0013-7227/91/1285-2369$03.00/0
Endocrinology

Vol. 128, No. 5
Printed in U.S.A.

Sexual Dimorphism of Growth Hormone-Releasing Hormone and Somatostatin Gene Expression in the Hypothalamus of the Rat During Development*

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ABSTRACT. The secretory pattern of GH secretion is markedly sexually dimorphic in the adult rat. The patterning of GH secretion is determined by the coordinated activity of somatostatin (SS)- and GH-releasing hormone (GHRH)-containing neurosecretory cells located in the hypothalamus. In this study we examined whether there is sexual dimorphism in the expression of the SS and GHRH genes and, if so, at what developmental stage this becomes evident. To address these questions, we measured SS messenger RNA (mRNA) levels in neurons of the periventricular nucleus and GHRH mRNA levels in the arcuate nucleus and ventromedial nucleus of the hypothalamus in male and female rats at 10, 25, 35, and 75 days of age. Using *in situ* hybridization and a computerized image analysis system, we measured SS mRNA and GHRH mRNA signal levels in individual neurons and compared these levels among the different age groups. We found that male animals had significantly higher levels of SS mRNA than females at every age. Similarly, males had higher GHRH mRNA levels than females; however, this difference was statistically significant only at 10 and 75 days of age. Developmental changes in GHRH mRNA levels were similar for both sexes, with GHRH message levels increasing gradually over the course of maturation. SS mRNA signal levels also changed over the course of development in both male and female animals. In the male rat, SS mRNA levels increased significantly between 10 and 25 days of age and declined significantly between 35 and 75 days of age. In the female rat, SS mRNA levels increased gradually between 10 and 35 days of age, then, as in the male, declined significantly between days 35 and 75. We conclude that sex differences and age-dependent changes in the expression of the SS and GHRH genes may subserve the sexual dimorphism and developmental alterations in the pattern of GH secretion in the rat. (*Endocrinology* **128**: 2369–2375, 1991)

THE INTERPLAY of two hypothalamic neuropeptides, somatostatin (SS) and GH-releasing hormone (GHRH), controls the release of GH from the anterior pituitary. Acting in a reciprocal manner, SS inhibits and GHRH stimulates GH secretion to coordinate its episodic release into the peripheral circulation (1). The patterning of GH secretion is markedly sexually dimorphic and, as a consequence, so too is the rate of systemic growth (2, 3). The adult male rat exhibits a regular and strikingly pulsatile pattern of GH secretion with high amplitude pulses occurring at intervals of approximately 3.5 h separated by low or undetectable baseline levels (4). In contrast, the mature female rat exhibits irregular, low amplitude GH pulses, superimposed on a significantly elevated baseline level (5, 6). GH secretory patterns are altered over development in both male and female rats and become clearly sexually dimorphic with the onset of puberty (6). However, the mechanism underlying the sex difference in GH secretion patterns is not well understood.

We have previously demonstrated that there is a sexual dimorphism in SS gene expression in the adult rat brain (7). In this study we sought to determine when in the course of development this sexual dimorphism in SS mRNA levels becomes manifest. In addition, we examined whether there is a sexual dimorphism in GHRH gene expression, and if so, when this difference becomes evident. We addressed these questions by measuring the messenger RNA (mRNA) for SS in individual neurons of the periventricular nucleus (PeN) and GHRH mRNA in neurons of the arcuate nucleus (ArcN) and ventromedial nucleus of the hypothalamus (VMH) of male and female rats at different ages.

Received December 13, 1990.
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Materials and Methods

Animals and accommodations

Sprague-Dawley rats were purchased (Tyler Laboratories, Bellevue, WA) and housed under constant light (12 h)-dark (12 h) conditions with lights on at 0600 h. The animals were given free access to rat chow and tap water. For the 10-day-old experimental group, pregnant females were obtained and allowed to give birth. The pups remained with their mothers until the day of death.

