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Ontogenic and Sexual Differences in Pituitary GnRH Receptors and Intracellular Ca^{2+} Mobilization Induced by GnRH

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The present experiments were designed in order to elucidate the participation of the developing hypophysis in determining the changing sensitivity of gonadotrophins to gonadotropin-releasing hormone (GnRH) during ontogeny in the rat. To that end, we chose two well defined developmental ages that differ markedly in sexual and ontogenic characteristics of hypophyseal sensitivity to GnRH, 15 and 30 d. In order to study sex differences and the role of early sexual organization of the hypothalamus, experiments were carried out in males, females, and neonatally androgenized females (TP females). We evaluated (1) the characteristics of pituitary GnRH receptors, and (2) associated changes in GnRH-induced mobilization of intracellular Ca^{2+} (a second messenger involved in gonadotropins exocytosis).
We measured binding characteristics of the GnRH analog D-Ser(TBu)6-des-Gly10-GnRH ethylamide in pituitary homogenates. We found that K_{ds} did not vary among the different sex groups. Total number and concentration of receptors decreased in the female rat from 15–30 d of age, whereas in the male and TP female, receptors/pituitary increased, and the concentration/mg tissue did not change. Also, at 30 days of age, males presented higher content and concentration of receptors than females, and higher content than TP females.
In order to evaluate if developmental and sexual differences in pituitary sensitivity to GnRH might be expressed through variations in the intracellular Ca^{2+} signal, we studied the mobilization of intracellular Ca^{2+} induced by GnRH (1×10^{-8} to $1 \times 10^{-11} M$) in a suspension of dispersed pituitary cells in the six groups. In cells from 15-d-old females, Ca^{2+} response was greater than in 30-d-old females at the doses of 10^{-8} to $10^{-10} M$, indicating that in the infantile female rat activation of highly concentrated GnRH receptors is reflected in an increase in signal transduction mediated by Ca^{2+}. In males and in female rats androgenized at birth, there was also a decrease in the magnitude of intracellular Ca^{2+} mobilization induced by GnRH (10^{-8} to $10^{-10} M$) from 15–30 d of age, even though the concentration of GnRH receptors did not change in the same period.
In conclusion, the present results suggest that high sensitivity to GnRH, which has been described in the female infantile rat, may be related to elevated concentration of hypophyseal receptors coupled to an increase of intracellular calcium response to GnRH, both parameters decreasing as the rat matures. In males, the greater sensitivity that has been described for GnRH at 30 d in comparison to 15 d is paralleled by an increase in the total number of GnRH receptors per pituitary (and not in their concentration), but not in an increase in the magnitude of Ca^{2+} mobilization induced by GnRH. On the other hand, neonatal sexual organization of the hypothalamus is involved in the differential expression of GnRH receptors, but does not modulate mobilization of intracellular Ca^{2+} induced by the decapeptide.

Key Words: GnRH receptors; pituitary; calcium; sexual differences; ontogeny.

Introduction

The hypothalamic–pituitary–gonadal system undergoes complex morphological and physiological changes during progression to puberty. For instance, during development, rats exhibit fluctuations in luteinizing hormone (LH), follicle-stimulating hormone (FSH), and steroids, which have profound influences on 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 at the neural, hypophyseal, and gonadal levels.

During d 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 gonadotropin-releasing hormone (GnRH) *(4)*. In the male, levels of FSH, LH,

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and hypophyseal sensitivity to GnRH 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 *(5)*. The intricacies of the mechanisms underlying such ontogenic changes are being studied.

GnRH content as well as GnRH mRNA in the hypothalamus increase with advancing age *(6–10)*. Alterations in GnRH secretion may be related to the synaptic circuitry connecting GnRH neurons to each other or to the relevant neurotransmitter systems. On the other hand, it can be hypothesized that the hypophysis *per se* may be involved in the developmental changes described. In particular, binding characteristics of GnRH receptors in the anterior pituitary and second messengers activated by the neuropeptide might modulate physiological changes in gonadotropic sensitivity to GnRH secreted by the hypothalamus. It has been described that development of GnRH receptors follows different timecourses between sexes *(11,12)*. Up to the moment, there are no data in the literature describing the development of GnRH receptors in female rats androgenized at birth (TP females). This experimental model evaluates the contribution of neonatal masculinization of hypothalamic structures controlling sexual differences in gonadotropins secretion and lordotic behavior *(13)*.

