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Sexual Dimorphism and Testosterone-Dependent Regulation of Somatostatin Gene Expression in the Periventricular Nucleus of the Rat Brain*

JULIE A. CHOWEN-BREED, ROBERT A. STEINER, AND DONALD K. CLIFTON

Departments of Obstetrics and Gynecology (D.K.C., R.A.S.), Physiology and Biophysics (J.C.B., R.A.S.), and Zoology (R.A.S.), University of Washington, Seattle, Washington 98195

ABSTRACT. Gender differences in hypothalamic somatostatin (SS) secretion may account in part for the sexually dimorphic patterns of GH secretion in rats. Since males have lower baseline serum GH levels than females, and SS inhibits GH secretion, we hypothesized that the SS neurons in the periventricular nucleus (PeN) of the male rat would have greater biosynthetic activity than those of the female. We tested this hypothesis by measuring SS mRNA in cells in the PeN of intact male and proestrous female rats. Using *in situ* hybridization and a computerized image analysis system, we measured SS mRNA content in individual cells in the PeN and compared signal levels (autoradiographic grains per cell) between male and proestrous female animals. The signal level of SS mRNA in cells of the PeN was significantly greater in males than in proestrous females (males, 210 ± 7 grains/cell; females, 158 ± 5 grains/cell; $P < 0.0005$), whereas no difference was observed in SS cells of the frontal cortex (males, 100 ± 0.8 grains/cell; females, 99 ± 5.9 grains/cell). This difference in SS mRNA levels is likely to be the result of different hormonal environments exerting an influence on neurons of the hypothalamus. To test the hypothesis that testosterone stimulates SS gene expression in neurons of the PeN, adult male rats were castrated and immediately implanted with either empty (sham; n = 3) or testosterone-containing (n = 3) Silastic implants of a size that would deliver physiological levels of testosterone (3.6 ± 1.5 ng/ml). We observed that castrated animals had significantly lower levels of SS mRNA signal in neurons of the PeN compared with intact animals (intact, 195 ± 3 grains/cell; castrated, 159 ± 6 grains/cell; $P < 0.003$) and that physiological levels of testosterone prevent this reduction in SS mRNA levels (castrated testosterone-replaced, 182 ± 4 grains/cell; castrated, 159 ± 6 grains/cell; $P < 0.003$). Furthermore, testosterone-treated castrates had SS mRNA signal levels indistinguishable from those of intact controls (intact, 195 ± 3 grains/cell; castrated testosterone-replaced, 182 ± 4 grains/cell). There was no significant difference in SS mRNA levels in neurons of the frontal cortex (intact, 98 ± 2 grains/cell; castrated, 98 ± 3 grains/cell; castrated testosterone-replaced, 102 ± 2 grains/cell). Based on these observations we infer that differences in the GH secretory profile between male and female animals may occur by virtue of sexually dimorphic SS gene expression in the hypothalamus; moreover, the observed sexual dimorphism in SS mRNA levels may be attributable in part to the different hormonal environments between the sexes with testosterone stimulating SS gene expression in neurons of the PeN. (*Endocrinology* **125**: 357–362, 1989)

THE SECRETORY pattern of GH exhibits a pronounced sexual dimorphism in the mature rat (1). Adult male rats exhibit high amplitude GH pulses approximately every 3 h superimposed on a low or undetectable baseline plasma GH level (2). Adult females, on the other hand, have an irregular pattern of low amplitude GH pulses superimposed on an elevated interpulse baseline plasma GH level (3, 4). These sexually dimorphic GH secretory patterns may be attributable to differences in hypothalamic function between the sexes. At least two neuropeptides, GHRH and somatostatin (SS), govern the episodic release of GH from the anterior pituitary. Acting in a reciprocal fashion, GHRH stimulates whereas SS inhibits GH secretion (5). SS is released into the portal vasculature primarily from neurons originating in the periventricular nucleus (PeN) (6, 7). This SS source is the major determinant of baseline plasma GH levels (8).