Experimental design

To assess when the sexual dimorphism in SS and GHRH gene expression becomes manifest, we measured SS mRNA levels and GHRH mRNA levels in male and female rats at 10, 25, 35, and 75 days of age (n = 4 in each experimental group). Only those female rats that did not present with vaginal opening by day 35 were used in the 35-day-old experimental group. In addition, because differences in the hormonal environment associated with the different stages of the estrous cycle could introduce a source of variability in neuropeptide gene expression, the estrous cycles of adult female animals were monitored by performing daily vaginal swabs. Only those animals exhibiting two consecutive 4-day cycles were used for this study. Adult female rats were killed on the afternoon of proestrus and were between 74 and 77 days of age. All animals were killed between 1300–1500 h.

Tissue preparation

On the day of death, animals were asphyxiated with carbon dioxide generated from dry ice and immediately decapitated. The brains were removed, rapidly frozen on dry ice, and stored intact at −80 C until processed for *in situ* hybridization histochemistry. The sex of immature animals was determined by surgical demonstration of sex organs after the animal had been killed.

For *in situ* hybridization the brains were allowed to equilibrate in the cryostat chamber (−15 C). Coronal sections were cut at 20 μm and thaw-mounted onto poly-L-lysine (50 μg/ml)-coated slides. Brain sections were collected beginning rostrally at the decussation of the anterior commissure and continuing caudally to the supramammillary nucleus. Tissue slices were stored at −80 C in airtight boxes until hybridization histochemistry was performed.

SS riboprobe synthesis and labeling

A 340 base pair (bp) ^{35}S-labeled complementary RNA (cRNA) probe, complementary to preproSS mRNA, was used for hybridization histochemistry. The original EV 142 DNA employed for preparation of this probe was previously described in detail (8). The probe was hydrolized 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 μg/ml·kilobase (kb) (*i.e.* 0.204 μg/ml), a concentration previously shown to saturate the reaction (9). This corresponded to a specific activity of approximately 2.76×10^8 dpm/μg.

GHRH riboprobe synthesis and labeling

The original plasmid, prGHRF-2, consists of a 360 bp EcoR1-Sal 1 fragment of a rat GHRH cDNA clone inserted into the a pUC8 vector. This plasmid was provided by Dr. Kelley Mayo (10). The EcoR1-Sal1 fragment contains the coding regions for GHRH-43 and a 30-amino acid peptide of unknown function, a 105-nucleotide 3′-untranslated region and the poly (A) tract. A 198 bp EcoR1-*Hin*dIII fragment was subcloned into the transcription vector pGEM4 (Promega Biotec, Madison WI). This fragment includes the entire GHRH-43 coding sequence and a portion of the sequence coding for the 30-amino acid peptide. Restriction mapping was used to confirm the orientation of the fragment. Antisense cRNA probes were made *in vitro* by linearizing this plasmid with EcoR1 and transcribing the insert sequence using the T7 promoter present on the plasmid vector. Sense cRNA probes were made by linearizing the plasmid with Sal1 and transcribing the sequence with SP6 polymerase.

The transcription reaction was performed at a uridine triphosphate (UTP) concentration of 50 μM and consisted of 9.9 μM ^{35}S-labeled α-thio UTP (New England Nuclear, Boston MA) and 40.1 μM unlabeled α-thio UTP. Residual DNA template was removed by digestion with DNase (Promega Biotec) and the cRNA probe separated from unreacted components on Nensorb 20 columns (New England Nuclear). Polyacrylamide gel electrophoresis was used to verify the identity and integrity of the transcript. This reaction gives a final probe specific activity of approximately $5.0–5.6 \times 10^8$ dpm/μg.

In situ hybridization

In situ hybridization was performed as previously described (9). Briefly, after fixation in 4% paraformaldehyde, tissue sections were pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The slides were rinsed in 2× SSC (1× SSC = 150 mM NaCl and 15 mM Na citrate) and blotted. Either the SS or GHRH cRNA probe was applied in 60 μl of hybridization buffer at a concentration of 0.6 μg/ml·kb and 1.2 μg/ml·kb, respectively. The slide was covered with a strip of parafilm and sealed with rubber cement. The sections were incubated overnight in moist chambers at 45 C (SS) or 56 C (GHRH). The following day the parafilm coverslips were peeled off with tweezers and the slides rinsed in 4× SSC. After RNase treatment and a series of washes in SSC of decreasing concentration, the slides were dehydrated in ethanol washes and air-dried.

