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Growth Hormone-Releasing Hormone Messenger Ribonucleic Acid in the Hypothalamus of the Adult Male Rat Is Increased by Testosterone*

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ABSTRACT. Since intact adult male rats have higher GH pulse amplitude than do castrated animals and since GH-releasing hormone (GHRH) secretion is predominantly responsible for the production of these GH pulses, we hypothesized that testosterone stimulates GHRH synthesis in neurons of the hypothalamus. To test this hypothesis, we compared GHRH mRNA content in individual neurons of the arcuate (ARC) and ventromedial (VMH) nuclei among groups of intact (n = 3), castrated (n = 5), and castrated testosterone-replaced (n = 5) adult male rats. Cellular GHRH mRNA content was measured by using semiquantitative *in situ* hybridization with an ^{35}S-labeled cRNA probe complementary to the coding sequence of rat GHRH mRNA. Castration resulted in an approximately 35% decline in GHRH mRNA signal relative to that in intact animals in both the ARC ($P < 0.005$) and VMH ($P < 0.005$). Replacement with testosterone at the time of castration completely prevented the decline in both areas. Testosterone can exert effects either through activation of the androgen receptor directly or through aromatization to estradiol; therefore, we also examined the effects on GHRH mRNA of replacement with 17β-estradiol (n = 5) or dihydrotestosterone (DHT), a nonaromatizable androgen (n = 4). Estradiol had no effect on the castration-induced decline in GHRH mRNA in either the ARC or VMH. In contrast, DHT partially prevented the postcastration decline in GHRH in the ARC ($P < 0.005$), while having no statistically significant effect on GHRH mRNA in the VMH.

These results clearly indicate that testosterone stimulates expression of GHRH mRNA in neurons of the hypothalamus. Furthermore, the failure of estradiol to substitute for testosterone and the ability of DHT to substantially support GHRH mRNA suggest that testosterone exerts its effects on GHRH gene expression predominantly through direct activation of the androgen receptor. (*Endocrinology* **127:** 1362–1368, 1990)

THE ULTRADIAN patterning of GH secretion in the rat is influenced by sex and developmental stage (1). While the adult male rat displays a secretory pattern characterized by high amplitude GH peaks and profound troughs, the female exhibits low amplitude GH pulses superimposed on a baseline which is elevated relative to that in the male. The GH secretory pattern of the prepubertal male rat is devoid of regular pulsatile activity and is characterized by a low but measurable baseline. The characteristic adult male secretory pattern becomes manifest with the onset of puberty. In addition, GH secretory patterns are markedly influenced by androgens during both the neonatal and pubertal periods (2–5). However, the mechanism of androgen action on the GH axis has not been clearly elucidated.

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The secretion of GH from the pituitary is predominantly controlled by two reciprocally acting neuropeptides, somatostatin (SS) and GH-releasing hormone (GHRH), the alternating release of which models GH troughs and peaks (for review, see Ref. 6). We hypothesized that the effect of androgens on GH secretory patterns is mediated at least in part by effects on one or both of these neuropeptides and that development of the profound secretory troughs and high amplitude GH peaks characteristic of the adult male reflects testosterone (T) stimulation of SS and GHRH gene expression, respectively. We (7) and others (8) have previously reported that SS gene expression is androgen sensitive. We now extend these studies and report evidence from *in situ* hybridization histochemistry that GHRH gene expression in the adult male rat is also androgen sensitive.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Tyler Laboratories, Bellevue, WA) were housed in group cages under constant light

conditions (12 h of light, 12 h of darkness; lights on at 0600 h) in the facilities of the Department of Animal Medicine of the University of Washington. The animals were given free access to rat chow and tap water.

Experimental design

The cellular content of GHRH in the arcuate (ARC) and ventromedial (VMH) nuclei was assayed by *in situ* hybridization and compared among five experimental groups of adult male rats. The groups compared were I, intact sham-castrated animals (n = 3); C, castrated animals receiving an empty implant (n = 5); T, castrated animals receiving a T-releasing implant (n = 5); DHT (dihydrotestosterone), castrated animals receiving a dihydrotestosterone-releasing implant (n = 4); and E, castrated animals receiving a 17β-estradiol-releasing implant (n = 5). On day 1, animals were operated upon and immediately received the appropriate implant. On day 5, all animals were killed between 0900–1000 h, and the brains were prepared for *in situ* hybridization as described below.

