Sexually dimorphic distribution of substance P in specific anterior pituitary cell populations

(somatotroph/thyroph/growth hormone/thyroid-stimulating hormone/estrogen)

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ABSTRACT Substance P (SP) immunoreactivity is detectable in the rat pituitary by RIA; however, immunolocalization has been difficult. We used a sensitive immunogold silver-enhancement staining technique to cytochemically locate SP in the gland. SP-immunoreactive (SP-ir) cells were seen in anterior pituitary (AP), and occasional SP-ir fibers and terminals were seen in both AP and posterior pituitary. Colocalization studies showed the vast majority of SP-ir cells in the male AP to be also immunoreactive for growth hormone (GH). These GH/SP-ir cells represent ~23% of the somatotroph population in the male. SP-ir cells did not colocalize with lactotrophs, gonadotrophs, or corticotrophs; however, rare thyroid-stimulating hormone/SP-ir cells were found in the male AP. Comparisons of pituitaries from males and females revealed that females have 70% fewer SP-ir cells and that only ~6% of the somatotrophs in the female express SP. This sexual dimorphism is diminished in 6-day ovariectomized rats because this treatment increases the GH/SP-ir cell population 3-fold. This result suggests that the previously reported estrogen-induced decrease in SP gene and peptide expression in the pituitary occurs, at least in part, in a subpopulation of somatotrophs. To test this hypothesis, distribution of SP-ir cells was examined in pituitaries from estrogen- and oil-treated ovariectomized rats. Estrogen reduced the percentage of somatotrophs with SP immunoreactivity by 70% compared with ovariectomized oil-treated controls, indicating that estrogen most likely regulates SP levels in the pituitary by acting on a subpopulation of somatotrophs to suppress SP expression. Estrogen does not appear to alter SP immunoreactivity that is detected in the additional population of SP cells that colocalize with thyroid-stimulating hormone. These SP-expressing thyrotrophs were seen 6-fold more frequently in the female than in the male pituitary, regardless of steroid status. These studies reveal that males have more total SP-ir cells in the AP than do females and that there is a sexually dimorphic pattern of SP distribution in the gland. Males have a higher percentage of SP-ir GH cells, whereas females have more SP-ir thyrotrhops than do males. Identification of independently regulated SP-ir somatotroph and thyrotrhop populations provides a basis for investigating the roles of SP in autocrine or paracrine regulation of pituitary hormone secretion.

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MATERIALS AND METHODS

Animals, Surgery, and Hormone Treatments. Adult male (n = 7) and female (n = 9) Sprague–Dawley rats, 200–350 g, were obtained from Sasco (Omaha, NB) and housed in the animal care facility at Washington University School of Medicine. Males and females were caged separately, and animals were allowed ad libitum access to food and water. Lights were on a 12 hr/12 hr schedule, with lights on at 0700 hr.

For steroid manipulations, random cycling females were bilaterally ovariectomized (OX) under light ether anesthesia between 1500 and 1800 hr. These OX rats were then injected s.c. with either 2 µg of estradiol benzoate (Sigma) or oil

Abbreviations: SP, substance P; SP-ir, SP-immunoreactive; AP, anterior pituitary; GH, growth hormone; TSH, thyroid-stimulating hormone; OX, ovariectomized; PRL, prolactin; TRITC, Texas Red isothiocyanate; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate.

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vehicle \((n = 2 \text{ per treatment})\) daily for 6 days after surgery. Other \(O_X\) rats \((n = 2)\) were left untreated for 6 days before perfusion.

**Immunohistochemistry.** Rats were anesthetized with an overdose of sodium pentobarbital and perfused through the heart with 100 ml of isotonic saline followed by 150–200 ml of cold Bouins’ fixative. Pituitaries were removed and post-fixed for 1 hr in Bouins’ fixative, rinsed in 0.15 M phosphate-buffered saline, pH 7.4 (PBS), and stored at 4°C in 10% sucrose. Pituitaries were frozen and mounted in embedding medium by submersion in liquefied cryogen spray, sectioned with a cryostat in 10-μm sections, and thaw-mounted onto gelatin-coated glass slides. Sections were then stored in boxes with desiccant at \(-20°C\) until immunohistochemical analysis.