Autoradiography and histological staining

Slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) that had been diluted 1:1 with 600 mM ammonium acetate and heated to 45 C in a water bath. They were allowed to air-dry for 30 min, then were further dried in a moist chamber at room temperature for 45 min. Slides were placed in light-tight boxes containing small tubes of desiccant, and exposed for 3 days (SS) or 6 days (GHRH) at 4 C. Slides were developed, counterstained with cresyl violet, and coverslips were then applied.

Anatomical matching of slides

To compare anatomically similar regions between the sexes and among the age groups, slides were matched by reference to the rat brain atlas of Paxinos and Watson (11) as an anatomical guide.

Matching of SS mRNA slides. The PeN was divided into 3 areas of approximately equal length in the rostral-caudal plane. Four tissue sections from each area (areas 1–3) were selected from each animal (12 slices per animal). These areas corresponded to the following descriptors.

Area 1 began rostrally at the decussation of the anterior commissure (atlas plate 20) where SS-positive cells first appear in the PeN and continued caudally to the appearance of the paraventricular nucleus (approximately plate 24). (See Ref. 11 for plate identification.)

Area 2 was continuous with area 1, beginning approximately at plate 24 and continuing caudally to the disappearance of the paraventricular nucleus at plate 27.

Area 3 began with the disappearance of the paraventricular nucleus and continued caudally to the disappearance of the PeN (approximately plate 29).

Matching of GHRH mRNA slides. The arcuate nucleus was divided into 3 areas of approximately equal length in the rostral-caudal plane. Four tissue sections from each area from each animal were selected for analysis (12 slices per animal). These areas were as follows.

Area 1 began rostrally with the appearance of the arcuate nucleus and GHRH positive cells (plate 26) and continued caudally to plate 29.

Area 2 was continuous with end of area 1 and continued caudally to the appearance of the dorsomedial nucleus (plate 31).

Area 3 began with the appearance of the dorsomedial nucleus and continued caudally until the disappearance of GHRH positive cells (plate 33).

Image analysis

Anatomically matched slides were assigned a random three-letter code, alphabetized, and read in random order with an automated image processing system by an operator unaware of the animal's experimental group. 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 Inc., Michigan City, IN) attached to an ACM Zeiss Photomicroscope (Zeiss, New York, NY) equipped with a special 40× epi-illumination dark-field objective. In each section for SS mRNA analysis, the number of grains over each identifiable cell contained within that section of the PeN that could be isolated from other SS-positive neurons was determined. For GHRH mRNA analysis, every identifiable cell was analyzed and the anatomical location recorded (ArcN or VMH). Cells were identified by the presence of a cresyl-stained nucleus associated with an isolated cluster of silver grains. The number of grains per cell is also referred to here as mRNA signal level. It should be borne in mind that the grains per cell measurement is a semiquantitative estimate of message levels and, although there should be a direct relationship between signal level and the amount of message contained within a cell, the relationship is most likely not a linear one. We have previously published a detailed description of this methodology (9).

Control experiments

SS probe specificity. Control experiments have been performed and previously reported to demonstrate the specificity of the binding of the preproSS cRNA probe, as well as to determine the optimal probe concentration for these assays (9). 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 an RNA species of approximately 850 nucleotides.

GHRH probe specificity. To demonstrate the specificity of binding of the GHRH antisense probe used in these studies, brain slices were hybridized with a GHRH sense cRNA probe of identical specific activity. Use of the GHRH sense probe resulted in the absence of autoradiographic grain clusters. The ability of the GHRH antisense probe to bind specifically to RNA was demonstrated by preincubation of brain slices with RNase A. This also resulted in the absence of autoradiographic grain clusters. In addition, probe specificity is also inferred by the demonstration of anatomical specificity. Brain slices were examined to ensure that GHRH-positive cells were restricted to areas previously shown to contain GHRH immunoreactive neurons (12).

The optimal probe concentration for these assays was previously determined by performing a saturation curve (13). Saturation of GHRH mRNA was found to occur at approximately 0.6 μg/ml·kb, and all subsequent experiments were performed at a probe concentration which was in excess of saturation (*i.e.* 1.2 μg/ml·kb).

