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Rapid Changes in Anterior Pituitary Cell Phenotypes in Male and Female Mice after Acute Cold Stress

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The anterior pituitary (AP) is made of five different cell types. The relative abundance and phenotype of AP cells may change in different physiological situations as an expression of pituitary plasticity. Here, we analyze in detail the phenotype of mouse corticotropes and the effects of acute cold stress on AP cell populations. The hormone content and the expression of hypothalamic-releasing hormone (HRH) receptors in all the five AP cell types were studied in the male and female mice at rest and after a 30-min cold stress. Expression of HRH receptors was evidenced by imaging the single-cell cytosolic Ca^{2+} responses in fura-2-loaded cells. Hormone contents were studied by multiple, simultaneous immunofluorescence of all the five hormones. Corticotropes displayed a striking sexual dimorphism, even in the resting condition. Male corticotropes showed the orthodox phenotype. They were monohormonal, storing only ACTH, and monoreceptorial, responding only to CRH. In contrast, female corticotropes were made of about equal parts of orthodox cells and multifunctional cells, which co-stored additional AP hormones and expressed additional HRH receptors. Cold stress did not modify the number of ACTH containing cells, but, according to immunostaining, it increased the relative abundance of other AP cell types at the expense of the pool of cells storing no hormones. Cold stress also modified the response to CRH and other HRHs. Most of these phenotypical changes presented a strong sexual dimorphism. These results indicate that pituitary plasticity is even larger than previously thought. (*Endocrinology* 149: 2159–2167, 2008)

THE ANTERIOR PITUITARY (AP) gland is made of five different cell types, each one storing a different AP hormone. In addition, each cell type would be controlled by a specific hypothalamic-releasing hormone (HRH) and would selectively express receptors for this HRH. For example, corticotropes express adrenocorticotropic hormone (ACTH) and CRH receptors. This cell organization underlies the conceptual frame for division of the endocrine system into several independent axes (1). The hypothalamic-pituitary-adrenal (HPA) axis is activated by different stressful stimuli, which promote CRH release in the median eminence. CRH triggers ACTH secretion in AP, and ACTH stimulates glucocorticoid secretion by the adrenal cortex. Glucocorticoids execute the metabolic adaptation to stress and feed back at brain and pituitary sites to limit the CRH and ACTH responses (2).

However, this orthodox view of the AP cell organization is becoming less and less clear-cut, and AP cell subpopulations differing from this pattern are often described. Thus, each one of the main types of endocrine cells in AP may have a number of subtypes, and, additionally, there is a significant degree of sexual dimorphism. What perhaps is less well understood is the plasticity of these postmitotic cells. For example, it has been reported that some pituitary cells may store and release more than one AP hormone (poly-hormonal cells). Well-established examples include mammo-somatotropes that store and release GH and prolactin (PRL) (3, 4), poly-hormonal corticotropes (5), somato-gonadotropes (6, 7), cells that store and release LH and PRL (8), and poly-hormonal thyrotropes containing both TSH and GH (thyro-somatotropes) (9). In addition, cells co-storing and co-secreting ACTH and TSH during cold stress have been reported (10). Consistently, single-cell RT-PCR studies revealed that a large pool of AP cells expressed mRNAs for multiple AP hormones (11–13). The source of poly-hormonal cells remains obscure. Although there is no direct evidence, poly-hormonal cells have been regarded as an intermediate stage in the conversion of one cell type into another, *e.g.* conversion of somatotropes into mammotropes. This phenotypical switch between different mature cell types without cell division was called transdifferentiation (3, 7, 9). Transdifferentiation is thought to happen in AP in situations of high hormone demand as, for example, lactation or adaptation to stress.

On the other hand, a large fraction of rat and mouse AP cells bears multiple HRH receptors (multiresponsive cells) (14–17). For example, somatotropes may express transiently LHRH receptors, and LHRH may stimulate GH secretion (6). CRH is able, in turn, to stimulate TSH secretion in chicken AP cells *in vivo* acting directly on type 2 CRH receptors (18). Together, the aforementioned studies indicate that the AP contains multifunctional cells (multiresponsive and/or poly-hormonal) whose stimulation could occasion paradoxical secretion, *i.e.* secretion of a given hormone driven by a non-corresponding factor. Paradoxical secretory responses are common in nonnormal pituitaries, especially pituitary tumors (19–22), but they have also been sporadically reported in normal pituitaries, both *in vitro* and *in vivo* (23–25).

The exact phenotype of mouse corticotropes relative to

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Abbreviations: AF, Alexa Fluor; AP, anterior pituitary; $[Ca^{2+}]_C$, cytosolic free calcium concentration; Dichroic, dichroic mirror; Em, emission filter; Ex, excitation; HPA, hypothalamic-pituitary-adrenal; HRH, hypothalamic-releasing hormone; PRL, prolactin.

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hormone storage and HRH receptor expression has not been reported yet. In addition, it is not known whether the corticotrope's phenotype shows a dimorphic pattern that may account for the reported sexual differences in response to stress. It is well established that male and female mammals respond to stress differently at several points of regulation (26, 27). For example, female mice secrete more corticosterone than males, and this behavior does not change throughout the estrous cycle (28). In addition, other sex-related differences in resting pituitary-adrenal function in the rat have been reported (29). In rats, blood plasma corticosterone levels in basal HPA axis function were higher in the female than in the male, but the differences were not statistically significant (30). In addition, a variety of stimuli cause more HPA responsiveness in female than in male rats (31). However, whereas female rats often show a greater absolute increase in HPA axis activity in response to stimulation, male rats release more corticosterone relative to control values in response to certain stimuli (28, 31). Cold exposure is one of the most common stressful situations studied. Acute cold stress for only 30 min may also promote CRH and TRH release, as well as changes in the population of AP cells storing ACTH and/or binding of CRH (32). These rapid changes in AP cell composition with cold led authors to postulate the existence of reserve cells that may be sensitive to certain levels or types of stimuli (33). In addition, we have recently reported that age profoundly influences the expression of HRH receptors and the relative abundance of multiresponsive cells (17). Thus, the AP gland seems to show a unique degree of cell plasticity that allows changes of cell composition during high hormone demands, such as lactation, sexual cycle, and protracted hypothyroidism (4, 6, 9).

