

Anterior pituitary thyrotropes are multifunctional cells

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Villalobos, Carlos, Lucía Nuñez, and Javier García-Sancho. Anterior pituitary thyrotropes are multifunctional cells. *Am J Physiol Endocrinol Metab* 287: E1166–E1170, 2004. First published June 29, 2004; doi:10.1152/ajpendo.00194.2004. Anterior pituitary (AP) contains some unorthodox multifunctional cells that store and secrete two different AP hormones (polyhormonal cells) and/or respond to several hypothalamic-releasing hormones (HRHs; multiresponsive cells). Multifunctional cells may be involved in paradoxical secretion (secretion of a given AP hormone evoked by a noncorresponding HRH) and transdifferentiation (phenotypic switch between different mature cell types without cell division). Here we combine calcium imaging (to assess responses to the four HRHs) and multiple sequential immunoassay of the six AP hormones to perform a single-cell phenotypic study of thyrotropes in normal male and female mice. Surprisingly, most of the thyrotropes were polyhormonal, containing, in addition to thyrotropin (TSH), luteinizing hormone (40–42%) and prolactin (19–21%). Thyrotropes storing growth hormone and/or ACTH were found only in females (24% of each type). These results suggest that costorage of the different hormones does not happen at random and that gender favors certain hormone combinations. Our results indicate that thyrotropes are a mosaic of cell phenotypes rather than a single cell type. The striking promiscuity of TSH storage should originate considerable mix-up of AP hormone secretions on stimulation of thyrotropes. However, response to thyrotropin-releasing hormone was much weaker in the polyhormonal thyrotropes than in the monohormonal ones. This would limit the appearance of paradoxical secretion under physiological conditions and suggests that timing of hormone and HRH receptor expression during the transdifferentiation process is finely and differentially regulated.

anterior pituitary; hypothalamic releasing factors; paradoxical secretion

THE ANTERIOR PITUITARY (AP) contains five main cell types, somatotropes, mammotropes, corticotropes, thyrotropes, and gonadotropes, which secrete growth hormone (GH), prolactin (PRL), ACTH, thyrotropin (TSH), and gonadotropins [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)], respectively. Thyrotropes are the least abundant cell type, amounting to 5–8% of all the AP cells. However, this cell type plays a pivotal role in control of metabolism through regulation of thyroid activity. In the classic view, each AP cell type stores a single hormone in which secretion is specifically regulated by a particular hypothalamic-releasing hormone (HRH). Thus thyrotropes are believed to store and release TSH and express functional thyrotropin-releasing hormone (TRH) receptors involved in hypothalamic control of TSH secretion. However, in the last decade, this one-cell-one hormone hypothesis has been challenged repeatedly. It has been reported, for example, that many pituitary cells

may store and release more than one AP hormone. Mammosomatotropes that store and release GH and PRL (13) have been regarded as an intermediate stage for conversion of somatotropes into mammotropes, a phenotypic switch between mature cell types without cell division called transdifferentiation (13, 31). Polyhormonal corticotropes (4), somatogonadotropes (5, 6, 8), and cells storing and releasing LH and PRL (14) have also been reported. Polyhormonal thyrotropes containing both TSH and GH (thyrosomatotropes) cells have been observed after thyroidectomy (18) or protracted primary hypothyroidism (30). In addition, the existence of a cell pool that costores and cosecretes ACTH and TSH during cold stress has been reported (9). Finally, single-cell RT-PCR has also revealed that 30% of pituitary cells display mRNAs for multiple AP hormones (17, 25–28) in rats, mice, and monkeys. It is remarkable that most of the cells expressing TSH-mRNA (11%) actually costored it with other AP hormone mRNAs (9%), whereas cells expressing only the TSH-mRNA were much less abundant (2%; see Ref. 26). These data suggest that most thyrotropes are potentially multifunctional cells, although multiple mRNA-containing cells do not necessarily translate the multiple hormone mRNAs into functional proteins (26). Dual-protein hormone content has been reported in corticothyrotropes (4).