Statistical analysis

The mean number of grains per cell for each neuropeptide in each individual animal was determined from the analysis of approximately 150 cells per animal. The mean grains per cell for each individual animal was then used to determine the mean ± SEM for each experimental group. Therefore, the n in all statistical analyses refers to the number of animals in that group. A Kruskal-Wallis one-way analysis of variance was then performed to determine if differences between the various ages existed within a sex. Comparisons between the sexes at each age group and between ages within each sex were made with a Mann-Whitney U test. The rejection criterion was chosen at $\alpha = 0.05$.

Results

Sexual dimorphism of SS mRNA signal levels

We have previously reported that adult male rats have significantly more SS mRNA signal in individual neurons of the PeN than adult proestrous females. We have confirmed this observation and now show that this dif-

ference persists throughout the course of development. Male rats had significantly more SS mRNA than female animals at all age groups examined (Fig. 1). Differences between the sexes were the greatest during the earlier stages of development, with the male having 65% and 69% more SS mRNA signal than the female at 10 and 25 days of age, respectively. The difference in mRNA signal levels between males and females at 10 days of age can be visualized in Fig. 2. At 35 and 75 days of age the male had 33% and 29% more SS mRNA signal than the female, respectively.

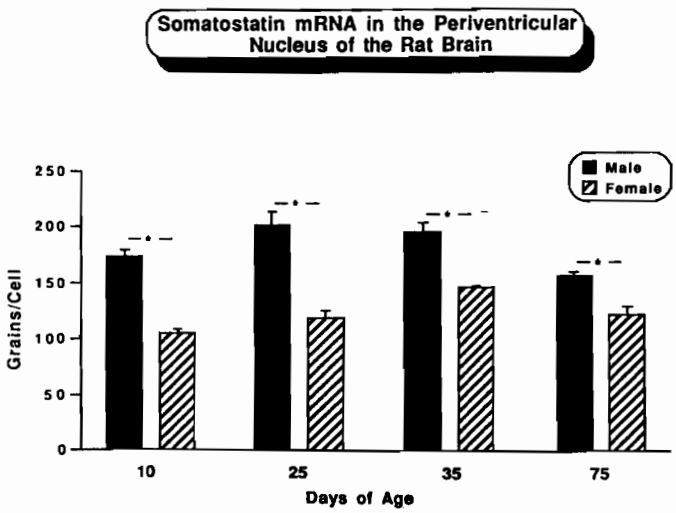

FIG. 1. Relative amounts of SS mRNA, as reflected in grains per cell, in the PeN of male and female rats at various developmental stages. Values are given as mean ± SEM. Males have significantly more SS mRNA signal at every age examined (* $P < 0.05$).

Sexual dimorphism of GHRH mRNA levels

Analysis of the ArcN and VMH separately resulted in similar results in all experimental groups. Therefore, all results are reported as hypothalamic GHRH mRNA signal levels.

Although male animals tended to have more GHRH mRNA signal than female animals at all time points, this difference was statistically significant at only two ages. There was a significant difference in GHRH mRNA signal levels between the sexes at 10 and 75 days of age with male rats having 13% and 27% more GHRH mRNA signal than the females, respectively. The difference in GHRH mRNA signal levels between males and females at 10 days of age can be visualized in Fig. 3.

Developmental changes in SS mRNA levels

In the male rat SS mRNA content changed significantly throughout maturation. SS mRNA signal levels increased by 17% between 10 days and 25 days of age (10D: 173 ± 7 grains per cell *vs.* 25D: 202 ± 13 grains per cell) and declined (19%) after the onset of puberty (35D: 195 ± 9 grains per cell *vs.* 75D: 158 ± 4 grains per cell). This decline resulted in the adult animal having significantly fewer grains per cell than the 25- and 35-day-old groups.

In the female rat SS mRNA levels also changed significantly over the course of development. SS mRNA signal levels were not significantly different between 10 and 25 days of age (10D: 106 ± 4 grains per cell *vs.* 25D: 119 ± 7 grains per cell). Levels increased significantly (23%) between 25 and 35 days of age (147 ± 4 grains per

