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Regulation of Luteinizing Hormone Pulse Frequency and Amplitude by Testosterone in the Adult Male Rat*

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ABSTRACT. Our objective was to gain a better understanding of the role of testosterone (T) in regulating the minute to minute dynamics of LH secretion in the adult male rat. To this end, we examined the patterns of blood LH levels in intact animals and evaluated the effect of small physiological doses of T on mean blood LH and FSH levels and on LH pulse frequency and amplitude in the castrate animal. The intact rat exhibited low frequency (period, ~145 min) and low amplitude (~16 ng/ml) LH pulses. After castration, LH pulse frequency (period, ~20 min) and amplitude (~118 ng/ml) increased dramatically over that of intact animals. T, administered to castrate rats through Silastic implants, caused a dose-dependent and parallel reduction in mean blood LH and FSH levels. The lowest T dose, which increased mean plasma T levels to 0.5 ng/ml above those of the sham-treated castrates, produced a significant reduction in LH pulse frequency, with a significant increase in pulse amplitude. The next highest T dose caused a reduction in pulse amplitude to a value significantly lower than that in the sham-treated castrates. The highest T dose, which produced steady state mean plasma T levels (~1.6 ng/ml) less than the mean level of the intact group (~2.2 ng/ml), caused a profound reduction in pulse frequency to lower than that of the intact group. These observations demonstrate that T can exert a complex, dose-dependent effect on LH secretory dynamics and imply that one important site of T-mediated negative feedback is the brain's LHRH pulse generator. (*Endocrinology* 111: 2055, 1982)

REGULATION of testicular function by the gonadotropins depends upon the operation of a hormonally mediated negative feedback control loop between the testis and the brain-pituitary axis. An important component of this control system is the feedback relationship between testosterone (T) and LH secretion. A careful study of this relationship in the male rat by Damassa *et al.* (1) has established that physiological doses of T suppress the postcastration rise of LH in a dose-dependent fashion. Whereas the foregoing study has provided important insights into the general quantitative relationships that characterize the LH-T axis in the rat, we understand relatively less about the role of T in regulating the minute to minute secretory patterns of LH.

In 1972, Gay and Sheth (2) demonstrated that LH is released from the pituitary of the castrate rat in episodic bursts. They described a sawtooth pattern of plasma LH levels over time, with peaks occurring at 20- to 60-min intervals. This pulsatile LH release pattern appears to be driven by a LHRH pulse generator located in the medial basal hypothalamus (3–5). The relationship between plasma T levels and the operation of the LHRH pulse generator is poorly understood. Our inability to accurately and quantitatively describe the signal characteristics, *i.e.* amplitude and frequency, of pulsatile LH secretion has thwarted progress in elucidating the dynamics of this hormonal control system. Using a new mathematical technique for analyzing the pattern characteristics of pulsatile LH secretion (6), we have studied the minute to minute secretory dynamics of LH in the intact male rat and evaluated the effects of small doses of T on the frequency and amplitude of pulsatile LH secretion in the castrate male rat. We report finding evidence for 1) low frequency-low amplitude LH pulses in intact animals and 2) a dose-dependent modulatory effect of T on the frequency and amplitude of pulsatile LH secretion in the castrate rat.

Materials and Methods

Animals and accommodations

Adult male Sprague-Dawley rats (Tyler Laboratories, Bellevue, WA), weighing 200–250 g, were housed in the Department of Animal Medicine animal care facilities at the University of Washington Health Sciences Center. Animals were maintained in air-conditioned, constant temperature rooms (22 C) with

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artificial light, alternating on a 12-h light, 12-h dark schedule (lights on at 0600 h). Food and water were supplied *ad libitum*.

Surgical procedures

Using small scrotal incisions, we performed bilateral orchidectomies while maintaining the animals under ether anesthesia. Animals were treated postoperatively with 30,000 U (im) procaine penicillin (Eli Lilly Co., Indianapolis, IN).

To permit the collection of rapid sequence blood samples, animals were implanted with indwelling atrial catheters on the day before blood sampling. We used a modification of the cannulation technique described by Terkel and Urbach (7). Dow-Corning Silastic tubing (id, 0.020 in.; od, × 0.037 in.; Dow-Corning Corp., Midland, MI), precut to terminate in the right atrium, was cemented to small rectangle (3 mm^2) of reinforced Silastic sheeting (0.020 in. thick). While maintaining the animals under ether anesthesia, we inserted the tubing into the jugular vein just as it enters the pectoralis muscle. With a single suture, the Silastic sheeting was fastened to the pectoralis muscle, and the distal end of the tubing was led sc up and out through a small incision in the midscapular region. We filled the tubing with heparinized saline and plugged the end with a short piece of stainless steel.

