BIOLOGY OF REPRODUCTION 50, 680–685 (1994)

Continuous Gonadotropin-Releasing Hormone Infusion Stimulates Dramatic Gonadal Development in Hypogonadal Female Mice[1]

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ABSTRACT

Adult hypogonadal (*hpg*) mice, lacking GnRH, have infantile reproductive systems and levels of pituitary gonadotropins that are lower than normal. The mutant mice respond to brain grafts containing GnRH neurons with gonadal development and increased production of gonadotropins. In view of the substantial literature regarding the nature and necessity of pulsatile GnRH stimulation of gonadotropins, we were not surprised in earlier studies to find that the majority of *hpg* mice with successful grafts have pulsatile LH secretion. It is not known, however, why LH pulsatility was undetectable in some animals with significant gonadal development. The present experiment was intended to determine the degree to which *hpg* mice respond to continuous infusion of GnRH via osmotic minipumps. Unexpectedly, female *hpg* mice exhibited dramatic ovarian and uterine growth after 15 or 30 days of continuous exposure to GnRH, with five- and eightfold increases in ovarian and uterine weights, respectively. Despite evidence of increased gonadotropin secretion in the treated *hpg* mice, pituitary stores of FSH and LH remained low. Similar treatment of normal female mice for 15 days also depleted pituitary concentrations of LH and FSH without significantly altering gonadal weights or plasma gonadotropin levels. It is clear from the present findings that inferences of pulsatile GnRH secretion based on stimulation of gonadal development in *hpg* mice should be made with caution.

INTRODUCTION

It is accepted that episodic secretion of GnRH into the portal circulation stimulates pulsatile LH secretion, which supports gonadal development. The *hpg* mouse, deficient in GnRH due to a deletion in the gene [1], shows increased gonadotropin production and gonadal growth in response to exogenously administered GnRH pulses [2, 3] or after receiving intrahypothalamic grafts of tissue containing GnRH cells [4, 5]. The majority of male [6] and female [7] *hpg* mice bearing such grafts show pulsatile LH secretion, with pulse frequencies that do not differ from those in normal control animals. We have presumed that those *hpg* mice that exhibited significant gonadal development after receiving grafts, but did not exhibit detectable LH pulses, were pulsing infrequently or below the limit of the assay. However, the possibility remained that GnRH from the grafts in some *hpg* mice was being secreted more or less continuously, due to a failure of the putative "pulse generator."

The present study was designed to test whether continuous administration of GnRH, delivered via osmotic minipump, would affect gonadotropin production and gonadal development in *hpg* female mice. We now report that constant administration of less than 1 μg/day GnRH stimulated gonadotropin secretion and dramatic gonadal development in *hpg* female mice. Pituitary gonadotropin stores were depleted in both *hpg* and normal mice.

Accepted November 1, 1993.

Received July 27, 1993.

[1]This work was supported by NIH grant HD 19077 to M.J.G. and a Sigma Xi-Rudin Foundation Summer Fellowship to H.K.

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MATERIALS AND METHODS

Animals

Normal adult female mice (C3H/HeH × 101H F1 hybrid intercrosses) and *hpg* mice of the same stock were housed in a colony room under a 14L:10D light cycle. Animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals. Food and water were available ad libitum. Osmotic minipumps (Model 2002, mean volume 235 μl; Alza Corp., Palo Alto, CA) secreting at a mean rate of 0.48 μl/h were filled with saline or with GnRH (Calbiochem, La Jolla, CA) diluted in saline, and equilibrated in saline at 37°C for 18–24 h before use. Minipumps were implanted s.c. in the intrascapular region of mice anesthetized with Metofane (Pitman-Moore, Inc., Washington Crossing, NJ). Vaginal cytology was assessed daily. At the end of each experiment, mice were given an overdose of chloral hydrate (800 mg/kg, i.p.), and blood for RIA of LH and FSH was obtained via intracardiac puncture. Pituitaries were removed, homogenized in 1 ml PBS, flash frozen, and stored at −20°C for subsequent RIA of LH and FSH. Aliquots were obtained for protein determinations [8]. Ovaries and uteri were dissected and weighed, and the ovaries were fixed in Bouin's for subsequent histology.