Here, we asked whether the corticotrope subpopulation contains multifunctional cells and whether their phenotype is sexually dimorphic. In addition, we investigated whether a brief cold stress *in vivo* could induce phenotypical changes in corticotropes and other AP cell types, and whether there are gender differences. For this end, we have developed a new methodology for multiple, simultaneous immunocytochemistry for the five main AP cell types. Our results reveal a striking sexual dimorphism in the phenotype of resting corticotropes. In addition, the cold stress promoted dramatic changes in AP cell composition, again in a sexually dimorphic manner.

Materials and Methods

Materials

Antisera against mouse PRL (no. AFP131078Rb), rat β-TSH (no. AFP1274789), rat GH (no. AFP411S), rat β-FSH (no. AFPHSFSH6Rb), rat β-LH (no. AFP571292393R), and rat ACTH (no. AFP71111591GP) were generous gifts from Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). The antirat reagents work in mouse just as well as in rat (National Hormone and Peptide Program and Parlow, A. F., personal communication). Fura-2/AM, Oregon Green 488-isothiocyanate, and the succinimidyl esters of Cascade Yellow, Pacific Blue, and Alexa Fluor (AF) (AF350, AF568, and AF633) were purchased from Molecular Probes Europe BV (Paisley, UK). The HRHs were obtained from Sigma-Aldrich (Madrid, Spain).

Cold stress protocol

Four-month-old male and randomly cycling female mice (Swiss) were used in this study. Animal experimentation was conducted in accord with accepted standards of human animal care and the Valladolid University Ethical Committee. Mice were placed in different cages during 48 h for a habituation period. Control mice were then immediately removed from the cage and killed by cervical dislocation. At the same time, stress mice were placed during 30 min at 4 C (cold stress) before being killed for corticosterone determination and pituitary cell dispersion. Because placing mice in the cold room requires moving them to a new environment, the *in vivo* stress situation used here may be a mixture of cold and novel environment stress.

Measurement of corticosterone

Four control and four stressed mice were killed by cervical dislocation, and, immediately, the blood was collected by cardiac puncture and placed in individually labeled 1.5-ml microcentrifuge tubes. All blood was kept for 4 h at room temperature and centrifuged for 5 min at 3000 rpm, and the serum was obtained. Serum was transferred to clean, labeled 0.5-ml microcentrifuge tubes. All serum samples were stored frozen at −20 C until the determination of corticosterone. Corticosterone was measured by ELISA (Immunodiagnostic Systems Ltd. OCTEIA Corticosterone kit; sensitivity 0.23 ng/ml; Immunodiagnostic Systems Ltd., Tyne & Wear, UK). The procedure was performed following the kit protocol, and the samples were measured by duplicate.

AP cell culture

Cell culture was performed as previously reported (16, 34, 35). Mice were killed by cervical dislocation, and the AP glands were quickly dissected and extracted. After removing the neurointermediate lobe, the glands were chopped into little pieces (~1 × 1 mm) with small dissecting scissors and dispersed by incubation with 1 mg/ml trypsin (Sigma-Aldrich) in Hanks' balanced salt solution (Life Technologies, Inc., Gaithersburg, MD) at 37 C with gentle shaking for 30 min. Every 15 min, pieces were passed repeatedly through a fire-polished siliconized Pasteur pipette to triturate the tissue into single cells and to help digestion. The cells were then sedimented by centrifugation at 200 × *g* for 7 min, washed twice with Hanks' balanced salt solution, and counted. About 1×10^6 cells per pituitary were obtained. Dead cells were less than 5% as measured by Trypan blue exclusion. Cells from different animals of the same gender and condition (either control or stressed) were mixed to avoid the influence of animal to animal variations and the sex cycle. Monodispersed cells were finally plated onto coverslips previously coated with 0.01 mg/ml poly-L-lysine and incubated in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum and antibiotics, and used within 2–4 h for calcium imaging and/or fixed for multiple immunocytochemistry. Cell viability was also assessed at this stage by simultaneous staining of living and dead cells using fluorescein diacetate and propidium iodide, respectively. The percentage of dead cells was 3 ± 2% of all the cells, and this proportion was not affected by gender or condition. Cells were fixed by incubation with 4% paraformaldehyde in PBS and then permeabilized with Triton X-100. Permeabilization was optimized by testing different concentrations, and best results were achieved by treatment with 0.3% Triton X-100 for 5 min, followed by washing with PBS.

Calcium imaging

Single-cell responsiveness to the four HRHs (20 nM) was assessed from the changes of cytosolic free calcium concentration ($[Ca^{2+}]_C$), which were measured in fura2/AM-loaded cells by digital imaging fluorescence microscopy as described previously (16, 17, 34, 35).

Multiple, simultaneous immunofluorescence

Antibodies against AP hormones (GH, LH, PRL, TSH, and ACTH) were labeled with fluorophores in such a way that fluorescence emission could be dissected spectrally, using different filter settings. Fluorescent antibodies were prepared from antisera provided by the National Institute of Diabetes and Digestive and Kidney Diseases and purified over a protein A-Sepharose column (15, 16). Antibody-fluorophore combi-

TABLE 1. Cross talk between the different fluorescent channels used for multiple immunocytochemistry

	Ch	Ex	Em	Cross talk				
				Ch 2	Ch 3	Ch 4	Ch 5	Ch 6
Bright-field	1	na	na	na	na	na	na	na
Pacific Blue	2	390/40	460/50		0.07			0.20
Oregon Green 488	3	490/20	535/35					0.08
AF568	4	546/12	605/55		0.04			0.05
AF633	5	615/40	675/50		0.04	0.25		0.05
Cascade Yellow	6	380/15	535/35	0.05	0.04			

Ex and Em wavelengths are in nm. The cross talk of each dye in the noncorresponding channels (Chs) was subtracted from the crude images. For example, the corrected image of AF568 was obtained as: channel 4 − 0.04 × channel 3 − 0.05 × channel 6. Cross talk below 4% was not corrected. na, Not applicable.