Since male rats have lower baseline plasma GH levels and SS inhibits GH secretion, we proposed that PeN neurons of males have higher levels of SS gene expression than those of females and that this occurs at least in part by virtue of different hormonal environments between the sexes. First, we compared the level of SS mRNA in individual PeN neurons of male and proestrous female rats. We report that the SS mRNA content of PeN neurons of male rats is significantly greater than that of proestrus female rats. Second, to determine if testosterone could be involved in the stimulation of SS gene expression in PeN neurons, we compared SS mRNA signal levels in intact, castrated, and castrated testoster-

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Address all correspondence and requests for reprints to: Dr. Donald K. Clifton, Department of Obstetrics and Gynecology RH-20, University of Washington, Seattle, Washington 98195.
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one-replaced male rats. We report that intact animals have significantly higher SS mRNA levels than castrated animals. Furthermore, replacement with physiological levels of testosterone immediately after castration prevents this decline in SS message.

Materials and Methods

Animals and accommodations

Adult (90-day-old) male and female Sprague-Dawley rats were purchased from Tyler Laboratories (Bellevue, WA) and housed under constant temperature and light (14 h)-dark (10 h) cycles, with lights on at 0700 h. The animals were given free access to tap water and rat chow.

Experimental design

In the first experiment we examined whether there was an inherent difference between male and female rats in the level of SS mRNA in neurons of the PeN. To control for variance in SS message that might occur over the estrous cycle, all female animals were killed on the day of proestrous, when SS peptide content is lowest (9). Vaginal smears were performed daily between 0830 and 1000 h. Male rats were also handled at this time. Only females exhibiting four consecutive 4-day estrous cycles were used for this study. On the afternoon of proestrus, between 1400 and 1500 h, females were paired with males and killed. Animals were sedated with ketamine HCl (100 mg/kg) and xylazine (10 mg/kg) and perfused through the heart with 0.9% saline followed by 4% paraformaldehyde (100 ml/animal), and then 4% paraformaldehyde containing 10% sucrose (100 ml/animal). The brains were removed, rapidly frozen on dry ice, and stored at −80 C.

In the second experiment we assessed whether testosterone modulates SS mRNA levels in neurons of the PeN in the male rat. Male rats were either castrated or sham operated under ether anesthesia. Immediately after castration, animals received a 30-mm Silastic capsule (od, 3.18 mm; id, 1.57 mm; Dow-Corning, Midland, MI) that was either empty (n = 3) or contained crystalline testosterone (n = 3). Four days after surgery, animals were asphyxiated with carbon dioxide generated from dry ice and decapitated. The brains were removed onto dry ice, quickly frozen, and stored intact at −80 C until processed for *in situ* hybridization histochemistry. Trunk blood was collected, and the serum was stored at −20 C before RIA for testosterone.

Tissue preparation

For *in situ* hybridization, the brains were allowed to equilibrate in the cryostat chamber (−18 C), trimmed, and embedded in Tissue-Tek OCT (Miles Scientific, Naperville, IL). Coronal brain sections were cut at 10 μm (Exp 1) or 20 μm (Exp 2) beginning rostrally at the decussation of the anterior commissure and continuing caudally to the recession of the optic chiasm. Sections were thaw-mounted on poly-L-lysine (50 mg/ml)-coated glass slides and stored at −80 C until hybridization histochemistry was performed.

SS cRNA probe synthesis and labeling

A 340-basepair ^{35}S-labeled cRNA probe, complementary to pre-pro-SS mRNA, was used for hybridization histochemistry. The original EV142 DNA used for preparation of this probe, previously described in detail (10), was kindly provided by Richard Palmiter. The probe was hydrolyzed in 100 mM bicarbonate buffer (pH 10.2) to yield fragments of approximately 150 bases in length, heat denatured at 80 C, rapidly cooled, and added to hybridization buffer to obtain a final concentration of 0.6 mg/ml·kilobase (*i.e.* 0.204 μg/ml). This corresponded to a specific activity of approximately 2.76×10^8 dpm/μg for Exp 1 and 3.29×10^8 dpm/μg for Exp 2.

