. For instance during development, rats exhibit fluctuations in LH, FSH, and steroids, which have profound influences in the reproductive system and which are different in males and females (1, 2). It has been proposed that these ontogenic alterations might be linked to events occurring both at neural and gonadal levels.

During Days 12–18 in the female rat there are high levels of FSH (3), sporadic LH peaks (1), and a high sensitivity of the hypophysis to the releasing effect of GnRH (4) as well as to nonspecific depolarization with KCl (5). In

age-matched males, levels of FSH, LH, and hypophyseal sensitivity to GnRH and K^+ are low. As the rat progresses to a prepubertal stage conditions vary markedly. Around the fourth or fifth week of life FSH and LH levels as well as hypophyseal sensitivity to GnRH decrease in females, whereas the same parameters increase in males (6). At the hypothalamic level, alterations in the morphology and connection of GnRH neurons, or in their capacity for synthesizing and releasing the decapeptide could be related to such pattern of gonadotropin release. GnRH content as well as GnRH mRNA in the hypothalamus increase with advancing age (7–11). Alterations in GnRH secretion may be related to the synaptic circuitry connecting GnRH neurons to each other and to the relevant neurotransmitter systems. We postulate that the plasmatic membrane of the GnRH cell may also have different sensitivity to depolarization between sexes and during development, as we described for gonadotropes (5, 12). To study this hypothesis, the capability of the isolated hypothalamus of releasing GnRH from hypothalami of females, neonatally androgenized females, and males of 15 and 30 days of age was evaluated. Hypothalami were perifused *in vitro,* and the release of GnRH was measured in the effluent, both in basal conditions and after the exposure to increasing concentrations of a depolarizing stimulus induced by $K.^+$ These data were correlated with serum FSH and LH levels measured in the same rats. Neonatally androgenized females were included as marked sexual differences in gonadotropin secretion have been encountered during development. This experimental model allows us to study the participation of the sexual organization of the hypothalamus, which occurs in the first days of life under the influence of testosterone and estradiol (13) and determines gonadotropin secretion in the adult rat.

Materials and Methods

Animals. Sprague-Dawley rats were housed in an air-conditioned room with lights on at 07.00 and off at 19.00 hr. The had free access to laboratory chow and tap water. Pregnant rats were kept in individual cages. On the day of birth, half of the newborn females were androgenized by administration of 100 ug testosterone propionate sc (Sigma Chemical Co., St. Louis, MO) dissolved in 50 μl of corn oil (TP females). Mothers fostered 3 males, 3 females, and 3 TP females until litters were used for experiments or weaned at 22 days of age. Groups of rats of 15 or 30 days of age were used.

Hypothalamic Perifusions. Rats were sacrificed by decapitation, and their brains were quickly removed and placed on ice. Trunk blood was collected and sera separated by centrifugation and frozen at −20°C for RIA determinations. Hypothalami, including mediobasal hypothalamus and the suprachiasmatic-preoptic area (MBH), were immediately dissected out as previously described (14), cut in sagittal halves, and transferred to incubation chambers. Three or four MBH were placed into each of the eight chambers that were submerged in a 37°C thermostatic bath. Chambers were perifused with Medium 199 (Sigma), 25 m*M* Hepes (Sigma), 0.1% bovine seroalbumin (Sigma), pH 7.2 at a rate of 0.05 ml/min by means of a multichannel perifusion Manostat cassette pump. After a 60-min preincubation period, fractions were collected at 5-min intervals for 30 min. A three-way valve was then switched in order to infuse a 5-min pulse of KCl (effective concentration at 2.5 min of 11 m*M,* calculated as previously described (15)), followed 105 min later by a second 5-min pulse of 56 m*M* KCl. Fractions were lyophilized and kept at −20°C until GnRH RIAs were performed. The response of each channel was normalized by the average baseline release of the channel during the first 30 min of incubation as described (pg released at time t × 100/average pg released during Minutes 5 to 30) (15). Perifusion was continued until the chambers were empty of liquid medium. The chambers were immediately frozen for RIA determination of remaining GnRH in the hypothalami.

