Effects of cyclic steroid hormone replacement on prolactin and luteinizing hormone surges in female rats

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Abstract

The ability of steroid hormones to produce an LH or prolactin (PRL) surge was determined in rats ovariectomized at 6, 9 or 13 weeks of age and subjected to one, three or six cycles of estrogen and progesterone replacement. Sensitivity to steroid replacement was dependent on the age of the animal at the time of ovariectomy. Repeated cyclic steroid hormone replacement significantly increased the magnitude of the PRL response, but not the LH response, in animals ovariectomized at 6 weeks. The LH response was significantly altered by cyclic steroid replacement only in animals ovariectomized at 13 weeks. These results indicate that the mechanisms involved in the regulation of PRL secretion are influenced by steroid hormone replacement and that cyclic steroid hormone exposure increases the magnitude of the PRL secretory response.

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Introduction

Puberty is a complex process during which the prepubescent female progresses into a reproductively mature state, marking the onset of regular cyclicity and ovulation. A number of neural changes occur during embryonic and prepubertal development that permit activation of gonadotropin-releasing hormone (GnRH) neurons, and therefore the onset of puberty (see Gore 2002 for review). Circulating gonadal steroid levels gradually rise as GnRH pulse frequency and amplitude increase during pubertal development (Sisk *et al.* 2001), but puberty occurs due to GnRH neuronal activation, independent of gonadal steroids (see Gore 2002 for review).

A luteinizing hormone (LH) surge can be induced in mature ovariectomized females by several different methods, ranging from sequential injections of estrogen and progesterone (e.g. Hiemke *et al.* 1987, Bonavera *et al.* 1993) to steroid implants (e.g. Le *et al.* 1997, Yen & Pan 1998). Simultaneous treatment with both estrogen and progesterone appears to suppress the steroid-induced LH surge (Legan *et al.* 1973, McPherson *et al.* 1975, Attardi 1981). One limitation of administering steroids by implanting pellets is that constant high levels of estrogen and/or progesterone desensitize the pituitary gland to subsequent estrogen or GnRH stimulation (for review see Gharib *et al.* 1990). The method that most closely resembles the estrous cycle of the rat (estrogen followed by progesterone replacement 1–3 days later) is effective at producing an LH surge in the mature female rat from 1

day to 8 weeks after ovariectomy (Legan *et al.* 1973, Caligaris *et al.* 1974, Blake 1977, Adler *et al.* 1983, Clough & Rodriguez-Sierra 1983, Rubin *et al.* 1985, Pi 1986, Bonavera *et al.* 1993). The interval between ovariectomy and steroid replacement is important, because after ovariectomy, circulating gonadotropin levels rise due to the decline in steroid hormones. This causes the pituitary gland to become more responsive to GnRH (Legan *et al.* 1973), resulting in a higher LH peak response to estrogen and progesterone treatment over time. Furthermore, King & Letourneau (1994) reported that by 3 weeks post-ovariectomy, the sex differences in GnRH terminals in intact male and female rats no longer existed and the percentage of GnRH terminals containing GnRH decreased.

The aim of this study was to determine if the age of female rats at the time of ovariectomy affected the magnitude of the prolactin (PRL) or LH surge induced by steroid hormone replacement. Steroids were administered to rats ovariectomized at 6, 9 or 13 weeks of age. We hypothesized that age at ovariectomy would not influence the magnitude of the LH or PRL surge when repeated cyclic steroid hormone replacement was given.

Materials and Methods

Animals and treatments

Female rats (*Ratus rattus*) of the Sprague–Dawley strain were purchased from Harlan Laboratories (Indianapolis,

IN, USA). All rats were ovariectomized. The ages of rats at the time of ovariectomy were: 6 weeks (puberty was initiated and all animals had vaginal opening), 9 weeks (pubescent), or 13 weeks (mature). All animals were housed in the animal facility at Miami University on a 12 h light:12 h darkness cycle (lights on at 0700 h). Food and water were freely available. All procedures were performed in accordance with the National Institutes of Health (NIH) guidelines and were approved by the Miami University Institutional Animal Care and Use Committee (IACUC).