Sections were thawed and preincubated in blocking buffer (1.0% bovine serum albumin/0.3% Triton/0.2% nonfat dry milk/0.01% sodium azide in PBS) at room temperature for 10 min. SP immunoreactivity was detected by a two-step silver-enhancement staining (Amersham), as described previously (12, 30). Briefly, a rabbit antiserum (R-5), directed against the amidated C-terminal region of SP and which crossreacts minimally with other tachykinins (31), was provided by J. A. Kessler (Albert Einstein College of Medicine) and was used for most experiments. Some studies were done with another SP antiserum of different specificity, R-140 (32, 33), provided by M. R. Hanley (University of Cambridge). Sections were incubated overnight at 4°C with the R-5 SP antiserum at 1:5000 (female) or 1:10,000 (male) dilution in blocking buffer. Because SP immunoreactivity in the female AP is much less than in the male (1–4), more concentrated dilutions of the SP antiserum had to be used to optimize signal-to-noise ratio. After three washes in PBS at room temperature, 5 min each, gold-labeled goat anti-rabbit IgG serum was diluted in blocking buffer (1:40) and applied to the sections for 1 hr at room temperature. Sections were then washed in PBS for 15 min and in \(H_2O\) for 5–10 min. The silver-enhancement process was done according to the manufacturer’s protocol, and development was monitored under a microscope; development was stopped by placing the slides in \(H_2O\). SP antiserum specificity was confirmed when sections incubated with nonimmune normal rabbit serum, or with SP antiserum that was incubated with 1 μM synthetic SP, showed no staining. However, incubation of the SP antiserum with 10 μM TSH or growth hormone (GH), provided by the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (which also provided pituitary hormone antisera), did not block staining.

To identify different pituitary cell populations in these SP-stained sections, subsequent incubations were done with primary antisera directed against pituitary hormones detected by immunofluorescence. Because the silver-developing process destroys antigenicity of the initial primary antibody–antigen complex (12, 30), subsequent incubations could be done with antisera raised in the same species. Therefore, rabbit anti-rat β-TSH (S-5), rabbit anti-rat β-luteinizing hormone (S-10), rabbit anti-rat β-folicue stimulating hormone (S-11), rabbit anti-rat PRL (S-9), and rabbit anti-human adrenocorticotropin (IC-1) were detected with either a Texas Red isothiocyanate (TRITC)-labeled goat anti-rabbit secondary serum (Sigma) or, for quadruple-labeling studies, a 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated donkey anti-rabbit secondary serum (The Jackson Laboratory). Monkey anti-rat GH (S-5) antiserum was detected with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary serum (Cappel Laboratories). In quadruple-labeling studies, a mouse anti-oopioic antibody (34) labeled with a rhodamine isothiocyanate (RTIC)-tagged goat anti-mouse secondary serum (The Jackson Laboratory) was used to identify the opioid-expressing corticotrophs. This monoclonal antibody (3-E7) recognizes the sequence Tyr-Gly-Gly-Phe (34) common to all opioid peptides, including β-endorphin, and was from E. Weber (University of California, Irvine). Primary incubations were done overnight at 4°C with antisera diluted in blocking buffer as follows: 1:1000 for anti-gonadotropin, -TSH, and -adreno-corticotropin; 1:2000 for anti-PRL and -opioid; and 1:10,000 for anti-GH. Sections were then washed in PBS for 15 min, and secondary incubations were done in dilutions of 1:500 (TRITC, RTIC, FITC) or 1:320 (AMCA) for 1 hr at room temperature, washed in PBS, and coverslipped with 50% (vol/vol) glycerol in PBS. Some slides were lightly counterstained with hematoxylin. Specificity of the antisera that colocalized with SP, anti-TSH, and anti-GH, was confirmed when staining was blocked by preincubating the antisera with 10 μM of the appropriate hormone, and when no staining was apparent by using normal rabbit or normal monkey sera. However, blocking with 10 μM SP did not diminish staining with either of these antisera.