In situ hybridization

For Exp 1, sections were baked at 37 C for 2 h and then pretreated with proteinase-K (1.0 μg/ml) and 0.25% acetic anhydride in 0.1 M Tris (pH 8.0) for 10 min before application of 60 μl probe mixture to the tissue. For Exp 2, which used fresh tissue, sections were postfixed with 4% paraformaldehyde, followed by the acetic anhydride treatment and application of 60 μl probe mixture to the tissue. (All subsequent steps of the procedure were identical for both experiments). Parafilm coverslips were applied to each slide, which were then incubated overnight at 45 C in moist chambers. After removal of the coverslips, slides were rinsed in 4 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M Na citrate) and then subjected to treatment with RNase, followed by a series of washes in SSC of increasing stringency. The slides were dipped in Kodak NTB2 photographic emulsion and exposed at 4 C for 6 days (Exp 1) or 3 days (Exp 2). Developed slides were counterstained with cresyl violet, and a coverslip was placed over the tissue. [We have previously described this methodology in greater detail (10).]

Image analysis

Sections throughout the PeN, beginning rostrally at the decussation of the anterior commissure and continuing caudally to the plane of the retrochiasmatic area, were anatomically matched (12/animal) across animals to insure that the same 12 planes of section throughout the PeN were analyzed in each animal. Slides from all of the animals were assigned a random 3-letter code, alphabetized, and then read blindly in random (alphabetical) order with an automated image-processing system. This system consisted of a Data Cube IVG-128 video acquisition board (Datacube Inc., Peabody, MA) attached to an IBM AT computer. Video images were obtained by a Dage model 65 camera (Dage-MTI, Inc., Michigan City, IN) attached to a Zeiss Photomicroscope 1 (Zeiss, New York, NY), equipped with a special ×40 epiillumination dark-field objective. In each section, the number of silver grains over each of 10 cells contained within the PeN was determined. Analysis of background grain density was performed simultaneously during the reading of each cell and automatically subtracted from the grains per cell value recorded by the computer. Cells were identified by the presence of a cresyl-stained nucleus associated with a cluster of silver grains. To avoid a bias in the selection of cells, we analyzed the first identifiable and readable cell encountered in each of 12 fields of an individual section of the

PeN. To be selected, a cell had to have had a cresyl-stained nucleus, an isolated cluster of silver grains, and be free of artifacts, including fixation and staining artifacts as well as the presence of debris. The various fields of view, and therefore the cells selected for analysis, were evenly distributed throughout the PeN in a given tissue section. The number of silver grains over a cell is directly related to the amount of radioactively labeled probe present in that cell. However, the number of silver grains associated with a cell cannot be assumed to be linearly related to the number of SS mRNA molecules in that cell. Hence, quantitative differences in the number of copies of SS mRNA molecules cannot be inferred from these data. However, differences in relative amounts of SS mRNA can be assessed with this methodology, for which we have previously published a detailed description (11).

Control experiments

Probe specificity. Control experiments have been performed and previously reported to demonstrate the specificity of the binding of the pre-pro-SS cRNA probe as well as to determine the optimal probe concentration for these assays (10). Briefly, all controls performed (*i.e.* RNase pretreatment, excess unlabeled probe, and test with a nonspecific probe) resulted in the absence of grain clusters, suggesting that this probe is binding specifically to the SS mRNA. By Northern blot analysis, this probe recognizes a single band corresponding to a RNA species of approximately 850 bases.

Anatomical specificity. To ascertain whether a significant change in mean grains per cell is specific to the nucleus of interest (*i.e.* the PeN), SS mRNA levels were analyzed in cells of the frontal cortex, an area of the brain in which SS mRNA levels were not expected to differ between male and female animals or in response to castration. SS mRNA levels were assessed in individual cells (10 cells/section) of the frontal cortex located in the same tissue sections in which PeN neurons were analyzed.

Perfused tissue vs. fresh tissue. To evaluate whether there might be an improvement in signal conferred by perfusing the brains *in situ*, we conducted a test assay to determine if there was a significant difference in SS mRNA signal level between animals perfused as described above compared with that in which the brain had been fresh frozen and postfixed. No significant difference in signal was observed between the two protocols (perfused, 106 ± 5 grains/cell; fresh, 115 ± 4 grains/cell).

