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EFFECTS OF STEROID IMPLANTS ON THE PREPUBERTAL INCREASE IN CIRCULATING GONADOTROPINS AND SEXUAL RECEPTIVITY IN THE FEMALE RABBIT

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Summary—The pubertal increase in gonadotropins in the female rabbit was inhibited 14–42-fold with Silastic implants of progesterone (P4) testosterone propionate (TP), estradiol benzoate (EB) or P4/EB placed subcutaneously on Day 24 of life. Rabbits with empty implants showed the normal prepubertal increase in circulating gonadotropins. By contrast, rabbits with implants of P4 only, had a 2-fold decrease in LH secretion when peak areas were compared. However, FSH secretion though slightly depressed was not significantly different from controls. The prepubertal increase in circulating gonadotropins was completely suppressed by implants of EB, TP and combined P4/EB. At 115-days-of-age, sexual receptivity and mating were absent in EB-treated animals and significantly suppressed in P4-treated ones when compared to controls, all of which mated. Mating was not completely inhibited in TP and combined P4/EB animals. Corpora lutea were found in all rabbits that mated. In the sexually non-receptive does, vaginal stimulation induced an LH surge in 2 of 15 animals. Ovarian weights and follicular development were significantly suppressed in rabbits with EB implants. Ovarian estradiol content was significantly increased in P4- and TP-treated rabbits. Maximum specific binding for [^{3}H]naloxone was suppressed in the hypothalami of P4-treated rabbits. These results suggest that the prepubertal increase in circulating gonadotropins may have an essential role in the control of sexual maturation in the female rabbit.

INTRODUCTION

Previous studies from our laboratory indicated that there is a prepubertal increase in gonadotropin secretion around days 35–60 of life [1]. This increase in gonadotropins correlated very well with a corresponding increase in opiate binding in the hypothalamus [2]. The female rabbit is able to mate, conceive and have a litter around 100-days-of-age and at a weight of 3 kg [3–5]. Whether the prepubertal increase in gonadotropins around day 35–60 has a role in sexual maturation is not known. To examine this question we have used the approach of inhibiting the prepubertal gonadotropin increase with implants of steroids and determining the sexual receptivity of the rabbits at about 100-days-of-age. In addition, we sought to determine whether chronic steroid treatment can influence opioid binding in prepuberty.

MATERIALS AND METHODS

New Zealand white rabbits were obtained from local breeders and kept in individual cages on a 12 h light:12 h dark schedule with food and water available *ad libitum*.

Female rabbits, 22 days *post partum*, were purchased. On Day 24 subcutaneous steroid implants were inserted under local anaestheisa. Dow Corning Silastic tubing (i.d. 0.078 in.) was used to give 2-cm implants. Steroids used to fill the tubing were estradiol benzoate (EB), testosterone propionate (TP), or progesterone (P4). The implants were kept in 3% BSA containing 0.1% sodium azide for at least 24 h before insertion under the skin. Controls received empty tubing.

Five groups of 6 females each were used to determine the effects of chronic steroid treatment on gonadotropin profiles and sexual receptivity. The treatments were: control—empty implants, EB, P4, TP and combined P4/EB. After insertion of the implants blood samples were taken on a weekly basis until they were 106-days-old and greater than 3 kg in weight, when they were checked for sexual receptivity with two or more proven bucks. If no mating occurred with 2 different bucks on two separate occasions vaginal stimulation was performed with a glass rod. Blood samples were taken prior to exposure to males and 1.5 h after coitus or vaginal stimulation. Animals were killed 24–72 h after mating or vaginal

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stimulation, uteri weighed and one ovary fixed for histological examination. The other ovary was frozen for steroid determinations. The histological presence of corpora lutea combined with an elevated LH level 90 min after coitus or vaginal stimulation were the criteria used for sexual maturation.

Radioimmunoassays

LH and FSH were measured by the double antibody radioimmunoassay technique previously described [6,7]. The LH standard was WP 360A (Dr A. F. Parlow), 1 ng of which was equivalent to 30 pg pure rabbit pituitary LH (EX 130 GB, Dr H. Papkoff). All LH results are expressed in terms of this pure standard. The first antibody was guinea-pig anti-rabbit LH, 7F GP ā LH (Dr R. J. Scaramuzzi). The antigen used was LER-1056-C2 (Dr L. E. Reichert, Jr). The sensitivity of the assay was 42 pg with intra- and inter-assay coefficients of variation of 4.8 and 18% respectively.