Quantitation was done by counting SP-positive cells in random fields of the AP at ×500 magnification with a Zeiss microscope. A minimum of 10 random fields from two or more pituitaries from each group of animals was examined. Cell counts were averaged across fields, and the data are expressed as the mean percentage of SP-ir cells of either the somatotroph or thyrotroph populations ± SEM.

**RESULTS**

Immunogold silver-enhancement staining revealed a number of randomly distributed SP-ir cells in the male AP not detectable when the SP antiserum was incubated with 1 μM SP or when normal rabbit serum was used (Fig. 1 A and B). Furthermore, we also observed SP-ir fibers and terminals in the AP and in the posterior pituitary (data not shown), which have been described in rat, monkey, and human (7–12). No staining of cells or fibers was seen in the intermediate lobe with the SP antiserum. Immunofluorescent detection of the different pituitary hormones revealed distinct populations of cells that varied in distribution and morphology.

Colocalization studies showed that most SP-ir cells within the male AP were double-labeled with GH antiserum (Fig. 1 D, E, and F). This SP-ir cell population represents =23% of the somatotroph population in the male AP (Fig. 2). Nearly all (98%) of the SP-ir cells in the male AP contained GH immunoreactivity and did not appear to colocalize with any of the other pituitary hormones, except for rare SP-ir cells containing TSH immunoreactivity (Fig. 2). The SP cells make up =12% of the total AP cell population in the male, as determined by cell counts of hematoxylin-counterstained SP-ir fields. Immunohistochemical analysis of APs from random cycling females revealed almost 4-fold fewer SP-ir cells compared with the male AP (Fig. 1C). As in the male, many SP-ir cells in the female colocalize with GH (Fig. 1 G, H, and I), and these amounted to =75% of the SP-ir cells in the AP. These cells represent =6% of the somatotroph population (Fig. 2). The remaining population of SP-ir cells (25%) colocalized with TSH (Fig. 1 G, H, and I), representing =8% of the thyrotrophs in the female (Fig. 2). The SP-ir cell population in the female represents =3% of the total AP cell population.

Because there is a sexual dimorphism in SP content in the pituitary—in part due to estrogen suppressing SP expression in the female AP (2–4) and because females have 75% fewer somatotrophs with SP immunoreactivity than males (Fig. 2), it seemed that estrogen acts on a subpopulation of somatotrophs to down-regulate SP content. To test this
hypothesis, we performed SP immunohistochemistry on glands isolated from estrogen- and oil-treated OX rats. There were ≈3- to 4-fold more SP-ir cells in APs isolated from oil-treated OX rats compared to those from estrogen-treated OX rats or from normal cycling females (data not shown). There were no obvious differences between SP-ir cells in random cycling females and estrogen-stimulated OX rats. Most of these SP-ir cells in APs of oil-treated OX rats colocalize with GH, representing an increase in the SP-expressing somatotroph population to almost 20% of all somatotrophs (Fig. 3). There was no apparent effect of OX or estrogen replacement on the SP-ir thyrotroph population (Fig. 3). The quantitative analysis here, however, is based on the presence or absence of immunoreactivity and does not attempt to distinguish between relative amounts of immunoreactivity. Consequently, estrogen-induced alterations in the amounts of SP immunoreactivity within thyrotrophs would not be detected in this analysis. Furthermore, it is possible that OX increased the number of SP-ir thyrotrophs but that levels of SP expression were below sensitivity of the immunogold staining technique.