T administration

After castration, each animal received either a sham (empty) Silastic capsule or one containing T implanted sc along its dorsal hindquarter. We made the capsules by packing 10-, 20-, and 30-mm lengths of Silastic tubing (id, 0.062 in.; od, 0.125 in.) with crystalline T and sealing both ends with Silastic cement.

Blood sampling, processing, and replacement

On the day of blood sampling, animals were fitted with extension tubing attached to the distal end of the indwelling catheter. We began sampling in all groups between 0800 and 0900 h. From the castrate, sham-treated, and 10-, and 20-mm T-implanted groups, blood samples (15–30 μl) were obtained at 3- to 4-min intervals over a period of 4–5 h. Catheter lines were flushed between samples with a volume of saline equal to that of the sample taken. Hematocrit did not change significantly during the sampling period. All blood samples were split into two equal aliquots and placed into assay buffer. The diluted samples were then frozen and stored at −20 C until assayed. From the intact and 30-mm T-implanted groups, individual animals were bled at 10-min intervals for 8 h. Each blood sample was obtained by withdrawing a 400-μl volume, after which a blood replacement mixture (see below) was infused at equal volume. Each 400-μl blood sample was placed in a tube and centrifuged at 1500 × *g* at 4 C for 15 min. The plasma was collected, and 50-μl duplicates were dispensed into assay tubes, frozen, and stored at −20 C until assayed.

Clinical laboratory evaluation of electrolytes, blood urea nitrogen, creatinine, CO_2, and glucose levels measured in the first and last samples of the experiment indicated that the blood replacement procedure caused no significant alterations in these parameters; moreover, the animals showed no apparent disturbances in behavior, grooming, sleeping, drinking, *etc.*, during the experiment. At the end of each experiment, an additional 1-ml sample was taken for the measurement of plasma T concentration. Plasma aliquots drawn from these samples were also stored at −20 C until assayed.

Blood replacement mixture

Adult male rats were killed by guillotine for collection of red blood cells for the blood replacement mixture. The donor blood was funneled into 50-ml glass centrifuge tubes containing ice-cold Krebs-Ringer (K-R) solution, such that the final volumes in the tubes were 5 parts blood to 1 part K-R. To minimize clotting during the collection process, we added heparin to achieve a final concentration of 100 IU/ml. Blood was centrifuged (4 C; 750 × *g*; 15 min), and the supernate was removed. Cells were resuspended in heparinized K-R and centrifuged a second time. The supernatant was removed, and the cells were swirled in the tube just before pouring them over a nylon sheet (Nitex no. 243) cut to fit a Buchner funnel. A final rinse with K-R was used on the net filter, followed by a last centrifugation. The final supernatant was discarded, and the cells were resuspended in a mixture composed of human plasma protein fraction (Protenate, Travenol Laboratories, Deerfield, IL), glucose, sodium heparin, and penicillin. To remove trace steroid molecules, the commercial Protenate was extracted with Norit charcoal (MCB Labs, Cincinnati, OH) by incubation at 55 C for 10 min in a Dubnoff shaker bath, followed by an initial filtration through Whatman filters (Whatman, Inc., Clifton, NJ), and finally through a 0.22-μm Swinnex (Millipore Corp., Bedford, MA). Proportions of the final blood replacement mixture were 50 ml plasma protein fraction for each 45 ml cells, 0.5 mg glucose/ml, 2 IU sodium heparin/ml, and 100 U penicillin/ml. The mixture was prepared the day before usage and stored at 4 C. Immediately before use, an additional amount of sodium heparin was added (40 parts blood replacement mix to 1 part 1000 IU/ml sodium heparin), and the mixture was maintained in a water bath at 37 C for the duration of the replacement procedure.