Modulation of gonadotropin secretion during development may include an action at the GnRH signal transduction system. After binding to its receptor, GnRH stimulates phosphoinositide hydrolysis and the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which mobilize calcium from intracellular sources and activate protein kinase C, respectively *(14)*. In single gonadotropes, the GnRH-induced increase of intracellular Ca^{2+} exhibits dose-related response profiles. Since ovarian steroid hormones affect GnRH-induced Ca^{2+} mobilization and secretory responses in gonadotropes in vitro *(15,16)*, it was reasonable to speculate that variations in Ca^{2+} induced mobilization by GnRH might underlay developmental and sexual characteristics of gonadotrope function.

Therefore, we measured concentration, total content, and affinity of pituitary GnRH receptors, and intracellular Ca^{2+} mobilization induced by GnRH in cells from female, male, and TP female rats at 15 (infantile period) and 30 (prepubertal period) d of age to evaluate the participation of such parameters in ontogenic and sexual differences described for gonadotropins secretion.

Results

Pituitary GnRH Binding Sites of Females, TP Females and Males at 15 and 30 D of Age

Total number of receptors, expressed as fmol/pituitary, increased significantly with age both in TP females and in males, differences being more pronounced in this last group

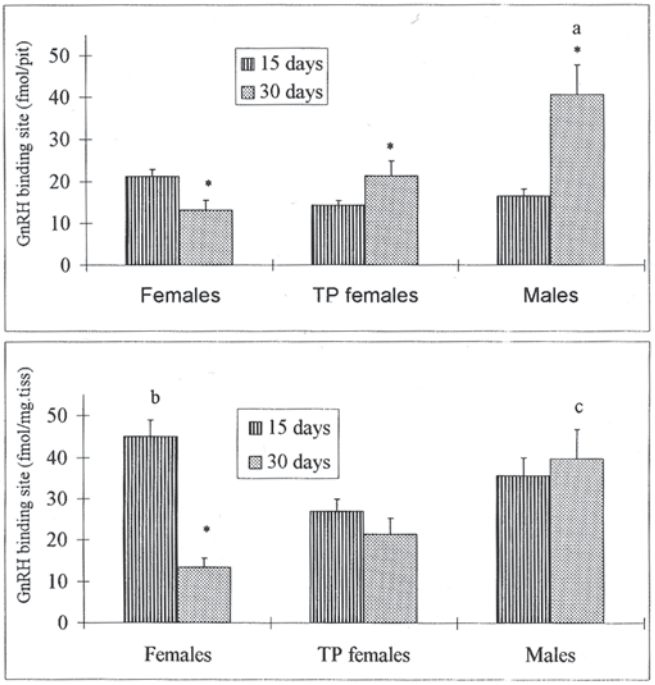

Fig. 1. *Upper panel:* Number (in fmol) of GnRH receptors/pituitary in females, TP females, and males at 15 and 30 d of age. *Represents significant differences between ages in a same-sex group ($p < 0.05$). a: $p < 0.05$ vs females and TP females in the same age group. For this and the following figure, the height of the bar represents the average and the line on top ±SE ($n = 11–18$). *Lower panel.* Concentration of GnRH receptors (fmol/mg tissue) in hypophyses from females, TP females, and males at 15 and 30 d of age. *Represents significant differences between ages in a same-sex group ($p < 0.05$). b: $p < 0.05$ vs TP females of the same age group. c: $p < 0.05$ vs females of the same age group ($n = 11–18$).

(Fig. 1, upper panel). Such an increment paralleled pituitary weight increase so that the concentration of receptors expressed as fmol/mg pituitary remained constant from 15–30 d of age in these two groups (Fig. 1, lower panel). In contrast, total number as well as concentration of receptors decreased significantly with age in females (Fig. 1) in spite of the increasing weight of the pituitary.

When we analyzed sexual differences within each developmental group, we found that at 15 d of age, there were no significant differences in fmol bound/pituitary, but that the concentration of receptors/mg hypophysis was greater in females than in TP females. At 30 d of age, males had a higher number and concentration of receptors than age-matched females, and a higher total number of receptors when compared to TP females.