Bilateral ovariectomies were performed under isoflurane/oxygen gas anesthesia. Following a 7-day recovery period, rats were injected with 17- β -estradiol (50 μ g/kg, s.c.) at 1000 h (day 1). Two days later (day 3), animals received progesterone (2.5 mg/kg, s.c.) at 1000 h. Animals receiving a single cycle of steroid hormones were used in experiments on day 3. Animals receiving multiple cycles did not receive any steroids for 2 days (days 4 and 5) and the injection schedule was then repeated starting on day 6. This steroid replacement regimen was designed to mimic the 5-day estrous cycle. Animals from each age group received either one, three or six consecutive cycles of steroid replacement following this same injection procedure. Steroids were solubilized in sesame oil, and were purchased from Sigma.

The day after the final estrogen injection, in-dwelling jugular catheters were surgically implanted in each rat under isoflurane/oxygen gas anesthesia as previously described (Bryant *et al.* 1998). The following day, each rat received a final progesterone injection at 1000 h and blood samples were withdrawn at 1200, 1400, 1500, 1600, 1700, 1900 and 2100 h. Blood volume was immediately replaced with an equal volume of sterile, heparinized (50 U/ml) saline. Following the last blood sample, rats were killed. All blood samples were stored at 4°C until centrifuged (1000 g). Plasma was collected and stored at -20°C until subjected to radioimmunoassay.

Immediately after the rats had been killed (2100 h), the uterus (including the cervix) was removed from each rat and the fat was removed. Uteri were blotted on absorbent paper, and wet uterine weights were recorded. Wet uterine weights are reported as a percentage of body weight.

Radioimmunoassay

Plasma samples were assayed in duplicate for LH and PRL using reagents provided by the National Hormone and Peptide Program (NHPP), the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), and Dr A F Parlow. Hormone levels were expressed in terms of rat PRL RP-3 or rat LH RP-3. Goat anti-rat gamma globulin was purchased from Antibodies Inc. (Davis, CA, USA) and 125 I-labeled PRL or 125 I-labeled LH was obtained from Covance Laboratories (Vienna, VA, USA). Intraassay and interassay coefficients of variation were less than 4% and 7% respectively.

Statistical analysis

Data for serial LH and PRL levels, and wet uterine weight/body weight, were analyzed using a repeated measures ANOVA. Maximum LH and PRL levels were defined as the highest respective hormone levels between 1400 and 1900 h. For consistency, a 'surge' in LH or PRL was achieved if that value exceeded the group mean basal level by more than six standard deviations (surge > mean_{basal levels} + 6S.D._{basal levels}). Group means were compared using a Bonferroni multiple comparison *t*-test and overall error rates were controlled at $\alpha = 0.05$. The relationship between the number of cycles of steroid hormone replacement and the tendency to reach surge levels was analyzed using Fisher's exact test for categorical data. All statistical analyses were performed on the Miami University SAS system.

Results

Multiple cycles of steroid replacement significantly increased the maximum PRL levels in females ovariectomized at 6 weeks, as well as the maximum LH level in females ovariectomized at 13 weeks (Table 1). In females ovariectomized at 6 weeks, maximum PRL levels were significantly higher following six cycles of steroid replacement compared with one cycle ($P = 0.0001$). The probability that steroids induced a PRL surge was greater following multiple cycles of replacement only in animals ovariectomized at 6 weeks ($P = 0.0357$) (Table 2). Multiple cycles of steroid treatment increased maximum LH levels only in females ovariectomized at 13 weeks, with a significant increase occurring after three cycles of estrogen and progesterone (E + P) compared with one ($P = 0.0019$) and six cycles ($P = 0.0003$) (Table 1). The probability that multiple cycles of steroid replacement would induce an LH surge was not significantly affected by age of the animal at the time of ovariectomy. There were no significant differences in basal levels of LH or PRL between steroid-replaced and non-treated animals. Basal LH levels in non-steroid-treated animals ranged from 8.3 to 42 ng/ml (mean, 25.5 ± 14.6). Basal PRL levels in non-steroid-treated animals ranged from 0.8 to 12.47 ng/ml (mean, 3.29 ± 2.95).