LH RIA

We used the NIAMDD RIA kit for measuring LH in both whole blood (sham, 10-mm T, and 20-mm T groups) and serum samples (intact and 30-mm T groups). In the whole blood assays, we corrected for the presence of red cells in the unknowns by adding an equivalent volume of washed cells to all standard tubes. The minimum sensitivities of the whole blood and serum assays were 0.5 ng/tube (33 ng/ml; at 15 μl) and 0.25 ng/tube (5 ng/ml; at 50 μl), respectively. The interassay coefficients of variation were 7% for the whole blood assay and 15% for the serum assay. In the results reported here, the serum values have been converted to equivalent whole blood concentrations by multiplying by 0.654, a value determined empirically by comparing identical samples in both the whole blood and serum assays (n = 15).

FSH RIA

Reagents supplied by the NIAMDD were used for the double antibody RIA of serum FSH levels. A partially purified preparation of rat pituitary gonadotropins (RP-1) was used as the

reference standard. Preparation I-5 was used for iodination. The first antibody was S-10. The sensitivity of the assay was 10 ng/tube (100 ng/ml using 100 μl serum). Intra- and interassay coefficients of variation were 4% and 11%, respectively.

T RIA

Using reagents supplied by the WHO Matched Reagent Programme, we measured basal plasma T levels in each animal. The antiserum was raised against T linked at the 3-position by carboxymethyl-oxime to BSA. This antiserum exhibits a cross-reactivity of 14% with dihydrotestosterone, 6% with 5α-androstanediol, and 2% or less with all other steroids tested. We extracted all serum samples with ether and used dextran-coated charcoal to separate free from bound hormone. The minimum sensitivity of the assay was less than 10 pg/tube (0.1 ng/ml). The intra- and interassay coefficients of variation were 5% and 10%, respectively.

Experimental design

Intact male rats (n = 11) were surgically implanted with jugular catheters and bled 24 h later, as described above. Paired sham and T-treated animals were castrated on day 0 and simultaneously received either empty 10-mm Silastic implants (n = 25) or T-filled implants in 10-mm (n = 12), 20-mm (n = 15), or 30-mm lengths (n = 12). Three weeks after castration, animals were surgically implanted with jugular catheters, and on the following day, they were bled as described above.

Data analysis

Sequential samples, collected from individual animals, were analyzed by a two-part procedure. The first part consisted of a one-way analysis of variance to determine whether the data contained any significant hormone fluctuations over time. The analysis of variance was also used to estimate the signal to noise ratio (SNR). The SNR is an indicator of the relative contribution of real variations in hormone concentration (signal) and measurement variability (noise) to the overall pattern observed in the experimental data.

The second part of the analysis involved the use of cycle detection to assess pulse frequency (or interpulse interval) and amplitude (6). Briefly, one complete cycle is defined as two increases greater than a threshold value, separated by a decrease which is also greater than the threshold. The difference in hormone concentration between the peak and nadir of a cycle is the cycle amplitude, and the time between peaks of successive cycles is the interpulse interval. An initial estimate of the frequency and amplitude is obtained with the threshold set at 2.7 times the SD of the assay error. The threshold is then readjusted as a function of both frequency and amplitude to equalize the probability of obtaining a false cycle with the probability of missing a true cycle. The empirically derived formula used for the threshold adjustment is: $T = A\ (0.7 - 1.75\ Nc/Ns)$, where T is the new threshold, A is the average cycle amplitude, Nc is the number of cycles detected, and Ns is the number of samples. This process is repeated 20 times or until the estimated parameters A and Nc stabilize. The cycle detection procedure has been validated by computer stimulation of both sawtooth and sinusoidal wave forms; it works well for SNRs as low as 1.5.

The Mann-Whitney U test was used to detect significant differences in amplitude, frequency, and average levels between the treatment groups. All variance measures reported here represent the SEM.

Results

Plasma T levels

Results of T measurements for each study group are presented in Fig. 1A. In the intact male group, plasma T levels averaged 2.19 ± 0.02 ng/ml. In the sham implant and 10-, 20-, and 30-mm T implant groups, plasma T levels averaged 0.11 ± 0.01, 0.63 ± 0.04, 1.10 ± 0.05, and 1.61 ± 0.17 ng/ml, respectively. All groups differed significantly from one another ($P < 0.01$).

LH patterns in intact males

Blood LH levels in the intact male group averaged 8 ± 1 ng/ml, calculated by averaging all 10-min interval blood samples from each animal. Eight of the 11 animals sampled exhibited 1 or more LH pulses over the 8-h bleeding period. For these 8 animals, the mean interpulse interval was 145 ± 27 min, and the mean pulse amplitude was 16 ± 3 ng/ml. One example of an intact animal exhibiting LH pulses is illustrated in Fig. 2A. Note the scale differences among the panels of Fig. 2. See Fig. 1 for data summary.