Reagents for the assay of FSH were provided by Dr A. F. Parlow. The antigen AFP-9688-C was used for iodination and standards. The antibody, AFP-4-7-21-76, was prepared in guinea-pigs. The sensitivity of the assay was 80 pg with intra- and inter-assay coefficients of variation of 13.2 and 21% respectively.

The radioimmunoassay for progesterone, testosterone and estradiol was the same as previously described [8].

Analysis

Results were subjected to one-way analysis of variance or t-test as appropriate. A P-level of 0.05 was considered significant. LH and FSH levels during development were also subjected to another type of analysis. The concentrations for each animal was plotted on grid paper and the areas under the curves computed with the Bioquant digitizing morphometric system [9]. The mean areas for each group were then compared. The results of the mating experiments were subjected to Chi-square analysis using the "Stats Plus" (Human Systems Dynamics, Northridge, Calif.) program for the Apple IIe.

The Bioquant System was also used to perform morphological analyses of the histological sections as described by Jarrell *et al.*[9]. Every fifth section was analyzed and only follicles with an oocyte were counted. Seven ovarian features were quantitated—follicles with granulosa cell layers of 2–3 cells, 4–6 cells, >6 cells; antral follicles, atretic follicles, corpora lutea, and anovulatory corpora lutea.

In another series of experiments, opioid binding in the hypothalamus was determined by the method described by Wilkinson and YoungLai[2]. Four series of experiments were performed. Implants of EB, P4 and diethystilbestrol (DES) were placed on Day 23. Animals were killed between 38–47 days and the hypothalami examined for [^{3}H]naloxone binding. The means of each series of experiments were then pooled and analyzed for differences among treatment groups.

RESULTS

Figure 1 shows the mean peripheral levels of LH and FSH in female rabbits bearing steroid implants. All steroid treatments effectively prevented the prepubertal increase in LH as seen when the LH areas for the period day 24–72 were analyzed (Table 1). On the other hand, FSH levels were not significantly suppressed by P4 but by EB, TP and P4/EB. In spite of the increases shown in the LH levels in the control animals analysis of variance indicated that there were no significant differences among the means within this group. However, LH levels within the P4-treated group were significantly different.

The results of testing for sexual receptivity are shown in Table 2.

One animal from each group was removed from analysis because of loss of implant or unexplained spinal injury. Both EB and P4 separately prevented coitus. None of the EB-treated group mated and vaginal stimulation failed to cause an increase in LH. Histological examination of the ovaries of EB-treated

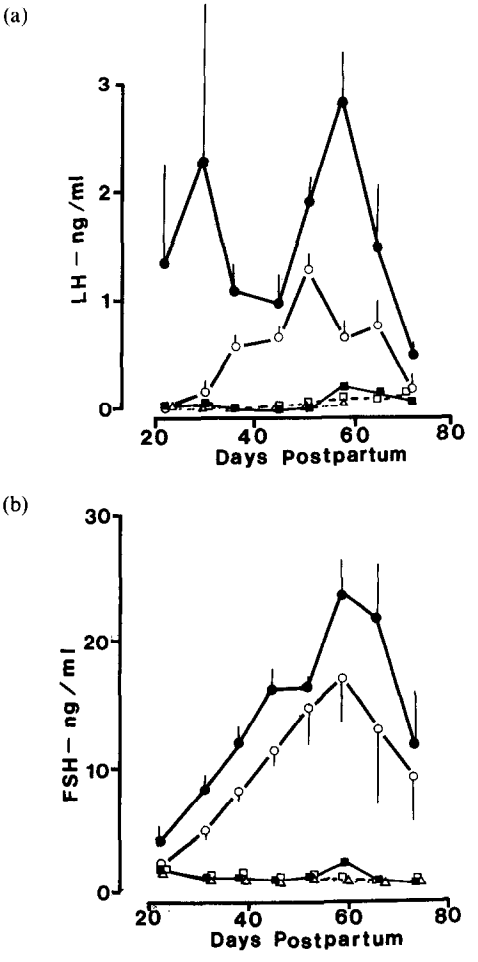

Fig. 1. Plasma (a) LH and (b) FSH in female rabbits bearing steroid implants. Solid circles = empty implants; open circles = progesterone; solid squares = estradiol benzoate; open squares = estradiol benzoate and progesterone; open triangles = testosterone propionate.

