

# Administration of Gonadal Steroids to the Castrated Male Rat Prevents a Decrease in the Release of Gonadotropin-Releasing Hormone from the Incubated Hypothalamus

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**ABSTRACT** The influence of testosterone on gonadotropin-releasing hormone (GnRH) secretion was assessed indirectly by altering the serum testosterone concentration of male rats and measuring GnRH release from their incubated hypothalami 1 wk later.

GnRH release from hypothalami of castrated rats was  $13.4 \pm 1.2$  (SE) pg/h, compared to  $35.3 \pm 3.8$  pg/h from hypothalami of intact rats ( $P < 0.001$ ). GnRH release from the hypothalami of castrated rats treated with testosterone propionate, 100 or 500  $\mu$ g daily, was  $25.0 \pm 3.4$  pg/h and  $27.9 \pm 3.6$  pg/h, which is significantly greater ( $P < 0.05$  and  $P < 0.01$ , respectively) than that from hypothalami of castrated rats treated only with sesame oil.

A similar decrease in GnRH release from hypothalami of hypophysectomized rats and prevention of this decrease by treating the hypophysectomized rats with testosterone propionate is evidence that the observed effects of testosterone are not mediated via luteinizing hormone and(or) follicle-stimulating hormone secretion. Treatment of castrated rats with either dihydrotestosterone propionate or estradiol benzoate also prevented the decrease in GnRH release from the hypothalami of castrated rats.

We conclude that testosterone, dihydrotestosterone, and estradiol all prevent the decrease in GnRH release from hypothalami of castrated rats treated with these steroids. The possibility exists that these steroids may also maintain GnRH secretion in vivo.

## INTRODUCTION

Secretion of luteinizing hormone (LH)<sup>1</sup> and of follicle-stimulating hormone (FSH) by the pituitary gland

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<sup>1</sup>Abbreviations used in this paper: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

are stimulated by gonadotropin-releasing hormone (GnRH) and are inhibited by testosterone. The inhibitory effect of testosterone on GnRH-stimulated LH secretion occurs, at least in part, directly at the level of the pituitary gland, since this effect can be demonstrated with incubated pituitary glands (1) and with pituitary cell cultures (2, 3). Little data is currently available concerning the possible effects of testosterone and other gonadal steroids on GnRH secretion from the hypothalamus. Castration of male rats has been shown to decrease hypothalamic GnRH content, and testosterone treatment of castrated rats to prevent the decrease in GnRH content (4). These changes in hypothalamic GnRH content could reflect changes in either GnRH synthesis or secretion, or both.

The purpose of this study was to determine the effect of testosterone on GnRH secretion indirectly by altering the serum testosterone concentration of adult male rats and then measuring GnRH release from their incubated hypothalami. GnRH release in the hypothalamic incubation system employed here has been shown to depend on membrane depolarization and calcium uptake (6), as does the secretion of most hormones.

## METHODS

**Animals.** Male Sprague-Dawley rats, 200–250 g, were used for all experiments. Immediately after either castration or hypophysectomy (the latter performed at Charles River Breeding Laboratories, Wilmington, Mass.), the animals were injected subcutaneously with 0.1 ml sesame oil, either alone or with added steroid. The steroids (Steraloids, Pawling, N. Y.) included testosterone propionate, 100 or 500  $\mu$ g; dihydrotestosterone propionate, 20 or 100  $\mu$ g; and estradiol benzoate, 1.0  $\mu$ g. All hypophysectomized animals also received subcutaneous injections of 250  $\mu$ g of hydrocortisone in sesame oil and 4.0  $\mu$ g of L-thyroxine in saline (7). All injections were continued daily for a total of 7 d. On the day after the seventh injection the treated rats, and a control group of intact rats for each experiment, were sacrificed by decapitation. Trunk blood was obtained for measurement of serum LH, FSH, and testosterone.

