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Changes in Expression of Hypothalamic Releasing Hormone Receptors in Individual Rat Anterior Pituitary Cells during Maturation, Puberty and Senescence

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Anterior pituitary (AP) is formed by five different cell types, each one producing a different AP hormone whose secretion is regulated by a specific hypothalamic-releasing hormone (HRH). On the other hand, a significant number of AP cells express multiple HRH receptors (multiresponsive cells). Plastic changes in expression of HRH receptors in individual AP cells are involved in critical endocrine events. Here we have characterized the changes in functional responses to CRH, LHRH, TRH, and GHRH in individual AP cells throughout the whole life span of the rat. To this end, calcium responses to the HRHs were followed by single-cell imaging in freshly dispersed AP cells prepared from rats of different ages (0–540 postnatal days). Three different cell pools were identified: 1) monoresponsive cells, holding a single class of HRH receptor; 2) multiresponsive cells; and 3) nonresponsive cells. The relative abundance of each pool changed with age. Nonresponsive cells were abundant at birth, multiresponsive cells were abundant at puberty, and monoresponsive cells dominated at senescence. The relative abundance of each HRH receptor changed largely with age but not gender. In addition, the contribution of monoresponsive and multiresponsive cells to responses to each HRH changed very much with age. Thus, the anterior pituitary shows large changes in cell populations typed by functional responses to HRHs during maturation, puberty, and senescence. (*Endocrinology* 146: 4627–4634, 2005)

THE ANTERIOR PITUITARY (AP) gland controls the endocrine system through the release of multiple hormones including GH, prolactin (PRL), ACTH, TSH, and gonadotropins (LH and FSH) that target multiple tissues and endocrine glands. It was formerly considered that each AP hormone is secreted by a different AP cell type including somatotropes, lactotropes (or mammotropes), corticotropes, thyrotropes, and gonadotropes. However, the existence of AP cells storing more than one AP hormone has been reported. An important control of AP hormone secretion is driven by several hypothalamic-releasing hormones (HRHs) including GHRH, TRH, CRH, and LHRH. There is much evidence indicating that changes in expression of pituitary receptors for HRHs (HRH receptors) are involved in critical endocrine changes occurring during life, such as postnatal development (maturation), start of sex cycling (puberty), and aging (senescence). For example, changes in GH secretion that occur during post natal development and aging are associated with changes in pituitary GHRH binding sites and GHRH sensitivity (1, 2). Expression of LHRH receptors may also be influenced by age, gender, and gonadal steroids (3–6). The hypothalamic-pituitary-adrenal axis has been reported to be impaired during the neonatal period and aging, and this effect has been attributed to lack or decrease of CRH receptor mRNA, respectively (7–9). Likewise, it has been reported that TRH binding sites change with aging (10). Most studies in this regard have focused on expression of a single HRH receptor in a particular physiologic situation such as aging or puberty, and receptor expression has been estimated at the level of mRNA in tissue extracts rather than the functional protein at the single cell level. Thus, a comprehensive study of functional responses to several classic HRHs in individual AP cells throughout the entire life span is lacking.

It was formerly considered that each AP cell subpopulation is stimulated by an specific HRH. However, in the last few years, it has been shown that a population of rat and mouse AP cells actually bear multiple HRH receptors (11–16). No data are yet available in healthy humans, but multiresponsive cells are quite abundant in human pituitary adenomas (17). The physiological role of multiresponsive cells is unclear. It has been proposed that they may contribute to pituitary plasticity processes such as paradoxical secretion and transdifferentiation. Paradoxical secretion refers to the release of a given AP hormone induced by a noncorresponding HRH (13, 17). It has also been proposed that multiresponsive cells may arise during transdifferentiation (cell phenotype change from an AP cell type to another without cell division) occurring in particularly demanding physiological or pathophysiological situations such as lactation, ovulation, or protracted hypothyroidism (18, 19). Despite the putative importance of multiresponsive cells in the physiology of the AP gland, changes in their relative abundance during life is unknown. Owing to these reasons, it is advisable to include analysis of multiresponsive cells in our study of functional responses to HRHs. We determined expression of four important HRH receptors in individual rat AP cells throughout the whole life span. To this end, we imaged calcium re-

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Abbreviations: AP, Anterior pituitary; $[Ca^{2+}]_{cyt}$, cytosolic free calcium concentration; $\Delta[Ca^{2+}]_{cyt}$, increase of $[Ca^{2+}]_{cyt}$; HRH, hypothalamic-releasing hormone; pnd, postnatal day; PRL, prolactin.

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sponses induced by sequential presentation of HRHs to freshly dispersed rat AP cells. It is well known that stimulation of AP cells with HRHs increases the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in target cells (20–23). We assessed functional responses to HRHs at birth and after 7, 15, 30, 45, 60, 90, and more than 540 d of life. We have chosen these ages to have detailed information of critical periods, such as postnatal development and maturation [1–15 postnatal days (pnd)], puberty (30–60 pnd), and senescence (>540 pnd).

Materials and Methods

Preparation of anterior pituitary cells

Male and female Wistar rats (0, 7, 15, 30, 45, 60, 90, and more than 540 pnd) were first weighed and then killed by cervical dislocation following the procedure approved by the Valladolid University School of Medicine Ethics Committee. Then AP glands from at least three rats were quickly removed, chopped into little pieces (about 1 × 1 mm) with small dissecting scissors, and dispersed by incubation with 1 mg/ml trypsin (Sigma, Madrid, Spain) in Hank's balanced salt solution (Life Technologies, Inc., Paisley, UK) at 37 C with gentle shaking for 15 (newborn rats), 30 (7–90 pnd rats), or 45 min (aged rats). Every 5 min, pieces were passed repeatedly through a fire-polished siliconized Pasteur pipette to triturate the tissue into single cells and help digestion. The cells were then sedimented by centrifugation at 200 × *g* for 5 min and washed twice with Hanks' balanced salt solution. Monodispersed cells were finally plated on coverslips previously coated with 0.01 mg/ml poly-L-lysine and incubated in DMEM (Gibco, Barcelona, Spain) supplemented with 10% fetal bovine serum and antibiotics and used within 2–4 h. We have shown previously that the response to the HRHs by these freshly dispersed cells is better than the one of cells maintained in primary culture for 1–3 d (13).