Table 1. Comparison of areas under peak LH and FSH values for rabbits with steroid implants ($n = 5$) mean ± SEM

	LH area (cm^2)	FSH area (cm^2)
Controls	12.68 ± 4.82^a	28.6 ± 6.04^a
P4	$6.19 \pm 1.57_b$	21.95 ± 8.90^a
EB	0.74 ± 0.42^c	2.16 ± 0.48^b
P4/EB	0.38 ± 0.25^c	2.37 ± 0.30^b
TP	0.30 ± 0.14^c	2.42 ± 0.25^b

Values with the same superscript are not significantly different. Areas under peak LH and FSH values from Day 24–72 were computed with a Bioquant Digitizing morphometric system.

Table 2. Mating response of female rabbits on Day 113–117 after having steroid implants on Day 24

Implant	Mated	Not mated	P
Empty	5	0	—
EB	0	5	0.002
P4	1	4	0.009
P4/EB	2	3	0.164
TP	3	2	0.435

Results were analyzed by Chi-square. Responses to vaginal stimulation are not included in the mating responses.

rabbits showed that follicular development was retarded with preantral follicles dominating and very few antral follicles. Call-Exner bodies were frequently seen in the follicles.

Of the five rabbits in the P4-treated group, one successfully mated, had a post-coital LH level of 52 ng/ml and 3 corpora lutea in one ovary. The remaining four animals were vaginally stimulated and one of these had a post-stimulation LH level of 12.6 ng/ml and 2 corpora lutea in one ovary. Ovaries from the other 3 rabbits had numerous cystic follicles. The two animals that mated in the P4/EB group, had LH values 90 min after mating, of 1 ng/ml compared to the controls which were 26.4 ± 4.3 ng/ml. No corpora lutea were found in the ovaries of these two rabbits. The other 3 rabbits were vaginally stimulated but no increase in LH could be elicited. Antral follicles were found in one ovary. The other ovaries had anovulatory corpora albicans, well developed nests of interstitial tissue and follicles which had more than four layers of granulosa cells.

In the TP group all the recovered Silastic tubings were empty, indicating that all the steroid had diffused out. Two does mated and had 90 min LH levels of 5.6 and 3 ng/ml but only one had fresh corpora lutea in its ovary. The ovary of the other rabbit that mated had many cystic follicles. The remaining rabbits were vaginally stimulated and one of these had an LH level of 29 ng/ml and 4 corpora lutea were present in its ovary the day after. A number of ovaries showed evidence of intraovarian follicular rupture. A similar finding was discovered in 2 ovaries of rabbits with EB implants.

The weights of the uteri and ovaries compared to body weights are shown in Table 3. The body weights of the combined P4/EB-treated group were significantly different from those of controls. Uterine weights of the TP group were significantly suppressed and the overies of all steroid treated animals were significantly decreased. Ovarian estradiol was elevated in P4 and TP groups.

Peripheral steroid levels are shown in Table 4. Progesterone levels were significantly increased in the P4 and TP groups while estradiol was increased in the TP group.

The results of the morphometric analysis of the ovaries are shown in Table 5. A significant decrease in the number of follicles having 4–6 layers of granulosa cells was observed in the EB. No corpora lutea were found in rabbits with implants of EB.

Opioid binding was depressed in hypothalami of P4-treated rabbits (Table 6). There was a slight decrease in naloxone binding in EB-treated rabbits but this was not as significant as in the DES treated rabbits. There was no significant difference in the dissociation constant for all treatment groups.

DISCUSSION

The results of the present study indicate that the chronic administration of ovarian steroids to female rabbits can cause a developmental delay in sexual maturation. At the time of testing for sexual receptivity all does were greater than 3 kg and older than 100 days, two criteria previously used for sexual maturation [3, 4]. The mechanism for this delay appears to be due to suppression of the prepubertal increase in gonadotropins, particularly LH. Chronic exposure to P4 resulted in a significant suppression of LH while not altering FSH secretion. Only one rabbit mated in the P4-treated group suggesting that the unaltered FSH secretory pattern may not have a major role in regulating sexual receptivity whereas the suppressed LH may have. Further support for this notion is seen in the mating behaviour of the TP and combined P4/EB groups where both LH and FSH secretion