**Incubation.** Hypothalami were removed and incubated as described (6). The hypothalami were placed, one per tube, in incubation medium at 37°C. The medium contained Krebs-Ringer salts, except for 2.7 mM NaHCO<sub>3</sub>, and also contained 20 mM Hepes, 56 mM glucose, Eagle's amino acids and vitamins, and 20 mM L-glutamine. The pH was adjusted to 7.0.

The hypothalami were preincubated in 2 ml medium for 30 min in a Dubnoff incubator in room air. After the preincubation, the medium was aspirated and the hypothalami were washed twice with 1 ml incubation medium. The hypothalami were incubated in 1 ml medium for 60 min. The medium was then aspirated and frozen until assay of GnRH, and the hypothalami were frozen until extraction.

**Extraction of hypothalami.** The hypothalami were homogenized in 2 ml 0.05 M Na<sub>2</sub>HPO<sub>4</sub>–0.15 M NaCl, pH 7.2, with a motor-driven, Teflon (DuPont Co., Wilmington, Del.) pestle at 830 rpm. After the addition of 10 ml 95% ethanol and mixing, the homogenate was centrifuged at 2,000 rpm for 15 min. The supernate was dried in a 60°C water bath overnight. The dried extract was kept at –20°C until immediately before GnRH assay. To determine the efficiency of recovery of GnRH by this extraction procedure, 30 male Sprague-Dawley rats, 200–250 g, were sacrificed and their hypothalami were isolated. 15 of the hypothalami were extracted in the manner above; the other 15 were extracted in a similar fashion, but 10,000 pg synthetic GnRH was added before homogenization. Recovery of the exogenous GnRH was 88%.

**Assays.** GnRH was assayed by a modification of the method of Nett et al. (8), with anti-GnRH generously provided by them. The modification was as described (6), except that the oxidizing agent used for the preparation of [<sup>125</sup>I]iodo-GnRH was chloramine T (5 µg), rather than lactoperoxidase, because the stability of [<sup>125</sup>I]iodo-GnRH prepared with lactoperoxidase varied with the batch of lactoperoxidase. Binding of [<sup>125</sup>I]iodo-GnRH to anti-GnRH was optimum at pH 7.0–7.2; assay reagents were therefore titrated to pH 7.2 (6). The lower limit of sensitivity of the assay was 1.5–2.0 pg/assay tube. Since duplicate 200-µl aliquots of the 1.0-ml incubation were assayed, the lower limit of detection of GnRH

release was 7.5 pg/h. The intra- and interassay coefficients of variation were 6 and 8%.

Serum LH was measured by the ovine-ovine-rat LH assay described by Niswender et al. (9), with his anti-ovine LH No. 15, purified ovine LH for iodination (LER 1056-C2), and rat LH standard (RP-1) from the National Institute of Arthritis and Metabolic Diseases. The lower limit of sensitivity of this assay was 1–2 ng/assay tube, and the intra- and interassay coefficients of variation were 7 and 13%. Serum FSH was measured by immunoassay, with reagents provided by the NIAMDD. The lower limit of sensitivity of this assay was 20 ng/assay tube, and the intra- and interassay coefficients of variation were 4 and 10%. Testosterone was measured as described (1).

Statistical analyses were performed with Duncan's (11) multiple range test.

## RESULTS

**Castration and treatment with testosterone propionate (Table 1).** Hypothalami taken from male rats castrated 7 d earlier released significantly less GnRH into the medium during the 1-h incubation than did hypothalami from a group of similar rats that had not been castrated. Hypothalami of rats that had been castrated and then given either 100 or 500 µg of testosterone propionate daily for 7 d released significantly more GnRH into the medium than hypothalami of castrated rats and similar amounts to that released from hypothalami of intact rats. Hypothalami of castrated rats contained significantly less GnRH than those of intact rats. Treatment of castrated rats with either 100 or 500 µg of testosterone propionate daily prevented the decrease in GnRH content.