Scatchard plots of the corresponding groups confirmed the above results and showed that there were no differences in the affinities of the receptors among groups ($K_d \pm$ SE [n*M*]): 0.061 ± 0.021; 0.065 ± 0.027; and 0.053 ± 0.03 for females, TP females, and males 15 d of age, respectively, and 0.059 ± 0.029, 0.061 ± 0.04, and 0.057 ± 0.025 for the same groups at 30 d).

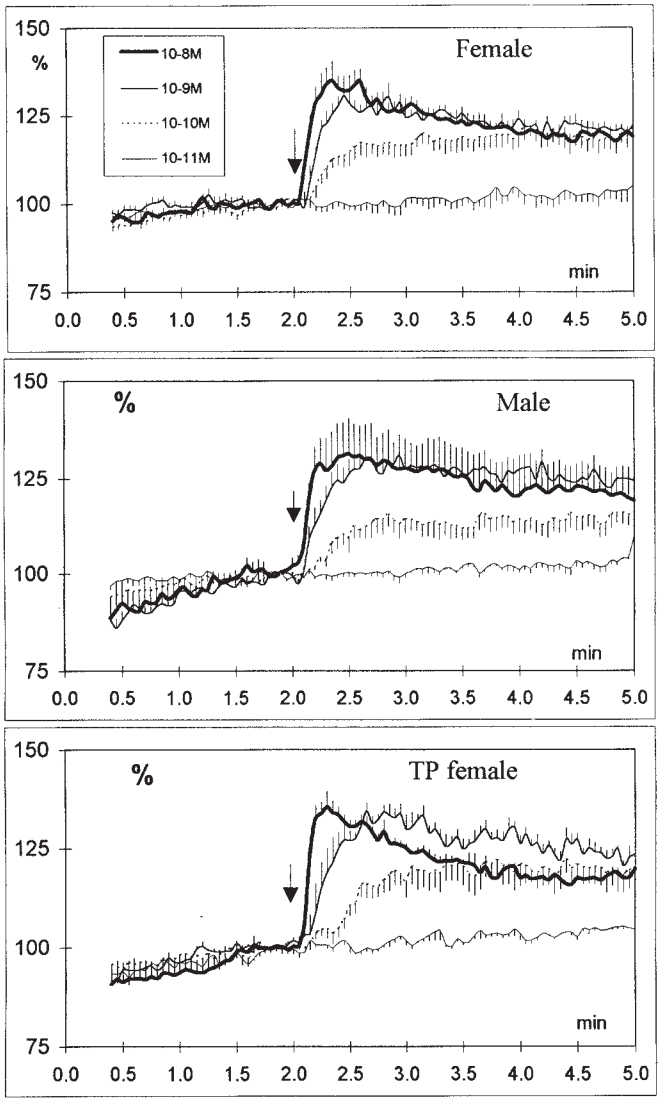

Fig. 2. Mobilization of intracellular Ca^{2+} monitored by FURA-2AM method in dispersed adenohypophyseal cells from 15-d-old females *(upper panel)*, males *(middle panel)*, and TP females *(lower panel)*. For this and the following figure, arrows indicate the stimulus of GnRH (10^{-8}, $^{-9}$, $^{-10}$, or $^{-11}$ *M*) (minute 2). Results expressed were normalized (values in n*M* * 100/basal level [in n*M*], for each cellular suspension; basal level: average of 1 min, 45 s to 2 min, 0 s). Curves indicate the average of five to six homogenates (each homogenate corresponded to a pool of six to nine rats). Lines over curves express ±SE.