Serum testosterone assay

Testosterone was measured in serum by RIA, as previously described (12). The sensitivity of this assay was less than 0.1 ng/ml, and the intra- and interassay coefficients of variations were 3.0% and 8.5%, respectively.

Statistical analysis

The mean number of grains per cell for each individual animal was calculated from the analysis of approximately 120 cells/animal. This number, *i.e.* the mean number of grains per cell for each individual animal, was then used to determine the mean ± SEM for each group. Therefore, n refers to the number of animals in each group, and this was the number used for statistical analysis. In the first experiment statistical comparison of the grains per cell/group was performed using Student's *t* test. In the second experiment the effect of castration and testosterone replacement on SS mRNA content was evaluated by one-way analysis of variance. Individual differences were then analyzed by the Duncan's new multiple range test at $\alpha = 0.05$.

Results

Male rats (n = 5) had approximately a 33% greater number of grains per cell (and, by implication, SS mRNA) in neurons of the PeN than proestrous female rats (n = 4; $P < 0.0005$). Relative mRNA levels of male and proestrous females are illustrated in Fig. 1A.

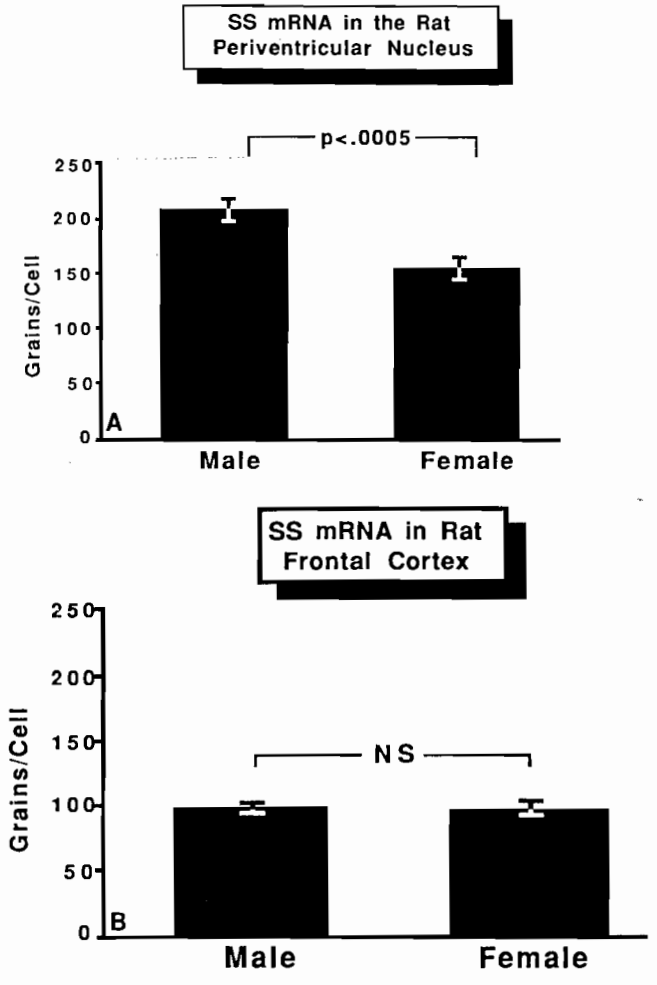

FIG. 1. A, Relative amounts of SS mRNA, as reflected by grains per cell, in the periventricular nucleus of male (n = 5) and female (n = 4) rats. Values given are the mean ± SEM. The signal level of SS mRNA is significantly greater ($P < 0.0005$) in males (210 ± 7) than in proestrous females (158 ± 5). B, Relative amounts of SS mRNA in neurons of the frontal cortex of male and female rats.

Whereas cellular SS mRNA levels in the PeN differed between males and females, no difference in SS mRNA content of frontal cortex neurons was observed between the sexes, as shown in Fig. 1B.