nations were as follows: α-rat ACTH Pacific Blue or Cascade Yellow, α-rat GH Cascade Yellow or Pacific Blue, α-rat LH Oregon Green 488, α-rat TSH AF568, and α-mouse PRL AF633. p-formaldehyde-fixed cells (overnight at 4 C, as detailed previously) were incubated with the labeled antibodies at the following dilutions (expressed in all the cases relative to their concentration in the original serum): ACTH, 1:350; GH, 1:350; LH, 1:1400; TSH, 1:700; and PRL, 1:2000. After washing, the coverslips were mounted over microscope slides using Vectashield (Vector Laboratories, Burlingame, CA). For each sample, specific fluorescence images corresponding to each fluorophore were captured to reveal the stained cells. The samples were studied using an Axioplan2 imaging Zeiss microscope (Carl Zeiss, Inc., Jena, Germany), Axiocam MRM digital camera (Carl Zeiss, Inc.), and Zeiss AxioVision system for capturing the images. The excitation (Ex), Ems, and dichroic mirrors (Dichroics) used were the following: Pacific Blue: Ex BP390/40, Em BP 460/50, Dichroic FT420; Oregon Green 488: Ex 490DF20, Em 535DF35, Dichroic 505 DRLP (Dichroic reflector, long pass); Cascade Yellow: Ex 380HT15, Em 535DF35, Dichroic 505 DRLP; AF 568: Ex BP546/12, Em BP605/55, Dichroic FT580; and AF 633: Ex HQ615/40, Em HQ675/50, Dichroic Q645LP. The images obtained were analyzed with ImageJ Plugins (National Institutes of Health, Bethesda, MD) developed *ad hoc* to correct the background and the overlapping of the different dyes. For this end, fluorescence images with the different settings were captured in cells stained with a single antibody, and coefficients for the cross talk between the different channels were obtained. The values used are shown in Table 1. The amount of labeled antibody used was carefully corrected to yield similar fluorescence intensity for each acquisition setting. Specificity controls were performed as reported previously (16). Single immunocytochemistry assays yielded the same percentage of stained cells than the multiple assay described here.

Statistical analysis

All data reported derive from at least three completely independent experiments for each sex and condition studied. When only two situations were compared, the Student's *t* test for paired population was used. For more than two groups, the statistical significance of the data was assessed by ANOVA and compared using Bonferroni's multiple comparison test. Differences were considered significant at $P < 0.05$ and $P < 0.01$. Data in the text and figures are expressed as the mean ± SE.

Results

Figure 1A illustrates the new methodology for the immunocytochemical, single-step, phenotypical characterization of the five AP cell types. Each antibody is shown in one panel coded in a different color: blue, ACTH Pacific Blue; green, LH

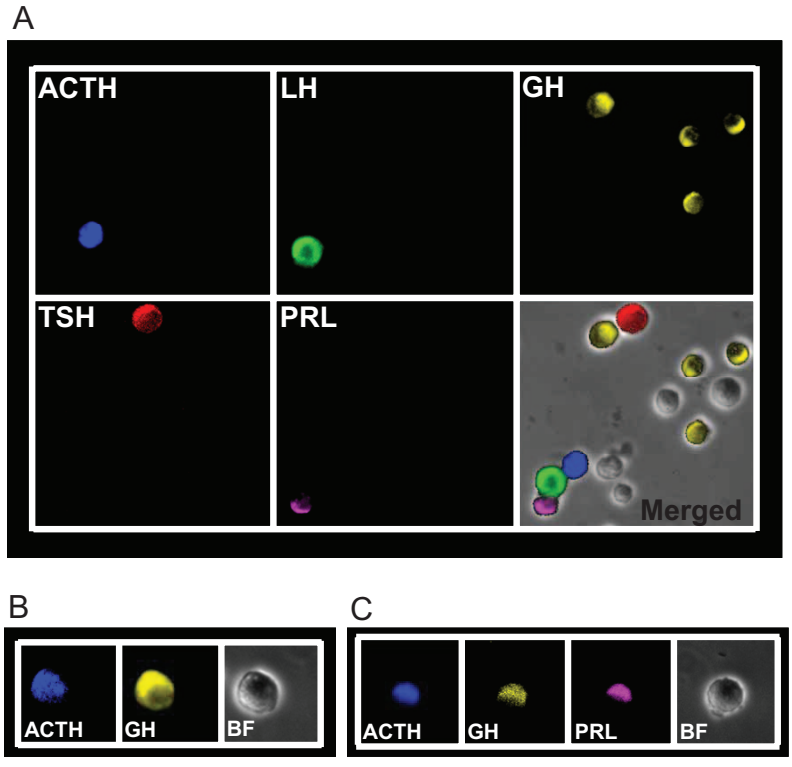

FIG. 1. Multiple simultaneous immunocytochemistry for phenotyping the five AP cell types in a single step. AP cells were obtained from Swiss mice and plated on glass coverslips. Cells were fixed with paraformaldehyde and subjected to multiple immunocytochemistry against five different AP hormones (ACTH, TSH, LH, PRL, and GH). Specific antibodies had been labeled with five different fluorophores (Pacific Blue, Cascade Yellow, Oregon Green 488, AF568, and AF633). See *Materials and Methods* for details. A, Images for each antibody in the same 150 × 150-μm microscopic field. Staining by each antibody is coded in *pseudocolor*. All the cells in this particular field were monohormonal or stored no hormone. *Bottom right*, The merge image of all five hormones, superimposed to the bright-field (BF) image revealing both hormone containing cells and cells storing no hormone. B, Example of a poly-hormonal cell containing ACTH and GH in another microscopic field together with the corresponding bright-field image. C, Example of a poly-hormonal cell containing ACTH, GH, and PRL in another microscopic field together with the corresponding bright-field image.

Oregon Green; yellow, GH Cascade Yellow; red, TSH AF568; and purple, PRL AF633. The panel at the bottom right corner shows the merge of all the aforementioned panels, superimposed to the bright-field image. The field contains 12 individual cells. Four cells did not express any of the AP hormones, and the remaining eight were monohormonal. We have analyzed the hormonal phenotypes of 3991 male and 3476 female mouse AP cells using the new methodology. Of the cells, 36–53% contained no hormones, and the rest distributed among the five different phenotypes (Table 2). The frequencies found for the different cell types were similar to the ones reported before, determined by multiple sequential, primary immunocytochemistry (16) or simple immunocytochemistry (15). Most of the cells, especially in male, were monohormonal. Figure 1A shows the example of a field that contained only monohormonal cells or cells storing no hormone. Examples of poly-hormonal cells are shown in Fig. 1B (cortico-somatotrope cell) and Fig. 1C (cortico-lacto-somatotrope cell).