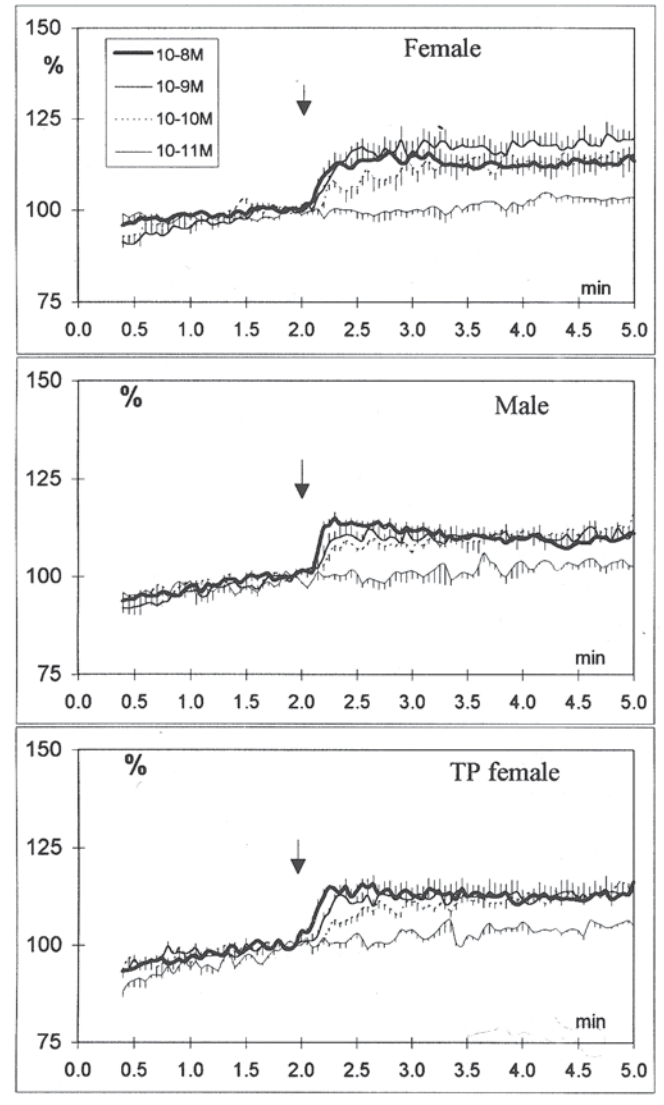

Fig. 3. Mobilization of intracellular Ca^{2+} monitored by FURA-2AM method in dispersed adenohypophyseal cells from 30-d-old females *(upper panel)*, males *(middle panel)*, and TP females *(lower panel)* For more details, *see* legend to Fig. 2.

Effect of GnRH on Mobilization of $[Ca^{2+}]_i$ in Dispersed Pituitary Cells from 15- and 30-D-Old Rats

Changes in intracellular concentration of Ca^{2+} induced by GnRH ($1 * 10^{-8}$–$1 * 10^{-11}$ *M*) were monitored in a suspension of adenohypophyseal cells from the different groups. Both at 15 and 30 d of age, the response was related to the concentration (Figs. 2 and 3), and no significant increase was encountered at the lowest concentration. When we integrated the area under the initial phase of response (% increase over basal levels [100%]) starting at 2 min, 15 s and ending at 3 min/10 s, we found that the concentration of 10^{-9} *M* already produced a maximal response that was not significantly different from that of 10^{-8} *M*. Furthermore at 15 d, Ca^{2+} increases were significantly greater than those at 30 d of age, for the three groups tested (Fig. 4), at the concentrations of 10^{-8}–10^{-10} *M*.

Basal unstimulated levels of intracellular concentration of Ca^{2+} were not different in the studied groups (Table 1).

Discussion

We studied the role of the developing hypophysis in the determination of the changing patterns of gonadotropin sensitivity to GnRH from the infantile to the prepubertal period in both sexes. To that end, we chose two well-defined developmental ages that differ in sexual and ontogenic