Ovariectomy at 6 weeks

Only one of five females ovariectomized at 6 weeks of age had a significant LH or PRL surge following one cycle of E + P replacement and the PRL surge was very high (Fig. 1, Table 2). Following three consecutive E + P treatments, two of six rats showed an LH surge, while four had a significant PRL surge. After receiving six cycles of E + P, only three of five rats (60%) had an LH surge, but all of these animals had a PRL surge. Representative LH and PRL surges for each treatment group are shown in Fig. 1.

Table 1 LH and PRL levels in female rats following steroid replacement.

Age at OVX (weeks)	E + P replacement (no. of cycles)	LH basal level (ng/ml)	LH surge level (ng/ml)	PRL basal level (ng/ml)	PRL surge level (ng/ml)
6 (n = 5)	1	7.7 ± 1.3	18.7 ± 42.6	2.0 ± 0.7	37.1 ± 54.6
6 (n = 6)	3	8.9 ± 1.8	80.2 ± 38.9	11.8 ± 5.0	216.0 ± 49.8
6 (n = 5)	6	9.2 ± 2.1	90.5 ± 42.6	10.8 ± 6.8	450.0 ± 54.6*
9 (n = 6)	1	7.5 ± 1.7	13.5 ± 38.9	4.1 ± 0.4	76.5 ± 49.8
9 (n = 6)	3	11.4 ± 2.9	43.6 ± 38.9	5.5 ± 1.4	157.6 ± 49.8
9 (n = 5)	6	13.9 ± 2.0	21.8 ± 42.6	6.4 ± 1.8	230.2 ± 54.6
13 (n = 7)	1	12.0 ± 1.8	32.8 ± 36.0	4.2 ± 3.2	186.0 ± 46.1
13 (n = 5)	3	31.0 ± 6.3	163.1 ± 38.9†	22.4 ± 10.4	253.9 ± 49.8
13 (n = 8)	6	15.9 ± 3.4	19.8 ± 14.7	17.1 ± 7.6	208.1 ± 66.5

n, number of animals in each treatment group.

*In females ovariectomized (OVX) at 6 weeks, six cycles of E + P produced a significant increase in maximum PRL levels compared to animals receiving one cycle ($P = 0.0001$).

†In females ovariectomized at 13 weeks, maximum levels of LH following three cycles of steroid replacement was greater than after either one ($P = 0.0019$) or six ($P = 0.0003$) cycles.

Ovariectomy at 9 weeks

One cycle of steroid hormone replacement did not induce an LH surge in any animal ovariectomized at 6 weeks of age. However, after three or six cycles of steroid replacement, some animals had a significant increase in LH. Only one female had an LH surge after six cycles of steroid hormones and this surge occurred earlier than expected. Significant PRL surges were observed in some animals in all treatment groups. See Fig. 2 for representative LH and PRL surges in animals ovariectomized at 9 weeks of age (see also Table 2).

Ovariectomy at 13 weeks

Only one of seven rats that received a single cycle of E + P exhibited an LH surge, whereas four animals in this group had PRL surges. After receiving three cycles of E + P, 60% had an LH surge, and all rats had a PRL surge. When females received six cycles of E + P, six of eight rats had a PRL surge, but none had an LH surge.

Table 2 Summary of the effects of repeated cycles of steroid replacement on the LH and PRL surges (ng/ml) in female rats ovariectomized at different ages.