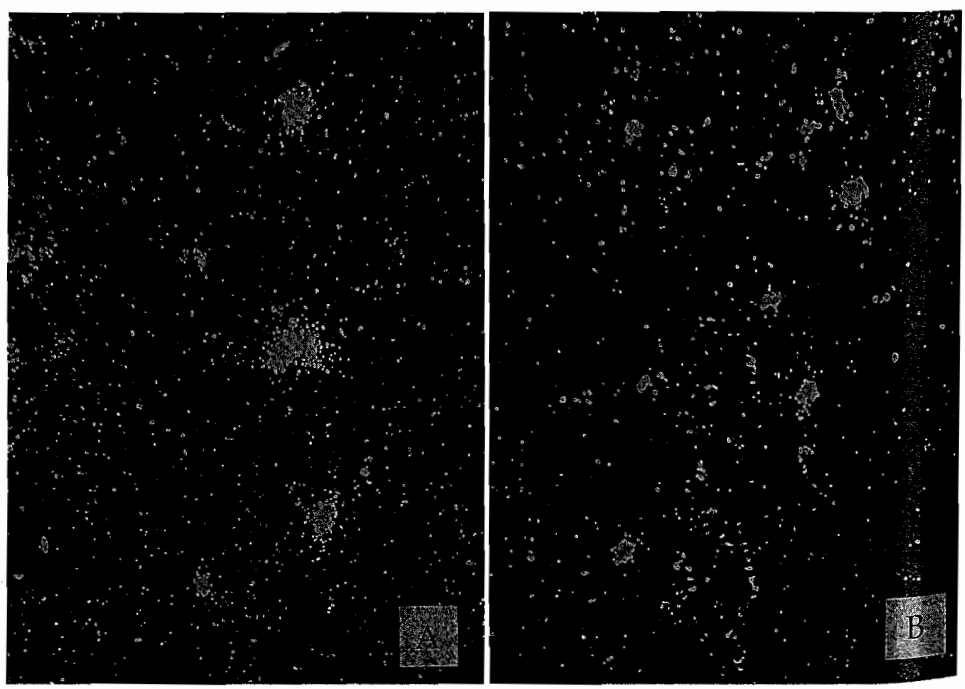

FIG. 2. Darkfield photomicrograph of the PeN showing cells labeled with a cRNA probe for SS mRNA in anatomically matched sections of a 10-day-old male (A) and a 10-day-old female (B) rat. Cells labeled with the cRNA probe for SS mRNA appear as bright clusters of silver grains.

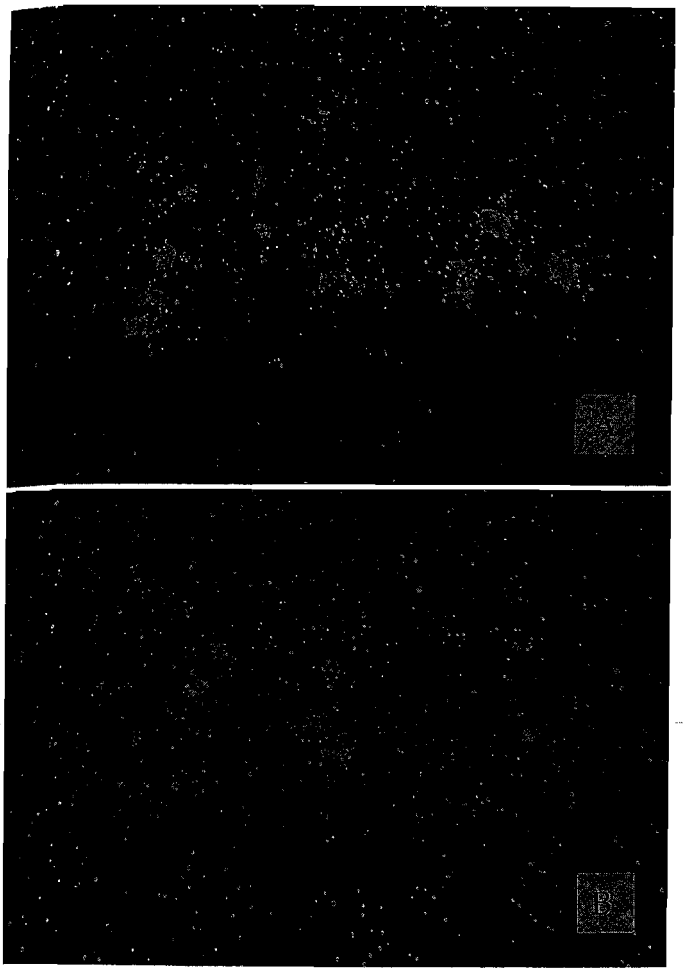

FIG. 3. Darkfield photomicrograph of the ArcN of anatomically matched sections from a 10-day-old male (A) and a 10-day-old female (B) rat. Cells labeled with the cRNA probe for GHRH mRNA appear as bright clusters of silver grains.

cell) and declined significantly (17%) after the onset of puberty (between days 35 and 75), so that the average SS mRNA signal level of 75-day-old animals (122 ± 8 grains per cell) was not significantly different from the earliest two time points.

