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TIME-COURSES OF THE APPEARANCE/DISAPPEARANCE OF NUCLEAR ANDROGEN + RECEPTOR COMPLEXES IN THE BRAIN AND ADENOHYPOPHYSIS FOLLOWING TESTOSTERONE ADMINISTRATION/WITHDRAWAL TO CASTRATED MALE RATS: RELATIONSHIPS WITH GONADOTROPIN SECRETION

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Summary—We characterized the temporal dynamics of brain and pituitary cell nuclear androgen receptor binding and serum androgen and gonadotropin levels associated with the implantation and removal of testosterone (T)-filled Silastic capsules into preformed s.c. flank pouches of castrated, awake male rats. These capsules produced serum T levels in the physiologic range. The number of cell nuclear androgen + receptor complexes, as measured in an exchange assay using [^{3}H]R1881, increased 15-fold at 0.5 h after capsule insertion in the HPAS (combined hypothalamus, preoptic area, amygdala and septum) and anterior pituitary gland, but then showed a second progressive rise within the next 8 h. This pattern suggests that T exerts an initial action in the tissues to alter the affinity and/or number of available androgen receptors. There was a lag time of 2–4 h to the first indication of negative feedback suppression of LH secretion. Serum LH levels declined only slightly at 4 h after capsule insertion but continued to fall thereafter, reaching undetectable values by 24 h. In contrast, serum FSH levels declined only slightly after 24 h of T exposure. After removal of the T capsules, serum T levels declined to castrate values within 2 h at which time the level of androgen + receptor complexes had fallen to 60% in the brain and pituitary. Serum LH and FSH concentrations were unchanged at 2 h after capsule removal, but rose significantly within the next 2 h. The data indicate that the occupation of androgen receptors rapidly changes in response to variations in circulating T in a fashion that implicates their involvement in the expression of this steroid's negative feedback actions on gonadotropin secretion.

INTRODUCTION

In the male rat, the secretion of testosterone (T) is an important mechanism whereby the testes regulate LH and FSH release by the anterior pituitary gland. In gonadally intact males, circulating gonadotropin levels are low and removal of the testes results in postcastration rises in their secretion rates; serum LH and FSH levels fall when physiologic levels of serum T are restored by androgen injections or the implantation of T-filled Silastic capsules [1–4]. T appears to regulate gonadotropin secretion by intracellular mechanisms which involve specific interactions with cell nuclear androgen receptors in the anterior pituitary gland and certain brain regions [3, 5, 6]. Hypophysial receptors appear to be concentrated in gonadotrophs [7–9]. Androgen receptor concentrations are also high in neurons in the ventral hypothalamus, medial preoptic area, medial amygdala and septum, brain regions implicated in the regulation of GnRH secretion [10–14]. Although T is converted to estrogens in these same brain regions [15], this conversion does not play a role in the regulation of basal gonadotropin release [1, 3]. Similarly, 5α-reduction of T in the brain and pituitary does not appear to be obligatory for T to suppress gonadotropin release [16, 17].

In an attempt to extend our understanding of the temporal and functional interrelationships between circulating androgens, androgen receptor occupation in hypophysial and neural tissues, and the regulation of gonadotropin secretion in male rats, we have measured serum T, cell nuclear levels of androgen + receptor complexes and LH and FSH levels at various times after acute exposure to and withdrawal from circulating T levels in the physiological range.

MATERIALS AND METHODS

Adult, male Sprague–Dawley rats (300–350 g body wt; Charles River Breeding Laboratories,

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Wilmington, Mass) were housed in group cages on a 14:10, light:dark cycle (lights on: 0500–1900). Food and water were freely available.

The animals were castrated under ether anesthesia 2–3 weeks prior to use. On the day before T replacement, the animals were shaved and outfitted with small s.c. pouches (approximately 0.25–0.5 inch incision) over the left flank under ether anesthesia. The pouches were not closed to permit the rapid (10 sec) insertion or removal of 1 cm long, T-filled Silastic capsules (i.d.: 0.058 in; o.d.: 0.077 in; Dow Corning, Midland, Mich.) with a small hemostat while the animals were awake and freely moving. The use of these open flank pouches minimizes stress-related adrenal activation associated with anesthesia or extensive handling and has been used to study estrogen-induced LH release in female rats [18].