RIAs and Statistical Analysis

RIAs for LH and FSH were performed with the NIH kits for rat (r) LH and rFSH (courtesy NIDDK), expressed according to RP-2 standards. The mean intraassay coefficients of variation for the LH and FSH assays were 6.5% and 4.9%, respectively; the interassay coefficients of variation were 16.2% and 3.8%, respectively. Plasma samples (25 μl) were assayed in duplicate as previously described [7, 9]. Undetectable plasma values were assigned the limit of detection

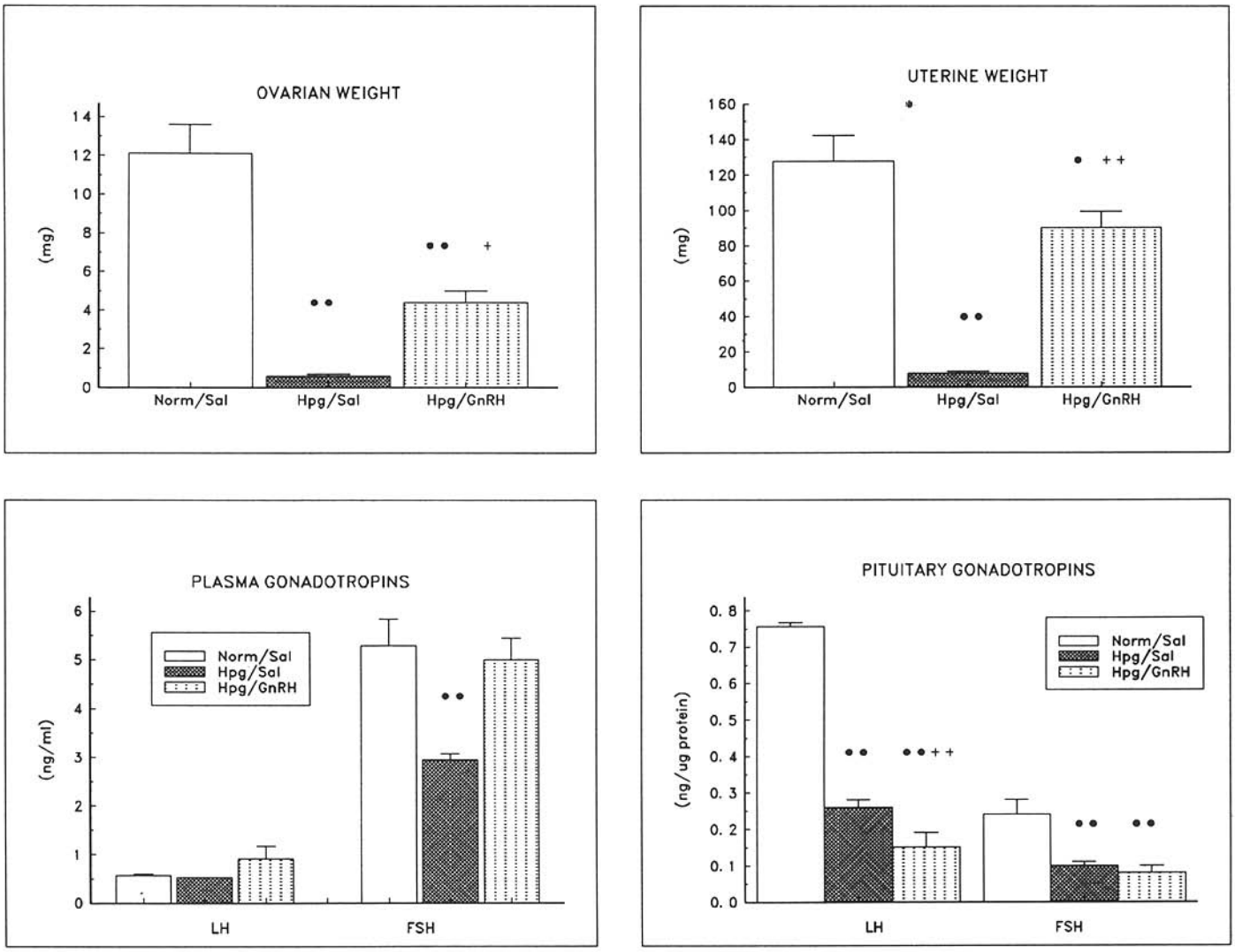

FIG. 1. Effect of chronic treatment with GnRH (40 ng/h, via osmotic minipump) for 15 days on ovarian and uterine weights and plasma and pituitary gonadotropin concentrations of *hpg* mice (Hpg/GnRH). Control normal (Norm/Sal) and *hpg* (Hpg/Sal) mice received saline via minipumps. •, $p < 0.05$; ••, $p < 0.01$ vs. Norm/Sal; +, $p < 0.05$; ++, $p < 0.01$ vs. Hpg/Sal.

of the assay (0.50 ng/ml for LH and 3.0 ng/ml for FSH) for statistical analysis. Statistical analyses were performed with ANOVA; logarithmic transformations of data were used when appropriate for unequal variances, and Tukey's test was used for post hoc comparisons if $p < 0.05$. All data are presented as means ± SEM.

RESULTS

Effect of Continuous Treatment with GnRH for 15 Days on hpg Female Mice

Normal mice 5–7 mo of age bearing saline minipumps (Norm/Sal, n = 11) continued to show ovarian cyclicity as evidenced by vaginal cytology, while *hpg* mice (Hpg/Sal, n = 8) of the same age had tiny vaginal orifices throughout the test period with sparse numbers of leukocytes and cornified cells in vaginal lavages. The *hpg* mice with minipumps secreting 40 ng GnRH/h (Hpg/GnRH, n = 12) had vaginal cytology similar to that of the Hpg/Sal mice for the first few days; but fully cornified cells were present by 5–12 days (median: 7 days) after pump implantation in 11 of 12 mice, accompanied by development of enlarged vaginas comparable to those in normal female mice. In two Hpg/GnRH animals, variations in vaginal cytology raised the possibility of ovulation, but this was not confirmed by the presence of CL when ovaries were studied after hematoxylin and eosin staining. In contrast to the undeveloped ovaries of the Hpg/Sal mice, those of the Hpg/GnRH mice frequently had large antral follicles (not shown).

Ovarian and uterine weights were significantly increased in Hpg/GnRH mice in comparison to Hpg/Sal mice (Fig. 1). Plasma LH levels were not significantly different among