Developmental changes in GHRH mRNA levels

GHRH mRNA signal levels changed significantly over the course of development in the male rat. Between 10 and 25 days of age in the male rat there was no apparent change in GHRH mRNA signal (10D: 86 ± 3 grains per cell *vs.* 25D: 85 ± 1 grains per cell). However, between 25 and 35 days of age, there was an 18% increase in the average GHRH mRNA signal level (35D: 100 ± 4 grains per cell) followed by a further 21% increase between 35 and 75 days of age (75D: 121 ± 4 grains per cell). In the female rat, as in the male rat, GHRH mRNA signal levels in neurons of the hypothalamus increased over the course of development; however, the increase in the

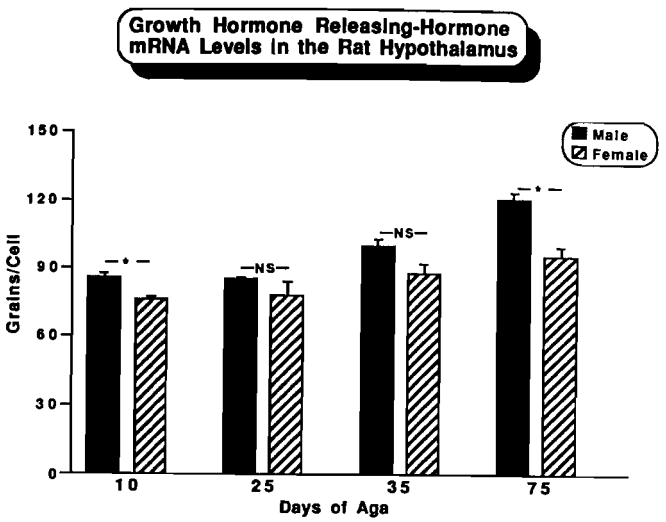

FIG. 4. Relative amounts of GHRH mRNA signal level in the hypothalamus of male and female rats at different developmental stages. Values are given as mean ± SEM. Males have significantly more GHRH mRNA signal at 10 and 75 days of age (* $P < 0.05$; NS, not significant).

female was more gradual and attained a lower maximum than in the male animals (10D: 76 ± 2 grains per cell; 25D: 79 ± 6 grains per cell; 35D: 88 ± 4 grains per cell; 75D: 96 ± 5 grains per cell) (Fig. 4).

Discussion

We have previously reported that there is a sexual dimorphism in SS gene expression, with neurons in the PeN of adult male animals containing more SS mRNA than those of proestrous females (7). We now report that this sex difference is manifest throughout earlier stages of development and is identifiable by at least 10 days of age. Since there appears to be a critical period during neonatal development when testosterone participates in the organization of the adult GH secretory pattern (14–16), the early appearance and persistence throughout maturation of sexual dimorphism in SS gene expression could reflect an organizational event occurring under the influence of sex steroids. It is conceivable that exposure to testosterone during the neonatal period induces a high level of SS mRNA in neurons of the PeN, and this induction persists throughout juvenile life into adulthood, independent of exposure to testosterone beyond the critical period. The fact that sexual dimorphism in SS gene expression persists even during the juvenile stage (25 days of age), when plasma levels of testosterone are low in both sexes (17, 18), supports the concept of an organizational event occurring during neonatal life.

In addition, androgens may also play an activational role in the adult animal, as evidenced by the fact that testosterone exposure is required postpubertally to achieve and maintain an adult male GH secretory pattern (14, 16). Our previous work has shown that testosterone

also stimulates SS gene expression in neurons of the PeN of adult male rats (7, 19–21) and that this effect is exerted through an androgen-dependent mechanism (21). Together with the present study, these observations suggest both organizational and activational roles for androgens to influence SS gene expression in the male rat; moreover, we infer that this induction may play a role in determining the ability of the adult male rat to secrete GH in its characteristic pulsatile fashion.