Age at OVX (weeks)	E + P replacement (no. of cycles)	No. of animals exhibiting LH surge	No. of animals exhibiting PRL surge*
6	1	1/5 (20%)	1/5 (20%)
6	3	2/6 (33%)	4/6 (67%)
6	6	3/5 (60%)	5/5 (100%)
9	1	0/6 (0%)	5/6 (83%)
9	3	2/5 (40%)	3/5 (60%)
9	6	1/5 (20%)	4/5 (80%)
13	1	1/7 (14%)	4/7 (57%)
13	3	3/5 (60%)	5/5 (100%)
13	6	0/8 (0%)	6/8 (75%)

*There was a greater probability ($P = 0.0357$) that PRL levels would reach defined surge values following multiple cycles of E + P in females ovariectomized (OVX) at 6 weeks.

Representative LH and PRL surges are shown in Fig. 3 (see also Table 2).

It is important to note that in every case, regardless of age or treatment, females that had an LH surge also had a concomitant PRL surge; however, many animals had a PRL surge without a concomitant LH surge.

Figure 1 Representative LH and PRL levels in female Sprague-Dawley rats ovariectomized at 6 weeks of age and treated with one, three or six cycles of E + P. **Figure 2** Representative LH and PRL levels in individual female Sprague-Dawley rats ovariectomized at 9 weeks of age and treated with one, three or six cycles of E + P.

Figure 3 Representative LH and PRL levels in individual female Sprague-Dawley rats ovariectomized at 13 weeks of age and treated with one, three or six cycles of E + P.

Wet uterine weight and body weight

Body weight increased gradually with age in all groups (data not shown). Both age and the number of E + P replacement cycles significantly affected wet uterine weight. Following three cycles of E + P treatment, rats ovariectomized at 13 weeks showed a significant increase ($P=0.0001$) in uterine weight over 6-week-old rats receiving the three cycles (Fig. 4). In addition, among rats ovariectomized at 13 weeks there was a significant increase in uterine weight after three cycles of steroid treatment compared with one ($P=0.0001$) or six cycles ($P=0.0013$).

Discussion

This study is the first to demonstrate that a PRL surge can be induced by steroids in young rats, and that the amplitude of the surge significantly increases with repeated cyclic exposure to steroids. Although LH levels showed a similarly increasing trend with age, this trend

Figure 4 Wet uterine weight at the time of killing (2100 h) as a percentage of body weight for animals ovariectomized at 6, 9 or 13 weeks of age and replaced with E + P for one, three or six cycles. In rats ovariectomized at 13 weeks, there was a significant increase in uterine weight after three cycles of steroid treatment compared with one ($P=0.0001$) or six cycles ($P=0.0013$). Values with the same letter are not significantly different. *Significant increase ($P=0.0001$) compared with 6-week-old rats receiving three cycles of E + P.

was not significant. Wet uterine weight, an index of estrogen sensitivity in females (Ojeda *et al.* 1976), increased significantly with steroid treatment only in animals ovariectomized at 13 weeks.

Although the cyclic steroid replacement regimen used in this study was unique, the low doses of E + P were similar to those used by others (Blake 1977, Hiemke & Ghraf 1984, Rubin *et al.* 1985, Pi 1986, Hiemke *et al.* 1987, Lee *et al.* 1990, Bonavera *et al.* 1993). Adult females, regardless of the doses of E + P administered, showed much larger LH surges (Blake 1977, Rubin *et al.* 1985, Pi 1986, Lee *et al.* 1990) than were observed in our study. In addition, dose and/or timing of steroid treatment, the age of the animal at ovariectomy (Adler *et al.* 1983, Clough & Rodriguez-Sierra 1983) as well as the recovery period (Legan *et al.* 1973) were different. In agreement with our results, Aguan *et al.* (1996) reported that female rats ovariectomized at 10 weeks of age had low LH levels in response to steroid replacement, even when higher doses of E + P were administered. Bonavera *et al.* (1993) also reported that LH surges were lower when doses of steroids were similar to those used in this study. PRL levels were not reported in any of the previous studies. Indeed, the only studies of the steroid-induced PRL surge in ovariectomized females were conducted in adults (Chen & Meites 1970, Gudelsky *et al.* 1981, Steele & Myers 1990, Mai *et al.* 1994, Yen & Pan 1998) and results varied widely, with maximum PRL levels similar to, or larger than, those reported here.