The present study has focused on corticotropes comparing the phenotypes in male and female mice. Figure 2A compares the relative abundance of monohormonal corticotropes, and the different types of poly-hormonal corticotropes in male (*filled bars*) and female (*white bars*). Most of the male corticotropes were monohormonal, storing only ACTH. In contrast, most of the corticotropes of females were poly-hormonal cells, co-storing both ACTH and other additional AP hormones (Fig. 2A). These results confirm our previous findings using another methodology (16, 34, 35). The contents of the co-stored hormone were not traces but similar to the normal content of that hormone in the monohormonal cells, as revealed by quantification of the different immunoreactivities (Fig. 2B). The hormones most frequently co-stored with ACTH were GH and PRL (Fig. 2A).

We have also studied the expression of the different HRH receptors in the different corticotrope populations. For this end, we have used the combination of our multiple sequential immunocytochemistry with calcium imaging (16). Figure 3A shows that most monohormonal corticotropes responded only to CRH. However, the poly-hormonal corticotropes responded to other HRHs, sometimes more intensely than to CRH. For example, cortico-somatotropes responded quite often to GHRH, whereas cortico-gonadotropes also responded to LHRH. Figure 3B shows the size of the $\Delta[Ca^{2+}]_C$ induced by each HRH in each cell type. The profile of the distribution was similar to the one obtained plotting the percentage of responsive cells (compare Fig. 3, A and B). The largest Ca^{2+} response was the one elicited by CRH in monohormonal corticotropes. GHRH also elicited a large response in some cortico-somatotropes.

TABLE 2. Frequencies of the different cell phenotypes in male and female AP

Hormone	Male	Female	All together
ACTH	4.3 ± 0.4	4.4 ± 1.0	4.4 ± 1.1
TSH	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
PRL	15 ± 3	19 ± 1.4	18 ± 2
GH	49 ± 2	25 ± 2	36 ± 2
LH	4.4 ± 0.3	4.1 ± 0.5	4.3 ± 0.4
None	36 ± 3	53 ± 5	44 ± 4

Figures correspond to percentages (mean ± SEM) of all the cells, including the ones containing no hormones, and come from the analysis of 3993 (males) and 3476 (females) cells.

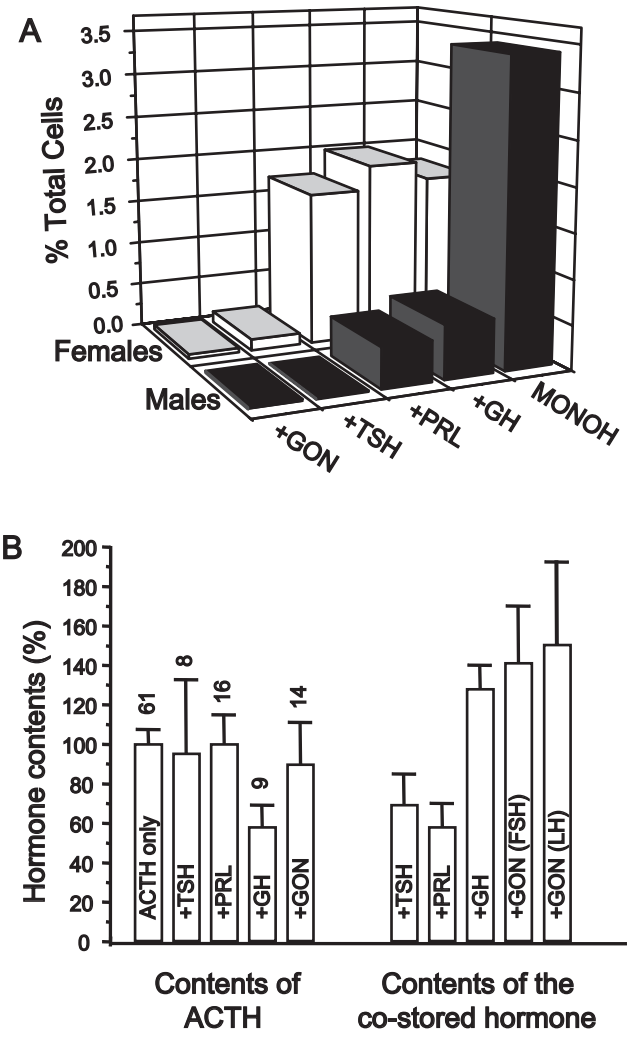

FIG. 2. Frequency distribution of ACTH-containing cells in the AP of male and female mice. A, The phenotype of 3991 male AP cells and 3476 female AP cells was studied by multiple simultaneous immunocytochemistry, as shown in Fig. 1. The relative abundance (%) of cells containing only ACTH (monohormonal; MONOH) and cells containing other hormones in addition to ACTH (poly-hormonal) is shown for males and females. Most male cells contained only ACTH, whereas most female cells stored additional AP hormones, particularly GH and PRL. +GON, Cells containing gonadotropins in addition to ACTH. B, Relative expression of different hormones in poly-hormonal corticotropes. The relative contents of ACTH in cells co-storing other hormones and the relative contents of co-stored hormones (mean ± SEM) are shown. Relative contents were computed by quantification of the fluorescence for each hormone in each individual cell. The values of fluorescence are expressed as a percentage (%) of the fluorescence value in the corresponding monohormonal cells, taken as 100%.