RIA Determinations. Perifusate fractions were resuspended in 200 μl of distilled water and the GnRH concentration was determined using the Anti GnRH (EL 14; final dilution 1:500000) kindly provided by Dr. William Ellinwood (Oregon Health Sciences, Portland, OR). Intra- and interassay coefficients of variation were 9.3 ± 1.5 and 13.4 ± 2.3% respectively. The content of the incubation chambers was transferred to a vial containing 1 ml of 0.1 *M* acetic acid and sonicated in ice. After centrifugation 10 μl of the supernatant were diluted wtih PBS-BSA 0.1% and assayed in duplicate for GnRH content as previously mentioned. Serum samples were measured in duplicate for LH and FSH with RIA kits provided by the NIDDK. Results were expressed in terms of LH-RP2 and FSH-RP2. Intra- and interassay coefficients of variance were 7.2 and 12.8% and 8.0 and 13.2% for LH and FSH respectively.

Statistical Analysis. Results of perifusions were analyzed by two-way analysis of variance to evaluate the effect of sex and concentration of $K.^+$ If F of interaction was found significant ($P < 0.05$), individual means were compared by Scheffé's test, if it was not significant, groups of means were analyzed by the same test. Basal GnRH and serum FSH and LH were also analyzed by two-way analysis of variance for the effects of age and sex.

Results

Basal Release of GnRH. Basal release of GnRH (in pg/5 min) from *in vitro* perifused hypothalami increased with age in the three experimental groups. No sexual differences were found (Fig. 1).

Response to K^+ Depolarization. Hypothalami of the different groups were tested for releasability of GnRH induced by two concentrations of $K,^+$ 11 and 56 m*M*. In Figure 2 the profile of percentage GnRH released in the perifusates of 15-day-old hypothalami is shown. The lowest concentration of K^+ produced a weaker release of GnRH in comparison to the concentration of 56 m*M* (see areas under peak in Fig. 4). Release of GnRH in 30-day-old perifusates

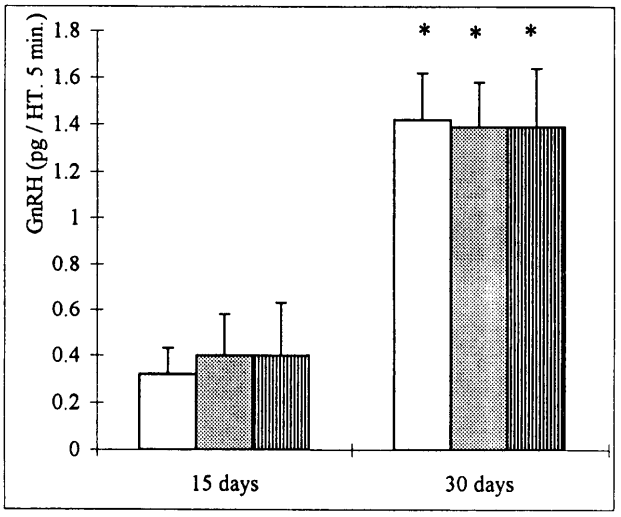

Figure 1. Absolute basal levels (average of the first 30 min of perifusion, pg/hypothalamus/5 min) of GnRH secreted during perifusions in females (open bars), males (gray bars), and TP females (hatched bars). Height of bars indicates means and line on top ± SE (n = six or seven channels per group; *P < 0.05 30 days vs 15 days).

