Neonatal Androgenization Potentiates the Inhibitory Effects of Blood Withdrawal on Ovulation in the Rat (44059)

JIN-FANG LIN, KIRSTEN M. VOGELSONG, AND ROBERT T. CHATTERTON, JR.[1]

Department of Obstetrics and Gynecology, Section of Reproductive Endocrinology and Infertility, Northwestern University Medical School, Chicago, Illinois 60611

Abstract. **Rats treated on Day 5 of life with testosterone propionate (TP) were tested for their ovulatory response to pregnant mare's serum gonadotropin (PMSG) on the 30th day of life with and without cardiac puncture under ketamine/xylazine anesthesia. TP without cardiac puncture in doses of 0.625, 1.25, or 2.5 μg did not inhibit ovulation, but 5.0 μg of TP inhibited ovulation in 15 of 16 rats. When cardiac puncture was performed at 1900 hr in rats not given TP, seven of eight ovulated on Day 33. However, none of the rats that received either 1.25 or 2.5 μg of TP ovulated after cardiac puncture at 1900 hr, and only one of seven given the 0.625-μg dose ovulated. Thus, the stress of bleeding greatly enhanced the inhibitory effect of otherwise ineffective doses of TP. The preovulatory luteinizing hormone (LH) surge was delayed and diminished by neonatal TP in a dose-related manner in animals from which blood was drawn from a previously inserted catheter. When blood drawn from similarly TP-treated animals by cardiac puncture, serum levels of LH were further reduced. Progesterone, given at 1100 hr on Day 32, was capable of partially overcoming the inhibitory effects of the combined treatments on both ovulation and serum LH levels at all but the highest dose of TP tested (5.0 μg). We conclude that neonatal exposure to androgen sensitizes rats to ovulation-inhibiting factors, such as bleeding, in a manner which appears to delay as well as inhibit the preovulatory release of LH. Such early exposure to androgen, therefore, may determine the susceptibility of individuals to reproductive failure in adult life.** [P.S.E.B.M. 1996, Vol 213]

High-dose neonatal androgenization of the female rat results in an inability of the hypothalamus to respond to estrogen with an increase in luteinizing hormone (LH) secretion in adulthood (1, 2). Estrogen administration fails to stimulate an increase in norepinephrine (NE) turnover in the medial preoptic area (MPOA) or median eminence (ME) of the androgen sterilized rat (ASR) as occurs in normal animals (3). This is not due to a decrease in estrogen receptor numbers or binding affinities in the preoptico-hypothalamic region (4), and induction of progesterone receptors by estradiol is normal in ASR (5). However, if NE is infused in the third ventricle in the presence of estradiol, a normal luteinizing hormone (LH) response is observed. Thus, it appears that neonatal androgen treatment of the female rat results in a defect in the NE response to increasing estradiol titers.

Although progesterone does not increase the magnitude of the LH response to either estradiol (3) or electrical stimulation (6) in normal female rats, it increases the LH response in ASR rats to that of the normal control animals. However, in normal rats progesterone does advance the time of the LH surge by 1–2 hr (7).

The ability of the pituitary to respond to LH-releasing hormone (LHRH) is apparently not affected

[1] To whom requests for reprints should be addressed at Department of Obstetrics and Gynecology, Northwestern University Medical School, 333 East Superior Street, Chicago, IL 60611.

Received December 27, 1995. [P.S.E.B.M. 1996, Vol 213]
Accepted July 14, 1996.

Dr. Jin-fang Lin was supported by the Shanghai Medical University throughout the course of this study.

0037-9727/96/2133-0268$10.50/0

in ASR, although the LH requirement in ASR for ovulation is increased 5- to 10-fold over that of normal rats (8).

Stress may also result in suppression of gonadotropin secretion in female rats. Footshock (9) and immobilization but not several other stressors (10) result in suppression of LH secretion in male or ovariectomized female rats. Biggio *et al.* (11) have reviewed the function of GABA receptors in the brain and conclude that these receptors play a major role in the pathophysiology of stress and anxiety. Since antagonism of either GABA-A or GABA-B receptors has been shown to greatly amplify the LH response to NE, these inhibitory systems may represent a point of convergence of the suppression of LH by both neonatal androgen treatment and by stressors in adult life.