Determination of cycling start

Vaginal smears of female rats were taken at 30, 45, and 60 pnd. Samples were collected with a cotton-tipped swab moistened with PBS from the vaginal cavity of rats. Samples were fixed with cold methanol, stained using GIEMSA (Analema, Vigo, Spain), and analyzed by light microscopy. This procedure was intended to determine when the females hit puberty and not to obtain AP cells at any particular time of the estrous cycle.

Functional responses of individual cells to hypothalamic releasing hormones

Responses of single AP cells to the HRHs were assessed from their changes in $[Ca^{2+}]_{cyt}$, which were measured by digital imaging fluorescence microscopy as described previously (12, 13). Briefly, cells were loaded with fura 2-AM (4 μM) for about 1 h at room temperature in standard medium of the following composition (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; HEPES, 10; glucose 10 (pH 7.4). Cells were then washed with the same medium, placed in a controlled temperature (37 C) chamber on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan), and perfused with standard medium prewarmed at 37 C. Cells were epiilluminated alternately at 340 and 380 nm, and light emitted above 520 nm was recorded using a Magical Image processor (Applied Imaging, Newcastle, UK). Pixel by pixel ratios of consecutive frames were produced and $[Ca^{2+}]_{cyt}$ was estimated from these ratios by comparison with fura 2 standards. The cells were perfused sequentially with the four test solutions containing HRHs (20 nM) during 30-sec periods at the times indicated. A depolarizing solution containing high K^+ (150 mM; replacing an equiosmotic amount of Na^+) was perfused for 10 sec at the end of the experiment to assess normal responsiveness of each cell preparation. Cells not responding to high K^+ (~20 and 5% of the whole AP population in newborn and adult rats, respectively) were excluded from the analysis. The procedure is illustrated in Fig. 1A, in which four images of fura 2-loaded AP cells taken during sequential addition of the several hypothalamic releasing hormones CRH, LHRH, TRH, and GHRH are shown. The *arrowheads* identify three multiresponsive cells. The scale on the left is the pseudocolor code for $[Ca^{2+}]_{cyt}$, from *dark blue* (50 nM) to *red* (1000 nM). Cells were considered responsive to a given HRH when a rise in $[Ca^{2+}]_{cyt}$ larger than 100 nM was obtained. This $[Ca^{2+}]_{cyt}$ rise threshold allows to distinguish real responses from noise variations. Most responses were actually much larger, as evidenced by average values of increases in $[Ca^{2+}]_{cyt}$ induced by HRHs. As reported previously (14), changing the order of stimulation with the different HRHs did not modify the responses. This procedure has been assessed and described in detail earlier (12, 13). At least three independent experiments from cells derived from two to six animals were carried out for each gender at each postnatal period. All experiments were carried out in the same year season to avoid seasonal influences.

Statistical analysis

All data reported are results of at least three completely independent experiments for each sex and age studied. A two-way ANOVA setting gender and age as independent factors was used to analyze the data, and the means were compared using Bonferroni's multiple comparison test. Differences were considered significant at $P < 0.05$.

Results

We assessed the functional responses to four classic HRHs including CRH, LHRH, TRH, and GHRH in individual rat AP cells. To this end we monitored the changes in intracellular $[Ca^{2+}]$ after sequential stimulation with the HRHs by digital imaging fluorescence microscopy of freshly dispersed AP cells loaded with fura 2. Figure 1 illustrates this strategy. Pictures (Fig. 1A) show calcium images (coded in pseudocolor) taken during sequential stimulation with the four HRHs. Cells were obtained from rat pituitaries at birth (0) or after 7, 15, 30, 45, 60, 90, or more than 540 pnd of life. Figure 1B shows the weight of pituitary donors at each age. The $[Ca^{2+}]_{cyt}$ responses of six representative AP cells are also shown (Fig. 1, C–H). Some cells responded specifically (with a $[Ca^{2+}]_{cyt}$ increase) to only one single HRH (monoresponsive cells, Fig. 1, C and D). These cells can be considered as the orthodox or classic cell type. We found cells that did not respond to any of the HRHs tested but responded to stimulation with high K^+ medium (nonresponsive cells, Fig. 1E). Finally, we found cells responding to multiple HRHs (Fig. 1, F–H): two (Fig. 1F), three (Fig. 1G), or even all four HRHs (Fig. 1H). These cell phenotypes cannot be considered as orthodox AP cells and will be referred to as multiresponsive cells.

Using the above strategy, we studied changes in the relative abundance of monoresponsive, multiresponsive, and nonresponsive AP cells throughout life from birth to senescence. After calcium-imaging experiments, cells were typed as nonresponsive, monoresponsive, or multiresponsive as described above and their relative abundance (percent) was calculated (Fig. 2). A two-way ANOVA was used to assess significance of the data, and the means were compared using the Bonferroni test. The relative size of the three cell pools changed with age but not with gender ($P > 0.05$). The nonresponsive cell pool (Fig. 2, *white* part of the bars) was most abundant at birth (35–42%) and then diminished significantly ($P < 0.05$) to 10% at 7 and 15 pnd reaching 18–20% just before puberty (30 pnd) and decreased again ($P < 0.05$) after puberty (45–60 pnd), remaining as low as 10% of the cells in the aged animals. The monoresponsive cell pool (Fig. 2, *dashed* part of the bars) was quite constant along the whole life span, representing 40–50% in all age periods. However,