Table 3. Uterine and body weights of female rabbits 24–48 h after mating or cervical stimulation on Days 113–117 after having steroid implants on Day 24

Implant	Body wt (kg)	Uterine wt (g)	Ovarian wt (mg)	Estradiol (pg/mg protein)
Empty	3.47 ± 0.06	11.92 ± 1.38	195 ± 23	1.26 ± 0.30
EB	3.54 ± 0.13	16.28 ± 4.67	$42 \pm 5^{**}$	2.75 ± 0.28
P4	3.38 ± 0.11	8.26 ± 3.38	$147 \pm 16^{*}$	$3.74 \pm 0.86^{*}$
P4/EB	$3.80 \pm 0.11^{*}$	13.39 ± 2.85	$73 \pm 18^{*}$	2.85 ± 0.30
TP	3.56 ± 0.25	$6.06 \pm 1.32^{**}$	$105 \pm 5^{*}$	$3.67 \pm 0.67^{*}$

$^{*}P < 0.05$, $^{**}P < 0.005$ compared to controls.

Table 4. Steroid levels prior to mating or vaginal stimulation

	Progesterone (pg/ml)	Testosterone (pg/ml)	Estradiol (pg/ml)
Empty	557 ± 49	125 ± 21	52 ± 11
EB	556 ± 100	93 ± 5	58 ± 3
P4	1062 ± 223[a]	122 ± 33	50 ± 12
P4/EB	563 ± 89	114 ± 17	48 ± 5
TP	734 ± 70[a]	172 ± 48	158 ± 53[a]

For peripheral steroid levels between groups, values with the same superscript are not significantly different from each other (Duncan's multiple range test).

Table 6. Specific binding of [^{3}H]naloxone to hypothalamic slices of 38–47-day-old female rabbits bearing steroid implants from Day 23. ($n = 4$)

Steroid treated	B_{max}	K_d
Control (empty implants)	2086 ± 190	2.71 ± 0.43
EB	1571 ± 157	3.65 ± 1.17
P4	1123 ± 273*	1.79 ± 0.09
DES	1156 ± 102*	1.62 ± 0.26

For B_{max} there was a significant interaction between the groups ($P = 0.01$, ANOVA); *$P < 0.05$ compared to controls. There was no significant difference among the treatment groups for K_d (ANOVA).

were suppressed but some matings still occurred. These latter results would suggest that the prepubertal increase in both gonadotropins between days 35–60 may be necessary for sexual receptivity at an age greater than 100 days. However, it is difficult to dissociate the direct effects of steroids on mating behaviour and their effects on the suppression of gonadotropin release. Previous studies have indicated that testosterone and combined P4/EB can elicit sexual receptivity in ovariectomized rabbits [10, 11]. It is, therefore, possible that the matings seen are direct effects of steroids. The absence and small number of corpora lutea found in these mated rabbits support this interpretation.

Preferential suppression of the prepubertal increase in LH without a significant effect on FSH secretion with progesterone implant is also a very interesting finding. It is not known what role, if any, ovarian inhibin has on prepubertal gondotropin levels. However, the fact that EB, TP and EB/P4 suppressed FSH to undetectable levels, indicates that inhibin may not be an important factor at this age. Since sexual receptivity i.e. mating was significantly suppressed in this group it can be concluded that FSH is not required for sexual receptivity. Although ovarian weight was suppressed in these animals (Table 3) follicular development was similar to controls (Table 5) but a 43% increase in antral follicles was found in the progesterone group indicating that FSH was effective.

The effect of the steroid implants could also be seen at the various target organs. The combined P4/EB treatment significantly increased body weight of all steroid-treated animals over controls. Uterine weight was suppressed in TP-treated animals. Ovarian weights of all steroid-treated animals were suppressed reflecting an apparent diminished stimulation by gonadotropins. Although there was a general increase in ovarian estradiol this was only significant in the P4- and TP-treated animals. Androgens have been shown to increase ovarian estradiol and progesterone formation [12–14]. Increased circulating levels of progesterone and estradiol in TP implanted rabbits confirm the findings in the ovaries. These data, therefore, provide additional support for the role of testosterone in stimulating estrogen and progesterone synthesis. These products can further act to suppress gonadotropin release from the pituitary.