LH patterns in castrate males

Castrate males had mean blood LH levels of 350 ± 13 ng/ml, representing an average blood level more than 40 times greater than that of the intact group (Fig. 1B). All animals exhibited a distinct pulsatile LH secretion pattern, with an average interpulse interval of 20.2 ± 1.1 min and an average pulse amplitude of 118 ± 7 ng/ml (see Fig. 2B). Thus, in castrate animals, LH pulse frequency and amplitude were greater by a factor of approximately 8 compared to normal intact animals. See Fig. 1 (C and D) for data summary.

Effects of T on LH patterns

T suppressed mean blood LH levels in a dose-dependent fashion (Fig. 1B). Treatment with 10-, 20-, and 30-mm T implants significantly reduced average blood LH levels to 65%, 55%, and 6% of the castrate, sham-implant group, respectively. All of these groups differed significantly from one another in mean plasma LH levels ($P < 0.01$). Although the average LH levels in the 30-mm T implant group exceeded that of the intact animals, 8 of 12 animals in the 30-mm group had mean plasma LH levels within 1 SD of the intact group mean, and the

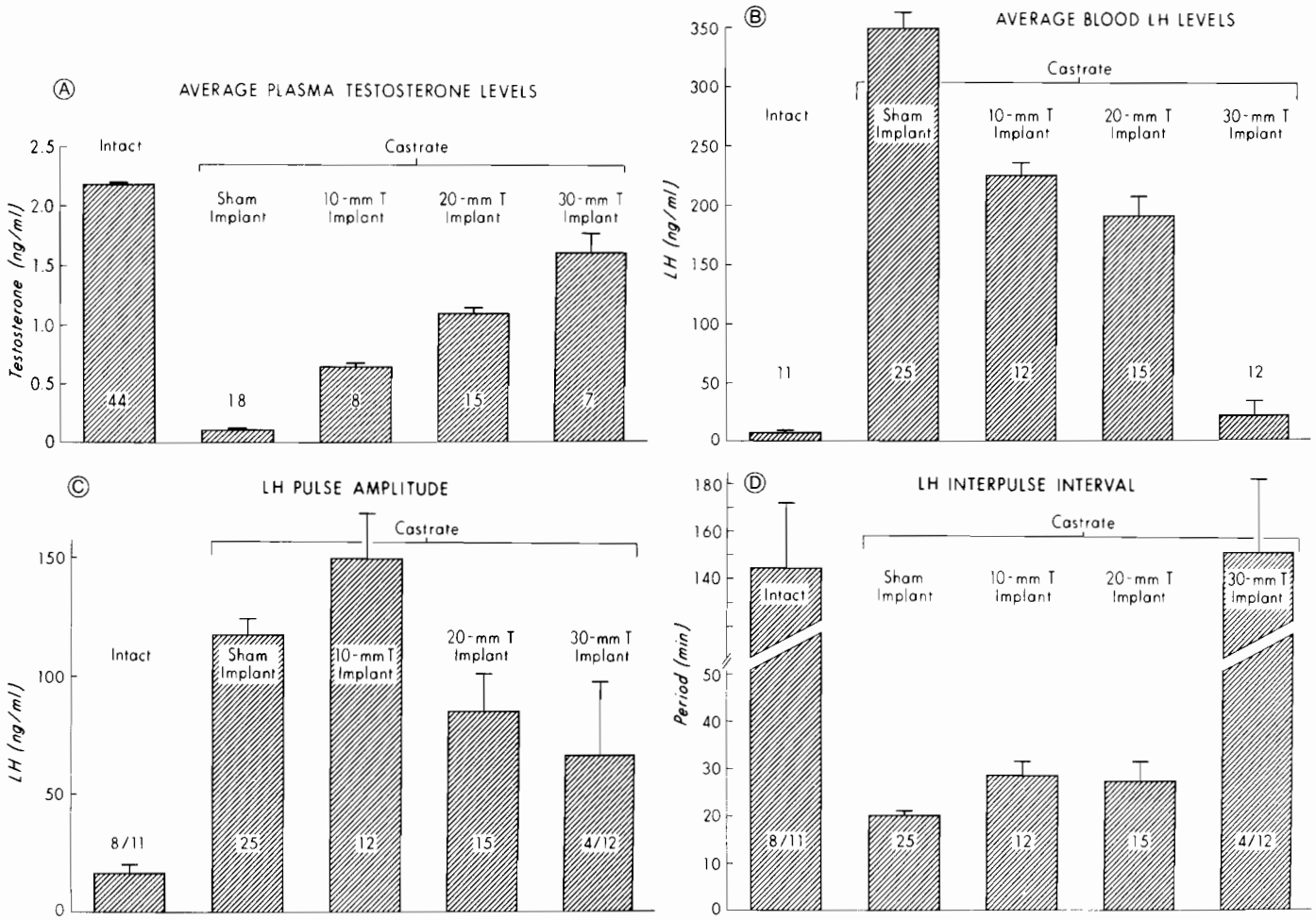

FIG. 1. A, Average plasma T levels in each treatment group. For the intact group, the mean value represents the average derived from single blood samples taken at random times during the day (n = 33) plus the 11 intact animals sampled for the serial 8-h bleeding, which were measured for T in the late afternoon blood samples. The number in each group is indicated either within or above each *bar*. The variance represents the SEM. B, Average blood LH levels in each of the treatment groups. C, The average LH pulse amplitude. D, The average interpulse interval (period) for each group. In the cases of the intact and 30-mm T groups, the fraction indicates how many animals studied exhibited detectable LH pulses. Note the break in the scale.