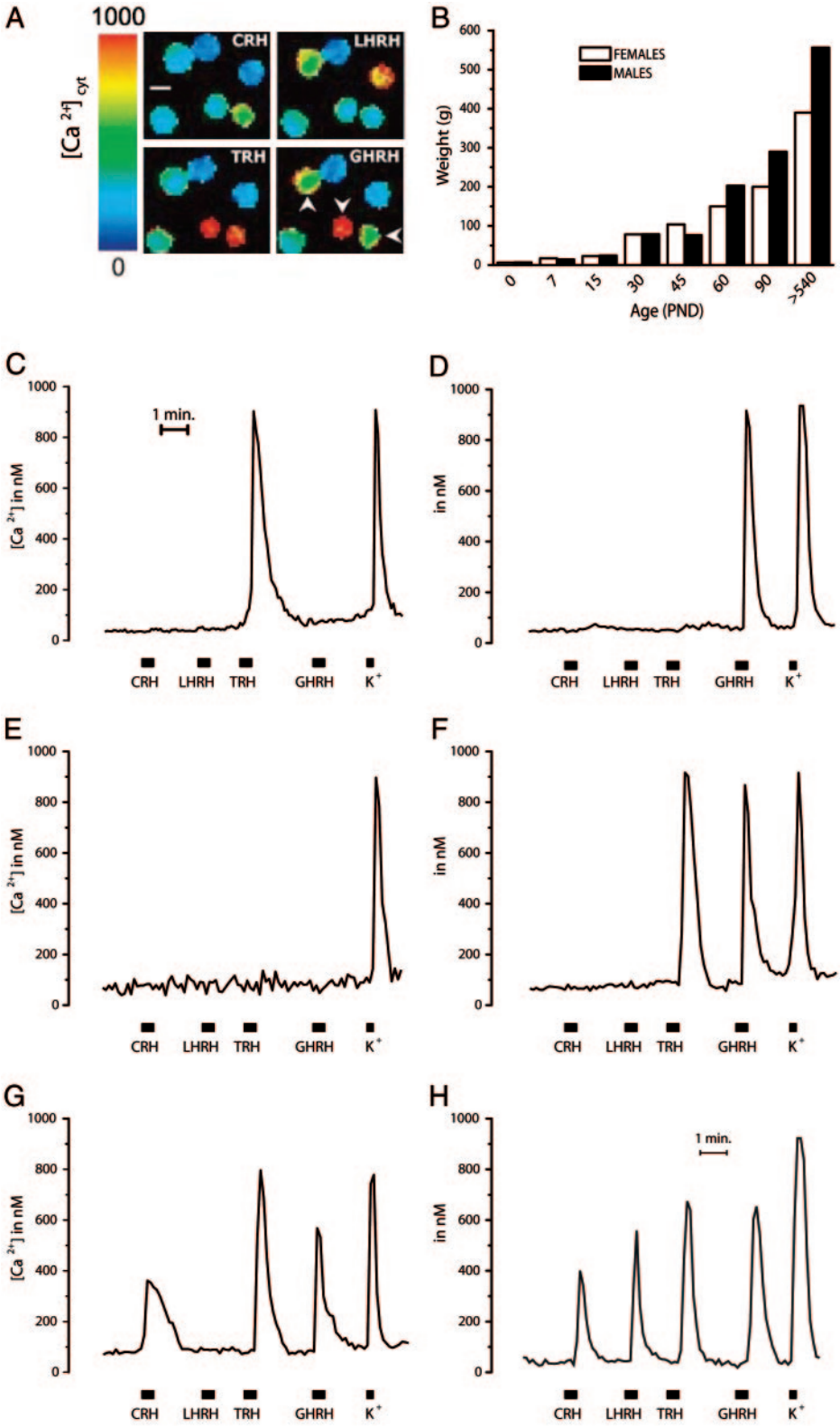

FIG. 1. Expression of functional HRH receptors in individual rat AP cells. Freshly obtained AP cells attached to glass coverslips were loaded with fura 2 and stimulated sequentially with the four HRHs (20 nM). $[Ca^{2+}]_{cyt}$ measurements were performed by digital imaging fluorescence microscopy. A, Fura 2 ratio images taken during sequential addition of the four HRHs. The *scale* on left is pseudocolor code for $[Ca^{2+}]_{cyt}$ from *dark blue* to *red*, 0–1000 nM. *Arrowheads* point to cells responding to more than one HRH (multiresponsive cells). *Calibration bar*, 10 μm. B, Pituitary donors were weighed just before pituitary removal. *Bars* represent average weight in male (*black*) and female (*white*) animals at 0, 7, 15, 30, 60, 90, and more than 540 pnd. C–H, Representative $[Ca^{2+}]_{cyt}$ traces of individual AP cells in response to sequential stimulation with the four HRHs (20 nM) and depolarizing high-K^+ solution (150 mM). C and D traces correspond to cells responding to a single HRH (monoresponsive cells), TRH in C, and GHRH in D. E, A nonresponsive cell. F–H, Traces correspond to multiresponsive cells. Results are representative of 3360 cells studied in 54 different experiments.

during aging (>540 pnd), the monoresponsive cell pool increased significantly to nearly 80% of all cells. The multiresponsive cell pool (Fig. 2, *black* part of the bars) represented 20–30% of all the cells at birth, increased significantly to 40–65% during maturation and at the start of cycling, and decreased significantly to 20% at senescence. Note that the figures at senescence and at birth were similar. Thus, the increase in multiresponsive cells during maturation (birth to puberty) was associated with an equivalent decrease in nonresponsive cells. Conversely, the decrease in multiresponsive