Surgery and steroid treatment

All manipulations were performed under ether anesthesia. Immediately after castration or sham castration, a Silastic (id, 1.57 mm; od, 3.18 mm; Dow-Corning, Midland, MI) capsule containing the appropriate steroid was inserted sc in the mid-capsular region. Preparation of the steroid capsules has been described previously (9). T capsules (30 mm; Sigma, St. Louis, MO) and DHT capsules (40 mm; Sigma) contained crystalline T and crystalline DHT respectively. Estradiol capsules (5 mm; Sigma) contained a 1:1 (wt/wt) mixture of cholesterol and 17β-estradiol.

Tissue preparation

On the day of death, animals were rapidly anesthetized with carbon dioxide vapor and immediately decapitated. The brains were removed directly onto dry ice, rapidly frozen, and stored intact at −80 C until processed for *in situ* hybridization histochemistry. Trunk blood was collected at the time of decapitation and separated by centrifugation, and the serum was stored at −20 C before RIA for steroids.

Before cutting, brains were allowed to equilibrate in the cryostat (−15 C), blocked, and embedded in OCT (Tissue-Tek, Elkhart, IN). Coronal sections (20 μm) were thaw-mounted onto poly-L-lysine-coated slides. Brain sections were collected through the hypothalamus caudally to the recession of the optic chiasm. Tissue slices were stored at 80 C in airtight boxes until hybridization.

Probe preparation

The plasmid prGHRF-2 was generously provided by Dr. Kelly Mayo (10). This plasmid consists of a 360-basepair *Eco*RI-*Sal*I fragment of a rat GHRH cDNA clone inserted into the vector pUC8. The *Eco*RI-*Sal*I fragment contains the coding region for GHRH-43 as well as a region coding for a 30-amino acid peptide of unknown function, a 105-nucleotide 3′-untranslated region, and the poly(A) tract. We further subcloned a 198-basepair *Eco*RI-*Hin*dIII fragment, which includes the entire GHRH-43-coding sequence and a portion of the coding sequence for the 30-amino acid peptide, into the transcription vector pGEM4 (Promega Biotec, Madison, WI). The orientation of the fragment was confirmed by restriction mapping. Antisense cRNA probes were made *in vitro* by linearizing this plasmid with *Eco*RI and transcribing the insert sequence using the T7 promoter present on the plasmid vector. Sense cRNA probes were made by linearizing the plasmid with *Sal*I and transcribing the sequence with SP6 polymerase.

The transcription reaction was performed at a UTP concentration of 50 μM, consisting 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-I (Promega Biotec), and the cRNA probe was separated from unreacted components on Nensorb 20 columns (New England Nuclear). The identity and integrity of the transcript were verified by polyacrylamide gel electrophoresis against known standards. The reaction gave a final probe specific activity of approximately 5.0–5.6 × 10^8 dpm/μg.

In situ hybridization

In situ hybridization was performed as previously described (11). Briefly, sections were fixed in 4% paraformaldehyde and pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. After slides were rinsed in 2 × SSC, probe was applied in 60 μl hybridization buffer. The slides were covered with parafilm, sealed with rubber cement, and incubated overnight in moist chambers at 56 C. On the following day, slides were treated with RNAse-A and rinsed in a series of washes of increasing stringency. The final wash was in 0.1 × SSC at 66 C. Slides were dehydrated in alcohols and air dried.

After hybridization, slides were dipped in Kodak NTB-2 emulsion (45 C; Eastman Kodak, Rochester, NY) diluted 1:1 with 600 mM ammonium acetate. Slides were allowed to air dry for 30 min, followed by further drying in moist chambers at room temperature for 45 min. Slides were then stored with dessicant in light-tight boxes at 4 C for 6 days. Slides were developed in Kodak D-19 developer and counterstained with cresyl violet before application of coverslips.