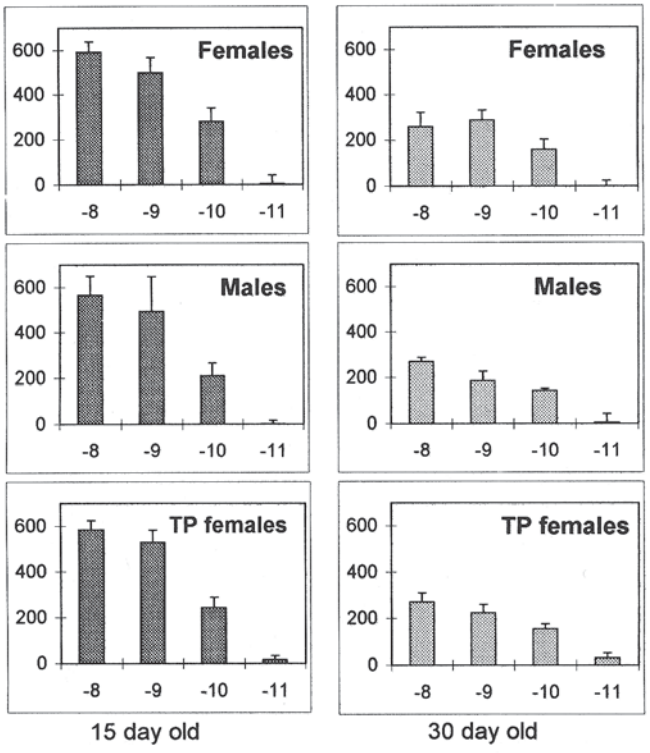

Fig. 4. Area (in %) under the initial peak of phase of intracellular Ca^{2+} induced by GnRH (10^{-8}, $^{-9}$, $^{-10}$, or $^{-11}$ *M*) in dispersed cells from 15- *(left panel)* and 30-d-old *(right panel)* female, male, and TP females. Values were considered to be integrating the area under the curves in Figs. 3 and 4, from 2 min, 15 s to 3 min, 10 s (*see* Material and Methods).

Table 1
Absolute Basal Levels of Intracellular Ca^{2+} (in n*M*) in Suspensions of Adenohypophyseal Cells from 15- and 30-D-Old Female, TP Female, and Male Rats[a]

	Females	TP Females	Males
15 D	93.7 ± 4.3 (*n* = 14)	106.0 ± 6.6 (*n* = 9)	99.6 ± 4.3 (*n* = 12)
30 D	106.4 ± 2.5 (*n* = 8)	102.7 ± 7.1 (*n* = 8)	85.7 ± 2.6 (*n* = 8)

[a]No significant differences were found.

characteristics of gonadotropins regulation, 15 and 30 d of age *(1,2)*. In the first age, which can be defined as infantile, serum LH and FSH levels are low in males, but in females, FSH levels are high and sporadic unsynchronized peaks of LH are found. 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 d serum 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 females. In the male, there is an increased sensitivity of the hypophysis to the releasing effect of GnRH.

Since marked sexual differences are encountered in gonadotropins regulation during development, we evaluated not only males and females, but also neonatally androgenized females. In this experimental model, the participation of neonatal sexual differentiation of the hypothalamus in conditioning responses in males and females can be evaluated *(13)*.

Sexual and ontogenic differences in gonadotropins secretion could arise from changes at the hypothalamic or pituitary level. At the pituitary level, changes in GnRH receptor or its transduction systems, in gonadotropin storage, synthesis and release, or in the different trophic factors that modulate the gonadotrope could participate in the differential regulation of gonadotropins. The present set of experiments studies the effect of sex and age on the characteristics of pituitary GnRH receptors and GnRH-induced intracellular Ca^{2+} mobilization in adenohypophyseal cells.

We measured binding characteristics of the GnRH analog D-Ser(TBu)6-des-Gly10-GnRH ethylamide in pituitary homogenates of the different groups and found that the K_ds of the GnRH receptor did not vary during development or among females, males, and TP females, and that its value was similar to that described in adult animals *(12)*. Total number and concentration of receptors decreased in the female rat from 15 to 30 d of age, whereas in the male and TP female, receptors/pituitary increased, and the concentration/mg tissue did not change. Also, at 30 d of age, males presented higher content and concentration of receptors than females, and higher content than TP females. Our results are highly concordant with data from the literature regarding development of GnRH receptors *(11,12)*, though no comparison was previously made with the TP female rat. Time-course of changes in receptors agrees well with the results of previous studies on LH and FSH responsiveness to GnRH *(4,5,17,18)*.