Castration caused the expected decrease in serum

TABLE I  
Effects of Castration of Male Rats and Treatment with Testosterone Propionate on GnRH Release from Their Incubated Hypothalami

	Intact rats	Castrated rats treated with		
		Sesame oil	Testosterone propionate, 100 µg/d	Testosterone propionate, 500 µg/d
<i>n</i>	18	18	18	18
GnRH release, pg/h	35.3±3.8	13.4±1.2*	25.0±3.4§	27.9±3.6 <sup>¶</sup>
GnRH content, pg/hypothalamus	2,289±203	1,420±109†	1,976±132§	2,432±193 <sup>¶</sup>
Serum testosterone, ng/ml	4.5±0.5	0.72±0.04*	2.0±0.1 <sup>¶</sup>	6.4±0.5 <sup>¶</sup>
Serum LH, ng/ml	30.8±3.3	187±19.7*	82.1±22.1§	7.6±0.5 <sup>¶</sup>
Serum FSH, ng/ml	244±17	600±37*	418±53§	214±8 <sup>¶</sup>

Male rats, 200–250 g, were left intact or castrated. Castrated rats were treated with either sesame oil alone or with sesame oil containing testosterone propionate, 100 or 500 µg daily for 7 d. The animals were then decapitated, and the hypothalami incubated, as described in Methods, and blood collected. Values are means±SE. Statistical significance:

\*  $P < 0.001$  vs. intact rats.

†  $P < 0.01$  vs. intact rats.

§  $P < 0.05$  vs. oil treated.

<sup>¶</sup>  $P < 0.01$  vs. oil treated.

<sup>¶</sup>  $P < 0.001$  vs. oil treated.

TABLE II  
Effects of Hypophysectomy of Male Rats and Treatment with Testosterone Propionate on GnRH Release from Their Hypothalami In Vitro

	Intact rats	Hypophysectomized rats treated with	
		Sesame oil	Testosterone propionate, 500 µg/d
n	13	13	14
GnRH release, pg/h	37.5±4.2	20.9±1.3*	44.9±3.1§
GnRH content, pg/hypothalamus	2,240±180	1,200±80†	1,950±140§
Serum testosterone, ng/ml	3.8±0.3	0.22±0.01†	8.0±0.5§
Serum LH, ng/ml	24.5±3.2	8.4±0.3*	7.8±0.2

Male rats, 200–250 g, were left intact or hypophysectomized. All hypophysectomized rats were treated with L-thyroxine and hydrocortisone (in Methods). One group of hypophysectomized rats was also given sesame oil, and the other group of hypophysectomized rats was given sesame oil with testosterone propionate, 500 µg daily for 7 d. The rats were then decapitated, and the hypothalami incubated, as described in Methods, and blood collected. Values are means±SE. Statistical significance:

\*  $P < 0.01$  vs. intact rats.

†  $P < 0.001$  vs. intact rats.

§  $P < 0.001$  vs. oil treated.

testosterone and increase in serum LH and FSH concentrations. Testosterone propionate, 100 µg daily, partially maintained serum testosterone levels and

partially suppressed the castration-induced rises in LH and FSH. The 500-µg dose of testosterone propionate produced supraphysiologic serum testosterone levels and suppressed the serum concentrations of LH and FSH.

*Hypophysectomy and treatment with testosterone propionate (Table II).* Hypothalami isolated from rats hypophysectomized 7 d earlier released significantly less GnRH than did hypothalami of intact rats. Hypothalami of rats that had been hypophysectomized and treated with 500 µg of testosterone propionate daily released a similar amount of GnRH as did hypothalami of intact rats and significantly more than hypothalami of hypophysectomized rats. Testosterone propionate treatment did not prevent the decrease in GnRH release from hypothalami of hypophysectomized rats, however, unless the testosterone was begun immediately after hypophysectomy and unless thyroxine and hydrocortisone were also given. Administration of hydrocortisone and thyroxine, without testosterone propionate, to hypophysectomized rats did not prevent the decrease in GnRH release. Hypothalamic content of GnRH was also decreased significantly by hypophysectomy, and the decrease prevented by testosterone treatment. Hypophysectomized rats had significantly lower serum LH and testosterone concentrations than did the intact rats. Testosterone propionate, 500 µg daily, increased the serum testosterone concentration above that of intact rats.