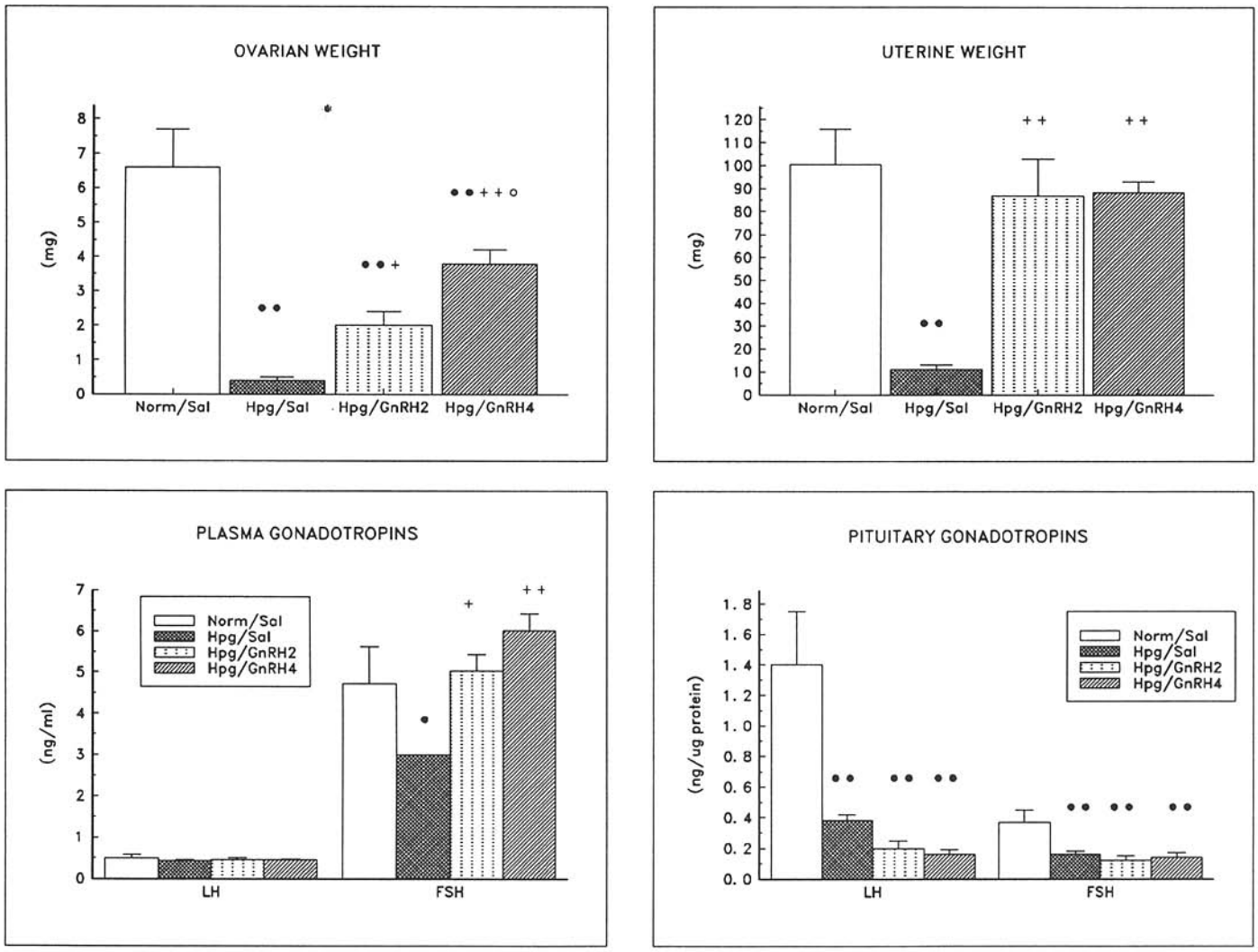

FIG. 2. Effect of chronic treatment with GnRH (33.3 ng/h, via osmotic minipump) for 2 (Hpg/GnRH2) or 4 (Hpg/GnRH4) wk on ovarian and uterine weights and plasma and pituitary gonadotropin concentrations of *hpg* mice. Control normal (Norm/Sal) and *hpg* (Hpg/Sal) mice received saline via minipumps. •, $p < 0.05$; ••, $p < 0.01$ vs. Norm/Sal; +, $p < 0.05$; ++, $p < 0.01$ vs. Hpg/Sal; ○, $p < 0.05$ vs. Hpg/GnRH2.

the groups, but LH was detectable in the plasma of 6 of 12 Hpg/GnRH mice vs. 0 of 8 Hpg/Sal and 3 of 11 normal mice. Plasma FSH increased to normal concentrations in Hpg/GnRH animals. In contrast, pituitary concentrations of both gonadotropins were significantly less than normal in both *hpg* groups, with LH levels lower in the Hpg/GnRH mice than in the control Hpg/Sal mice.

Effect of Continuous Treatment with GnRH for 30 Days on hpg Female Mice

Normal (Norm/Sal, n = 5) and *hpg* (Hpg/Sal, n = 6) female mice, 3–4 mo old, were given saline-filled minipumps; two groups of *hpg* mice were given minipumps secreting 33.3 ng/h GnRH. After 2 wk, one GnRH-treated group (Hpg/GnRH2, n = 5) and both saline-treated groups were killed. The pumps in the other GnRH-treated group (Hpg/GnRH4, n = 6) were replaced under Metofane anesthesia, and the mice were killed after a total of 4 wk of GnRH treatment.

GnRH treatment continued to significantly increase ovarian weights in the *hpg* mice, with greater stimulation after 4 than after 2 wk (Fig. 2). Uterine weights and plasma and pituitary gonadotropin levels, however, were similarly affected by 2 and 4 wk of continuous exposure to GnRH.