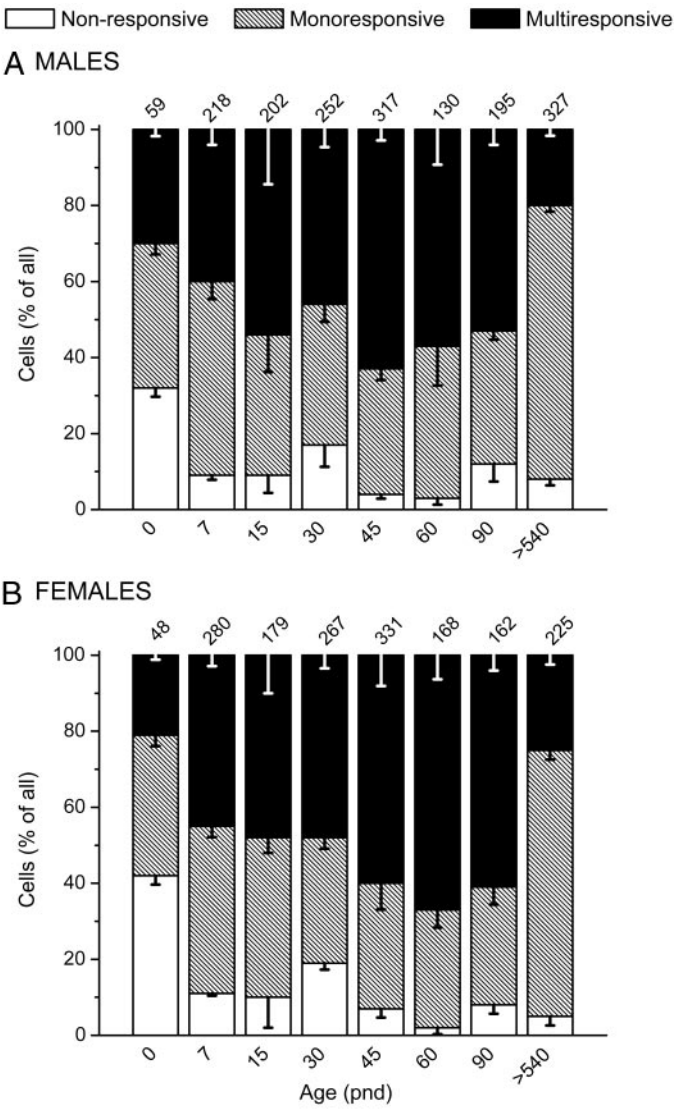

FIG. 2. Changes in the relative abundance (percent) of nonresponsive, monoresponsive, and multiresponsive AP cells along life. Male and female AP cells obtained from rats at birth (0) and after 7, 15, 30, 45, 60, 90, and more than 540 pnd were studied as in Fig. 1 and typed as nonresponsive, monoresponsive, and multiresponsive. Female rats started cycling at 45 pnd. Data are normalized to 100% and are the mean ± SEM of at least three independent experiments carried out with cells obtained from two to six rats in each period. *Numbers on top of the bars* represent the number of cells studied in each period and gender. Only *downward error bars* are shown for every cell pool. For comparisons a two-way ANOVA was performed and the mean values were compared by Bonferroni's test. Differences were considered significant at $P < 0.05$. No significant difference was found between male and female cells in any age group. The abundance of nonresponsive cells at birth (pnd = 0) was significantly larger than in any of the remaining age groups. The abundance of nonresponsive cells at 45 and 60 pnd was lower than that at 30 pnd. Monoresponsive cells were more abundant at senescence (>540 pnd) than at any of the other age groups. Finally, the abundance of multiresponsive cells was larger at 15, 45, 60, and 90 pnd than at birth. In aged rats, multiresponsive cells were less frequent than in any of the other age groups but birth.

cells during senescence was balanced by an equivalent increase in monoresponsive cells.

We also analyzed the relative abundance of the different cell populations pooled according to their responses to each HRH (Fig. 3). The *black* part of each bar represents the size of the monoresponsive cell pool in each category (*e.g.* cells responding only to GHRH), whereas the *white* part of the bar represents the contributions of multiresponsive cells (*e.g.* cells responding to multiple HRHs). We used a two-way ANOVA to compare not only the relative abundance of cells responding to each HRH but also the changes in the contribution of monoresponsive and multiresponsive cells to each cell population. The size of cell pools responding to each HRH was not significantly different between male and female pituitaries ($P > 0.05$) but, in some cases, showed striking changes with age as detailed below.

The CRH-responsive cell subpopulation (Fig. 3) ranged from 5 to 25% of all the cells and was composed mainly by multiresponsive cells throughout the whole life span in both males and females. At birth, only 0–5% of the cells showed functional responses to CRH. The size of the CRH-responsive cell pool increased significantly ($P < 0.05$) to 12–25% after puberty (60 pnd) remaining constant with age.

The LHRH-responsive cell population (Fig. 3) ranged from 5 to 20% of all cells and was also composed mainly by multiresponsive cells. The relative abundance of LHRH-responsive cells as well as the contribution of multiresponsive cells to this cell pool were quite constant and did not show any significant change throughout life.

The TRH-responsive cell subpopulation (Fig. 3) was very abundant (30–80% of all cells). At birth, TRH-responsive cells ranged from 30 to 35% and increased significantly and steadily to reach 60% at puberty and 70–80% at senescence. Interestingly, the relative contribution of multiresponsive cells to the TRH-responsive population varied significantly with age. At birth, only 50% of the TRH-responsive cells were multiresponsive. The contribution of multiresponsive cells increased significantly ($P < 0.05$) at puberty (45 pnd) and remained high at 60 and 90 pnd. Surprisingly, in old rats, the contribution of multiresponsive cells decreased dramatically ($P < 0.05$) and most cells became monoresponsive. Thus, the rise in TRH-responsive cells during senescence was due to a significant increase in monoresponsive cells paralleled by a significant decrease in multiresponsive cells.

The GHRH-responsive cell subpopulation (Fig. 3) represented 20–30% of all the cells at birth and increased significantly and very quickly to reach more than 70% of all cells at 7 pnd. This rise was due to the increase in both multiresponsive and monoresponsive cells ($P < 0.05$ for both increases). The percentages of GHRH-responsive cells remained constant or decreased slowly before rising significantly again to nearly 90% by puberty. This rise was due to a significant increase in multiresponsive cells. The size of the GHRH-responsive pool decreased dramatically ($P < 0.05$) to only 10–15% of all the cells at senescence. This decrease was due to a significant decrease in both monoresponsive and multiresponsive cells ($P < 0.05$ for both decreases).

We also analyzed the combinations of functional responses to the HRHs in multiresponsive cells (Fig. 4). The most common multiresponsive cells were those responding to both TRH and GHRH (Fig. 4, A and B). This receptor combination was the most frequent one during all the periods tested except during senescence when these cells almost disappeared. Cells responding to TRH, GHRH, and another additional HRH (TRH+GHRH+X in Fig. 4, A and B) were also quite frequent,