Suppression of circulating gonadotropins by EB and P4/EB implants along with prevention of sexual receptivity in the EB-treated group may be due to effects on hypothalamic neurocircuitry. There are numerous reports in the literature which suggest that estrogens produce changes in the hypothalamic wiring pattern [15–19]. As a consequence of decreased gonadotropins development and maturation of ovarian follicles are prevented. Evidence for a change in the hypothalamus is seen in the binding of naloxone an opioid antagonist, which is suppressed in chronic steroid treatment (Table 6). Estradiol can cause irreparable damage to the arcuate nucleus of female rats [20]. However, chronic estradiol treatment has been found to enhance opioid binding in the female rat hypothalamus [21]. It would, therefore, appear that opioid tone in the female rabbit brain is decreased by exogenous steroids.

As far as we are aware there are no previous reports on the delay of sexual maturation with chronic steroid treatment in rodents. A recent report in sheep indicates that puberty in lambs can be delayed by chronic treatment with estradiol [22].

Table 5. Morphological analysis of ovaries from female rabbits treated with steroid implants from Day 22

	Follicles						
Treatment	2–3 cells	4–6 cells	>6 cells	Antral	Atretic	CL	Anovulatory CL
Control	353 ± 112	126 ± 40	15 ± 8	74 ± 7	41.2 ± 11.5	8.5 ± 2.3	9.5 ± 4.4
EB	266 ± 39	59 ± 15	10 ± 4	37 ± 6	24 ± 9	0	16.7 ± 8.7
P4	357 ± 86	170 ± 19	11 ± 2	106 ± 6	21.2 ± 0.5	1.2 ± 1.2	32.2 ± 2.7
P4/EB	315 ± 29	85 ± 14	9 ± 1	52 ± 11	11.7 ± 4.1	0	18.2 ± 5.7
TP	289 ± 69	159 ± 40	15 ± 5	87 ± 10	14.5 ± 2.6	3.2 ± 1.8	23.5 ± 3.0

CL = corpora lutea. 4–6 cells $P < 0.02$. ANOVA; P4 > P4/EB > EB and TP > EB (Duncan's multiple range). CL $P < 0.001$. ANOVA; control > TP > P4/EB > EB (Duncan's multiple range). For atretic follicles, the TP and P4/EB groups were significantly fewer than controls. Anovulatory CL were significantly higher in P4 rabbits than in controls. For antral follicles, the number in ovaries from P4-treated rabbits was greater than those in controls, combined P4/EB and EB. Antral follicles in EB-treated rabbits were significantly suppressed compared to all other treatment groups except the combined P4/EB.

Direct effects of chronic steroid treatment on the ovary cannot be completely ruled out since ovarian effect have been observed in the intact [12] as well as the hypophysectomized rat [23]. However, there are no available data on the effects of steroid treatment on the prepubertal increase in gonadotropins and subsequent follicular development or sexual receptivity. Morphometric analysis of serial sections of ovaries indicated that EB treatment had a dramatic effect in preventing further development of ovarian follicles. A direct ovarian effect is suggested by the similar inhibition of gonadotropin release in all the steroid treated rabbits except for the P4-treated animals. Further support for a direct ovarian effect is seen in the ovaries of TP-treated rabbits. A number of these had evidence of intraovarian follicular rupture. The consequences of all these ovarian activity would be a lack of follicular development to a stage which can be induced to ovulate or to prime the hypothalamus for lordosis induction.

In summary, our data suggest that the chronic treatment of juvenile rabbits with ovarian steroids can cause a developmental delay in sexual maturation and receptivity.