GnRH receptors in neonatally TP female rats have not been described before, though it has been shown that testosterone administered to immature males at different periods and time schedules inhibits the developmental rise in GnRH receptors *(19)*. We describe that GnRH receptors in the TP female rat follows a time-course similar to that of male rats (no variation in concentration and increase in total content from 15–30 d of age), though at 30 d, receptor content is lower than in male rats. Fink and Henderson *(20)* described that in the adult rat that had been neonatally androgenized, the response of gonadotropins to GnRH was lower than that in female proestrus rats and similar to that in males, and Barraclough and Turgeon *(21)* have shown that the responsiveness of the pituitary to GnRH in both immature and mature TP female rats is considerably reduced in comparison to untreated rats. This suggests that basal as well as GnRH-induced gonadotropin secretion depends on sexual differentiation of the hypothalamo–hypophyseal system. Decreased responsiveness of the pituitary of TP females would be related not only to the

lower concentration of receptors as described in the present work, but also to lower pituitary concentrations of LH and FSH in TP females in comparison to males or females at 15 d of age *(22)*. At 30 d, concentration and content of receptors in TP female rats are similar to those in females, and pituitary LH and FSH concentrations are also similar *(22)*, suggesting that altered responsiveness to GnRH induced by neonatal testosterone might not be permanent.

Increased GnRH binding in the pituitary gland may result from an increase in the number of GnRH receptors/gondotropes or to an increased number of gonadotropes present in the pituitary. Chen *(23)* describes that the percentage of immunostained LH cells was similar at 15 and 30 d in females, but in males, the percentage of immunostained LH cells decreased slightly from 15–30 d, and the percentage of plaque-forming cells increased with age. Both at 15 and 30 d of age, females presented a higher percentage of plaque-forming cells than males. We therefore cannot conclude that variation in concentration and number of GnRH receptors is mainly owing to changes in the proportion of gonadotropes in the hypophysis, but probably results from modulation exerted by gonadal steroids *(19)*, variations in GnRH secretion, and the proportion of gonadotropes in the pituitary.

GnRH receptor activation is coupled to mobilization of intracellular Ca^{2+} *(14)* and agonist-induced increases in cytoplasmatic calcium concentration play a pivotal role in regulated exocytosis. Modulation of developmental and sexual differences in pituitary sensitivity to GnRH may be expressed through variations in the intracellular Ca^{2+} signal in gonadotropes. For example, it has been described that the modulatory effects of ovarian steroids on GnRH-induced gonadotropin secretion includes a significant action on the Ca^{2+} signaling pathway *(16)*. We therefore studied the mobilization of intracellular Ca^{2+} induced by GnRH (1×10^{-8}–10^{-11} *M*) in a suspension of dispersed pituitary cells from the different groups.

In dispersed cells from 15-d-old females, Ca^{2+} response was greater than in 30-d-old females, indicating that in the infantile female rat, activation of highly concentrated GnRH receptors is reflected in an increase in the signal transduction, which probably participates in the increased sensitivity to GnRH described at this age and which is gradually lost as the female rat matures. Such parallelism between sensitivity of GnRH and mobilization of Ca^{2+} has been also described in the progesterone attenuation of LH release *(16)*. On the other hand, Tomic et al. *(24)* describe the ontogeny of GnRH-induced Ca^{2+} increases, but only in females and at ages that do not correspond to the present work, and also suggest that developmental and physiological changes in pituitary sensitivity to GnRH are expressed through modulation of Ca^{2+} signal in gonadotropes.

Both in males and in female rats androgenized at birth, there was also a decrease in the mobilization of intracellular Ca^{2+} induced by the different concentrations of GnRH from 15–30 d of age, even though the concentration of GnRH receptors did not change in the same period, and it has been described that agonist-induced LH and FSH release increases with age in males *(5)*. A similar case of the lack of parallelism in the secretion of LH and magnitude of response of Ca^{2+} mobilization would be that of estradiol, which augments agonist-induced gonadotropin secretion without altering intracellular Ca^{2+} responses to GnRH *(16)*. This discrepancy in the response of intracellular calcium and secretion induced by GnRH could be explained through participation of modulatory actions exerted by different transducers of the agonist-stimulated signaling pathway *(25)*. It has been described that the concerted actions of several proteins (such as PKC, Exo 1 and 2 proteins, cytoskeletal actin, 145-kDa cytosolic protein, and so forth) are required to translate or modulate the Ca^{2+} signal into the secretory response *(25)*. A rise in cytoplasmic calcium concentration *per se* is sufficient to initiate exocytosis, but to a lesser degree than that elicited by agonist stimulation *(26)*.