difference between the two was not significant ($P > 0.85$).

Treatment with the smallest T implant significantly reduced LH pulse frequency by 30% ($P < 0.01$) and significantly increased pulse amplitude by 27% ($P < 0.05$) compared to that of the sham-treated group (Fig. 1, C and D). Treatment with the 20-mm T implant had no additional affect on pulse frequency; however, in the 20-mm T implant group, LH pulse amplitude was significantly suppressed to 73% of the level observed in the sham-implant group ($P < 0.01$). Treatment with the 30-mm T implant profoundly slowed LH pulse frequency compared to that in the sham implant and 10-, and 20-mm T-implant groups ($P < 0.01$). Only 4 of the 12 animals in the 30-mm T implant group had identifiable LH pulses during the 8-h sampling period. In those 4 animals showing LH pulses, the mean interpulse interval was 152 ± 98 min, not statistically different from that of the intact animals that exhibited pulses; the mean pulse amplitude for the 30-mm implant group was 67 ± 31 ng/ml, significantly greater than that of the intact group ($P < 0.05$).

FSH levels

Castration led to a predictable 4-fold increase in mean serum FSH levels (Table 1). T administration produced a dose-dependent suppression of mean FSH levels in castrated animals (Table 1). Neither the intact *vs.* the 30-mm T groups nor the 10-mm T *vs.* 20-mm T groups differed significantly from one another ($P > 0.5$ and $P > 0.2$, respectively); all other group comparisons were significantly different ($P < 0.01$). The degree of FSH suppression was proportional to the suppression of mean LH levels at the various T dosages.

Discussion

Our findings have demonstrated that the intact male rat exhibited low frequency, low amplitude LH pulses.