Once we characterized the phenotype of corticotropes, we asked whether a short period of cold stress, a well-established model of acute stress (32), would induce a different response in male and female mice. Figure 4 shows that, at rest, male and female mice had a very different level of corticosterone in serum ($P < 0.05$). After cold stress, both

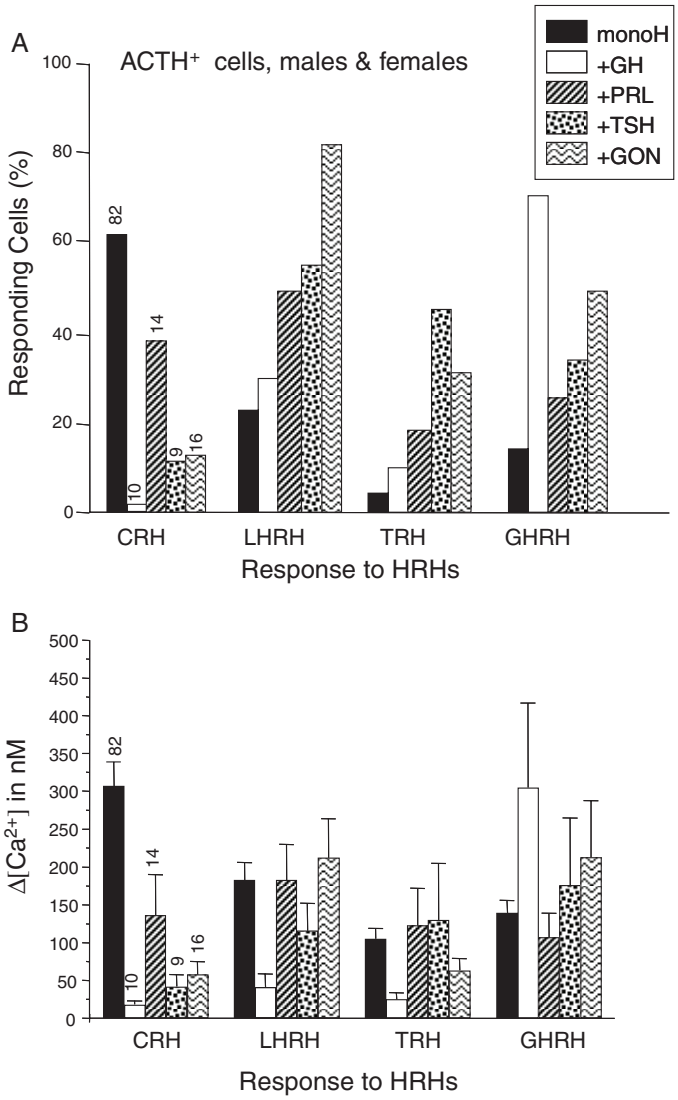

FIG. 3. Comparison of the responses of monohormonal and poly-hormonal corticotropes to HRHs. A, Percentages of monohormonal and poly-hormonal corticotropes responding to the different HRHs. Cells were considered responsive when they responded to a given HRH with an increase in cytosolic $[Ca^{2+}]$ larger than 50 nM as reported previously. Data from male and female were pooled because no significant differences were found. B, *Bars* represent responses to the HRHs, quantified as the increase in $[Ca^{2+}]_C$, $\Delta[Ca^{2+}]$ (mean ± SEM, in nM). The number of cells in each condition is given at the *top of the bars*. monoH and +GON stand for monohormonal cells and for cells containing gonadotropins in addition to ACTH, respectively.

male and female mice showed increased serum corticosterone levels. These results confirm previous reports (32).

Next, we asked whether cold stress would influence the fractional cell composition of the AP. Figure 5 shows the effects of cold stress on the relative abundance of corticotropes, and the other cell types in male and female mice. Each cell type was pooled into two groups, monohormonal and poly-hormonal cells. We found that cold stress did not change significantly the relative abundance of corticotropes or the proportional abundance of monohormonal and poly-hormonal corticotropes. It must be noted that ACTH storage in these experiments could be the net result of secretion and

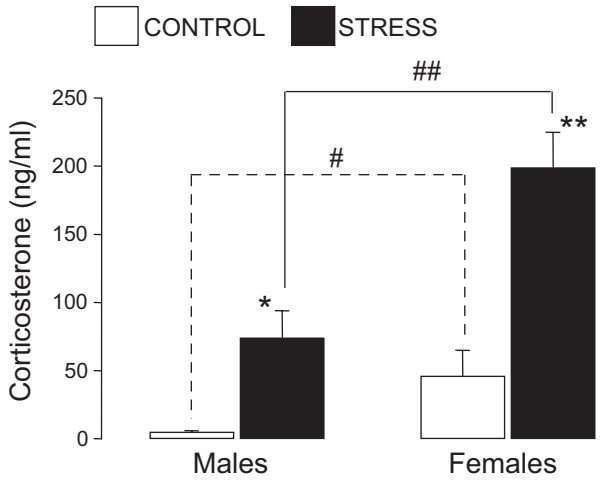

FIG. 4. Effects of cold stress on serum corticosterone values in male and female rats. Serum corticosterone values in male and female mice were measured before (*white bars*) and after (*black bars*) 30-min cold (4 C) exposure. Values are given as the mean ± SEM (n = 4 in each group). Data were analyzed using two-way ANOVA analysis together with the Bonferroni's test and the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$ *vs.* control; #, $P < 0.05$; ##, $P < 0.01$ comparing between gender.

synthesis, which could be faster in the stressed animals. Surprisingly, the abundance of other cell types was influenced by cold stress (Fig. 5). For example, in male mice, stress increased significantly the abundance of monohormonal gonadotropes (from 3 to 5%) and somatotropes (from 40 to 56%), with no change in their poly-hormonal counterparts. In females, cold stress increased significantly the abundance of monohormonal thyrotropes (from 0.5 to 1.2%), mammotropes (from 15 to 25%), and gonadotropes (from 4 to 7%). Again, stress did not change any of the pools of the corresponding poly-hormonal cells.

One of the advantages of the multiple immunocytochemistry procedure is that it allows not only identification of all cell types and cells storing multiple hormones at once but also of those cells storing no hormone. Because in all cases, the increases in monohormonal cells induced by stress were not balanced by decreases in their poly-hormonal counterparts, we asked whether stress can influence the abundance of cells storing no hormone. Figure 6 shows the relative size of the cell pools storing one hormone (monohormonal cells), several hormones (poly-hormonal), or no hormone before and after the cold stress for both male and female mice. Stress increased the abundance of the relative proportion of monohormonal cells, and this change was associated with an equivalent decrease in the abundance of cells storing no hormone. No changes were found for the population of poly-hormonal cells. Note that the changes were similar in male and females. These results suggest that the new monohormonal cells that appear after acute stress may originate from cells containing no hormones rather than from poly-hormonal cells.