Control experiments

In situ hybridization histochemistry with the cRNA probe for GHRH mRNA provided labeling of numerous cells and clusters of cells restricted to the ARC, VMH, ventromedial, and dorsomedial nuclei and a few cells arranged along the base of the hypothalamus lateral to the arcuate nucleus. No cells were seen in the cortex, basal ganglia, brain stem, or other areas of the hypothalamus. This distribution corresponds closely to previous studies of GHRH neuron distribution by immunocytochemistry (12). To further examine the specificity of the assay, sections were hybridized to a sense cRNA probe of identical specific activity under identical conditions. In addition, the effect of preincubation of the sections with RNase-A on binding with the antisense probe was examined to ensure that the probe was binding to RNA species. No binding was seen in either instance.

To ensure saturation of GHRH mRNA with radioactive

probe, hybridization was performed using probe concentrations from 0.1–2.5 μg/ml·kilobase (kb). As shown in Fig. 1, the probe reaches a saturating concentration at approximately 0.3 μg/ml·kb. As expected, background counts increase linearly, without evidence of saturation. To avoid probe-limiting conditions, all subsequent experiments were performed with a probe concentration of 0.6 μg/ml·kb.

Anatomical matching of sections

For purposes of ensuring matched anatomical representation for all animals, the arcuate nucleus was divided into 3 areas of approximately equal length by reference to the rat brain atlas of Paxinos and Watson (13). Four random tissue sections from each area from each animal were included in the analysis (12 sections/animal). These areas are as follows. Area 1 begins rostrally with the appearance of the arcuate nucleus and GHRH-positive cells (plate 26) and continues caudally 0.6 mm (plate 29). Area 2 is continuous with area 1 (plate 29) and continues caudally 0.5 mm to the appearance of the dorsomedial nucleus (plate 31). Area 3 is continuous with area 2 (plate 31) and continues caudally 0.5 mm to the splitting of the hypothalamic third ventricle (plate 33), which corresponded to the disappearance of GHRH-positive cells.

Image analysis

Slides selected for analysis as described above were assigned a random three-letter code and analyzed in alphabetical order by an operator unaware of the experimental group to which the animal was assigned. The automated image-processing system consists 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 ×40 epiillumination darkfield objective.

Grain clusters were identified by the operator, using darkfield illumination. If the cluster was associated with a single cresyl violet-stained nucleus under lightfield illumination and free from artifact, then it was considered to be a GHRH-positive cell and was analyzed by automated grain analysis, as previously described (11). All grain clusters in the hypothalamus that could be resolved as single cells were analyzed and assigned to an anatomical area.

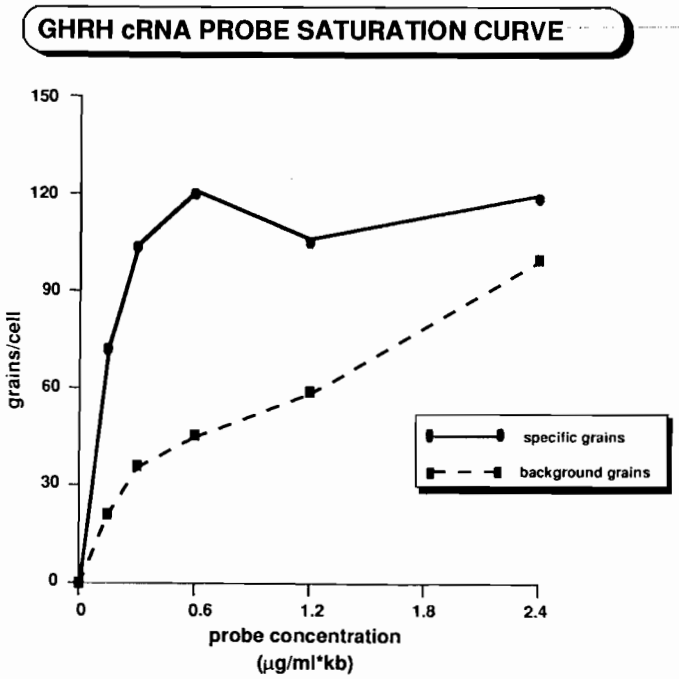

FIG. 1. GHRH *in situ* hybridization saturation curve. Effect of increasing GHRH cRNA probe concentration on specific binding (grain density over cells in the ARC) and nonspecific (background) binding.