On the other hand, a large fraction of rat AP cells bears multiple HRH receptors (multiresponsive cells; see Refs. 19, 32, and 33), and stimulation with different HRHs evokes paradoxical PRL secretion (32). It has been reported that gonadotropes may express growth hormone-releasing hormone (GHRH) receptors (7), that somatotropes may express transiently luteinizing hormone-releasing hormone (LHRH) receptors, and that LHRH may stimulate GH secretion (5). Regarding thyrotropes, it has been reported that corticotropin-releasing hormone (CRH) produces TSH secretion in chicken AP cells *in vivo*, acting directly on type 2 CRH receptors (10). Overall, the above studies indicate that the AP contains multifunctional (multiresponsive and/or polyhormonal) cells in which stimulation may give rise to paradoxical secretion. Paradoxical secretory responses are common in nonnormal pituitaries, especially pituitary tumors (3, 11, 21, 23), but they have also been sporadically reported in normal pituitaries, both *in vitro* and *in vivo*, including healthy humans (2, 12, 16). The expression of functional HRH receptors by thyrotrope subpopulations remains to be established.

In a recent study, we have attempted a phenotypic characterization of individual AP cells by the hormones they store and the HRH receptors they express (24). Multifunctional (multiresponsive and/or polyhormonal) cells were identified within all five AP cell subpopulations, but they were more

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frequent in thyrotropes than in the other cell types. Here we report the responses of the different polyhormonal thyrotrope subpopulations to the HRHs. We find that most thyrotropes show a striking phenotypic heterogeneity. In fact, most of them are multifunctional cells storing multiple AP hormones and bearing multiple HRH receptors.

MATERIALS AND METHODS

Pituitary glands were obtained from male and randomly cycling female mice (Balb/c, 12 wk old). Animal experimentation was conducted in accord with accepted standards of human animal care and the Valladolid University School of Medicine Ethics Committee. Pituitary glands were removed and digested with trypsin (1 mg/ml) for 30 min at 37°C, as described elsewhere (24). Cells were plated on poly-L-lysine-coated coverslips and used for the experiments within 2–4 h. We have shown before that responses to the HRHs by these cells are better than those maintained in primary culture for 1–3 days (32, 33). Single-cell responsiveness to the four HRHs (10 nM) was assessed from the changes of cytosolic free calcium concentration ($[Ca^{2+}]_c$), which were measured in fura 2-loaded cells by digital-imaging fluorescence microscopy, as described previously (24, 32, 33; also see Fig. 1).

At the end of the Ca^{2+} measurements, the hormonal contents of the cells were typed by multiple sequential primary immunofluorescence, combining three different fluorescent labels and image processing to resolve the five AP hormones (TSH, LH, PRL, GH, and ACTH) within ~2 h (Fig. 1). Briefly, cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.3% Triton X-100 in the above solution for 3 min, and washed with PBS for 5 min. Next, 10% goat serum in PBS was added. After 5 min, antibodies against three AP hormones (TSH, FSH, and LH) labeled with Oregon Green 488, Cascade Yellow, and Alexa Fluor 350, respectively, were added, and the incubation was continued for 30 min. After washing, specific fluorescence images corresponding to each fluorophore were captured

to reveal stained cells. This step enables one to type cells storing either TSH, LH, or FSH as well as those costoring combinations of these AP hormones. After capturing the first series of images, cells were washed and incubated again with antibodies against GH, PRL, and ACTH and labeled with Oregon Green 488 (PRL), Cascade Yellow (GH), and Alexa Fluor 350 (ACTH), and the incubation was continued for 30 min. After washing, three new fluorescence images were taken with the same fluorescence settings as above. This new series of images revealed cells stained by the second set of antibodies in addition to those stained with the first set. To reveal the specific staining by the second set of antibodies, the first series of fluorescence images was subtracted from the second ones. This procedure enabled detection of cells storing single or multiple AP hormones (Fig. 1) and typing of 90–93% of the cells present in the microscope field (701 male and 558 female cells studied; total number of cells determined by nuclear staining with Hoeschst 33258). See Refs. 1 and 24 for a more detailed description of the procedure.