In order to test the hypothesis of an interaction between stress and neonatal androgen, we exposed rats to doses of androgen below the range that results in the delayed anovulatory syndrome (DAS). DAS is induced by treatment of the neonate with testosterone propionate (TP) in doses between 5 and 10 μg. Anovulation occurs after an initial period of cyclicity and fertility (12, 13). Loss of the positive feedback response of the H-P axis to ovarian steroids eventually leads to a loss of the preovulatory LH surge and ovulation (1, 14). We therefore exposed rats to TP at doses of 5.0 μg and less. Animals were given pregnant mare's serum gonadotropin (PMSG) on Day 30 of life, to induce follicular development and ovulation, and were bled at 1900 hr by cardiac puncture on Day 32 before checking for ovulation on the morning of Day 33. To assess the effects of a stressor we initially chose to immobilize the animals. However, the bleeding under ketamine/xylazine anesthesia proved to be a sufficient stressor in itself. We also administered progesterone early on Day 32 to compare the effect on ovulation with that previously described in unstressed, neonatally androgenized rats.

Materials and Methods

Animals and Environment. Pregnant Sprague-Dawley rats were purchased at 16–18 days of gestation and maintained in air-conditioned quarters with a commercial standard diet and water available *ad libitum*. The lighting schedule was 14:10 hr light:dark, with lights on between 0600 and 2000 hr. The day of birth was defined as Day 1 if delivery was observed to occur before 1700 hr.

Experimental Design. On Day 5 of life, all females of a litter received a single sc injection of either 0.0, 0.625, 1.25, 2.5, or 5.0 μg of TP in 0.1 ml of sesame oil. All oil- or androgen-treated rats were weaned at 21 days and were given 12 IU of PMSG sc in 0.1 ml of isotonic saline on Day 30 between 0900 and 1000 hr.

We first investigated the dose dependency of the effect of neonatal TP on ovulation. Rats were sacrificed on the morning (1000–1100 hr) of the 3rd day after PMSG administration (Day 33). Certain rats also received 0.5 mg of progesterone in 0.1 ml of sesame oil sc at 1100 hr on Day 32. In each group given a particular dose of TP some rats were bled (0.5 ml) at 1900 hr of Day 32 by cardiac puncture within 10 min after an injection of ketamine and xylazine (87 mg/kg + 13 mg/kg body wt). Ovulation was ascertained by counting ova in the oviducts on the morning of Day 33 (15).

Additional neonatally androgenized and normal rats received cardiac catheters on Day 29. These catheters were inserted into the jugular vein, exteriorized through the skin at the back of the neck, and extended outside the animal's cages. The catheters were filled with a solution of heparin (5 units/ml) and physiological saline, and were sealed until blood samples were drawn on Day 32.

All procedures involving animals were approved by the Animal Care and Use Committee of Northwestern University.

Hormone Assays. Serum was separated from blood by centrifugation and was stored in sealed vials at −20°C prior to assay. Serum LH was measured by a double-antibody radioimmunoassay technique. Materials and the procedure were provided by the National Hormone and Pituitary Program of the NIDDK, NIH, at the University of Maryland. The reference for the LH assay was NIH-rLH-S25. The intra- and interassay coefficients of variation were 12% and 14%, respectively; the sensitivity of the assay was 0.02 ng/ml.

Statistical Analyses. Data are presented as means ± SEM. Differences between the numbers of animals ovulating in the several groups were evaluated by Fisher's exact test. Differences in ova counts were assessed by analysis of variance and Sheffe's *post hoc* test. Significance was considered to be reached at $P < 0.05$. There were 12 rats in the control group and 5 to 8 rats in each of the other groups.

Results

Effect of Neonatal Exposure to TP on Ovulation. The effect of neonatal exposure to TP on ovulation is presented in Table I. All rats given neonatal TP in the range of 0.625–2.5 μg ovulated following PMSG treatment. The average number of ova shed in the combined TP-treated groups did not differ from that of the controls that received the sesame oil vehicle. However, with the higher dose of 5 μg TP, ovulation occurred in only 1 of 16 rats, and that 1 released only five ova.

Combined Effects of Bleeding and Neonatal TP on Ovulation. Among rats that did not receive TP neonatally, 87% ovulated with 20 ± 2 ova per ovulat-

Table I. Suppression of Ovulation by Cardiac Puncture in Neonatally TP-Treated Rats and Its Reversal by Progesterone