Serum T assay

Serum T levels were determined with a commercially available RIA kit (Leeco Diagnostics, Southfield, MI), as previously described (14). The antibody used in this assay exhibits a cross-reactivity of 14% with 5α-dihydrotestosterone, 6% with 5α-androstanediol, and less than 2% with other steroids. The sensitivity of this assay was 5 pg/100 μl, and the intra- and interassay coefficients of variation were 3.0% and 8.5%, respectively.

Serum 5α-androstane-3α,17β-diol (3α-diol) assay

3α-Diol is the major circulating metabolite of DHT, and plasma levels of this metabolite provide a specific and sensitive index of circulating DHT levels (15, 16). Serum levels of 3α-diol were measured by RIA by Dr. David Damassa at Tufts University Medical Center, as previously reported (17). The antibody used in this assay has a cross-reactivity of less than 0.01% with 5αDHT, 5βDHT, T, and 17β-estradiol (18). All samples were assayed together, with an intraassay coefficient of variation of 9.6%.

Serum estrogen assay

Serum levels of 17β-estradiol were measured with a commercially available RIA kit (ICN Biomedicals, Carson, CA). The antibody used in this assay has a cross-reactivity of 20% with estrone, 1.5% with estriol, and less than 0.01% with other steroids. All samples were assayed together, with a sensitivity of 7.5 pg/ml and an intraassay coefficient of variation of 9.4%.

Statistical analysis

The mean number of grains per cell for each animal was determined from the analysis of 150–200 cells/animal. The mean number of grains per cell for each animal was then used to determine the mean ± SEM for each experimental group, and the n in all statistical analyses refers to the number of animals in the group. Comparison of mean grains per cell among groups was performed by one-way analysis of variance. If significant differences were found among the groups, a Scheffé F test (19) was performed to determine individual differences between pairs.

Results

Steroid levels

The results of steroid determinations are shown in Table 1. Serum concentrations of T in intact rats were confirmed to be within the normal physiological range, whereas castrated animals had a mean serum level of 0.4 ng/ml. Castrated animals that received T implants had circulating T levels that were not significantly different from those in the control animals.

Castration resulted in a 50% decline in the circulating concentration of estradiol. Estrogen treatment of castrated animals produced circulating estradiol levels approximately 3-fold greater than those in intact animals, while treatment with neither T nor DHT had any effect on estradiol levels in castrates.

Serum 3α-diol concentrations in castrated animals decreased 45% compared to those in intact controls. Replacement with DHT resulted in serum levels approximately 6-fold higher than those in intact animals, while replacement with either T or estradiol had no effect on 3α-diol levels.

Effects on GHRH mRNA in the ARC

As shown in Fig. 2, castration of adult male rats for 4 days resulted in a marked reduction in GHRH mRNA signal in neurons of the ARC compared to that in intact controls. This reduction was prevented by replacement with T at the time of castration. Figure 3 presents the results of semiquantitative analysis by automated image analysis, as described above. Castration resulted in a 34% reduction in GHRH mRNA signal in neurons of the ARC compared to that in sham-operated controls (118 ± 4 *vs.* 78 ± 2 grains/cell; $P < 0.005$). Replacement with physiological levels of T at the time of castration prevented this decline, and the GHRH mRNA signal in T-treated castrates (110 ± 4 grains/cell) was not significantly different from that in intact controls. In contrast to T, replacement with estradiol, even at a relatively high concentration, had no effect on GHRH mRNA in the ARC (74 ± 3 grains/cell; $P < 0.005$). Animals replaced with DHT had ARC GHRH mRNA signal levels (96 ± 5 grains/cell; $P < 0.01$) intermediate between those of the intact (or T-treated) animals and the castrated animals.