Antisera against mouse PRL (no. AFP131078Rb), rat β -TSH (no. AFP1274789), rat GH (no. AFP411S), rat β -FSH (no. AFPHSFSH6Rb), rat β -LH (AFP571292393R), and rat ACTH (no. AFP71111591GP) were generous gifts from Dr. A. F. Parlow. The anti-rat reagents work in mice just as well as in rats (National Hormone and Peptide Program and Dr. Parlow, personal communication). Fluorescent antibodies were prepared by labeling with either Oregon Green 488, Cascade Yellow, or Alexa Fluor 350 and purified over a protein A-Sepharose column. Fura 2-AM, Oregon Green 488-isothiocyanate, and the succinimidyl esters of Cascade Yellow and Alexa Fluor 350 were purchased from Molecular Probes Europe. The HRHs were obtained from Sigma.

RESULTS AND DISCUSSION

The combination of calcium imaging and multiple immunocytochemistry in the same cells has enabled the phenotypic characterization of thyrotropes for the expression of functional

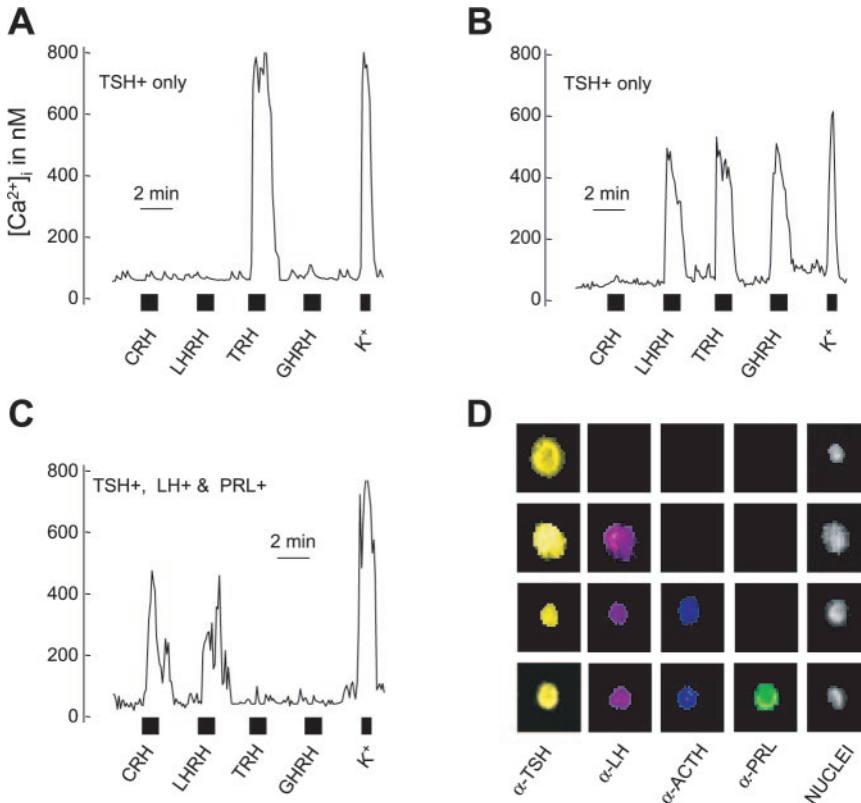


Fig. 1. Strategy for phenotypic characterization of thyrotropes. Mouse anterior pituitary (AP) cells were plated, loaded with fura 2, and subjected to digital imaging fluorescence microscopy. The effects of sequential stimulation with the four hypothalamic-releasing hormones (HRHs) on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) were monitored to reveal the functional expression of HRH receptors in monohormonal (A and B) and polyhormonal (C) thyrotropes. To type hormone storage, cells were fixed and subjected to multiple immunocytochemistry in the same microscopic field used for Ca^{2+} measurements. Examples of mono- and polyhormonal thyrotropes are shown in D. For more details on this procedure, see Ref. 24. CRH, corticotropin-releasing hormone; TSH, thyrotropin; LHRH, luteinizing hormone releasing hormone; TRH, thyrotropin-releasing hormone; GNRH, growth hormone-releasing hormone; LH, luteinizing hormone; PRL, prolactin.