Effect of Continuous Treatment with GnRH on Normal Female Mice

Groups of 3- to 4-mo-old normal female mice were given s.c. minipumps secreting saline (Norm/Sal, n = 6) or 33.3 ng/h GnRH (Norm/GnRH, n = 6) for 15 days. Ovarian (9.4 ± 1.1 and 7.8 ± 0.8 mg, respectively) and uterine (136.4 ± 17.3 and 108.0 ± 18.1 mg, respectively) weights in the Norm/Sal and Norm/GnRH groups were not significantly different. Plasma LH (Norm/Sal: 0.63 ± 0.04 ng/ml; Norm/

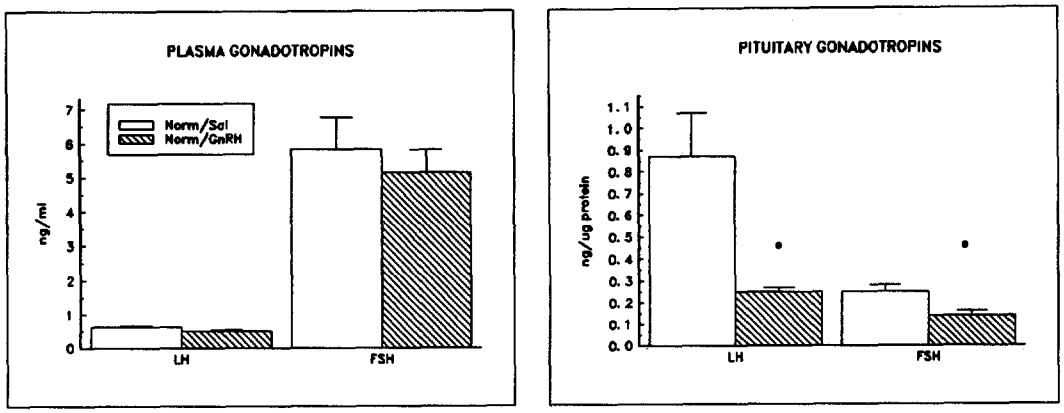

FIG. 3. Effect of chronic treatment with GnRH (33.3 ng/h, via osmotic minipump) for 15 days on plasma and pituitary gonadotropin concentrations of normal mice (Norm/GnRH). Control mice received saline (Norm/Sal) via minipumps. •, $p<0.05$ vs. Norm/Sal.

GnRH: 0.51 ± 0.03 ng/ml, $p \leq 0.06$) but not FSH levels were somewhat lower in the normal females receiving constant infusions of GnRH, while pituitary content of the two hormones was significantly diminished (Fig. 3).

DISCUSSION

Tonic administration of GnRH via osmotic minipump led to unexpectedly dramatic stimulation of gonadal development in *hpg* mice. The fivefold increase in ovarian weight after 15 days of continuous GnRH treatment was associated with follicular development and with steroidogenesis as reflected in vaginal morphology and cytology and vastly increased uterine weights. Elevated FSH levels and measurable LH in the plasma of many of the Hpg/GnRH mice indicated that gonadal development was stimulated by enhanced gonadotropin secretion. It is not clear whether gonadotropin synthesis was increased accordingly, as pituitary LH and FSH concentrations remained at or below those seen in the Hpg/Sal controls. The low concentrations of pituitary gonadotropins after 2 wk of chronic exposure to GnRH raised the possibility that the gonadotrophs of the *hpg* were becoming desensitized. The second study addressed this by maintaining GnRH administration for an additional 2 wk. Prolonged treatment resulted in continued increases in ovarian weights and FSH secretion and maintained uterine weights, while pituitary gonadotropin concentrations remained low. Interestingly, similar treatment of normal female mice for 2 wk also depleted pituitary concentrations of LH and FSH without significantly altering gonadal weights or plasma gonadotropin levels. In a dispersed rat pituitary perifusion system, it was reported that hourly pulses but not continuous infusion of GnRH resulted in stimulation of FSHβ mRNA over 12 h, while neither treatment affected LHβ mRNA levels; α subunit mRNA increased with either treatment [10]. In static culture of pituitary fragments with GnRH, only α subunit mRNA was increased, while hourly administration of GnRH in a perifusion system stimulated LHβ mRNA synthesis three- to fourfold over 6 h without affecting FSHβ mRNA [11].