Nuclear androgen receptor exchange assays

The animals were sacrificed for the exchange assays immediately prior to or 0.5, 2, 4, 8 or 24 h after T capsule insertion. Additional animals were exposed to T for 24 h and then killed 2, 4 or 8 h after removal of the capsules from the flank pouches. The number of cell nuclear androgen + receptor complexes was measured in combined hypothalamus, preoptic area, amygdala and septum (HPAS) and in the anterior pituitary gland (PIT) using a [^{3}H]R1881 (methyltrienolone; New England Nuclear, Boston, Mass) exchange assay as previously described by McGinnis *et al.*[13]. This assay does not measure unoccupied, or non-activated androgen receptors.

The animals were killed by decapitation, and blood was collected into iced tubes for later hormone assay. The brains were removed and the various regions were dissected on ice using landmarks previously described [13]. Tissues were homogenized in NIIDG (1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerole). Following centrifugation at 1000 *g* for 5 min and an additional wash in NIIDG, the crude nuclear fraction was resuspended in NIIDG and NIIIDG (1 mM KH_2PO_4, 2.39 M sucrose, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol) and centrifuged at 25,000 *g* for 20 min to obtain a purified nuclear pellet. Nuclear receptors were extracted from the pellet by sequential addition of TEDB (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 0.5 mM Bacitracin) and TEDBK (TEDB + 1.2 M KCl). Aliquots of the extract were incubated with [^{3}H]R1881 (sp. act.: 87 Ci/mmol) with and without an unlabeled 100-fold molar excess of 5α-dihydrotestosterone (DHT) under conditions previously shown to permit the exchange of [^{3}H]R1881 with receptor-bound androgens. Previous studies in this laboratory had indicated that co-incubation with triamcinalone acetonide, to block potential [^{3}H]R1881 binding to glucocorticoid and progestin receptors, was not necessary in this nuclear assay [13]. Receptor-bound [^{3}H]R1881 was isolated by passage through Sephadex LH-20 columns. DNA content was determined in the nuclear pellets [19], and binding results were corrected for non-specific binding and expressed as fmols [^{3}H]R1881 bound-mg DNA. Data from this assay was subjected to analysis of variance using Duncan's multiple range test for *post hoc* comparisons.

Radioimmunoassay of serum T, LH and FSH

Trunk blood was collected from the animals killed in the above study and centrifuged at 4°C; serum was harvested and stored at −20°C until assay. LH was assayed in duplicate using an antiserum to oLH (GDN No. 15) and a [^{125}I]oLH (LER 1056C2) tracer as previously described [3]. Results are expressed as ng rLH-RP1/ml; the intra-assay coefficient of variation was routinely < 10%. Serum FSH was assayed using the NIDDK rFSH kit. Values are expressed as ng rFSH-RP1/ml; intra-assay coefficient of variation was routinely < 10%. Serum T levels were assayed using GDN No. 250 antiserum generated against T-11-bovine serum albumin using [^{3}H]T tracer as previously described [20]. Serum T, LH and FSH data were analysed statistically as described in the preceding section.

Determination of episodic LH release

Two days prior to blood sampling, s.c. pouches were made and the left common carotid artery was catheterized with PE-50 tubing under methoxyflurane (Metofane; Pitman-Moore, Washington Crossing, N.J.) anesthesia. The catheter was tunneled s.c., exteriorized at the nape of the neck, filled with heparin-saline and plugged. On the day of sampling, 500 IU heparin/100 μl saline was administered and the catheter attached to an extension passed through a peristaltic pump. Blood samples (50 μl/6 min) were collected into iced tubes continuously for 4–6 h without fluid replacement. T-filled capsules were inserted into the pouches during the collection. Plasma was separated by centrifugation and the samples stored at −20°C until LH assay.

Episodic LH pulses were identified according to the coefficient of variation (CV) method of Gallo[21]. A subset of samples extending from nadir-peak-nadir was identified as a pulse if its CV was > 1.5 × intra-assay CV. Pulse amplitude was the difference between a peak value and its preceding nadir. Interpulse interval was set as the time elapsed between two identified pulse peaks. A paired *t*-test was used to test for significant differences in pulse parameters prior to and for approx. 2.5 h following T capsule insertion.