The probability of repeated steroid treatment inducing a PRL surge increased significantly in females ovariectomized at 6 weeks. Steroid hormones are known to increase the secretory ability of individual lactotroph cells. When adult, ovariectomized female rats received E + P replacement for 10–14 days, there was an increase in the number of lactotrophs that actively release PRL, as well as in the overall lactotroph cell number, and increased amounts of PRL were secreted by individual lactotrophs (Livingstone *et al.* 1998). We have found that pituitary cells from mature cycling females were more sensitive to angiotensin II stimulation of PRL release compared with young or aged females, or males of any age (Janik *et al.* 1997), suggesting that cyclic steroid exposure is important in the PRL response to secretagogues. GnRH may stimulate angiotensin II, thus contributing to increased PRL secretion (DePaul *et al.* 2000). The effects of steroids on lactotrophs at the time of puberty (~30 days) (Gore 2002) have not been examined after cyclic steroid replacement, but may be cumulative with successive numbers of E + P cycles.

The cyclic steroid replacement regimen used in this study prevented the post-ovariectomy rise in basal LH levels, indicating that the negative feedback control of estrogen was still maintained and that the high LH levels seen after this time were not artifacts of ovariectomy alone (Legan *et al.* 1973). The most responsive females were those ovariectomized at 13 weeks and subjected to three

cycles of steroid replacement. Indeed, following six cycles of replacement, steroids did not induce an LH surge in any of the animals ovariectomized at this age. The reason for this loss of responsiveness is not known, but one possibility is that the pituitary and/or hypothalamus may lose sensitivity to steroids. LH-releasing hormone (LHRH) immunoreactivity (Rubin & King 1994, 1995, Wise 1982), as well as c-fos nuclear expression in LHRH neurons (Lloyd *et al.* 1994, Rubin *et al.* 1994, 1995) decreased in middle-aged rats when regular cyclicity ceased. Although the rats used in our study were not middle-aged, the pattern of steroid replacement, while cyclic, is unlikely to mimic exactly the endogenous, cyclic patterns of secretion that occur in intact females. Furthermore, the only exposure to steroid hormones that these animals received was at doses sufficiently high to induce LH secretion (Blake 1977, Hiemke & Ghraf 1984, Rubin *et al.* 1985, Pi 1986, Hiemke *et al.* 1987, Lee *et al.* 1990, Bonavera *et al.* 1993). With multiple exposures at this level, the hypothalamus and/or pituitary may lose sensitivity to stimulation. Interestingly, the increase in wet uterine weight following successive E + P treatments was also most pronounced in animals ovariectomized at 13 weeks of age following three cycles of steroid replacement. These data suggest that by 13 weeks, the uterus is more responsive to steroid replacement and that the sensitivity decreases with greater exposure.

The LH response appeared to be less sensitive to steroid stimulation compared with the PRL response. All females in which a steroid-induced LH surge was detected also had a concomitant PRL surge, but females that had a PRL surge did not necessarily have a concomitant LH surge. This was not unexpected since the regulation of these hormones occurs through distinct neural pathways (Steele & Myers 1990). The PRL surges that were generated without concomitant LH surges were not stress induced, because even more frequent blood sampling did not significantly alter circulating hormone levels (Fox & Smith 1985) and similar blood sampling methods did not produce stress-induced PRL increases (Bryant *et al.* 1998).

In conclusion, this is the first report of the effects of cyclic steroid hormone replacement on LH and PRL levels in animals ovariectomized at different ages. As early as 6 weeks of age, the LH and PRL secretory systems are capable of producing surges in response to steroid hormones, and PRL, but not LH, has a greater response to multiple cycles of E + P. Since puberty occurs due to GnRH neuronal activation, independent of gonadal steroids (see Gore 2002, for review), the neural mechanisms controlling LH release are not affected by steroids, but those involved in PRL regulation are influenced by steroid hormone replacement.

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