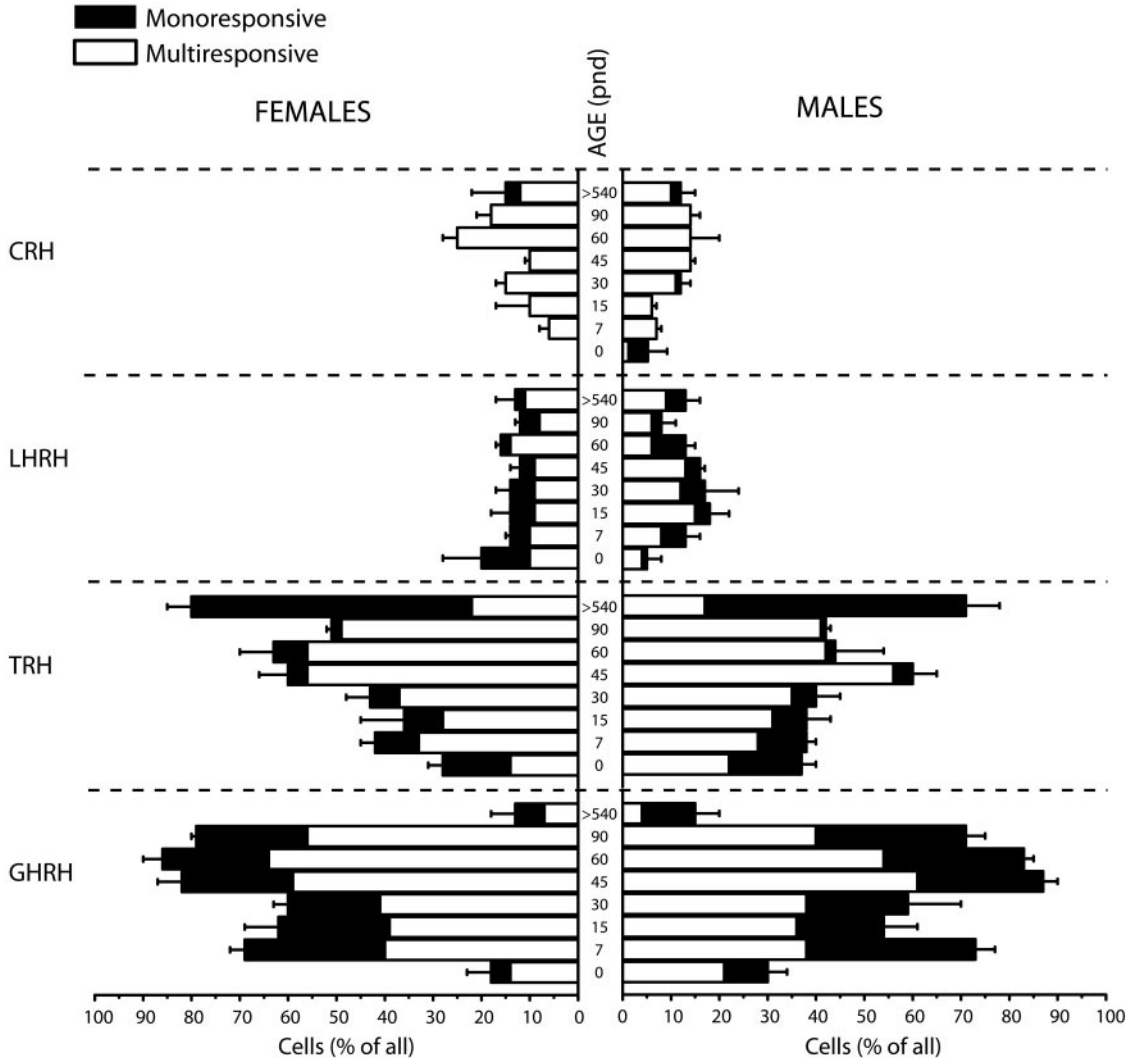

FIG. 3. Changes in the relative abundance of cells holding particular HRH receptors during the whole life span. The relative abundance of cells responding to each HRH is expressed as percentage of all cells. *Black bars* represent contribution of monoresponsive cells to each population, whereas *white bars* represent contribution of multiresponsive cells, responding to multiple HRHs. For each HRH and gender, the eight successive *bars* stand for data obtained (from bottom to top) at birth (0) and after 7, 15, 30, 45, 60, 90, and more than 540 pnd as shown in the figure. Data are mean ± SEM of at least three independent experiments carried out with cells derived from two to six rats. For simplicity, only *error bars* relative to the total percentage of cells responding to each HRH are shown. Differences among values were assessed by a two-way ANOVA and compared using Bonferroni's multiple comparison test. Differences were considered significant at $P < 0.05$. We found no significant difference with gender for any HRH-responsive population. See text for details about differences among age groups for each HRH-responsive population. Number of cells as in Fig. 2.

especially around puberty. For example, cells responding to GHRH, TRH, and LHRH increased from 1–2% at 7 pnd to 10–11% at puberty both in male and female animals. Other receptor combinations (Fig. 4, A and B, Others) were also frequent in different periods, especially senescence. Specifically, during maturation the fraction of cells responding to GHRH and LHRH represented about 6% of the cells (Fig. 4, C and D) and then decreased significantly by puberty and almost disappeared by senescence. About 2–5% of cells responded to both GHRH and CRH. This cell population increased significantly after puberty but also disappeared with aging (Fig. 4, C and D). Finally, during senescence, the most frequent multiresponsive cells were those responding to TRH and either CRH or LHRH (Fig. 4, C and D). Thus, demographics of multiresponsive cells changed very much with age.

We also studied whether the size of the $[Ca^{2+}]_{cyt}$ responses induced by each HRH was affected by gender, expression of additional receptors, and age. To this end, we measured the maximum increase of $[Ca^{2+}]_{cyt}$ ($\Delta[Ca^{2+}]_{cyt}$) during the 60-sec period after stimulation with each HRH. Then cells were grouped by the HRH response (CRH-, LHRH-, TRH-, and GHRH-responsive cells) and the average values of $\Delta[Ca^{2+}]_{cyt}$ and SEM were calculated for each group (Fig. 5). We found that gender did not significantly influence the maximum $\Delta[Ca^{2+}]_{cyt}$ elicited by each HRH. In addition, the $\Delta[Ca^{2+}]_{cyt}$ induced by each HRH was similar for monoresponsive and multiresponsive cells ($P > 0.05$, data not shown). Finally, our analysis revealed that age had little influence on the maximum $\Delta[Ca^{2+}]_{cyt}$ elicited by each HRH except for CRH (see legend for Fig. 5 for further details).