RESULTS

Serum hormone and cell nuclear concentrations of androgen + receptor complexes at varying times following T capsule insertion or removal are presented in Fig. 1. Serum androgen levels rose approximately 15-fold within 0.5 h after insertion of the T capsules and remained at 2–5 ng/ml, a range routinely

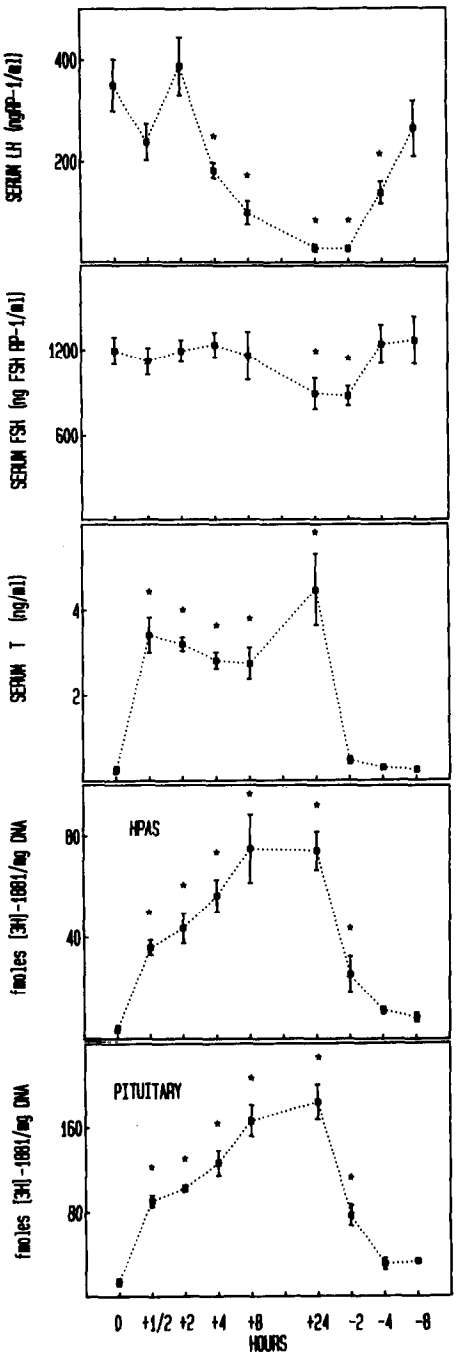

Fig. 1. Temporal patterns of serum levels of LH, FSH and testosterone (T) and cell nuclear androgen receptor occupation in HPAS (hypothalamus, preoptic area, amygdala and septum) and PIT (anterior pituitary gland) following the s.c. insertion (+1/2 to +24 h) and removal (−2 to −8 h) or T-filled Silastic capsules in castrated male rats. For the removal studies, the capsules were previously in place for 24 h. Each value represents the mean + SEM. For each LH and FSH value $n = 8$; for each T value $n = 10$–14; for each androgen receptor binding value $n = 3$–6 determinations. *Significantly ($P < 0.05$) different from the castrate control groups.

observed in this laboratory in gonadally intact rats, for the next 24 h. Serum androgens dropped to castrate values within 2 h after capsule removal.

The number of cell nuclear androgen + receptor complexes was significantly elevated at 0.5 h after T capsule insertion, rising about 10-fold in the HPAS and pituitary gland. However, these levels continued to rise thereafter in both tissues, approximately doubling by 8 h. There was no further increase in receptor levels between 8 and 24 h, and the values obtained at these times were in the range seen in gonadally intact male rats [22]. 2 h after T capsule removal, receptor number declined 60% in both brain and pituitary gland. Receptor levels in these tissues declined further at 4 and 8 h to values not statistically different from those in castrates.

Serum FSH levels did not change dramatically throughout the course of T replacement and withdrawal, showing only a slight, but significant, decline after 24 h of exposure to T and a return to castrate levels by 4 h after capsule removal. Serum LH levels, on the other hand, showed pronounced changes. LH levels at 0.5 and 2 h after T capsule insertion were not significantly different from castrate values (Fig. 1). LH release was episodic during this time (Fig. 2), and T capsules had no effect on interpulse interval (pre-T: 27 + 2 min; post-T: 28 + 3, $n = 7$) or peak amplitude (pre-T: 372 + 95; post-T: 340 + 60). However, serum LH values fell significantly after 4 h of exposure to T and then continued to decline at 8 and 24 h. LH secretory profiles after 8 h of T exposure were characterized by significantly smaller pulses with longer interpulse intervals (data not shown). Serum LH levels at 24 h are comparable to what we routinely observe in gonadally intact male rats. Circulating LH concentrations did not change at 2 h after T capsule removal, but rose significantly at 4 h and again at 8 h.

DISCUSSION

The use of preformed flank pouches for the atraumatic insertion and removal of T-filled Silastic capsules has allowed an examination of the temporal patterns of androgen receptor occupation and clearance in neuroendocrine tissues and their sequelae on gonadotropin release. Serum androgen levels significantly rose from and fell to castrate values within 0.5 and 2 h of capsule insertion and removal, respectively. This rapid decline in circulating androgens following acute T removal agrees with more detailed clearance patterns of serum T following castration in male rats [23].