SS mRNA signal levels were significantly reduced by 19% in castrated animals (n = 3) compared with those of intact controls (n = 3), whereas animals treated with physiological levels of testosterone immediately after castration (n = 3) did not differ significantly from intact control values. The effects of castration and castration plus testosterone replacement on SS mRNA signal levels in neurons of the PeN of the male are illustrated in Fig. 2A. No difference in SS mRNA content of frontal cortex neurons was observed among the three experimental groups, as illustrated in Fig. 2B.

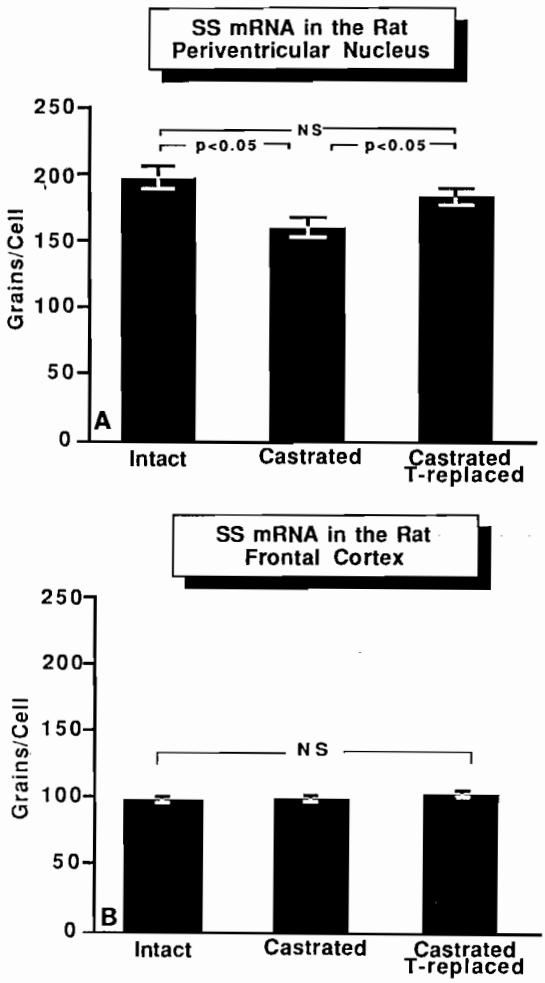

FIG. 2. A, Relative amounts of SS mRNA, as reflected by grains per cell, in the PeN of intact males (n = 3), castrated males (n = 3), and castrated testosterone (T)-replaced males (n = 3). Values given are the mean ± SEM. The signal level of SS mRNA is significantly reduced subsequent to castration. Replacement with physiological levels of testosterone prevents this decline in SS mRNA message. There was no significant difference between control and testosterone-treated animals. *Lines over bars* indicate results of Duncan's new multiple range test. B, Relative amounts of SS mRNA in neurons of the frontal cortex of intact males, castrated males, and castrated testosterone-replaced males.

In Exp 1 background values over an area the size of a cell were 34 ± 3 and 37 ± 3 grains/area for males and females, respectively. In Exp 2 background values over an area the size of a cell were 41 ± 3, 35 ± 1, and 34 ± 2 grains/area for intact, castrated, and castrated testosterone-replaced groups.

Plasma levels of testosterone in the three treatment groups were as follows: intact, 2.9 ± 0.7 ng/ml; castrate, 0.07 ± 0.03 ng/ml; and testosterone-treated castrates, 3.6 ± 1.4 ng/ml.

A darkfield photomicrograph taken of SS neurons in the PeN of an intact male rat is shown in Fig. 3A. Figures 3, B and C, are darkfield photomicrographs taken at the same level of the PeN as Fig. 3A of a castrated and castrated testosterone-replaced male rat, respectively.