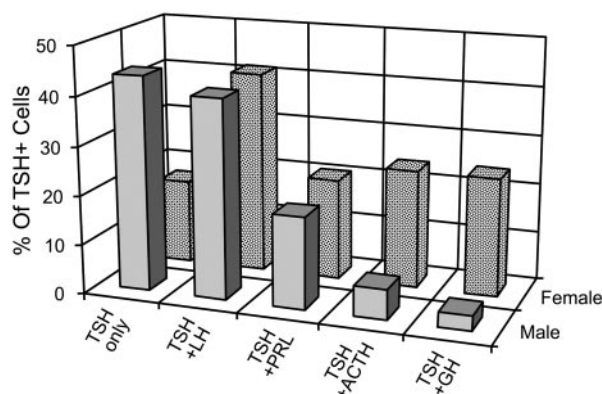


Fig. 2. Frequency distribution of TSH-containing cells in mouse AP. Costorage with other AP hormones is shown. Data are from analysis of 633 male and 520 female AP cells in 11 different experiments (6 males and 5 females). TSH-containing cells were 5.1–5.6% of all the cells.

HRH receptors and storage of different AP hormones. Figure 1 summarizes how characterization was achieved. Calcium imaging revealed that thyrotropes may express, in addition to TRH, other HRH receptors, as revealed by the increases in $[Ca^{2+}]_c$ induced by sequential perfusion with the four HRHs. Figure 1 also shows a representative multiple, sequential immunocytochemistry revealing that thyrotropes may store, in addition to TSH, other AP hormones. Monohormonal thyrotropes may respond to TRH or to multiple HRHs (Fig. 1, A and B). Polyhormonal thyrotropes may also show multiresponsiveness (Fig. 1C). When we assayed the content of different hormones, we were surprised to find that most thyrotropes were actually polyhormonal, i.e., they costored other hormones together with TSH. Figure 2 compares the profiles of hormonal contents in male and female mice. In the males, >50% of the cells were polyhormonal. LH was costored with TSH in 41% of the cells. PRL was costored in another 19% of the cells, and ACTH and GH were costored with TSH in 6 and 3% of the cells, respectively. In the females, the abundance of polyhormonal cells was even larger (83%). The dominant cell type was also the gonadothyrotrope (41%), followed by cells costoring ACTH or GH (24% each one) and cells costoring PRL (21%). Some cells stored three or more hormones (31% in the male and 45% in the female). Moreover, we must keep in mind that monohormonal and polyhormonal thyrotropes were identified after a sequence of stimulatory events (series of releasing hormones). Because of the possibility that a cell could release all of its stores of a particular hormone, the actual multihormonal state seen after the multiple-releasing hormone stimulation could be an underestimate of that seen in the basal state.

It has been reported before that somatotropes can transdifferentiate to thyrotropes after thyroidectomy (18) or protracted hypothyroidism (30). Cells containing both hormones, GH and TSH, were observed during this process (18, 30). We find here that thyrosomatotropes are already present in the AP of normal mice, especially in females, but other hormonal combinations, for example thyrogonadotropes, were much more frequent. This suggests that the appearance of bihormonal cells is not aimless. Somatotropes are much more frequent than gonadotropes in mice pituitary (24), and, at random, the combination TSH-GH should be much more likely. On the other hand,

thyrosomatotrope formation should be specifically favored by the pressure of low plasma thyroid hormone levels in hypothyroidism. As with other polyhormonal cells (24), polyhormonal thyrotropes were more frequent in females than in males, suggesting that the sexual cycle may favor transdifferentiation. It is remarkable that sex differences did not affect gonadotropins (LH) or PRL, which were similarly costored with TRH in males and females. However, costorage of ACTH and GH by thyrotropes was affected by sex (Fig. 2).

To estimate the single cell contents of each stored hormone, the fluorescence emission with the different antibodies was quantified and standardized, and values obtained in mono- and polyhormonal cells were compared. Results are summarized in Fig. 3. The contents of TSH in polyhormonal thyrotropes ranged between 70 and 118% of the values found in monohormonal thyrotropes. On the other hand, the contents of the different costored hormones in TSH-containing cells was 93–144% of the contents measured in the corresponding monohormonal cells. Therefore, hormonal contents of the costored hormones were not traces but amounts comparable to the ones contained in the corresponding monohormonal cells (Fig. 3). This output suggests that considerable mix-up of hormones could happen when thyrotropes are stimulated to secrete, and this could be related to paradoxical secretion.