In this study we have observed that male rats have higher GHRH mRNA signal levels than do females at every age examined; however, these differences were statistically significant only at 10 and 75 days of age. GHRH mRNA signal levels were significantly higher in males compared with females only at those stages of life when the male animals would be experiencing high levels of circulating testosterone (17), suggesting an activational effect of androgens on GHRH gene expression. Consistent with this argument, we have recently reported that testosterone stimulates GHRH mRNA levels in the adult male rat, which, like the effects of testosterone on SS gene expression, occurs through an androgen receptor-dependent mechanism (13). Thus, we would infer that sexual dimorphism in GHRH gene expression may be the result of the different hormonal environments existing in males and females.

We have observed a significant sexual dimorphism in cellular levels of both SS and GHRH mRNAs at 10 days of age. The existing literature indicates that mean plasma GH concentrations do not differ between the sexes at this age (22); however, GH secretory patterns have not been assessed at this early stage of development. The earliest age at which GH secretory patterns have been assessed is 22 days, when a significant sexual dimorphism is not demonstrable (6). Nevertheless, it is conceivable that a sexual dimorphism in the episodic release of GH does exist in the neonatal period; this possibility warrants re-examination. The neuroendocrine reproductive system does become active during the neonatal period (23). In fact, similar to gonadotropin plasma levels, plasma GH levels are high during neonatal life compared with the later juvenile period. The results reported here, showing that there is a sexual dimorphism in both SS and GHRH gene expression at 10 days of age, suggest that a sexual dimorphism in GH secretory patterns could also exist at this early stage of development.

The developmental changes in GHRH mRNA we report here for both sexes are consistent with other observations about this system. We demonstrate a steady rise in GHRH mRNA levels throughout development in both male and female rats, which corresponds to previous reports of maturational increases in GHRH immunoreactivity in the median eminence (24, 25). Together, these observations indicate an increase in the activity of hypothalamic GHRH neurons that may be partially responsible for the developmental rise in mean plasma GH levels and GH pulse amplitude.

The greatest developmental change in cellular SS mRNA content occurs around the time of puberty onset, when SS message levels decline, especially in the male. The biological significance of this decline is unknown, but it is conceivable that it is involved in generating high amplitude GH pulses, characteristic of the male rat at the time of puberty onset. The mechanism responsible for the observed decrease in SS mRNA is enigmatic, especially in light of the fact that circulating sex-steroid levels and GH levels are rising, both of which have been shown to increase SS mRNA levels in neurons of the PeN (7, 9, 19–21).

In the female rat, we have observed that SS mRNA levels remain relatively constant throughout development, with the notable exception at 35 days of age, when there is a significant increase in SS message, which diminishes again by 75 days of age. We note that it is at this time, near the time of vaginal opening, that female rats exhibit a GH pulse pattern similar to that of the male animal, *i.e.* higher pulse amplitude and a decreased baseline, only to return to the characteristic female GH pattern by adulthood (6). Although the factors responsible for these observed changes are not well understood, changes in the level of SS gene expression may be involved in mediating the alterations in GH pulse patterns in the pubescent female.

In summary, we have demonstrated that in the adult rat there is a sexual dimorphism in the relative amounts of both SS mRNA and GHRH mRNA within cells of the PeN and ArcN/VMH, respectively. In addition, we have shown that the dimorphism in SS mRNA levels is evident as early as 10 days of age and persists throughout development, whereas GHRH gene expression is clearly sexually dimorphic only in the late neonate and adult. We have also demonstrated that the mRNA levels for both of these neuropeptides change significantly over the course of development in both male and female rats. We infer that the developmental and sexually dimorphic pattern of GH secretion observed in the adult rat may be partially attributable to differences between the sexes in the expression of the SS and GHRH genes.

Acknowledgments

We thank Pamella Kolb and Emilia Kabigting for their excellent technical assistance. We are grateful to Dr. Kelly Mayo and Dr. Richard Palmiter for their kind gifts of the GHRH cDNA and SS cDNA sequences, respectively.

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