*Treatment with dihydrotestosterone propionate (Table III).* Administration of 20 µg of dihydrotestosterone propionate daily to castrated rats increased GnRH release from incubated hypothalami compared to GnRH release from hypothalami of castrated rats

TABLE III  
Effects of Castration of Male Rats and Treatment with Dihydrotestosterone Propionate on GnRH Release from Their Hypothalami In Vitro

	Intact rats	Castrated rats treated with	
		Sesame oil	Dihydrotestosterone propionate, 100 µg/d
n	10	12	12
GnRH release, pg/h	30.2±4.7	14.0±1.2*	25.9±5.1§
GnRH content, pg/hypothalamus	2,196±326	1,102±93†	2,167±174 <sup>  </sup>
Serum LH, ng/ml	23±6.4	129±17	57.4±20.8§
Serum FSH, ng/ml	228±9	590±27†	659±25

The experimental procedure was similar to that described in Table I, except that dihydrotestosterone propionate, 20 or 100 µg, was administered instead of testosterone propionate. Values are means±SE. Statistical significance:

\*  $P < 0.01$  vs. intact rats.

†  $P < 0.001$  vs. intact rats.

§  $P < 0.05$  vs. oil treated.

<sup>||</sup>  $P < 0.001$  vs. oil treated.

TABLE IV  
Effects of Castration of Male Rats and Treatment with  
Estradiol Benzoate on GnRH Release from  
Their Hypothalami In Vitro

	Castrated rats treated with		
	Intact rats	Sesame oil	Estradiol benzoate, 1 µg/dl
n	8	8	8
GnRH release, pg/h	33.3±4.0	20.1±2.3*	43.3±5.0§
GnRH content, pg/hypothalamus	3,139±270	1,410±320†	2,310±240 <sup>#</sup>
Serum LH, ng/ml	19.3±3.4	145±12.0†	104.4±25.1
Serum FSH, ng/ml	220±10	636±34†	51.6±34

The experimental procedure was similar to that described in Table I, except that estradiol benzoate, 1.0 µg daily, was administered instead of testosterone propionate. Values are means±SE. Statistical significance:

\*  $P < 0.05$  vs. intact rats.

†  $P < 0.001$  vs. intact rats.

§  $P < 0.001$  vs. oil treated.

<sup>#</sup>  $P < 0.05$  vs. oil treated.

treated only with sesame oil. Administration of 100 µg dihydrotestosterone propionate daily caused an even greater rise in GnRH release. The lower dihydrotestosterone dose did not affect hypothalamic GnRH content, but the higher dose resulted in maintenance of a level similar to that of intact animals. The two doses of dihydrotestosterone caused a stepwise decrease in the LH rise after castration, but only the larger dihydrotestosterone dose caused a significant decrease in FSH.

*Treatment with estradiol benzoate (Table IV).* Administration of 1 µg of estradiol benzoate daily to castrated rats prevented the decrease in GnRH release from incubated hypothalami. The GnRH content of the hypothalami from the estradiol-treated castrated rats was also significantly higher than that of the castrated rats treated only with sesame oil and similar to that of intact rats. This dose of estradiol benzoate was not effective, however, in suppressing significantly the LH and FSH rise after castration.

## DISCUSSION

Stimulation of LH and FSH secretion by GnRH and inhibition of LH and FSH secretion by testosterone has been amply demonstrated in several mammalian species. Little data are available, however, concerning the possible effect of testosterone on GnRH secretion. The reasons for the paucity of such data appear to be the

apparently low concentrations of GnRH in peripheral blood and the difficulty in obtaining blood from the hypothalamic-hypophyseal portal circulation. Neill et al. (12) found higher GnRH concentrations in the portal blood of ovariectomized than of intact monkeys, but Carmel et al. (13) did not. Slightly higher GnRH concentrations were found in the portal blood of castrated male rats than in intact male rats (14). With a bioassay, Seyler and Reichlin (15) reported higher GnRH concentrations in the serum of men who had been castrated more than 30 d previously, and even higher concentrations when those men were treated with estrogen. Mortimer et al. (16) found in extracted human serum that midcycle and postmenopausal women had detectable serum GnRH levels, but men and women in other phases of the menstrual cycle did not.