Discussion

We have shown that SS mRNA content in PeN neurons is greater in male rats than in proestrous females. Furthermore, castration of adult male rats results in a significant reduction in SS mRNA in this same population of neurons, an effect that can be prevented by administration of physiological levels of testosterone. Although it cannot be assumed either that all SS mRNA produced is translated into the mature peptide or that the peptide produced is actually released at the nerve terminal, mRNA availability is one important factor in protein biosynthesis. Hence, these results would suggest that there is sexual dimorphism in the capacity of PeN neurons to synthesize SS and that this difference in synthetic capacity is controlled at least in part by testosterone's ability to stimulate SS gene expression in neurons of the PeN. In addition, we would infer that the amount of SS available for release from the median eminence may also differ between the sexes. If this premise were correct, sex differences in GH secretory patterns and perhaps differences in growth rates (13, 14) between the sexes could be partially attributable to a difference in the ability of PeN SS neurons to produce this neuropeptide.

The hormonal milieu of the female animal changes over the course of the estrous cycle. To alleviate the confounding effect of possible variation within the female test group due to the hormonal cycles, only proestrous females were assayed for SS mRNA. The decision to focus on proestrous females introduces the possibility that this cycle stage may be the only time point during the estrous cycle that the difference in SS mRNA content between male and female animals is extant. It is possible that SS mRNA varies over the course of the estrous cycle, reflecting the changing hormonal status of the female reproductive system. Indeed, hypothalamic SS

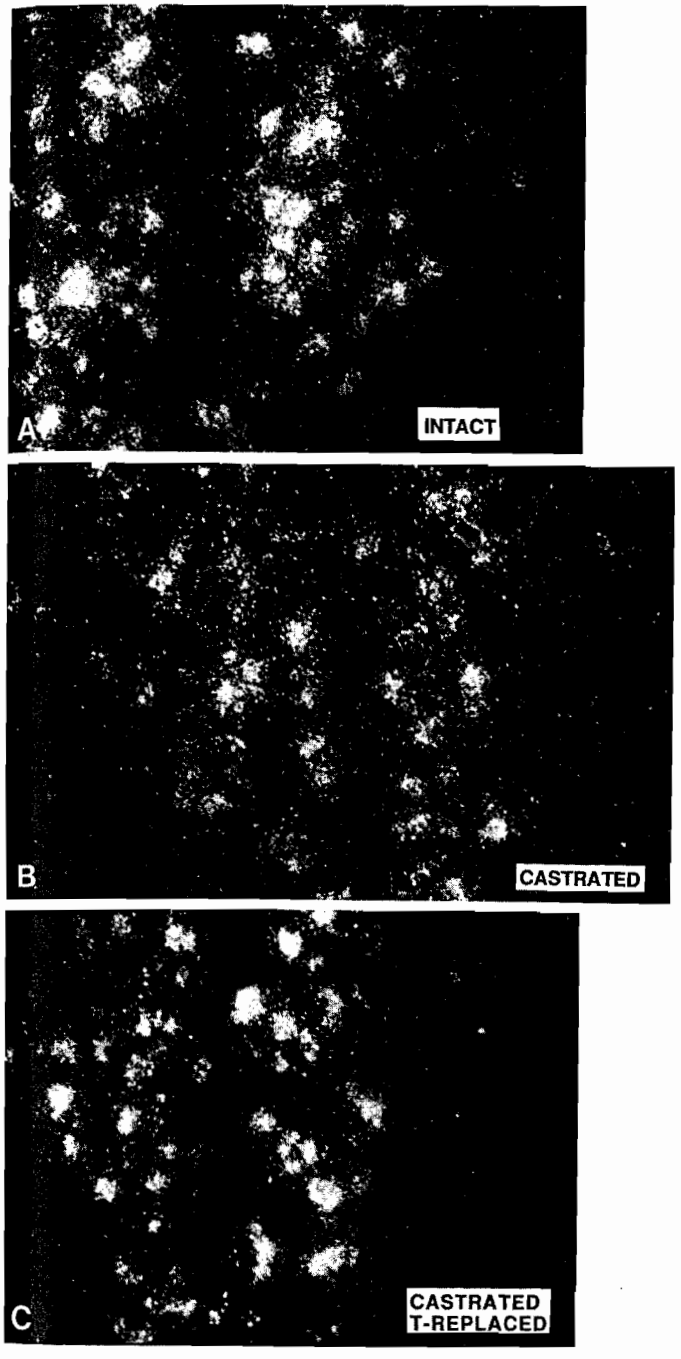

FIG. 3. Darkfield photomicrograph of *in situ* hybridization for prepro-SS mRNA in the PeN of an intact male (A), a castrated male (B), and a castrated testosterone-replaced male (C), all taken at the same anatomical plane of section.