TABLE 1. Serum steroid levels (mean ± SEM) in intact sham-operated (n = 3), castrated sham-replaced (n = 5), castrated T-replaced (n = 5), castrated DHT-replaced (n = 4), and castrated estradiol-replaced (n = 5) adult male rats

Group	T (ng/ml)	Estradiol (pg/ml)	3α-Diol (ng/ml)
Intact	2.6 ± 0.7	66.3 ± 2.4	1.08 ± 0.24
Castrated	0.4 ± 0.1	28.5 ± 6.0	0.60 ± 0.17
Castrated + T	2.6 ± 0.4	37.5 ± 7.7	0.61 ± 0.07
Castrated + DHT	0.9 ± 0.3	40.3 ± 12.3	6.68 ± 1.43
Castrated + estradiol	0.8 ± 0.2	142.9 ± 50.3	0.73 ± 0.14

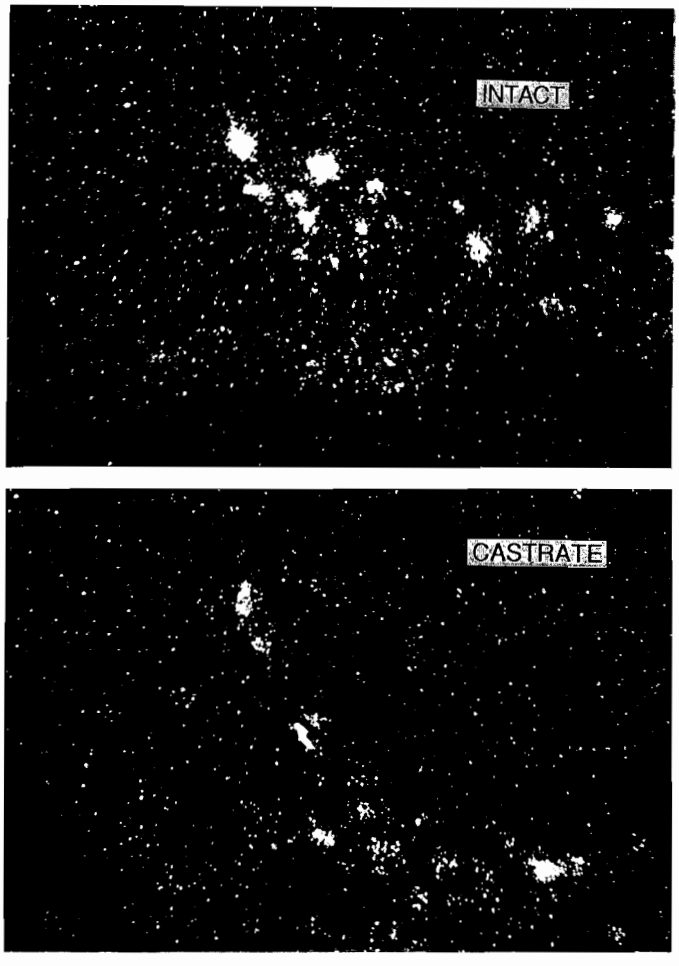

FIG. 2. Darkfield photomicrographs of half of the ARC showing cells labeled with a cRNA probe for GHRH mRNA in anatomically matched sections obtained from intact (A) and castrate (B) adult male rats.

Effects on GHRH mRNA in the VMH

As shown in Fig. 4, the effects of castration and steroid replacement on GHRH mRNA signal in the VMH were similar but not identical to the effects in the ARC. As in the ARC, castration resulted in a significant decline in GHRH mRNA signal compared to that in the intact controls (85 ± 5 *vs.* 127 ± 5 grains/cell; $P < 0.005$) and replacement with T at the time of castration prevented this decline (117 ± 6 grains/cell). Replacement with 17β-estradiol had no effect on the castration-induced decline in GHRH signal (90 ± 4 grains/cell). DHT had no significant effect on the castration-induced decline in GHRH signal (96 ± 6).