**DISCUSSION**

SP is present in nerve fibers and cell bodies in the posterior pituitary and in the AP of several species (5–12). Because SP may act directly at the level of the AP to induce PRL secretion (18, 19, 22, 27) and perhaps alter the release of other pituitary hormones (18–29), these effects could arise from SP released locally from nerve terminals and/or from secretory cells in the AP. Identification of the pituitary cells that express and perhaps utilize SP as a local regulatory peptide would help elucidate the pituitary hormonal system(s) that communicate via SP. Although SP has generally been diffi-
FIG. 2. Distribution of SP-ir cells in the somatotroph and thyro-
troph populations in the male and female pituitary. SP/pituitary 
hormone colocalization studies were done as described. Immu-
nogold-labeled SP-ir cells were counted in 10 or more random fields 
\((x300)\) from at least two pituitaries from normal male (m) and cycling 
female (f) rats. Fluorescently labeled GH- or TSH-ir cells were also 
counted in each field, and the percentage of SP-ir cells per each of 
the somatotroph and thyrotrhop cell populations was determined. 
Data are represented as the mean percentage of all fields counted \(\pm \) 
SEM. Note that the SEM represented here reflects variability 
between randomly selected fields, as well as between pituitaries. 
Males had a greater percentage of SP-ir somatotrophs than did 
females, whereas females had a greater proportion of SP-ir thyro-
trophs than did males. Because the somatotrophs are much more 
abundant than thyrotrhops, however, this graph does not reveal the 
fact that females had more SP-ir somatotrophs than SP-ir thyro-
trophs. (SP/GH cells represent 75% of all SP cells in the female AP.) 

FIG. 3. Distribution of SP-ir cells in the pituitary of estrogen- 
and oil-treated OX females. Female rats were OX and treated with either 
estradiol benzoate (EB; m) or oil vehicle (f) for 6 days after surgery 
as described. Pituitaries were processed for immunohistochemistry 
to identify SP-ir cells by immunogold and the five pituitary cell types 
by immunofluorescence. Quantitation of the distribution of SP-ir 
cells was done as described in the legend to Fig. 2.

(12) that SP does not colocalize with GH, and in guinea pig 
(5) and human (12) AP SP immunoreactivity is detected in 
TSH cells. Moreover, one study reported the presence of SP 
immunoreactivity in lactotrophs and gonadotrophs in rat (6). 
However, these investigators based pituitary cell type iden-
tification on ultrastructural evidence of granule size, whereas 
the present study used the more specific criterion of immu-
nohistochemical localization of pituitary hormones. Further-
more, we have seen similar SP/GH colocalization using a 
different SP antiserum, R-140 (32, 33), as well as a goat 
anti-human GH serum that crossreacts with rat GH (data not 
shown), and all criteria for antisera specificity were fulfilled 
by antigen-blocking controls.

Multiple-labeling studies revealed a rare SP-ir mammo-
somatotroph in the male AP (i.e., a cell triple-labeled with SP, 
GH, and PRL antisera). Such cells, believed to be progeni-
tors of mature lactotrophs and somatotrophs (35), were not 
usually SP-ir. In fact, such expression would suggest that 
these rare cells were in the process of differentiating into 
somatotrophs, the cell type displaying most SP immunoreac-
tivity.

SP-expressing thyrotrhops in the female pituitary were 
much more common than in the male. Males had an occa-
sional SP/TSH dual-positive cell, which probably represents 
the 2% of nonsomatotrophic SP cells in male AP. But such 
cells were never present to the degree seen in female pitu-
ity, even when lower SP antiserum dilutions were used 
\((1:1000)\). The sex difference in SP/TSH distribution might 
reflect a sexual dimorphism in thyroid status because thyroid 
hormones can regulate SP expression in the pituitary (13–17).