Dose of TP	Cardiac puncture	Progesterone	Number ovulating	Ova per ovulating rat
0.0	No	−	12/12	22 ± 3
0.0	1900 hr	+	7/8	20 ± 2
0.625	No	−	5/5	23 ± 2
0.625	1900 hr	−	1/7	6
0.625	1900 hr	+	6/6	14 ± 2
1.25	No	−	9/9	23 ± 2
1.25	1900 hr	−	0/9	0
1.25	1900 hr	+	6/6	13 ± 2
2.5	No	−	5/5	17 ± 2
2.5	1900 hr	−	0/10	0
2.5	1900 hr	+	5/5	9 ± 0.4
5.0	No	−	1/16	5
5.0	No	+	5/5	12 ± 2
5.0	1900 hr	−	0/8	0
5.0	1900 hr	+	1/5	11

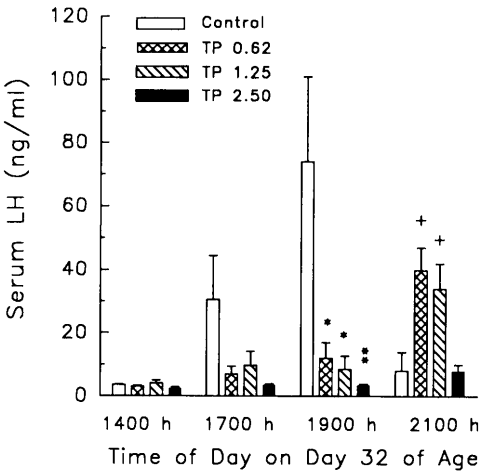

Figure 1. Serum LH on Day 32 after PMSG (12 IU) on Day 30 in rats treated neonatally with different doses of TP. Blood was drawn from cardiac catheters that had been implanted on Day 29. $^{*}P < 0.01$ and $^{**}P < 0.001$ relative to control at 1900 hr; $^{+}P < 0.01$ relative to control at 2100 hr. The number of rats in the control, 0.625, 1.25, and 2.50 μg TP groups was five, seven, and seven, respectively. Vertical lines show one SEM.

ing rat after having been bled at 1900 hr on Day 32 (Table I). Among rats that received TP, only one rat that had been given the lowest dose of TP (0.625 μg) ovulated, and that one released only six ova. None of the rats that received doses of 1.25 μg of TP or greater and were bled under anesthesia at 1900 hr of Day 32 ovulated on Day 33.

Effect of Progesterone on Ovulation. After administration of 0.5 mg of progesterone on the morning (1100 hr) of Day 32, all rats given 5 μg of TP neonatally ovulated after the standard PMSG treatment, but with significantly fewer ova than animals not given TP. However, progesterone treatment was not capable of restoring ovulation in rats that had received 5 μg of TP

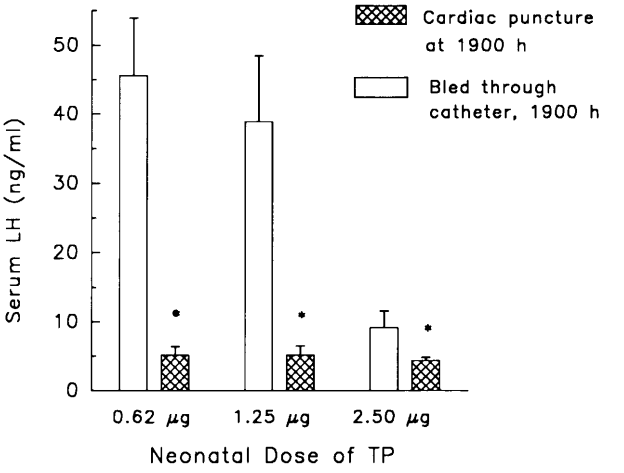

Figure 2. Serum levels of LH at 2100 hr in rats that were bled by cardiac puncture under anesthesia at 1900 hr or bled without anesthesia through an indwelling cardiac catheter at the same hour on Day 32 of age after receiving PMSG on Day 30. $^{*}P < 0.01$ between groups with cardiac puncture versus those bled through a catheter. There were between five and seven rats per group.

and were bled under anesthesia at 1900 hr of day 32. Only one of five such rats ovulated.

With lower doses of TP, however, progesterone was effective in rescuing ovulation in all rats that were bled at 1900 hr, albeit with a significantly lower number of ova than in control animals.

Luteinizing Hormone Levels in Neonatally Androgenized Rats. Plasma LH levels in unanesthetized cardiac-catheterized rats on the afternoon and evening of Day 32 after PMSG treatment are shown in Figure 1. In control rats, the peak of the LH surge occurred at 1900 hr. Doses of TP of 0.625 and 1.25 μg resulted in significantly lower LH levels at 1900 hr and with some increase at 2100 hr. The 2.50-μg dose, which resulted in release of fewer than the normal number of ova but did not inhibit ovulation in any such treated rats, did not exhibit an increase in LH within the time frame of the study.