Finally, we have investigated the effects of cold stress on the responses to the different HRHs, both in corticotropes and in the other AP cells. First, we asked whether cold stress does influence the size of the Ca^{2+} increase induced by each of the four HRHs used. We found that the sizes of the Ca^{2+}

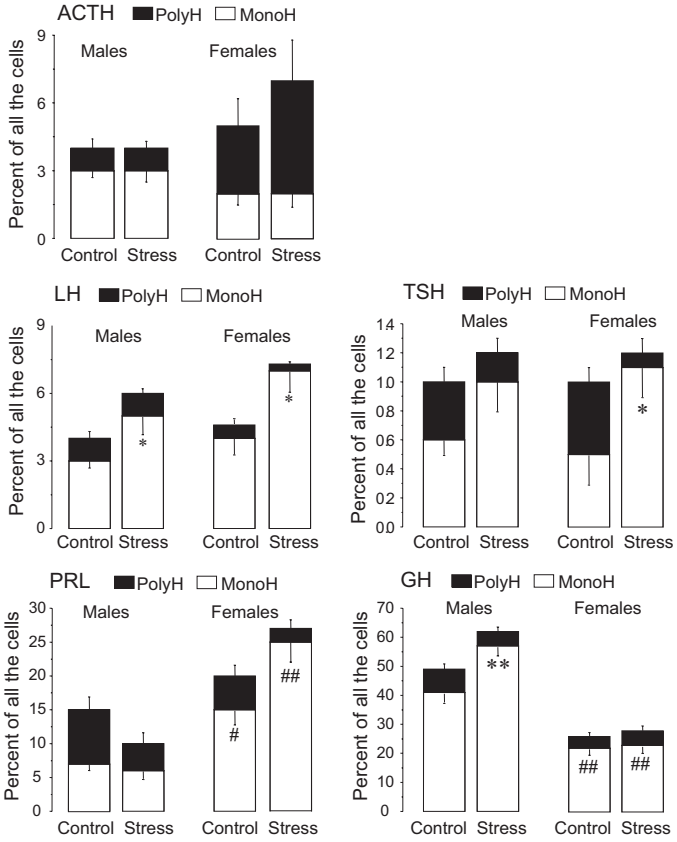

FIG. 5. Effects of cold stress on the relative abundance of monohormonal and poly-hormonal cells. The five AP cell classes (storing ACTH, LH, TSH, PRL, and GH) are shown. The relative abundance of cells storing each AP hormone is expressed as percentage of all cells. *White bars* represent contribution of monohormonal (MonoH) cells to each population, whereas *black bars* represent contribution of polyhormonal (PolyH) cells, storing multiple AP hormones. Data are mean ± SEM of nine independent experiments in each condition performed with cells derived from six male (three control and three stressed) and six female (three control and three stressed) mice, and a total of 14,957 cells (control males 3,991; stressed males 4,192; control females 3,476; and stressed females 3,298). Statistical significance was assessed by ANOVA: *, $P < 0.05$; **, $P < 0.01$ *vs.* control; #, $P < 0.05$; ##, $P < 0.01$ in comparison between genders.

responses to GHRH, TRH, and LHRH were not influenced by stress (results not shown). However, the size of the Ca^{2+} response to CRH was influenced by stress, again in a dimorphic manner. Interestingly, gender and stress did not influence significantly the maximum $\Delta[Ca^{2+}]_C$ induced by CRH in the multiresponsive cells, expressing other HRHs in addition to CRH (values ranged between 370 and 540 nM in all the cases). Specifically, the maximum $\Delta[Ca^{2+}]_C$ in monoresponsive cells, responding solely to CRH, increased rather dramatically from 166 ± 166 nM to 695 ± 23 nM after exposure to cold stress. This difference was significant ($P < 0.05$). Notice that this influence involved only cells from female mice because monoreceptor CRH cells disappear in males after stress (see Fig. 7 below). We also asked whether the relative abundance of cells responding to each HRH was influenced by stress. Figure 7 shows the percentage of cells responding to each HRH for male and female AP cells before and after cold stress. As shown before (16, 17), AP cells can be pooled in monoresponsive cells, responding to only one HRH (*white bars*), and multiresponsive cells showing responses to more than one HRH (*dark bars*). Under resting conditions, the CRH-responsive cell population was significantly larger in males than in females for both monoresponsive and multiresponsive cells ($P < 0.05$). Cold stress induced changes in the relative abundance of CRH-responsive cells in a gender-dimorphic manner. In males, the CRH-responsive cell pool decreased in response to cold exposure. This effect was due mainly to the disappearance of CRH-monoresponsive cells. In females, on the contrary, the CRH-responsive cell pool increased after stress, and this effect was entirely due to the increase in the cells that responded only to CRH. Thus, a simple 30-min exposure to cold changes the relative abundance of CRH monoresponsive cells in a gender dimorphic manner (Fig. 7).

Regarding the responses to other HRHs, cold stress increased the pool of LHRH-responsive cells in both males and females. The increase was made of multiresponsive cells. The TRH-responsive cell pool was not modified. Finally, cold exposure tended to increase the GHRH-responsive pool in males, and this increase was due to the increase in the GHRH-monoresponsive cell subpopulation. In females, on the contrary, stress decreased the GHRH-responsive cell subpopulation. Thus, cold stress also influences expression of HRH receptors other than CRH in a sexually dimorphic manner (Fig. 7).

Discussion

Here, we introduce a new methodology for phenotyping all five AP cell types based on single-step, multiple immunocytochemistry. Cells containing any combination of hormones, including cells storing no hormone, can also be identified. The present method offers several important advantages over our previous method for sequential, multiple immunocytochemistry (16, 22, 34, 35). First, it eliminates most of the image arithmetics (and derived errors) needed previously and reveals at the first sight all the AP cell types, including poly-hormonal cells and cell storing no hormones. In addition, the new method enables analysis of many microscopic fields in the same experiment, which makes possible a much larger sampling. This is particularly important when phenotyping scarce cell types, such as corticotropes here.