content changes over the estrous cycle (15), and it is plausible that this reflects changing SS mRNA levels and biosynthetic capacity. However, it is also possible that the reported change in SS content over the estrous cycle (15) is reflective of a change in the secretion of this peptide. Notwithstanding, we have shown that there is a significant difference between males and proestrous females in SS mRNA content in neurons of the PeN. Furthermore, it is conceivable that this difference in SS mRNA persists over the entire estrous cycle, reflecting a sexually differentiated organizational pattern in the SS system. In support of this argument, albeit indirect, is the fact that the GH secretory pattern of the female rat does not appear to change significantly over the estrous cycle (3); however, full resolution of this point will require an extensive analysis of SS mRNA in females throughout the entire estrous cycle.

Since gonadal steroids influence both hypothalamic SS content and GH secretory patterns (4, 15–17), the apparent sex difference in SS gene expression may be attributable to the differences in the hormonal environment of the PeN neurons between males and females. There is compelling evidence to suggest that gonadal hormones in general and testosterone in particular influence GH secretory patterns in the rat (4, 15–18). Castration of adult males results in an elevation of baseline plasma GH levels. Furthermore, gonadectomy of prepubertal male rats results in elevated basal GH levels when the animals become adults. Testosterone replacement lowers the elevated baseline GH levels to normal (4). Testicular feminized male rats, deficient in intracellular androgen receptors, have elevated baseline GH levels compared to normal male littermates (19). Together these observations underscore the importance of androgens in influencing SS, and possibly GHRH, which establish the low plasma GH baseline levels characteristic of normal male animals. We have shown that castration of adult male rats decreases SS mRNA levels in neurons of the PeN, and replacement with physiological levels of testosterone prevents this effect. These observations support the hypothesis that the male patterning of GH secretion may be attributable at least in part to the influence of testosterone on SS gene expression.

These results corroborate and extend those of Werner *et al.* (20), who reported that castration of both male and female rats resulted in a reduction of SS mRNA, as detected by both Northern blot analysis and *in situ* hybridization. The seminal report by Werner and colleagues documented that sex steroids are involved in the regulation of SS mRNA levels in the rat and established the precedent for the studies described in this paper. While this earlier report established that the effect of castration could be observed in 3–6 weeks, we now show that this effect becomes manifest within 4 days; moreover, we demonstrate that the decrease in SS mRNA can be prevented with the administration of physiological (*vis à vis* pharmacological) levels of testosterone. In addition, the method of analyzing *in situ* hybridization assays differs between the reports. We report results as autoradiographic grains per cell, as determined by an image analysis system in contrast to an overall densito-

metric analysis of autoradiograms (not on an individual cell basis). Our results establish that the increase in SS mRNA message is due at least partially to an increase in message levels in individual cells of the PeN and cannot be explained entirely by an increase in the number of cells expressing the message for SS. In addition, given the caveat of examining only proestrous females, we have presented a direct comparison between male and female animals, demonstrating that there is indeed a physiological difference between the sexes.

Together, the earlier study of Werner *et al.* and ours suggest that sex steroids play an important role in the regulation of SS gene expression in neurons of the PeN. It therefore seems likely that testosterone suppresses GH baseline levels through a stimulatory effect on SS gene expression. We conclude that the observed difference in SS mRNA in the PeN neurons between male and proestrous female rats represents a possible explanation for the sexually dimorphic pattern of GH secretion observed in rats. Furthermore, sex differences in growth rates at puberty may occur by virtue of the presence of testosterone in the male, its effect on SS gene expression, and, ultimately, the steroid-dependent patterning of GH secretion.

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