Discussion

In this study we have demonstrated that the GHRH mRNA content in both the ARC and VMH is signifi-

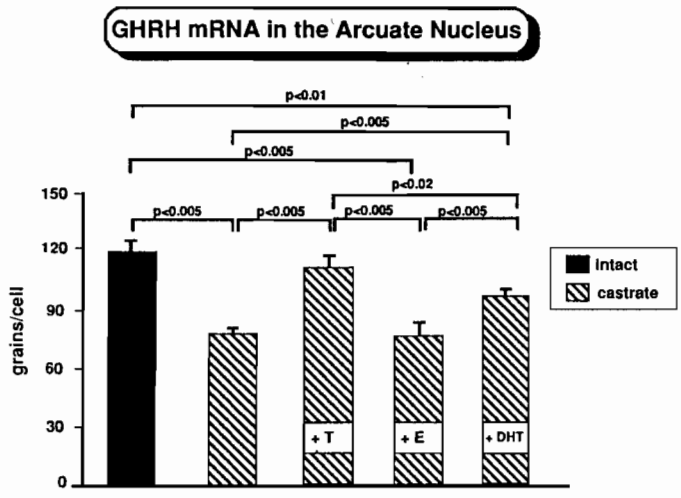

FIG. 3. Relative amounts of GHRH mRNA, as reflected by grains per cell, in the ARC of intact, castrated, castrated T-replaced (T), castrated estradiol-replaced (E), and castrated DHT-replaced (DHT) male rats. Values are given as the mean ± SEM. The level of GHRH mRNA signal is significantly reduced in castrated compared to intact animals. This reduction is completely prevented by replacement with T and partially prevented by replacement with DHT. Estradiol replacement did not prevent the castration-induced decrease in GHRH mRNA signal.

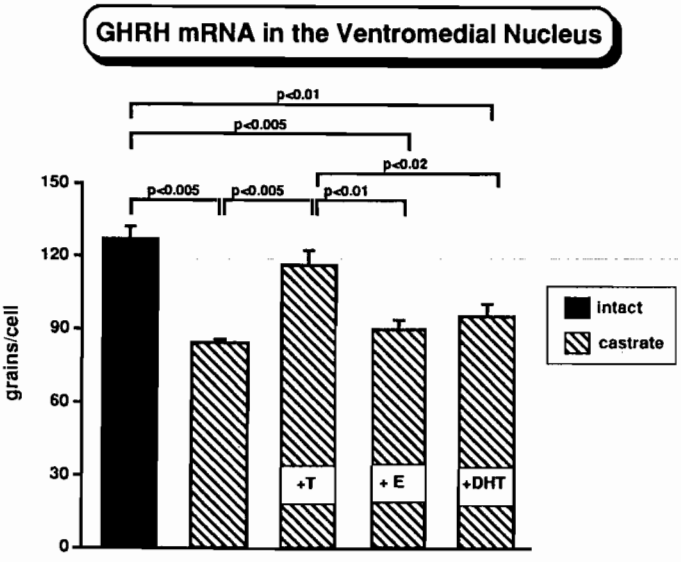

FIG. 4. Relative amounts of GHRH mRNA, as reflected by grains per cell, in the VMH of intact, castrated, castrated T-replaced (T), castrated estradiol-replaced (E), and castrated DHT-replaced (DHT) male rats. Values are given as the mean ± SEM. The level of GHRH mRNA signal is significantly reduced in castrated compared to intact animals. This reduction is completely prevented by replacement with T. Neither 17β-estradiol nor DHT had a statistically significant effect on VMH GHRH mRNA signal.

cantly reduced after orchidectomy in the adult male rat. Furthermore, this reduction can be prevented by administration of physiological doses of T at the time of castration. Although it cannot be assumed that all GHRH 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. Therefore, these results highlight the effect of androgens on the capacity of hypothalamic neurons to synthesize GHRH and, in conjunction with previous demonstrations of T stimulation of SS mRNA expression in the periventricular nucleus (7, 8), demonstrate that the hypothalamus may be a major site of androgen action on the GH axis. The stimulation by T of GHRH and SS mRNA expression may explain, at least in part, the observed effects of androgens *in vivo* as well as the sexual dimorphism and developmental changes observed in the pattern of GH secretion in rats. Thus, the stimulation by T of increased transcription and/or stability of mRNAs for these two neuropeptides may contribute to the well described effects of androgens to increase GH pulse amplitude (*i.e.* presumably reflecting, at least in part, increased GHRH activity) and to decrease the interpulse baseline (*i.e.* reflecting a presumed increase in SS secretory activity) (3–5, 20–22).