We have attempted here to perform a comprehensive phenotypical characterization of male and female mouse corticotropes, and to study the effects of acute, cold stress on AP cell composition. The phenotype of mouse corticotropes displayed a striking sexual dimorphism. Most male corticotropes showed a classical phenotype, storing only ACTH and responding only to CRH. The response to CRH was strong, with a large $\Delta[Ca^{2+}]$. In contrast, female corticotropes, although similar in relative abundance and ACTH storage to male corticotropes, displayed a much more heterogeneous phenotype. About half of the female corticotropes were classical, monohormonal cells, storing only ACTH and responding only to CRH. However, at variance with males, the $\Delta[Ca^{2+}]$ of the female monohormonal cells in response to CRH was small. In addition, the other half of the female

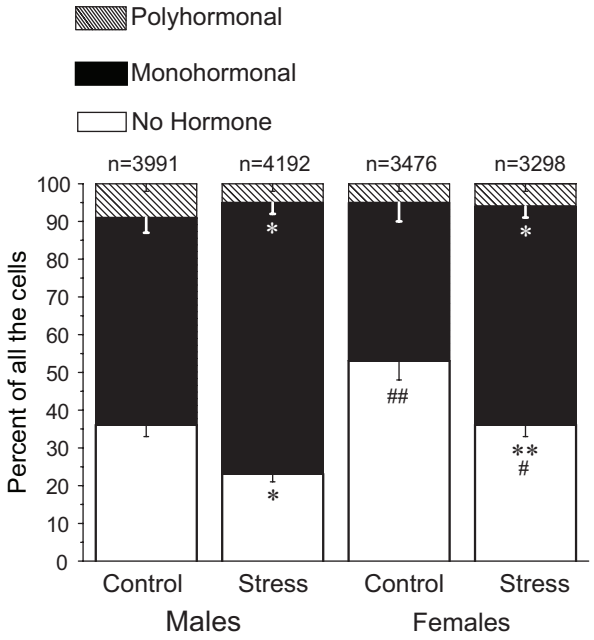

FIG. 6. Cold stress-induced changes in the abundance of the populations of monohormonal cells, poly-hormonal cells, and cells storing no hormones. *Figures* and *percentages* refer to all the cells in the gland, with no separation by cell types. Male and female AP cells obtained from mice in the resting condition and after cold stress situation were typed as either no hormonal (*white bars*), monohormonal (*black bars*), or poly-hormonal (*hatched bars*). The values shown are the mean ± SEM of nine independent experiments performed with cells obtained from three male and female mice before and after cold stress. Only *downward error bars* are shown for every pool. An ANOVA test was performed, and the mean values were compared by Bonferroni's test. Differences were considered significant at: *, $P < 0.05$; **, $P < 0.01$ *vs*. control; #, $P < 0.05$; ##, $P < 0.01$ comparing between gender. The number of individual cells analyzed in each case is shown on *top of the bars*.

corticotropes was made of poly-hormonal cells, co-storing other AP hormones together with ACTH and responding to other HRHs in addition to CRH. Thus, our data suggest that poly-hormonal cells are in a less differentiated or responsive state (to CRH) than the monohormonal corticotropes. The co-stored hormone was present in amounts comparable to ACTH. Therefore, whereas the male corticotropes were a homogenous and orthodox cell type, the female corticotropes were a mosaic of classical and multifunctional cells that share phenotypical characteristics with other AP cell lineages, particularly somatotropes. We have compared cell phenotypes of AP cells from male and randomly cycling females. Given that pituitary morphology varies much during the estrous cycle, changes in cell phenotypes along the estrous cycle can be expected (7). Thus, it must be considered that the use of randomly cycling donors might have increased the inherent variability in the system. The gender dimorphism of the corticotrope phenotype and the amount of hormone storage by poly-hormonal corticotropes suggest that storage of additional hormones is genuine and not due to uptake of released hormones, a possibility that is minimized by the extensive washout of cells after dispersion. The fact that noncorresponding hormones of poly-hormonal cells are stored in concentrations similar to the ones found in monohormonal cells suggest that this is not due to uptake of hormone released from other cells. It must be pointed out that although fresh primary cultures have provided important information about the physiology of the AP, including changes in corticotropes after acute cold stress, the fact that fresh dispersed cells may not be entirely representative of the post-stress situation *in vivo* must be acknowledged.

The aforementioned results disagree with those reported recently by Luque *et al*. (36) who used irreversible labeling of GH cell lineage by GH-promoter driven cAMP response element recombinase. These authors reported that GH cell lineage was confined to GH cells, a pool of PRL cells, and some hormone-free cells. Whereas the reasons for the discrepancy are not clear, multiple evidence by our group (16) and others (5, 6, 9, 12) indicates the existence of GH cells storing or expressing messenger for other hormones, including ACTH, LH, FSH, and TSH,