In conclusion, the present results suggest that high sensitivity to GnRH in the female infantile rat may be related to a high concentration of hypophyseal receptors coupled to an increase of intracellular calcium response to GnRH, both parameters decreasing as the rat matures. Also, the developmental pattern in GnRH binding in the pituitary of female infantile rats depends on early sexual organization of the hypothalamus. In males, the greater sensitivity that has been described for GnRH at 30 d in comparison to 15 d is paralleled by an increase in the total number of GnRH receptors per pituitary (and not in the concentration), but not in an increase in the magnitude of Ca^{2+} mobilization induced by GnRH. It is therefore probable that in this situation, alternative mechanisms may modulate GnRH response.

Materials and Methods

Animals

Sprague-Dawley rats were housed in an air-conditioned room with lights on from 0700 to 1900 h. They 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 sc administration of 100 µg testosterone propionate (Sigma, 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 d of age. Groups of rats of 15 or 30 d of age were used.

Preparation of [^{125}]Iodine GnRH Analog

(D-Ser[-tBu]^6-des-Gly^{10})-GnRH-*N*-ethylamide (buserelin, GnRH-a) (gift from Hoechst, Buenos Aires) was used as tracer and unlabeled hormone in the binding assay. GnRH-a was iodinated using a chloramine-T method.

Briefly, 2 μg of GnRH-a were iodinated in the presence of 2 mCi [^{125}]iodine (New England, MA) and 0.2 μg chloramine-T. Reaction proceeded for 2 min. The procedure was repeated once, and reaction was stopped by transferring to a carboxy-methyl cellulose column and eluting unbound iodine with 0.002 *M* ammonium acetate, and labeled analog with 0.060 *M* ammonium acetate. The iodinated analogue was stored at 4°C in this last buffer and used within 3 wk of preparation. The specific activity of each preparation was assessed by self-displacement in the receptor assay using a crude membrane fraction prepared from pooled pituitaries. Specific activities ranged from 600–1000 μCi/μg. Maximum binding of the trace determined by incubation with excess pituitary membranes was 40–65% *(27)*.

Assay of Pituitary GnRH Receptors

Rats were killed by decapitation and anterior pituitaries were quickly removed and stored at –70°C *(28)*. Pituitaries were thawed, weighed, and homogenized in 400 μL of assay buffer (Tris-HCl 10 m*M*, 0.1% bovine serum albumin [Sigma], and 1 m*M* dithiothreitol [Sigma], pH 7.6) at 4°C in a glass homogenizer. Homogenates were prepared immediately before addition to assay tubes. For saturation analysis, the homogenate from a single pituitary (30-d-old animals) or from two or three rats from the same group (15-d-old animals) was used to prepare five incubation mixtures (three for total binding and two for nonspecific binding). Each tube had approx 0.15–0.35 mg tissue (40–70 μg proteins) in 300 μL buffer. Pituitary homogenates were incubated with 6–8 × 10^4 cpm [^{125}I] GnRH-a in a total volume of 500 μL assay buffer. Ligand concentrations were near saturating, representing about 85% receptor occupancy. Nonspecific binding was determined by addition of 10^{-7} *M* unlabeled GnRH-a, and represented 5–8% of total iodinated tracer.

For Scatchard analysis, pituitary homogenates (300 μL) were incubated in the presence of increasing concentrations of labelled analog (5000–120,000 cpm) in a total volume of 500 μL. Parallel incubation mixtures contained 2×10^{-7} *M* unlabeled GnRH-A to assess nonspecific binding. Experiments for Scatchard analysis were repeated three times.

In all cases, tubes were incubated for 120 min on ice, and reaction was terminated by centrifugation at 16,000*g* for 20 min at 4°C. The supernatant was discarded, and the pellets were counted in a γ-spectrometer (efficiency 73%). Specific binding was expressed/pituitary or mg of tissue.