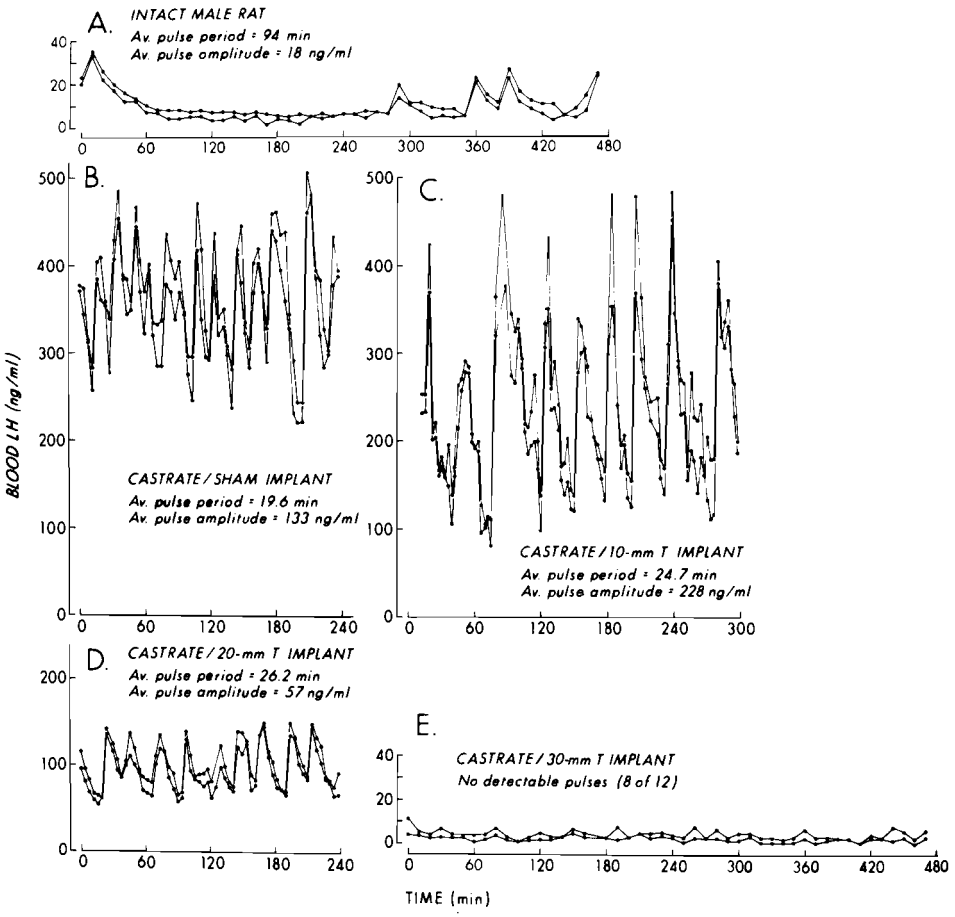

FIG. 2. Examples of pulsatile patterns of blood LH levels in intact (A); castrate, sham-implant (B); 10-mm T implant (C), 20-mm T implant (D), and 30-mm T implant (E) animals. Values at each time point represent the assay duplicates. Note the difference in scale between that for the intact and 30-mm T implant groups and that for the other groups.

TABLE 1. Serum FSH levels in intact rats and in castrated rats treated with Silastic implants containing various amounts of T

	Intact (n = 14)	Castrate			
		Empty implant (n = 13)	10-mm T implant (n = 5)	20-mm T implant (n = 11)	30-mm T implant (n = 9)
Serum FSH (ng/ml)	513 ± 30	2072 ± 77	1520 ± 126	1328 ± 109	527 ± 91

These pulses often appeared as multiple trains of discrete bursts, with three or four of them occurring over approximately 3-h intervals. These findings are consistent with the earlier report of Desjardins (8) illustrating a similar pattern in a single animal. Thus, like the male of a number of other species, including the mouse (8), ram (9), bull (10), monkey (11, 12), and man (13, 14), the intact male rat exhibits plasma LH patterns that reflect a predominantly pulsatile secretory mode.

We have shown here in the male rat that with castration, LH pulse frequency and amplitude as well as mean plasma levels increased dramatically over those of intact animals. T, when administered to castrate rats, produced a dose-dependent, parallel reduction in mean blood LH and FSH levels and a complex modulation of LH pulse frequency and amplitude. That the frequency of the LHRH pulse generator can be influenced by gonadal steroids, as we have shown, was first suggested by the early work of Yen and coworkers (15), who reported that the frequency and amplitude of pulsatile LH secretion might be varied over the course of the human menstrual cycle. Work by Baird (16) in the ewe and another report by Rahe *et al.* (17) in the cow demonstrate that frequency and amplitude modulation of pulsatile LH secretion occurs during the course of the estrous cycle in these animals as well; moreover, treatment of ovariectomized ewes with either estradiol or progesterone can modulate LH pulse frequency and amplitude (18). Gallo (19) has reported that in the female rat, LH pulse frequency and amplitude vary as a function of estrous cycle stage, and a preliminary report by Butterstein and Whitmoyer (20) suggests that estradiol can modulate LH pulse frequency in the ovariectomized female rat. Other studies would suggest that T, administered in physiological doses, can effect a reduction in LH pulse frequency in both human (21) and rhesus males (22). Work by Lincoln and Short (23) in the ram has demonstrated that LH pulse frequency and amplitude may be modulated over the course of the breeding season. Together, these studies under-