Stimulation of HRH receptors induces an increase of $[Ca^{2+}]_c$ and hormone release (15, 20, 22, 29). To quantify the responses to the HRHs, we measured the changes of $[Ca^{2+}]_c$ induced in the different cell types. Figure 4, A and B, compares the size of the responses ($\Delta[Ca^{2+}]_c$ in nM) of the monohormonal and the different polyhormonal thyrotropes with HRHs. The responses of the different cell types were very similar for males (Fig. 4A) and females (Fig. 4B), even though the frequencies of the different cell subtypes were very different among genders (Fig. 2). The largest Ca^{2+} response was the one elicited by TRH in the monohormonal thyrotropes. All the other responses were smaller. In general, male and female cells behaved similarly, except that the monohormonal cells were somewhat more sensitive to all the other HRHs in females. In males, the thyrogonadotropes were somewhat more sensitive to

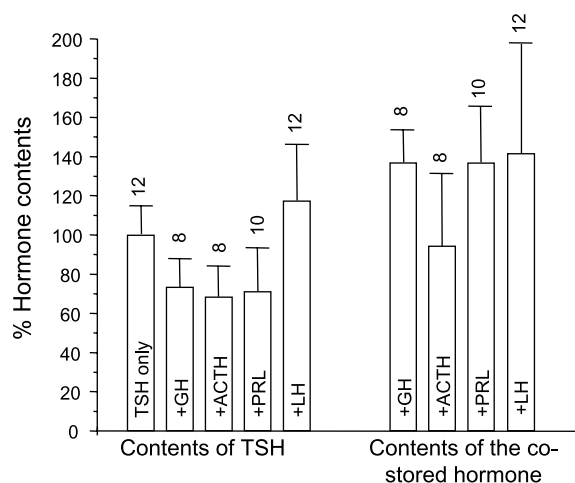


Fig. 3. Comparison of the hormonal contents of the monohormonal and polyhormonal thyrotropes. Hormone contents (means \pm SE) are expressed as %value found in the monohormonal cells of the same experiment. No. of cells analyzed is shown at top of the bars.