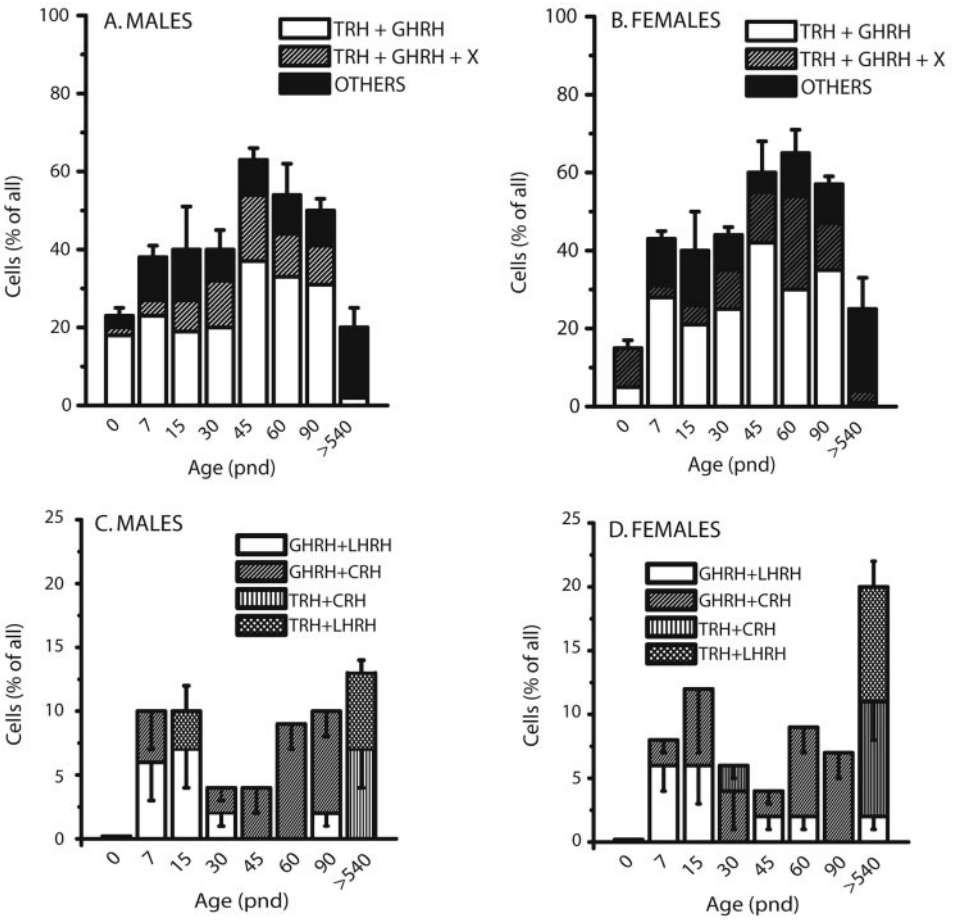

FIG. 4. Combinations of HRH receptors in multiresponsive cells. *Bars* represent multiresponsive cells (as percent of all the AP cells) in males (A) and females (B) for each age. Abundance of multiresponsive cells bearing either both TRH and GHRH receptors (*white*), another HRH receptor in addition to TRH and GHRH receptors (*hatched*), or any other combination of receptors (Others, *black*) are shown. These other combinations are further analyzed (C, males) and (D, females), and results of combinations of GHRH and TRH with LHRH or CRH are shown. The frequency of other combinations was less than 1%. Differences among groups were analyzed using ANOVA and Bonferroni's multiple comparison test. See text for details about differences among age groups. Number of cells as in Fig. 2.

Discussion

When classified according to the responses to HRHs, the rat AP is made up of three different types of cells: 1) monoresponsive, 2) multiresponsive, and 3) nonresponsive, depending on whether they respond to one or more of the four HRHs or they lack responses to the four HRHs tested here. Even though these nonresponsive cells responded to depolarization by high K^+ medium, indicating that they are viable, excitable cells, our results reveal remarkable changes in the size of these three cell pools during life. The three pools are similar at birth, whereas multiresponsive cells dominate at puberty and monoresponsive cells do so at senescence. The changes are similar in the male and female rats.

How do these changes take place? The increase in multiresponsive cells on maturation from birth to puberty is balanced by the decrease in the nonresponsive cell pool, whereas the increase of monoresponsive cells during senescence is balanced by the decrease in the multiresponsive pool. These results could be explained in terms of different turnover rates for the three cell pools. Thus, an increase in multiresponsive cells may be secondary to increased proliferation rate of these cells relative to that of the monoresponsive and the nonresponsive ones. However, if this were the only explanation, then the highly proliferative multiresponsive cells should switch to slowly proliferating to account for the changes observed at senescence. Because this seems unlikely, we will discuss below alternative mechanisms that may contribute to changes in AP cell pools. Multiresponsive, as well as polyhormonal AP cells (containing more than one AP hormone), have been proposed to arise by transdifferentiation among different cell types during demanding physiological situations (24–26). We reported previously that multiresponsive cells were much more abundant than polyhormonal cells in the mouse AP, suggesting that phenotypic changes involving changes in expression of HRH receptors are much more frequent than previously thought (14). It is tempting to speculate that multiresponsive cells could arise from nonresponsive cells during maturation and puberty just by sudden expression of multiple types of HRH receptors by the prior nonresponsive cells. This is the simplest explanation for the increase in multiresponsive cells balanced by a decrease in nonresponsive cells that is observed during maturation. In addition, monoresponsive cells could arise from multiresponsive cells by loss of HRH receptors during senescence. This could explain why the increase in monoresponsive cells is balanced by a decrease in multiresponsive cells during this period. The model is summarized in Fig. 6, in which the nonresponsive cells tend to express single or multiple HRH receptors during maturation and puberty. On the contrary, at senescence multiresponsive cells tend to lose HRH receptors to become monoresponsive. The TRH receptors tend to be better preserved so that TRH-sensitive