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REFERENCES

1. YoungLai E. V.: Age-related change in the concentration of serum gonadotropins and cholesterol in the female rabbit. *J. Endocr.* **109** (1986) 287–290.
2. Wilkinson M. and YoungLai E. V.: Development of opiate ([^{3}H]naloxone)-binding sites in female rabbit brain: correlation with prepubertal gonadotropin secretion. *Biol. Reprod.* **35** (1986) 572–578.
3. Hulot F., Mariana J. C. and Lebas F.: L'establissement de la puberte chez la lapine (folliculogenese et ovulation). Effet du rationnement alimentaire. *Reprod. Nutr. Dev.* **22** (1982) 439–453.
4. Kamwanja L. A. and Hauser E. R.: The influence of photoperiod on the onset of puberty in the female rabbit. *J. Animal Sci.* **56** (1983) 1370–1375.
5. de Turckheim M., Berger M., Jean-Faucher Ch., Veyssiere G. and Jean Cl.: Changes in ovarian estrogen and in plasma gonadotropins in female rabbits from birth to adulthood. *Acta Endocr. (Copenh.)* **103** (1983) 125–130.
6. Moor B. C. and YoungLai E. V.: Variations in peripheral levels of LH and tetosterone in adult male rabbits. *J. Reprod. Fert.* **42** (1975) 259–266.
7. Armstrong R. W., Gauldie J. and YoungLai E. V.: Effects of active immunization of female rabbits against testosterone. *J. Endocr.* **79** (1978) 339–347.
8. YoungLai E. V.: Administration of GnRH *in vivo* stimulates progesterone and inhibits androgen accumulation by ovarian follicles isolated from pubertal rabbits. *J. Steroid Biochem.* **22** (1985) 91–96.
9. Jarrell J. F., YoungLai E. V., Barr R., O'Connell G., Belbeck L. W. and McMahon A.: An analysis of the effects of increasing doses of ionizing radiation to the exteriorized rat ovary on follicular development, atresia, and serum gonadotropin levels. *Am. J. Obstet. Gynec.* **154** (1986) 306–309.
10. Beyer C., Vidal N. and Mijares A.: Probable role of aromatization in the induction of estrous behaviour by androgen in the ovariectomized rabbit. *Endocrinology* **87** (1970) 1386–1389.
11. McDonald P., Vidal N. and Beyer C.: Sexual behaviour in the ovariectomized rabbit after treatment with different amounts of gonadal hormones. *Horm. Behav.* **1** (1970) 161–172.
12. Armstrong D. T., Moon Y. S. and Leung P. C. K.: Uterotropic effects of testosterone and 5α-dihydrotestosterone in intact and ovariectomized immature female rats. *Biol. Reprod.* **15** (1976) 107–114.
13. Daniel S. A. J. and Armstrong D. T.: Site of action of androgens on follicle-stimulating hormone-induced aromatase activity in cultured rat granulosa cells. *Endocrinology* **114** (1984) 1975–1982.
14. Schomberg D. W., Stouffer R. L. and Tyrey L.: Modulation of progestion secretion in ovarian cells by 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone): a direct demonstration in monolayer culture. *Biochem. Biophys. Res. Commun.* **68** (1976) 77–81.
15. Arai Y. and Matsumoto A.: Synapse formation of the hypothalamic arcuate nucleus during post-natal development in the female rat and its modifications by neonatal estrogen treatment. *Psychoneuroendocrinology* **3** (1978) 31–45.
16. Gorski R. A.: Long-term hormonal modulation of neuronal structure and function. In *The Neurosciences, Fourth Study Program* (Edited by F. O. Schmitt and F. G. Worden). MIT Press, Cambridge (1979) 969–982.
17. Garcia-Segura L. M., Baetens D. and Naftolin F.: Synaptic remodelling in arcuate nucleus after injection of estradiol valerate in adult female rats. *Brain Res.* **366** (1986) 131–136.
18. Matsumoto A. and Arai Y.: Synaptogenic effect of estrogen on the hypothalamic arcuate nucleus of the adult female rat. *Cell Tissue Res.* **198** (1979) 427–433.
19. Matsumoto A. and Arai Y.: Sexual dimorphism in "wiring pattern" in the hypothalamic arcuate nucleus and its modifications by neonatal hormonal environment. *Brain Res.* **190** (1980) 238–242.
20. Brawer J., Naftolin F., Martin J. and Sonnenschein C.: Effects of a single injection of estradiol valerate on the hypothalamic arcuate nucleus and on reproductive function in the female rat. *Endocrinology* **103** (1978) 501–512.
21. Wilkinson M., Brawer J. and Wilkinson D. A.: Gonadal steroid induced modification of opiate binding sites in anterior hypothalamus of female rats. *Biol. Reprod.* **32** (1985) 501–506.
22. Foster D. L., Ryan K. D., Goodman R. L., Legan S. J., Karsch F. J. and Yellon S. M.: Delayed puberty in lambs chronically treated with estradiol. *J. Reprod. Fert.* **78** (1986) 111–117.
23. Hillier S. G. and Ross G. T.: Effects of exogenous testosterone on ovarian weight, follicular morphology and intraovarian progesterone concentration in estrogen-primed hypohysectomized immature female rats. *Biol. Reprod.* **20** (1979) 261–268.