Figure 2 shows a comparison of the serum LH levels at 2100 hr in animals that received cardiac puncture under ketamine/xylazine anesthesia at 1900 hr. All animals received PMSG treatment on Day 30. It is clear that there is an additional suppression in levels of LH at this time point which is attributable to the stress of the cardiac puncture procedure.

The effect of progesterone administration on serum LH, when administered on the morning of Day 32 at 1100 hr, in rats that had received PMSG on Day 30 is shown in Figure 3. Progesterone produced significant increases in serum LH concentrations which were inversely related to the dose of TP that the animals had received.

Discussion

The normal pattern of central nervous system development is affected by the presence of specific go-

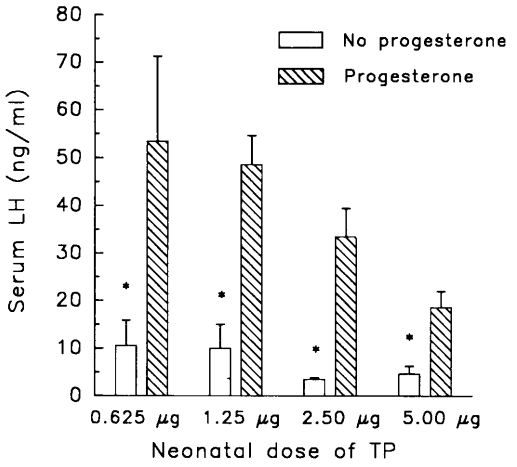

Figure 3. Serum LH measured at 1900 hr in rats bled by cardiac puncture on Day 32 after receiving PMSG on Day 30. Progesterone (0.5 mg) or vehicle was administered at 1100 hr on Day 32. *$P < 0.01$ between groups with and without progesterone. There were between five and eight rats per group.

nadal steroids during critical periods. Testicular testosterone secretion causes the brain to differentiate along male lines (16–18). Neonatal exposure of female rats to lower levels of TP, between 5 and 10 μg, results in a condition described as the delayed anovulatory syndrome, or DAS (12, 13).

In the present study, doses of TP less than 5.0 μg had no effect on the percentage of animals ovulating or on the number of ova released by immature rats in response to PMSG. The effect of bleeding under ketamine/xylazine at 1900 hr alone was minimal as would be expected, since this is well after the well-established critical period for LH release (1400 hr) 2 days after PMSG treatment (19, 20). Thus, in the present study the combination of an ineffective neonatal dose of TP and a bleeding procedure that was too late in the day to effect LH release (7, 19–21) resulted in almost complete inhibition of ovulation. Measurements of serum LH on the evening of Day 32 revealed that neonatal TP treatment had an effect on the time and magnitude of LH released in response to the PMSG treatment. Doses that were more than 10-fold lower than those used in previous studies still produced significant suppression of the LH surge.

The inhibition of ovulation by bleeding under ketamine/xylazine anesthesia in rats neonatally exposed to a low dose of androgen was an unexpected finding in this study. We were prepared to immobilize the animals to study the interaction of neonatal TP and stress, but the effect of bleeding at 1900 hr on the evening before ovulation itself inhibited ovulation, and so an additional stressor was not required. Neonatal TP treatment resulted in sufficient delay in the LH surge that the bleeding procedure, which was performed at 1900 hr, was actually performed before the LH surge. This permitted the two factors to act together to suppress the LH surge.

It is interesting to speculate about how neonatal TP treatment could act to increase the susceptibility of the animal to the effects of a stressor which was administered shortly before the release of the preovulatory LH. High-dose neonatal TP treatment is known to block the ability of estrogen to evoke increases in NE in the hypothalamus (3). Low-dose TP may partially inhibit this process such that the amount of NE released is limiting or nearly limiting. Any factor that also impinges on the availability of NE would reduce the amount of NE to a point that it is no longer sufficient to stimulate LHRH release. A stressor such as bleeding under anesthesia may act to decrease NE directly or to increase the turnover of GABA, an inhibitory neurotransmitter that is produced in the brain in response to stress (11).

Progesterone restored ovulation in all TP-treated rats even at the highest dose of TP. However, the numbers of ova were decreased, even with the lowest dose of TP, when the stress of bleeding was added to TP treatment. Since serum LH levels were restored to values that were not different from the group receiving 0.625 μg of TP without cardiac puncture, the low number of ova in this progesterone-treated group cannot be explained at this time.