In the HPAS and anterior pituitary gland, the changes in the number of nuclear androgen + receptor complexes temporally paralleled the patterns in circulating T with one significant exception. Following acute exposure of both tissues to T, the pattern of receptor occupation appeared to be biphasic: an abrupt and sizeable increase at 0.5 h followed by a gradual, but consistent, doubling over the next 8 h to a secondary level which persisted for the next 16 h. It is unlikely that this biphasic pattern is a

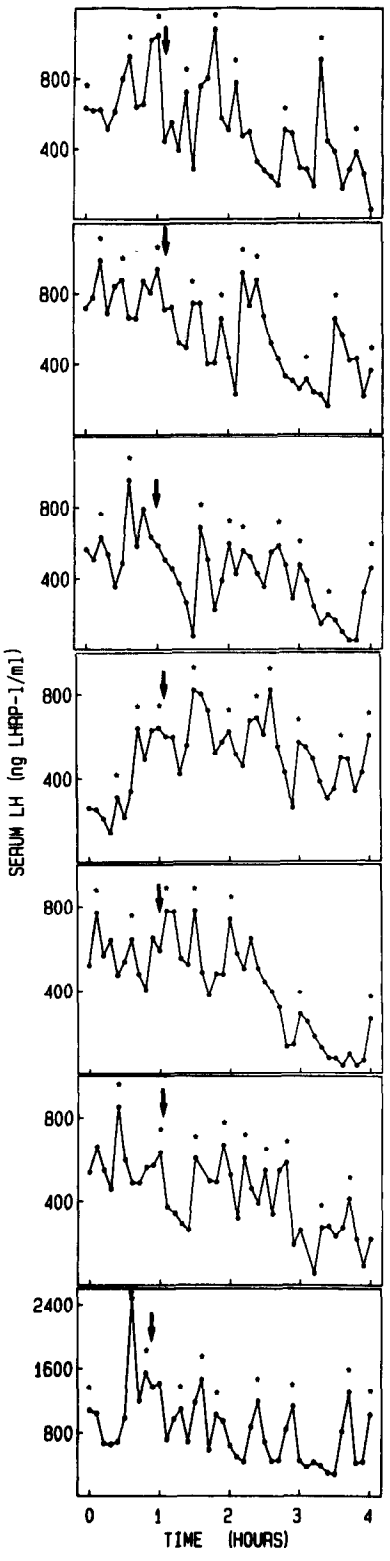

Fig. 2. Pulsatile patterns of serum LH in 7 castrate male rats before and after the insertion (arrow) of a T-filled Silastic capsule into a s.c. flank pouch. *Peaks of pulses identified according to Gallo[21].

procedural artifact, because samples from a variety of time points were processed simultaneously. We have found a similar temporal pattern of androgen receptor occupation *in vitro* following exposure of cultures of dispersed rat pituitary cells to [1,2,6,7-^{3}H]-T (10^{-8} M, 2.88 ng/ml, Krey and Kamel, unpublished observations). In these cells cultured in steroid-free medium for 72 h prior to [^{3}H]T treatment, increases in [^{3}H]androgen-labeled receptors levels was observed between 4 and 12 h. Significantly, Handa *et al.*[24] have reported similar findings in the pituitary gland following s.c. DHT injections to female rats, ovariectomized 3 days beforehand. In this study, both occupied and unoccupied androgen receptor levels in the anterior pituitary gland, as measured by a combined [^{3}H]R1881-exchange + cytosol assay, increased between 6 and 12 h after DHT administration despite a 60% decline in serum androgen levels.

When target tissues are exposed to physiologic concentrations of T or [^{3}H]T, 50–70% of available receptors are usually occupied [25–27]. Thus, the biphasic increase in the level of nuclear androgen + receptor complexes can be explained by an increase in either receptor affinity or number. The findings of Handa *et al.*[24] support a hypothesis that androgen receptor number increases shortly after androgen treatment and suggests that androgen receptor synthesis is an on-going process since cycloheximide injections suppressed receptor levels in steroid-free animals [24]. Indications of similar actions of cycloheximide to block the secondary rise in androgen + receptor levels in our T-replaced model would clearly imply that androgens acutely stimulate the synthesis of their own receptors in neuroendocrine target tissues.