In addition to these attempts at measuring GnRH secretion directly, indirect assessment of the effects of testosterone on GnRH secretion has been attempted by implantation of testosterone into the rat hypothalamus and measuring the serum LH concentration. The serum LH concentration falls after implantation of testosterone into the hypothalamus (17, 18), but whether this fall is a result of an action of testosterone on GnRH secretion or an action directly on the pituitary gland after release of testosterone into the systemic circulation (17) is difficult to ascertain.

We have recently described a hypothalamic incubation system that offers the possibility of investigating the factors that regulate GnRH secretion (6). GnRH release into the incubation medium in this system is linear for 1 h; stimulated by membrane-depolarizing concentrations of potassium and ouabain; and is inhibited by omitting calcium from the incubation medium or by blocking calcium uptake with verapamil. Of particular relevance to our experiments was the demonstration that omission of calcium from the medium diminished basal, as well as stimulated, GnRH release. Although release of GnRH into an incubation medium cannot be equated with secretion in vivo, the demonstration that GnRH release in this system depends on membrane depolarization and calcium influx suggests that GnRH release in this system is under the control of at least some of the same physiologic factors that influence the secretion of most hormones. These considerations are especially germane to interpreting the parallel results of GnRH release into the incubation medium and of hypothalamic GnRH content. This parallel relationship raises the possibility that the GnRH released merely leaked out. The demonstration that GnRH release is under physiologic control, at least to some extent, is evidence against the possibility of leakage.

Data obtained with this hypothalamic incubation system show that GnRH release from the hypothalami of rats castrated 1 wk earlier was significantly less than

that from hypothalami taken from a control group of intact rats. Testosterone treatment of castrated rats prevented the fall in GnRH release. These changes in GnRH release suggest that the previously demonstrated decrease in hypothalamic GnRH content after castration (4), confirmed by these studies, may have been caused by a decrease in GnRH synthesis, rather than an increase in GnRH secretion.

These changes in GnRH release also suggest the possibility that the well-documented rise in serum LH concentration after castration is the result of increased pituitary sensitivity to a less than normal concentration of GnRH. Administration of exogenous GnRH to castrated male rats has already been shown to cause a greater LH response than to intact rats (19). Administration of testosterone to castrated male rats for 1 wk decreases the LH response to GnRH (17).

The possibility that the decrease in hypothalamic GnRH release and content after castration was caused by the postcastration rises in LH and(or) FSH, rather than by a decrease in the serum testosterone concentration, was evaluated by lowering the serum testosterone concentration by hypophysectomy. The resulting decrease in GnRH release, and prevention of that decrease by the administration of testosterone, suggests that testosterone, or one of its metabolites, is responsible for the observed effects on GnRH release.

The demonstration that testosterone can be metabolized to dihydrotestosterone (20) and to estradiol (21) in the hypothalamus, as well as in other tissues, suggested the possibility that the stimulatory effect of testosterone on GnRH release might require the conversion of testosterone to dihydrotestosterone or estradiol. Treatment of castrated rats with either dihydrotestosterone or estradiol prevented the fall in GnRH release. The necessity of the conversion of testosterone to either of these metabolites to stimulate GnRH release, therefore, remains a theoretical possibility.

We conclude GnRH release from incubated hypothalami taken from castrated rats is less than that from intact rats, and that treatment of castrated rats for 1 wk with testosterone or dihydrotestosterone or estradiol prevents this decrease. These data suggest the possibility that these steroids may maintain GnRH secretion in vivo.

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