Cell Dispersion for Ca^{2+} Measurements

Rats were decapitated, and adenohypophyses were removed and placed in chambers containing freshly prepared Krebs-Ringer bicarbonate buffer (KRBGA) without Ca^{2+} or Mg^{2+}. Buffer contained 14 m*M* glucose, 1% bovine serum albumin (Sigma), MEM amino acids 2% (Gibco), and phenol red 0.025%, and was previously gassed during 15 min with 95% O_2 and 5% CO_2, and adjusted to pH 7.35–7.40. Buffer was filtered through a membrane (Nalgene) whose pore diameter was 0.22 μm. Hypophyses were washed three times with buffer KRBGA and then cut in 1 mm pieces. Obtained fragments were washed and incubated in the same buffer containing 0.2% trypsin for 30 min at 37°C, 95% O_2, and 5% CO_2. They were then treated for two additional minutes with DNase I (Sigma, 1 mg/mL), and digestion was ended adding 0.2% newborn calf serum (Gibco). Fragments were dispersed in individual cells by gentle tritruration through siliconized Pasteur pipets. Resulting suspension was filtered through a nylon gauze (160 μm), and centrifuged 10 min at 1200*g*. Before centrifugation, an aliquot of cellular suspension was taken in order to quantify hypophyseal cell yield, using a Neubauer chamber. Viability of cells determined by trypan blue was always > 95%. Pellet was redispersed for measurement of intracellular Ca^{2+}.

Intracellular Ca^{2+} Measurements

Fura-2/AM (tetracetoxymethylesther-Fura 2, Sigma) was used as a fluorescent indicator *(29)*. Pellets of adenohypophyseal cells of each experimental group (females, TP females, or males of 15 or 30 d of age) were redispersed. Experiments were performed on the heterogeneous population of pituitary cells, since previous studies on enriched and purified gonadotrophs have shown that GnRH receptors reside exclusively in gonadotrophs (30). Cells were incubated in a buffered saline solution (BSS: NaCl 127 m*M*, KCl 5 m*M*, KH_2PO_4 0.5 m*M*, $NaHCO_3$ 5 m*M*, $CaCl_2$ 1.8 m*M*, $MgCl_2$ 2 m*M*, HEPES 10 m*M,* pH = 7.5) in the presence of Fura-2/AM 1.5 μ*M*, 10 m*M* glucose, 0.1% BSA. Cells were incubated for 40 min at 37°C in an atmosphere of 5% CO_2, time during which Fura-2/AM was incorporated into the cells and converted to the fluorescent indicator Fura-2 in the cellular cytoplasm by endogenous esterases. After incubation, cells were washed twice in BSS without Fura-2/AM, and prepared at a density of 1.7–2 × 10^6 cells/mL. Fluorescence was measured in a spectrofluorometer (Jasco Corporation, Tokyo) provided with the accessory CA-261 to measure Ca^{2+} with continuous stirring, thermostat adjusted to 37°C, and injection chamber. Intracellular Ca^{2+} levels were registered every second by exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured. In this way, light intensities and their ratio (F340/F380) were monitored. Stability of the suspension was evaluated during the two initial minutes (basal values). At minute 2, 5 μL of GnRH solution (from 10^{-6}–10^{-9} *M*) were injected into the chamber (final concentration in cell suspension 10^{-8}–10^{-11} *M*), and 3 min later, KCl to test viability of the suspension (final concentration 56 m*M*). Two minutes after KCl injection the preparation was calibrated determining maximal fluorescence induced by 0.1% Triton X-100 (F_{max}) and minimal fluorescence (F_{min}) in the presence of 5 m*M* EGTA (pH

adjusted to over 8.3). $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. *(31)*. Values were corrected for dye leakage and autofluorescence. Resulting graphs were processed and quantified using software Ungraph 2.0, and Excel 5.0. Percentual increase induced by GnRH for each measurement was calculated considering basal level and the average of values during the 15 s before GnRH administration. To quantitate the spike response to GnRH, area under the curve between minutes 2 min, 15 s and 3 min, 10 s was integrated.

Statistical Analysis

Scatchard analysis of binding data was performed by a computer curve-fitting program (Ligand) for a single class of binding sites. Results in experiments of GnRH binding sites (saturation analysis) were analyzed by two-way analysis of variance for the effects of age and sex. If F of interaction was found to be 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 intracellular Ca^{2+} concentration was also analyzed by two-way analysis of variance for the effects of age and sex. Differences in areas under the curve in Ca^{2+} experiments were analyzed by two way analysis of variance, followed by Scheffé's test, for the effects of age and dose; $p < 0.05$ was considered significant in all cases.

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