The results presented here are consistent with previous studies of sexual dimorphism and developmental changes in rat GHRH peptide concentrations. GHRH immunoreactivity in the median eminence increases steadily with postnatal age, experiencing the most rapid increase during the pubertal period (22, 23), coincident with the rise in circulating androgen concentrations. Similarly, the hypothalami of adult male rats have higher GHRH contents than those of females (22–24). In contrast, castration had no effect on whole hypothalamic GHRH peptide content in adult males (23). However, if decreased GHRH synthesis in castrate males is accompanied by a decrease in GHRH peptide release from the median eminence, peptide content may not change, despite a decrease in the GHRH synthesis rate.

The question of whether the primary masculinizing effect of androgens resides at the level of the pituitary or hypothalamus has been controversial. While pituitaries from male rats have higher GH peptide content and greater response to GHRH than pituitaries from female animals (25–32), differences which are removed by gonadectomy and returned with T replacement (27, 30, 32), the evidence for a direct effect of androgens on the pituitary is limited and conflicting. T has been demonstrated to decrease (33), increase (34), or have no effect (35) on basal and/or GHRH-stimulated GH release from pituitaries *in vitro*. It has been suggested that the apparent effects of androgens on parameters of pituitary GH synthesis and release may be mediated indirectly through alterations in the pattern of hypothalamic GHRH and SS release to which the pituitaries have been exposed (36–39). The results presented here along with the earlier demonstration of androgen effects on hypothalamic SS mRNA lend further support to the notion of a major effect of androgens on hypothalamic components of the

GH axis. Furthermore, analysis by both autoradiography and quantitative binding assay reveals that the ARC is well supplied with androgen receptor peptide (40, 41).

Since T can exert its effects through either activation of the androgen receptor directly or aromatization to estradiol and activation of the estrogen receptor, we also examined the effects on GHRH mRNA of replacement with estradiol and the nonaromatizable androgen DHT. Estradiol, even at relatively high concentrations equivalent to circulating levels found in females at proestrus or to local concentrations that might be expected from the aromatization of T circulating in the nanogram range, did not prevent the castration-induced decline in GHRH mRNA in either the ARC or VMH. This concentration of estradiol, however, was sufficient to completely prevent the postcastration decline in POMC mRNA in the ARC of these animals (42).

Unlike estradiol, replacement with DHT at the time of castration partially, but not completely, prevented the castration-induced decline in GHRH mRNA in the ARC, yet DHT had little effect on GHRH mRNA-containing cells in the VMH. The reason for the partial effect of DHT on GHRH mRNA is unclear. This may represent an inadequate replacement concentration of DHT, a possibility that is hard to assess directly, since only the concentrations of DHT metabolites are readily assayed. However, previous analysis of SS mRNA in an identical paradigm demonstrated complete prevention of postcastration decline by DHT replacement at this level (43). Furthermore, this treatment is adequate to stimulate prostate growth in adult male rats (15). Therefore, the failure of DHT to fully support GHRH message in these animals suggests that the sensitivity of the response of the GHRH message to DHT is lower than that of SS mRNA. Alternatively, the partial effect of DHT replacement may reflect the need for estradiol as a permissive factor for androgen receptor-dependent action. Such an association between the actions of androgens and estrogens has been demonstrated in vasopressin peptide synthesis (44). Indeed, recent evidence suggests that estradiol may be required to support androgen receptor synthesis in specific brain regions (45). The absence of a DHT effect in the VMH suggests that the GHRH-containing cells in this area are either less sensitive to this dose of DHT replacement than corresponding cells in the ARC or are more dependent on estradiol synergy for DHT effects. Further clarification of these possibilities will require assessment of GHRH mRNA levels in animals replaced with both estradiol and DHT.

In conclusion, we have demonstrated that castration of adult male rats decreases GHRH mRNA levels in neurons of the hypothalamus, and replacement with physiological levels of T prevents this reduction. Furthermore, the failure of estradiol to substitute for T, even at supraphysiological doses, suggests that the ability of T to modulate the expression of GHRH mRNA is mediated through direct activation of androgen receptors. These results complement our previous studies of the effects of androgens on SS mRNA expression and may help to explain, at least in part, the mechanism by which sex and androgens influence the GH axis.

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