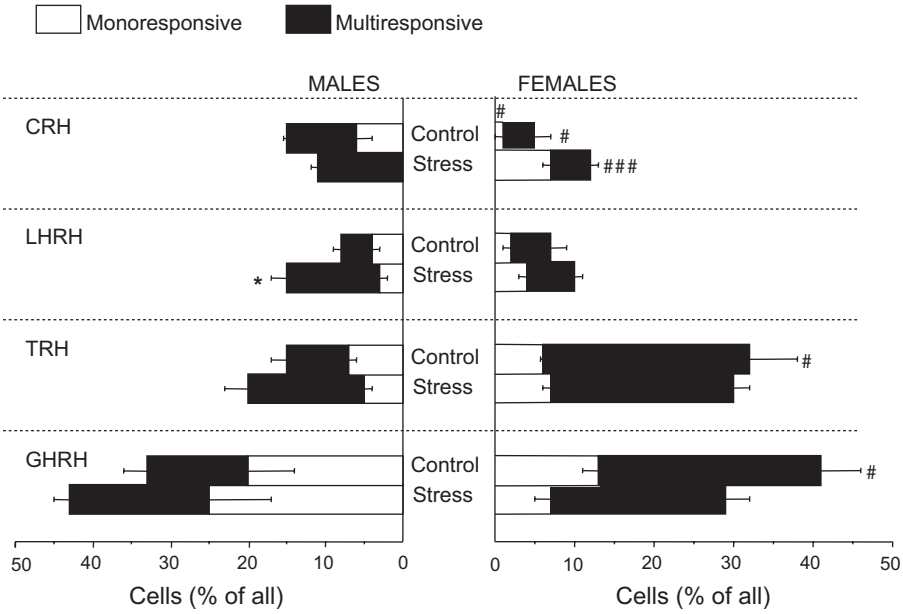

FIG. 7. Cold stress-induced changes in the responses to the different HRHs. Cells are grouped by type (hormone content). The percentage of cells responding to GHRH, TRH, LHRH, and CRH, expressed as percentage of all the cells, was determined for the control and stressed group. *White bar segments* represent contribution of monoresponsive cells, whereas *black bar segments* represent contribution of multiresponsive cells, responding to multiple HRHs. Data are mean ± SEM of six independent experiments performed with cells derived from three different mice. The total number of cells was 780 (control males 160; stressed males 178; control females 178; and stressed females 264). Significance was assessed with a two-sample independent *t* test: *, $P < 0.05$ *vs*. control; #, $P < 0.05$; ###, $P < 0.001$ comparing between gender.

despite the fact that none of cell phenotypes was found in the GH-promoter driven cell lineage. A possibility to reconcile these results is the finding that plurihormonal cells may follow a developmental pattern independent of that of monohormonal cells (13).

The resting corticosterone levels were lower in males than females, but cold stress induced a relatively larger increase of corticosterone in males. However, the absolute corticosterone levels after stress were larger in females than males. These results are consistent with previous findings (32). It has been proposed that the different corticosterone level in resting conditions is due to a permissive role of estrogen in females (37) and a nonpermissive role of testosterone in males (38). The different response to stress cannot be accounted for by differences in the abundance of corticotropes and/or the ACTH storage by corticotropes, which were similar in males and females. The differences in the phenotype of corticotropes reported here could, on the contrary, contribute to the observed gender dimorphism of the stress response. Although the dominant monohormonal corticotropes of males would be silent at rest, the multireceptorial corticotropes of females could be stimulated paradoxically by HRHs other than CRH because they express functional receptors for these HRHs. This would occasion a tonic activation of the HPA axis, and this may contribute to explain the larger corticosterone levels found in female mice at rest. After cold stress, CRH is released from the hypothalamic paraventricular nucleus and stimulates monohormonal corticotropes, more abundant and responsive in males. This would occasion a relatively larger response in males, as we have observed.

The finding that a simple 30-min exposure to cold induced a significant change in the relative abundance of cells other than corticotropes was unexpected. In addition, the changes observed were sexually dimorphic. In males the cold stress promoted the appearance of new classical gonadotropes and somatotropes, whereas in females the increase was made up of classical gonadotropes, thyrotropes, and mammotropes. However, these findings may be of no surprise considering that stress may involve an endocrine axis other than the HPA. For example, corticosterone may promote GH release, arginine vasopressin release after stress may stimulate TSH release, and stress-induced epidermal growth factor release may stimulate gonadotropes and other cell types. On the other hand, cold stress increased the abundance of monohormonal cells paralleled by a similar decrease in cells storing no hormone, with no changes in the abundance of polyhormonal cells. These results suggest that the new monohormonal cells derive from the pool of cells containing no hormones, rather than from the poly-hormonal pool, which seemed at first sight more likely (4, 6, 9). We have no evidence as to the phenotypical nature of cells storing no hormone. The value of 22–50% estimated for this kind of cells may seem rather high. However, these values cannot be compared directly with previous estimations because no simultaneous detection of all five AP hormones at once in the same cells had been performed before. We estimate that 10–15% of the cells may be either damaged ($3 \pm 2\%$) or nonexcitable (lacking Ca^{2+} responses to high K^+ medium; ~5% of cells). The remaining cells may include cells storing hormones in trace amounts not detected by our immunocytochemistry procedure or cells not storing hormones at all. As stated previously, the relative abundance of each cell type was similar in single and multiple immunocytochemistry, suggesting that the large number of hormone-depleted cells is not due to a lack of sensitivity of the procedure. However, these results must be taken with some caution because the sensitivity of a direct, multiple immunocytochemistry, such as the one developed here, may be lower than flow cytometry or electron microscopy studies that have shown a somewhat different relative abundance of individual AP cell types. Nevertheless, it must be considered that there is some degree of variability in estimates of specific cell types as exemplified by reports on the prevalence of immunocytochemically defined corticotroph cells in the rat pituitary (39). Electron microscopy immunostaining has shown the existence of cells containing more than one AP hormone (9, 40), but, to our knowledge, the attempts to quantify the relative abundance of the different cell types are scarce (41).

In addition to modifications of the AP cell composition, cold stress also induced changes in the responses to HRHs. Again, the HRHs involved included not only CRH, but also other HRHs. Finally, the changes presented a striking gender dimorphism. Nevertheless, it must be kept in mind that detecting hormone storage and HRH responsiveness may be partial dimensions in the identification of a cell, and to prove that a hormone depleted cell does not have a particular phenotype may also require additional single-cell tests as *in situ* hybridization.

Our findings pose the question as to where the new classical cells that show up after acute stress come from. Because the AP cells were studied right after the 30-min cold exposure period, it is unlikely that the changes in cell composition were due to changes in cell turnover, proliferation, or death. As discussed previously, our results are consistent with the possibility that the new monohormonal cells that show up after stress derive from cells not storing any AP hormone. Several possibilities can explain our results. First, it could happen that the new monohormonal cells store undetectable amounts of hormone at rest and that these amounts are dramatically increased after stress. Second, the new monohormonal cells may store no hormone but express the corresponding mRNA before stress and simply start to translate the messenger after stress. A last possibility would be that the new monohormonal cells did not store any AP hormone or express any hormone mRNA before stress but start expressing and storing a particular AP hormone after stress. Whether acute, cold stress activates differentiated cells (corticotropes) for hormone translation, promotes differentiation from committed or noncommitted cells, or a combination of several of these processes remains to be established. In any case, the overall reduction in cells that express nondetectable hormones shows the extent to which the pituitary has reserves to support each system. Our results indicate that AP cells are more plastic than previously thought, and could be continuously adjusting their phenotype according to intrinsic and extrinsic environmental influences, as simple as, for example, waiting in the cold for the bus.

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