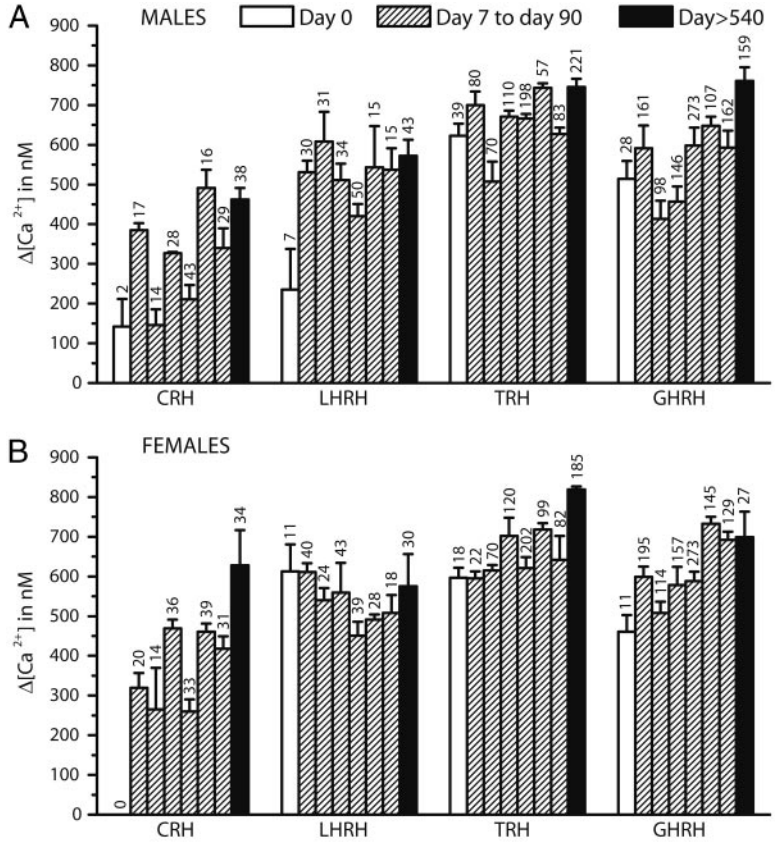

FIG. 5. Size of calcium responses during the whole life span. Responses to each HRH are expressed as $\Delta[Ca^{2+}]_{cyt}$ (during the stimulation period) in males (A) and females (B). *Bars* represent mean ± SEM. For each HRH, the eight successive *bars* stand for data obtained at the different ages from birth (*white bars*) to several time periods (*hatched bars* for 7, 15, 30, 45, 60, 90 pnd) and senescence (*black bars*, > 540 pnd). Differences among groups were analyzed using ANOVA and Bonferroni's multiple comparison test. Except for CRH (see text), the changes with age were small, but some of them were statistically significant. Specifically, no significant changes with age were found in the $\Delta[Ca^{2+}]_{cyt}$ induced by LHRH. The maximum $\Delta[Ca^{2+}]_{cyt}$ induced by both TRH and GHRH showed very small but significant increases after puberty ($P < 0.05$) and senescence ($P < 0.05$). Finally, the maximum $\Delta[Ca^{2+}]_{cyt}$ induced by CRH showed larger, significant increases during maturation ($P < 0.05$; 7 *vs.* 0 pnd, only in males) and after puberty ($P < 0.05$, 60 *vs.* 45 pnd) and to a lower extent during senescence ($P < 0.05$). The *figures on top of the bars* stand for the number of cells studied in each period and gender. Note that there was no CRH-sensitive cells at d 0 in females.

cells dominate at this stage. Finally, a combination of transdifferentiation and differences in turnover rate of the different cell pools could also account for the changes reported here.

The study of individual HRH receptors disclosed several unexpected outcomes. First of all, the relative abundance of cells expressing particular HRH receptors was much more dependent on age than gender. Moreover, each cell population typed by response to a particular HRH contained cells expressing multiple HRH receptors. Interestingly, multiresponsive cells were extremely abundant within specific cell types. For example, most of the CRH-responsive cells were responsive also to other HRHs. In some cases the contribution of multiresponsive cells also depended on age. One of the more striking examples was the TRH responsive population, in which about 50% of the cells were multiresponsive at birth; essentially all became multiresponsive at puberty and then predominantly monoresponsive with aging. The contribution of multiresponsive cells to the GHRH-responsive population also shows large changes throughout life. Therefore, both the relative abundance of cells holding a particular HRH receptor and the expression of additional HRH receptors may undergo large changes during maturation, puberty, or senescence. Because HRH receptors are the cornerstone of hypothalamic control of AP secretion, the changes in expression reported here may contribute to the endocrine events driven by this gland. For example, the decrease of growth during senescence coincides with a dramatic decrease of the fraction of cells expressing GHRH receptors (Fig. 3). In addition, the increase of GHRH-responsive cells by the start of the estrous cycle, which is accounted by the rise in multiresponsive cells, coincides with the appearance of a subset of multifunctional cells that share phenotypic characteristics with both somatotropes and gonadotropes (18). This cellular partnership may be relevant for GH demands during ovulation. Thus, it is possible that these cells be represented by cells responding to both GHRH and LHRH. Another example is the low number of CRH-responsive cells at birth that can contribute to explain the lack of functionality of the pituitary-adrenal axis at this stage.

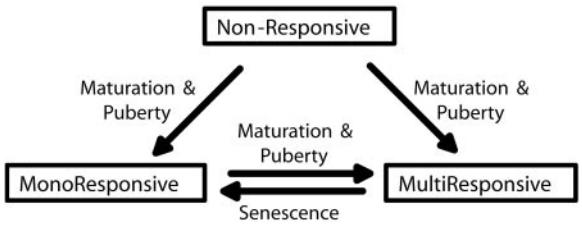

FIG. 6. A model to explain the changes in the nonresponsive, monoresponsive, and multiresponsive cell pools throughout life. Monoresponsive and multiresponsive cells may derive from nonresponsive cells during postnatal development (maturation) and puberty. Multiresponsive cells may also arise from monoresponsive cells. At senescence, monoresponsive cells may derive from multiresponsive cells.

Changes in the number of HRH receptors per cell could also modulate AP output. We have not measured the density of HRH receptors in individual cells as such, but the size of the HRH-induced $[Ca^{2+}]_{cyt}$ increase was, with few exceptions, little influenced by age or gender. In addition, coexpression of additional HRH receptors does not seem to influence the size of the $[Ca^{2+}]_{cyt}$ induced by any of the HRHs

except, perhaps, for CRH. Specifically, the size of the $[Ca^{2+}]_{cyt}$ induced by CRH was larger in multiresponsive than monoresponsive cells. Nevertheless, cells responding only to CRH were a rare cell pool. Thus, our results suggest that changes in responses to HRHs are driven by changes in the quantity and quality of cells holding a particular receptor. However, in the case of CRH, it is also possible that changes in the density of CRH receptors may contribute to differences with age. On the other hand, it is noteworthy that the $\Delta[Ca^{2+}]_{cyt}$ found here for both monoresponsive and multiresponsive cells are large enough to elicit hormone secretion. Thus, multiresponsive cells storing a particular AP hormone may undergo exocytosis in response to multiple HRHs. Consistently with this view, we reported previously that TRH induced PRL secretion from most mammotropes, as revealed by reverse hemolytic plaque assay. In contrast, the other HRHs including CRH, GHRH, and LHRH also induced PRL secretion but just from a subset of mammotropes. The proportions were consistent with the population of mammotropes holding other HRH receptors in addition to TRH (13). In addition, we also reported previously that all cell kinds typed by hormone storage contained subpopulations of multiresponsive cells (13). Thus, the $[Ca^{2+}]_{cyt}$ rises reported here for multiresponsive cells are large enough to explain paradoxical secretion in mammotropes and other cell types. Our present results are consistent with the existence of large changes in paradoxical secretion throughout life. Whether hormone storage by mono- and multiresponsive cells changes with age remains to be established.