score the importance of environmental and gonadal cues to the operational characteristics of the LHRH pulse generator and pituitary gonadotropin secretion. Our own findings in the adult male rat extend this general principle by showing that T can independently regulate various components of the LH signal, *i.e.* LH pulse frequency, amplitude, and mean plasma LH levels, as a function of its concentration. Based on the foregoing studies together with the recent work of Gross (24) and others documenting the marked influence of castration and T replacement on the immunoreactive LHRH content of the hypothalamus, we infer that at least a portion of the effect we are observing is attributable to an action of T on the central nervous system (CNS); however, neither the CNS site nor the mechanism of T-mediated frequency/amplitude modulation is known. Whether this effect represents a direct action of T or one mediated by a metabolite, such as dihydrotestosterone or estradiol, remains unknown.

The LHRH signal to the pituitary appears to be frequency coded, such that a change in LHRH pulse frequency signals new information to the pituitary (25). This concept is based on the following observations. 1) Pulsatile, but not continuous, LHRH stimulation of the pituitary sustains gonadotropin secretion (26). 2) LHRH frequency modulation may differentially control LH and FSH secretion (27, 28). 3) The frequency of LHRH secretion may influence the sensitivity of the pituitary to steroid-mediated negative feedback (29). These observations illustrate how a modulation in LHRH pulse frequency by gonadal and environmental cues could serve as a critical element in the regulation of the CNS-pitiutary-gonadal axis.

Measurement of FSH levels in the present study demonstrated the ability of T administration to suppress mean FSH levels in castrate animals in proportion to the suppression of LH levels. In spite of changing blood T levels and the induction of varying LH pulse frequencies and amplitudes, no significant dissociation between mean LH and FSH levels was found. A conclusive test of the hypothesis that frequency modulation of pulsatile LHRH secretion can differentially control LH and FSH secretion in the rat awaits the administration of LHRH to animals with experimentally induced deficiencies of this hypothalamic peptide.

It has been suggested that a pulsatile mode of LH secretion sustains testicular steroidogenesis more effectively than a continuous exposure to the gonadotropin. This concept is based on the observation that sustained elevations of either LH or hCG densensitize Leydig cells to further stimulation by LH, apparently due to a direct loss of gonadal LH receptors (30, 31) or perhaps indirectly by an induction of a local LHRH-like inhibitory factor (32). The degree of Leydig cell impairment also appears to be related to the qualitative and quantitative nature of the gonadotropin stimulus (31). Recent evidence suggests that changes in gonadotropin pulse amplitude and frequency may be important regulatory processes in controlling Leydig cell function. A report by Payne *et al.* (33) demonstrates the existence of two distinct populations (I and II) of Leydig cells, each differentially responsive to the temporal patterning of gonadotropin signal. Both cell populations have identical concentrations of LH receptors, but they exhibit different T responses to hCG challenges. Whereas repeated injections of low doses of LH result in a similar decrease in the concentration of LH receptors of both populations, only population I cells show an enhancement of steroidogenic responsiveness to hCG. These data suggest that the temporal and quantitative nature of the gonadotropin signal may represent important controlling variables for the regulation of normal testicular function. Our data in the male rat show that plasma T levels, in turn, can profoundly influence the feedback loop by regulating the frequency and amplitude of LH secretion.

In addition to its absolute plasma level, the qualitative features of the T feedback signal to the CNS-pituitary axis may also play a significant role in regulating gonadotropin secretion. T is not secreted in a steady state fashion in the adult male rat, but, rather, exhibits complex oscillations in its plasma concentration (34), which may undergo 3- to 8-fold variations in absolute magnitude over 3- to 6-h periods (Steiner, R. A., W. J. Bremner, and D. K. Clifton, unpublished observations). The T-containing implants produce relatively steady state plasma T concentrations, which probably do not simulate the short term waxing and waning of plasma levels occurring under normal physiological conditions. That the 30-mm T implant virtually abolished LH pulses, while maintaining average, steady state levels of T less than the intact group, suggests that a changing T signal, oscillating about a given mean, conveys different feedback information to the CNS-pituitary axis than does a steady state level maintained at the same mean. This difference between an oscillating and a steady state T feedback signal may also partially account for the observation that the 30-mm T implant did not completely reduce LH pulse amplitude to the levels seen in intact animals. Alternatively, testicular hormones other than T could also be important in regulating LH pulse amplitude and frequency.

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