Previous studies with *hpg* mice using periodic administration of 60 ng GnRH, delivered once every 2 h either by injection [2] for 15 days or by infusion pumps [3] for 18 days, showed significant stimulation of the reproductive system, while GnRH administered as a bolus of 1 μg/day was ineffective. Ovarian weights were not reported, but uterine weights ranged from about 35 to 48 mg in response to periodic GnRH, about half the value seen here with continuous implementation of 0.8–0.96 μg/day GnRH for 15 days. Serum LH increased within 15 min of an administered GnRH pulse [3] and was lower than normal at other times; serum FSH values were in the normal range [2]. In the absence of repeated measures of plasma gonadotropins with each treatment to assess possible dynamic changes, it is difficult to accurately compare pulsatile vs. tonic GnRH effects on LH secretion.

The increased FSH secretion seen in the present study may be sufficient to account for the notable ovarian development in the *hpg* mice chronically treated with GnRH. Treatment with FSH alone is capable of initiating ovarian growth in *hpg* mice as well as stimulating the development of antral follicles and steroidogenesis [12]. The possibility also remains that minipump-derived circulating GnRH may directly play a role in stimulating ovarian development. However, such an action is seen primarily in mature preovulatory follicles, and many inhibitory effects of GnRH on the ovary are also reported (reviewed in [13]).

The present findings are consistent with studies suggesting that FSH secretion is not as strictly dependent on the pattern of GnRH stimulation as is LH [14, 15]. For example, an elegant study of hypophysectomized, pituitary-grafted rats showed that LH but not FSH secretion is pulsatile in response to periodic GnRH administration [15] and that in this model estrogen inhibited LH but not FSH secretion. Uterine development, a bioassay for estrogen se-

cretion, suggests that significant levels of estrogen were secreted in the hypogonadal mice bearing minipumps and may have had a similar effect on LH but not FSH secretion.

Episodic GnRH essentially normalizes pituitary LH [2, 3] and FSH [2] concentrations in intact *hpg* female mice. The depletion in LH stores seen here in *hpg* mice in the first experiment, as well as in the normal females, was described in ovariectomized female [16] and intact male [17] rats exposed to continuous exogenous GnRH for 20 h [16] or six days [17]. Implantation of a potent GnRH agonist in s.c. depot into *hpg* male mice for seven days [18] reduced pituitary LH but not FSH content, while testes weights increased fivefold, suggesting stimulated gonadotropin secretion.

Since the demonstration that continuous infusion of a wide range of doses of GnRH fails to stimulate LH secretion in the ovariectomized, arcuate nucleus-lesioned rhesus monkey [19] while pulsatile administration quickly induces LH secretion, numerous reports have appeared regarding the effect of continuous vs. pulsatile GnRH administration on LH secretion both in vivo and in vitro. Women with idiopathic hypothalamic amenorrhea respond to continuous GnRH infusion with increased serum LH as measured by RIA but not by bioassay, and have unchanged serum FSH and estrogen levels [20]; however, pulsatile GnRH stimulates hormone production and ovulation [20, 21].

In contrast, continuous exposure to GnRH may be associated with normal reproductive function in several nonprimate species. Tonic infusion of GnRH induces ovulation in anestrous deer hinds [22], postpartum anestrous cows [23], seasonally anestrous mares [24], and anestrous bitches [25], in which ovulation occurs sooner with continuous than with pulsatile administration.

It is clear from the present findings that inferences of pulsatile GnRH secretion based on stimulation of gonadal development in *hpg* mice should be made with caution. We have now shown that female *hpg* mice may exhibit notable growth of their reproductive organs in response to chronic GnRH. Nevertheless, it is established that the majority of *hpg* mice bearing preoptic area grafts do show plasma LH pulsatility [6, 7] reflecting periodic GnRH secretion from their grafts. These mice may also ovulate reflexively [9] or (more rarely) spontaneously [26]. Further, in marked contrast to the effects of continuous exposure to GnRH, pituitary LH and FSH content is increased to within normal range in *hpg* female mice with GnRH cell-containing grafts [5, 9], as it is after administration of periodic GnRH to the mutant mice [2, 3].

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