In summary, we outlined here a comprehensive characterization of changes in functional responses to CRH, LHRH, TRH, and GHRH in individual male and female rat AP cells throughout the whole life span. The multiresponsive cells are present already at birth and contribute differentially to each AP cell subpopulation, depending on the age. They tend to increase during maturation and puberty but decrease very much at senescence. These changes are the expression of pituitary plasticity and likely contribute to endocrine changes observed during the life span, such as the endocrine decay that accompanies aging. Further research will be required to ascertain the role of multiresponsive cells in AP hormone secretion during particular physiological situations such as pregnancy, lactation, stress adaptation, and sex cycle.

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References

1. **Abribat T, Deslauriers N, Brazeau P, Gaudreau P** 1991 Alterations of pituitary growth hormone-releasing factor binding sites in aging rats. Endocrinology 128:633–635
2. **Kamegai J, Wakabayashi I, Kineman RD, Frohman LA** 1999 Growth hormone-releasing hormone receptor (GHRH-R) and growth hormone secretagogue receptor (GHS-R) mRNA levels during postnatal development in male and female rats. J Neuroendocrinol 11:299–306
3. **Duncan JA, Dalkin AC, Barkan A, Regiani S, Marshall JC** 1983 Gonadal regulation of pituitary gonadotropin-releasing hormone receptors during sexual maturation in the rat. Endocrinology 113:2238–2246
4. **Belisle S, Bellabarba D, Lehoux JG** 1989 Basal and stimulated receptor sites in the pituitary of aging female mice. Gynecol Endocrinol 3:183–192
5. **Lacau-Mengido JM, González-Iglesias A, Lux-Lantos V, Libertum C, Becu-Villalobos D** 1998 Ontogenic and sexual differences in pituitary GnRH receptors and intracellular Ca^{2+} mobilization induced by GnRH. Endocrine 8:177–183
6. **Shinkai T, Roth GS** 1999 Mechanisms of age-related changes in gonadotropin-releasing hormone receptor messenger ribonucleic acid content in the anterior pituitary of male rats. Exp Gerontol 34:267–273
7. **Heroux JA, Grigoriadis DE, de Souza EB** 1991 Age-related decreases in corticotropin-releasing factor (CRF) receptors in rat brain and anterior pituitary gland. Brain Res 542:155–158
8. **Tizabi Y, Aguilera G, Gilad GM** 1992 Age-related reduction in pituitary corticotropin-releasing hormone receptors in two rat strains. Neurobiol Aging 13:227–230
9. **Ceccatelli S, Calza L, Giardino L** 1996 Age-related changes in the expression of corticotropin-releasing hormone receptor mRNA in the rat pituitary. Brain Res Mol Brain Res 37:175–180
10. **Blanchard L, Barden N** 1986 Ontogeny of receptors for thyrotropin-releasing hormone in the rat brain. Brain Res 389:85–88
11. **Kasahara K, Tasaka K, Masumoto N, Mizuki J, Tahara M, Miyake A, Tanizawa O** 1994 Characterisation of rat pituitary cells by their responses to hypothalamic releasing hormones. Biochem Biophys Res Commun 1999:1436–1441
12. **Villalobos C, Núñez L, García-Sancho J** 1996 Functional glutamate receptors in a subpopulation of anterior pituitary cells. FASEB J 10:654–660
13. **Villalobos C, Núñez L, Frawley LS, García-Sancho J, Sánchez A** 1997 Multiresponsiveness of single anterior pituitary cells to hypothalamic-releasing hormones: a cellular basis for paradoxical secretion. Proc Natl Acad Sci USA 94:14132–14137
14. **Núñez L, Villalobos C, Senovilla L, García-Sancho J** 2003 Multifunctional cells of mouse anterior pituitary reveal a striking sexual dimorphism. J Physiol (Lond) 549:835–843
15. **Villalobos C, Núñez L, García-Sancho J** 2004 Anterior pituitary thyrotropes are multifunctional cells. Am J Physiol Endocrinol Metab 287:E1166–E1170
16. **Villalobos C, Núñez L, García-Sancho J** 2004 Phenotypic characterization of multifunctional somatotropes, mammotropes and gonadotropes of the mouse anterior pituitary. Pflügers Arch 449:257–264
17. **Senovilla L, Núñez L, de Campos JM, de Luis DA, Romero E, Sánchez A, García-Sancho J, Villalobos C** 2004 Multifunctional cells in human pituitary adenomas: implications for paradoxical secretion and tumorigenesis. J Clin Endocrinol Metab 89:4545–4552
18. **Childs GV** 2000 Growth hormone cells as co-gonadotropes: partners in the regulation of the reproductive system. Trends Endocrinol Metab 11:168–175
19. **Childs GV, Unabia G, Miller BT, Collins TJ** 1999 Differential expression of gonadotropin and prolactin antigens by GHRH target cells from male and female rats. J Endocrinol 162:177–187
20. **Gershengorn MC** 1986 Mechanism of thyrotropin releasing hormone stimulation of pituitary hormone secretion. Annu Rev Physiol 48:515–526
21. **Luini A, Lewis D, Guild S, Corda D, Axelrod J** 1985 Hormone secretagogues increase cytosolic calcium by increasing cAMP in corticotropin-secreting cells. Proc Natl Acad Sci USA 82:8034–8038
22. **Shangold GA, Murphy SN, Miller RJ** 1988 Gonadotropin-releasing hormone-induced Ca^{2+} transients in single identified gonadotropes require both intracellular Ca^{2+} mobilization and Ca^{2+} influx. Proc Natl Acad Sci USA 85:6566–6570
23. **Kato M, Hoyland J, Sikdar SK, Mason WT** 1992 Imaging of intracellular calcium in rat anterior pituitary cells in response to growth hormone releasing factor. J Physiol (Lond) 447:171–189
24. **Frawley LS, Boockfor FR** 1991 Mammosomatotropes: presence and functions in normal and neoplastic pituitary tissue. Endocr Rev 12:337–355
25. **Childs GV** 1991 Multipotential pituitary cells that contain ACTH and other pituitary hormones. Trends Endocrinol Metab 2:112–117
26. **Childs GV, Unabia G, Wu P** 2000 Differential expression of growth hormone messenger ribonucleic acid by somatotropes and gonadotropes in male and cycling female rats. Endocrinology 141:1560–1570