The 4-fold difference in the SP-expressing somatotroph 
population between males and females corresponds well to 
the sex difference reported (2, 3) by RIA in AP SP content-
males having 4- to 6-fold more pituitary SP than females. 
Because estrogen is, at least in part, responsible for the 
sexual dimorphism in SP content (2–4), we hypothesized that 
estrogen acts on a subpopulation of somatotrophs in the 
female pituitary to down-regulate SP expression. There is 
precedence for estrogen regulating somatotroph function. 
Estrogen stimulates GH secretion from the pituitary (36, 37), 
and somatotrophs have been shown to bind \(^{3}H\)estradiol in 
females in an ovarian-dependent fashion (38). Furthermore, 
in the present study OX caused a 4-fold increase in the 
SP-expressing somatotroph population over the percentage 
seen in cycling females and in OX estrogen-stimulated rats. 
Therefore, the estrogen effects reported (2–4) on pituitary SP 
content and SP mRNA expression probably occur directly on 
a subpopulation of somatotrophs to suppress SP synthesis. 
Though androgen effects on SP distribution in the pituitary 
were not examined, the increases in SP immunoreactivity due 
to androgen stimulation reported (1–4) could occur in the 
somatotroph population because GH cells represent most 
SP-ir cells in the male pituitary.

Although the SP immunoreactivity described may be partly 
due to uptake of SP supplied either from pituitary sources or 
via the portal circulation, it is highly unlikely that all SP 
immunoreactivity seen in somatotrophs represents only up-
take. SP mRNA is expressed within the AP (4, 13, 14), so 
those cells synthesizing it should display some SP immuno-
reactivity. Furthermore, pituitary SP mRNA is down-
regulated by estrogen (4), causing decreased SP content in 
the gland (2–4) similar in magnitude (\(\sim 50\%\)) to the estrogen-
induced suppression of somatotroph SP immunoreactivity 
observed in the present studies (\(\sim 70\%\)). Therefore, the 
estrogen-regulated SP immunoreactivity seen in somato-
trhops probably represents SP expressed de novo in those 
cells.

The SP immunoreactivity detected in thyrotrhops in fe-
males may also represent SP expression in those cells. 
Pituitary SP immunoreactivity in other species is contained in
thyrotrophs (5, 12), and thyroid hormones down-regulate SP mRNA expression in the rat pituitary (13, 14). But thyroid status may affect pituitary cell types other than thyrotrophs. For instance, thyroid hormone replacement can reverse thyrotophexin-induced decreases in [3H]estradiol binding in the hypothyroid state occur not only in thyrotrophs but in somatotrophs as well (39). This fact indicates that thyroid hormones may up-regulate estrogen receptors in the somatotroph population, as has been demonstrated also in liver and kidney (40, 41). This mechanism would provide one way by which thyroid hormones could decrease pituitary SP levels—i.e., by increasing the amount and/or availability of estrogen receptors in somatotrophs, so that more estradiol can inhibit SP mRNA expression.

The immunohistochemical evidence reported here shows that most SP immunoreactivity in the rat pituitary is associated with a subpopulation of somatotrophs that responds to estrogen by suppressing SP expression in the female pituitary. This result indicates that the GH-secreting cells in the pituitary use this steroid-regulated peptide perhaps as a local effector of pituitary function. SP may act in a paracrine fashion—that is, on cells different from the somatotrophs and thyrotrophs that presumably secrete it. For instance, it could act on lactotrophs because SP may locally induce PRL secretion (18, 19, 22, 27). On the other hand, perhaps SP serves an autocrine role, acting on the same cell types that synthesis and secrete it, and, in fact, effects of SP on GH and TSH secretion have been documented (18, 19, 24, 28). Vasoactive intestinal peptide has been shown to have such an autocrine function, being secreted from and acting on lactotrophs to induce PRL secretion (42). Identification of the cell type(s) that bind and presumably respond to SP will distinguish between these possibilities and help determine the pituitary hormonal system(s) that communicate by means of SP.

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