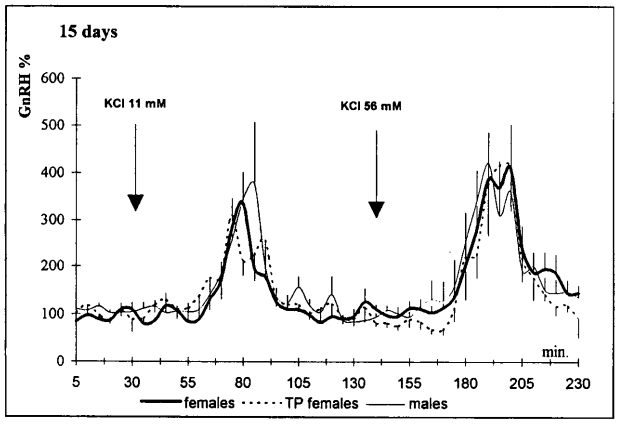

Figure 2. GnRH released during perifusion of hypothalami of 15-day-old rats. For this and the next figure: a) GnRH released in each fraction is expressed as a percentage of basal values (average between fractions 4 to 7). b) Lines over the curve are + SE. c) Number of channels for each group were six or seven, and there were 3–4 hypothalami in each chamber. d) Arrows indicate the beginning of the KCl pulse.

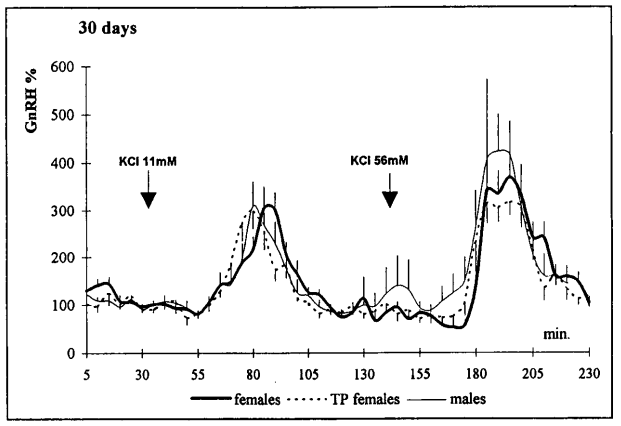

Figure 3. GnRH released during perifusion of hypothalami of 30-day-old rats.

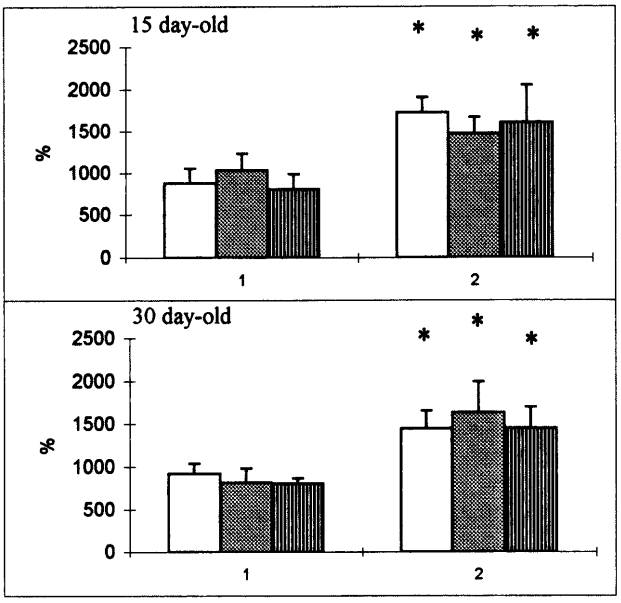

Figure 4. Area under the curve for 1st and 2nd peaks (1 and 2), in % ± SE over basal values during a period of 45 min of perifusion (beginning at 60 and 180 min) for hypothalami of 15-day-old animals (upper panel) and 30-day-old animals (lower panel). Females (open bars), males (gray bars), and TP females (hatched bars). (*P < 0.05 vs Peak 1)

is shown in Figure 3. At this age the profile of response to K^+ was also concentration dependent and similar to that found in 15-day-old animals (Fig. 4). The total GnRH released during perifusion was 5.2 ± 2.3% of the total content of GnRH (GnRH released × 100/GnRH released plus remaining GnRH in the hypothalamus) in the three groups (data not shown). As basal release increased with age (Fig. 1) the absolute amount of GnRH released per hypothalamus was greater in 30- than in 15-day-old rats of the three groups.