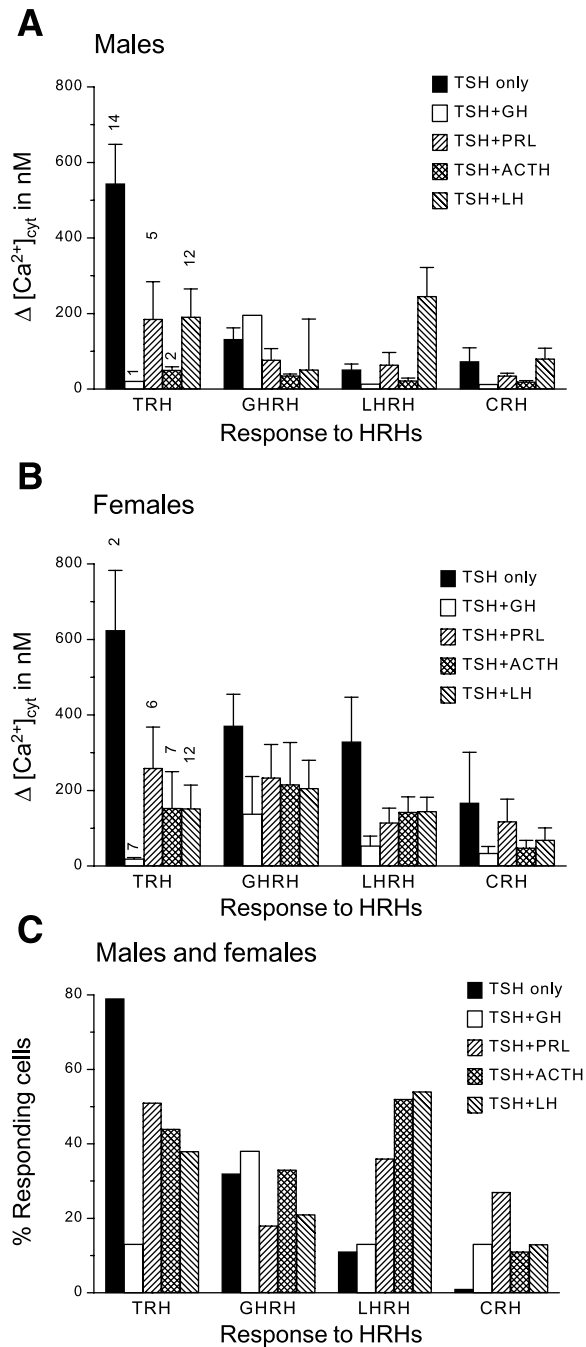


Fig. 4. Comparison of the responses of monohormonal and polyhormonal thyrotropes to the different HRHs. In *A* and *B*, bars represent the means of the peak increases of $[Ca^{2+}]_c$ ($\Delta[Ca^{2+}]_c$ in nM \pm SE). No. of cells in each class is given at top of the bars. *A*: males. *B*: females. In *C*, data from the same experiments were quantified as %responding cells. Responding cells are defined as those giving a $\Delta[Ca^{2+}]_c > 50$ nM after stimulation with the HRH. Data from males and females have been pooled in *C*.

TRH and LHRH than in females. In Fig. 4C, sensitivity to the different HRHs was quantified as a percentage of cells responding to the stimulus within each cell type. Responders were defined as cells showing a $\Delta[Ca^{2+}]_c > 50$ nM. Results from males and females are pooled in Fig. 4C. The results were generally consistent with the ones just discussed (Fig. 4, *A* and *B*) except that this procedure was more sensitive for detecting

small responders. For example, a large fraction of thyrogonadotropes (46% in males and 75% in females) responded to LHRH, although with a moderate $\Delta[Ca^{2+}]_c$ (mean value 246 nM in males and 145 nM in females). A large fraction of thyromammotropes (50–60%) responded moderately to TRH both in males and in females, and to LHRH only in females. Finally, female ACTH-containing cells showed moderate responses to LHRH and TRH. These results suggest that the mix-up of hormone secretion may be, after all, not as large as was inferred from the large percentages of polyhormonal cells, because polyhormonal thyrotropes are poor responders to HRHs, including TRH. Cells with mixed phenotypes could contribute, however, to gonadotropine and/or PRL secretion, especially in females if changes concentrate, as suggested by Childs (6), at a given moment of the cycle. Changes of TSH secretion during the sex cycle may not be physiologically relevant, since the secretion by the thyroid gland has a long temporal inertia. When blood thyroid hormone levels decrease, the formation of thyrosomatotropes seems favored (18, 30). Under these pathophysiological conditions, thyrosomatotropes could contribute to TSH secretion. It would be very interesting to investigate the functional expression of HRH receptors in these cells.

Multifunctional (polyhormonal and/or multiresponsive) cells have been proposed to be an intermediate stage in the transdifferentiation process. Thus cells costoring GH and PRL show up during transformation of somatotropes into lactotropes (13, 31). Transformation of somatotropes to gonadotropes involves not only dual hormone storage (GH and gonadotropins) but also multiple HRH receptor expression (GHRH and LHRH; see Ref. 6). It must be remarked that thyrogonadotropes and thyromammotropes are normal constituents of male and female pituitary under physiological conditions. Does that mean that transformations of these cell kinds are happening under the normal resting conditions? The significance of this finding is unclear at the moment. On the other hand, the fraction of thyrosomatotropes and thyrocorticotropes is much larger in the females than in males (Fig. 2). This observation suggests that transdifferentiation of GH and ACTH cells toward thyrotropes may be favored at some stage of the sex cycle.

In summary, our results indicate that thyrotropes are not a single cell type but rather a mosaic of different cell phenotypes whose relative abundance could vary in different physiological and pathophysiological conditions. However, monohormonal thyrotropes respond much better to TRH than the polyhormonal ones, thus explaining why paradoxical secretion is so elusive. In any case, thyrotropes from mice AP seem an excellent model for further studies on transdifferentiation.

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