To conclude, even though relatively low doses of TP (0.625–2.5 μg) do not affect PMSG-induced ovulation, they result in a delay of the proestrus LH surge by at least 4 hr. Because of the delayed LH surge, cardiac puncture under anesthesia at 1900 hr of proestrus was able to exert its effects before initiation of the LH surge. The combination of low-dose neonatal TP treatment and exposure to a mild stressor shortly before LH release, neither of which inhibit ovulation itself, inhibited PMSG-induced ovulation. Exogenous progesterone counteracted the effect of neonatal exposure to TP on ovulation but was less effective in inhibiting the superimposed effect of bleeding under anesthesia.

1. Harlan RE, Gorski RA. Steroid regulation of luteinizing hormone secretion in normal and androgenized rats at different ages. Endocrinology **101**:741–749, 1977.
2. Mennin SP, Gorski RA. Effects of ovarian steroids on plasma LH in normal and persistent estrous adult female rats. Endocrinology **96**:486–491, 1975.
3. Lookingland KJ, Wise PM, Barraclough CA. Failure of the hypothalamic noradrenergic system to function in androgen-sterilized rats. Biol Reprod **27**:268–281, 1981.
4. Barley J, Ginsberg M, MacLusky NJ, Morris ID, Thomas PJ. Sex differences in the distribution of cytoplasmic oestrogen receptors in rat brain and pituitary; Effects of gonadectomy and neonatal androgen treatment. Brain Res **129**:309–318, 1977.
5. Etgen AM. Estrogen induction of progestin receptor in the hypothalamus of male and female rats which differ in their ability to exhibit cyclic gonadotropin secretion and female behavior. Biol Reprod **25**:307–313, 1981.
6. Chappel SC, Barraclough CA. Plasma concentration changes in

LH and FSH following electrochemical stimulation of the medial preoptic area or dorsal anterior hypothalamic area of estrogen- or androgen-sterilized rats. Biol Reprod **15**:661–669, 1976.
7. McCormack CE, Strauss WF. Reversal by progesterone of phenobarbitone blockade of ovulation in the prepubertal rat primed with pregnant mare serum gonadotropin. J Endocrinol **65**:177–182, 1975.
8. Mennin SP, Kubo K, Gorski RA. Pituitary responsiveness to luteinizing hormone-releasing factor in normal and androgenized female rats. Endocrinology **95**:412–416, 1974.
9. Rivier C, Rivier J, Vale W. Stress-induced inhibition of reproductive functions: Role of endogenous corticotropin-releasing factor. Science **231**:607–609, 1986.
10. Blake CA. Effects of "stress" on pulsatile luteinizing hormone release in ovariectomized rats. Proc Soc Exp Biol Med **148**:813–815, 1975.
11. Biggio G, Concas A, Corda MG, Giorgi O, Sanna E, Serra M, GABAergic and dopaminergic transmission in the rat cerebral cortex: Effect of stress, anxiolytic and anxiogenic drugs. Pharmacol Ther **48**:121–142, 1990.
12. Gorski RA. Influence of age on the response to prenatal administration of a low dose of androgen. Endocrinology **82**:1001–1004, 1968.
13. Swanson HE, Van der Werff ten Bosch JJ. The 'early-androgen' syndrome: Its development and response to hemi-spaying. Acta Endocrinol **45**:1–12, 1964.
14. Harlan RE, Gorski RA. Correlations between ovarian sensitivity, vaginal cyclicity, and luteinizing hormone and prolactin secretion in lightly androgenized rats. Endocrinology **101**:750–759, 1977.
15. Rowlands IW, Williams PC. Production of ovulation in hypophysectomized rats. J Endocrinol **3**:310–315, 1943.
16. McLusky NJ, Naftolin F. Sexual differentiation of central nervous system. Science **211**:1294–1303, 1981.
17. McEwen BS. Gonadal steroids and brain development. Biol Reprod **22**:43–48, 1980.
18. Toran-Allerand CD. Gonadal hormones and brain development: Implications for the genesis of sexual differentiation. Ann NY Acad Sci **435**:101–111, 1984.
19. Blake CA. Differentiation between the "critical period," the "activation period" and the "potential activation period" for neurohumoral stimulation of LH release in proestrous rats. Endocrinology **95**:572–578, 1974.
20. Everett JW, Sawyer CH, Markee JE. A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cyclic rat. Endocrinology **44**:234–250, 1949.
21. Ching M. Correlative surges of LHRH, LH and FSH in pituitary stalk plasma and systemic plasma of the rat during proestrus. Neuroendocrinology **34**:279–285, 1982.