Serum Gonadotropins. Marked sexual and ontogenic differences were encountered in FSH levels. At 15 days of age FSH levels were higher in females than in males or TP females; besides TP females had higher serum FSH than males. At 30 days of age FSH titers in males were higher than in females and TP females. With regard to ontogenic variations, FSH decreased significantly from 15 to 30 days of age in females and TP females whereas a significant increase was observed in males.

Higher LH values were encountered in 15-day-old females in comparison to age-matched males, TP females, or older females. No significant differences were found at 30 days of age (Fig. 5).

Discussion

The present experiments were designed to elucidate the capability of the developing hypothalamus to release GnRH in response to membrane depolarization in the infantile and the prepubertal periods in both sexes. To that end we chose two well-defined developmental ages that differ markedly

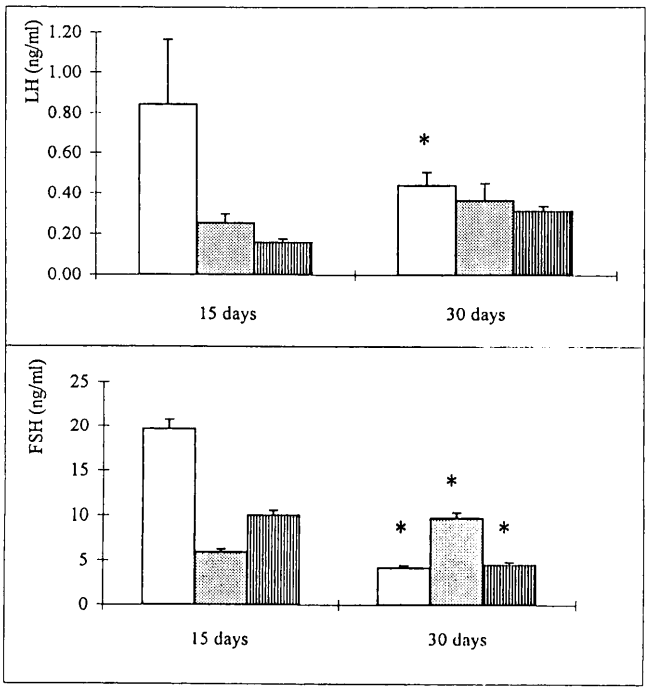

Figure 5. Serum concentration of LH and FSH in the three groups (females (open bars), males (gray bars) and TP females (hatched bars)) at 15 and 30 days. (*$P < 0.05$ for 15 vs 30 days within each sex group.) For differences within age groups, see Results.

in sexual and ontogenic characteristics of gonadotropins regulation, 15 and 30 days of age (1, 2). In the first age, which can be defined as infantile, serum LH and FSH levels are low in males, whereas in females FSH levels are high, and LH is secreted as sporadic unsynchronized peaks. At this age in the female, positive feedback to estradiol is absent, and there is a high sensitivity of the hypophysis to the releasing effect of GnRH. At 30 days, prepubertal stage in females, serum levels of gonadotropins change markedly, FSH decreases in the female, and increases in males, LH is secreted in a pulsatile synchronized pattern, and the positive feedback of estradiol is established. In the male there is an increased sensitivity to the releasing effect of GnRH. Sensitivity of the hypophysis to a depolarizing stimulus also presents developmental differences. Exposing isolated hypophyses to K^+ results in a higher release of LH and FSH at 15 days than at 30 days of life (5), suggesting a special sensitivity of the gonadotrope at that age.