Previous work has indicated that estrogen increases the number of androgen receptors in a variety of tissues, including the anterior pituitary gland and medial preoptic nucleus [3, 23, 28, 29]. However, it seems unlikely that T-derived estrogens underlie our observed increase in hypophysial receptor levels. The pituitary contains little or no aromatase activity [15] and shows no increase in estrogen-receptor occupation following T-replacement treatments identical to those employed in this study [3]. In contrast to the pituitary gland, the medial preoptic nucleus does contain aromatase activity [15], and one must expect that the neural estrogen receptors in this region are occupied under the experimental conditions employed in this study [27]. However, given the temporal and quantitative similarities between the hypophysial and neural patterns in receptor occupation, one might expect that the underlying cellular mechanisms would be identical.

We have used the same experimental approach to monitor the level of nuclear estrogen + receptor complexes following estradiol replacement to acutely ovariectomized female rats. Significantly, we saw no evidences of a biphasic pattern of receptor number in the preoptic area, hypothalamus or pituitary gland; receptor levels, as measured by exchange assay, were comparable at 0.5 and 24 h following the insertion of estradiol-filled capsules which generated

proestrous-like levels of serum estrogen [18]. In addition, the decline in nuclear estrogen receptor levels following removal of the capsules was appreciably slower than that reported here for androgen. Over 80% of the estrogen receptors were still occupied at 2 h after capsule removal, whereas at 4 h nuclear receptor levels were at 30–50%. Only at 8 h did receptor levels fall by 85% or more. In contrast, the number of androgen + receptor complexes in the brain–pituitary complex declined by 60% at 2 h and 85% at 4 h after capsule removal (Fig. 1). It is possible that these differences in androgen and estrogen receptor clearance can be accounted for by differences in the clearance of these two steroids from the circulation. Whereas serum androgen levels decline to castrate values at 2 h after capsule removal, serum estrogens only fall to castrate levels between 2–4 h. However, it should be noted that serum estrogen levels decline by ~90% at 2 h after capsule removal.

The increases in circulating T concentrations and in HPAS and hypophysial androgen receptor levels preceded any noticeable change in episodic gonadotropin secretion by 2–4 h. Such a lag time suggests that T exerts its negative feedback actions through genomic mechanisms. Clearly this lag time does not appear to be due to any castration-induced deficit in the intracellular processing of T within the HPAS–pituitary complex. Nuclear androgen receptor levels were comparable in the animals at the 2–4 h period when feedback suppression becomes evident. Moreover, resident aromatases and 5α-reductases have been shown to metabolize T in these tissues following acute [^{3}H]T injections to castrated animals [15, 30]. However, these metabolic alterations do not appear to be obligatory for the expression of T feedback [16, 17]. The observation that T suppression of serum LH and FSH levels dissipated only between 2–4 h after removal of the T-filled capsules, a time when occupied androgen receptor levels complete their fall to castrate values, is also consistent with a genomic activation hypothesis. Clearly, the rapidity with which serum LH/FSH levels return to castrate values in this animal model system indicates that, whatever the relevant gene product(s) might be, they possess a relatively short half-life. The same gene products need not be involved in T induction of male sex behaviors or other behaviors regulated by T-target neurons in the HPAS. It is important to note in this regard that, in a similar rat model, a virtually continuous (>21 h/day) exposure to T is required for 7 days in order for ejaculatory behavior to be restored [31].

Significantly, T suppression of serum LH levels was not accompanied temporally by changes in FSH. Moreover, whereas 24 h of exposure to T suppressed LH levels by 90%, to values normally observed in gonadally intact males in this laboratory, serum FSH levels were reduced by only 30%. Such a disparity in androgen influences on the release of these two gonadotropins has been observed by other investigators and suggests a prominent role for testicular inhibins in the control of FSH secretion in this species [32, 33].

In summary, the present study provides descriptive information on the temporal patterns of neural and hypophysial androgen receptor occupation following the imposition and withdrawal of a "constant" T stimulus in castrated male rats. A biphasic pattern of receptor number followed exposure to T and suggests that androgens rapidly stimulate the synthesis or mobilization of their own receptors. Relationships between the uptake and clearance patterns of receptor occupation and circulating gonadotropin concentrations reflect genomic activation in the negative feedback actions of T. When compared to the temporal patterns of testicular T secretion in rats and other species [23, 34], the present time-courses of uptake and clearance suggests that, under normal circumstances, androgen receptor occupation in neuroendocrine tissues is not constant but rather fluctuates dramatically. Whether such a discontinuous pattern influences the distribution or quality of the physiologically-relevant gene products is an interesting question for future consideration.

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