As marked sexual differences are encountered in gonadotropin regulation during development, experiments were carried out in males, females, and females androgenized at birth. In this experimental model the participation of the sexual differentiation of the hypothalamus in conditioning responses in adult males and females can be evaluated. It is well known that neonatal days constitute a critical period with respect to the sexual organization of the brain, and that it is the time at which there is a marked increase in the amount of biogenic amines in the external layer of the median eminence and when differentiation of hypothalamic nuclei reaches a peak. This process is modulated by testosterone and estradiol (13).

Sexual and ontogenic differences in gonadotropin secretion could arise from changes at the hypothalamic or the pituitary level. At the hypothalamic level alterations in the morphology of GnRH neurons, in their capacity to synthesize and release the decapeptide, or in the synaptic circuitry involved could be hypothesized. At the pituitary level changes in GnRH receptor or its transduction systems, in the storage, synthesis and release of gonadotropins, or in the different trophic factors that modulate the gonadotrope, could participate in the differential regulation of gonadotropins.

When mediobasal hypothalami of females, androgenized females, and males were perifused *in vitro* and GnRH measured in the effluent, it was found that basal secretion of the decapeptide increased with age in the three groups, no sexual differences being encountered. These results correlate with those of the literature if we consider that increased basal secretion is a result of increased GnRH concentration in the hypothalamus with advancing age. To this respect it has been described that GnRH content increases with age both in males and females (7–9). No sexual differences in such a parameter were described at 15 and 30 days of age (16). Furthermore transcription of mRNA of GnRH and its precursor increase during development in both sexes (9, 10).

When evaluating K^+-induced GnRH secretion we did not find any differences in sensitivity to the depolarizing agent among age or sex groups; nevertheless, the total amount released was greater in older rats. These findings do not correlate with the high rate of gonadotropin secretion in infantile females, or the marked sexual differences in gonadotropin secretion during development. In the hypophysis, in contrast, we have described that K^+-induced LH and FSH release are maximal at Day 15 (5). To our knowledge no comparative description of K^+-induced GnRH release in developing females and males, or in androgenized females, has been made in the literature. Results presented with regard to K^+-induced GnRH secretion in developing rats are somewhat contradictory. Bourguignon *et al.* (17) described an increase with age of GnRH release induced by depolarizing agents in males. Nevertheless if one considers percentage increases, release was similar at 15 and 30 days. On the other hand Hompes *et al.* (8) showed that in hypothalami of 12–16-day-old females, K^+-induced GnRH release was greater than that in 32-day-old females; and Sladek *et al.* (18) described opposite results. The lower concentration of K^+ used in the present experiments was near the minimal effective concentration (19); nevertheless we cannot rule out that the increasing levels of extracellular K^+ used may mask subtle age-related changes in the signal transduction or in intracellular mechanisms normally involved in the regulation of GnRH secretory activity. We must also consider that the net output of GnRH *in vivo* will be principally related to serotonergic, dopaminergic, and opioid regulation of GnRH secretion, which varies specifically during ontogeny and depends on sexual organization of the hypothala-

mus (14, 15, 20–23), and that it has been shown that specific neuroendocrine systems may alter K^+-induced GnRH release (24).

FSH levels decreased with advancing age in females and increased in males, as previously described (3). In TP females, at 15 days of age, levels were lower than in females but higher than in male littermates, and such levels decreased by 30 days showing a similar pattern to that observed in females. On the other hand LH levels also decreased in females with advancing age. Therefore, we can infer that the developmental and sexual differences in gonadotropin secretion are not due to variations in the secretory capacity of the isolated hypothalamus related to membrane depolarization. Similarly, in other experimental situations, such as after gonadectomy both in the male and in the female, neurosecretory activity of GnRH neurons does not correlate with hypersecretion of gonadotropins (25). Furthermore, in old rats and in the developing cockerel there is no correlation between basal and K^+-induced GnRH release and variations in plasma LH (26, 27).

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