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Prenatal Exposure of the Ovine Fetus to Androgens Sexually Differentiates the Steroid Feedback Mechanisms That Control Gonadotropin Releasing Hormone Secretion and Disrupts Ovarian Cycles

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Exposure of the female sheep fetus to exogenous testosterone in early pregnancy permanently masculinizes the reproductive neuroendocrine axis. Specifically, in utero androgens given to female lambs from day 30 to 90 of a 147 day pregnancy dramatically altered the response of the gonadotropin releasing hormone (GnRH) neuronal network in the hypothalamus to both estrogen (E) and progesterone (P) feedback. Elevated concentrations of estrogen stimulated a massive release of GnRH in gonadectomized female sheep; however, male and androgenized female lambs were unable to respond to high E concentrations by producing this preovulatory-like "surge" of GnRH. Further, the inhibitory actions of progesterone (P) were also sexually differentiated and adult males and androgenized females were much less responsive to P-negative feedback than normal ewes. The consequences of these abnormal steroid feedback mechanisms were reflected in the fact that only 72% of ovary-intact androgenized ewes exhibited normal estrous cycles in their first breeding season whereas none had a single estrous cycle during the second breeding season. In contrast, 100% of the control animals exhibited repeated reproductive cycles in both seasons. These data indicate that a relatively short exposure to male hormones during in utero life permanently alters the neural mechanisms that control reproduction and leads progressively to a state of infertility.

KEY WORDS: sexual dimorphism; estrogen; progesterone; LHRH; sheep.

The reproductive neuroendocrine axis of many species of mammal is sexually dimorphic as a result of exposure of the fetus/neonate to male steroid hormones in early pre- or postnatal life (Gorski, 1985). Androgens exert an organizing action on the central nervous system during a window of development that is species-specific as well as being unique for any sexually dimorphic trait. In the sheep, the "critical period" for sexual differentiation of the brain areas controlling reproduction has been well defined and occurs in the first half of pregnancy, lasting from approximately day 30 to day 90 of gestation (Clarke, Scaramuzzi, & Short, 1976; Short, 1974). More recently, a series of studies have shown that in utero exposure to androgens causes the gonadotropin releasing hormone (GnRH) neuronal network that controls luteinizing hormone (LH) release to develop in a male-typical manner (Wood & Foster, 1998). The studies described in this article show that ovariectomized ewe lambs that have experienced abnormally elevated concentrations of androgens during early fetal life have markedly reduced responsiveness of the GnRH system to both estrogen and progesterone feedback. The consequences of such abnormal regulation of GnRH secretion for the generation of estrous cycles in the ovary-intact androgenized ewe are explored.

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MATERIALS AND METHODS

Animals and prenatal androgen treatment. Estrous cycles were synchronized in ewes of the Dorset breed in October/November using vaginal Controlled Internal Drug Release (CIDR) devices (InterAG, New Zealand) for 7–12 days followed by an im injection of pregnant mare serum gonadotropin. Mating was detected using a raddled Poll Dorset ram and pregnancy confirmed and the number of fetuses determined by ultrasound at between 60 and 90 days of gestation (term 147 days). Fetuses were androgenized by giving the mothers twice weekly injections of testosterone propionate (Sigma; im; 100 mg/injection in vegetable oil) from day 30 to 90 of pregnancy. This encompasses the whole of the critical period for sexual differentiation. Controls received no injections. Lambs were born in late February/early March and maintained outdoors at the Babraham Institute. Procedures were carried out with the approval of the local animal welfare and ethics committee and under Home Office Licence PPL 80/1037. Three separate studies were performed. The first two used animals that were ovariectomized to determine the response of the GnRH neuronal network to ovarian steroid feedback, whereas the third explored whether ovary-intact androgenized animals could exhibit normal reproductive cycles.

Experiment 1

Response of the GnRH neuronal network to stimulation by estrogen. The aim of this study was to determine if adult sheep that have been exposed to either endogenous or exogenous androgens (males and androgen-treated females, respectively) during the critical period can generate a preovulatory-like surge of GnRH in response to elevated concentrations of estrogen. This study was performed on seven control ewes, seven androgenized ewes, and seven rams (12 months of age), all of which had been gonadectomized when less than 5 weeks old. At the time of gonadectomy, all of the animals were given a 1-cm long silastic capsule of crystalline estradiol to maintain low physiological concentrations of steroid (Goodman, Legan, Ryan, Foster, & Karsch, 1981). In an attempt to generate a surge of GnRH, circulating concentrations of estrogen were elevated to those experienced during the normal follicular phase of the cycle by implanting the animals subcutaneously with four 3-cm long silastic capsules of estradiol. Blood samples were collected from the jugular vein at hourly intervals from 6 to 30 hr after steroid implantation and assayed for LH as a reflection of GnRH secretion (Moenter, Caraty, & Karsch, 1990).

Experiment 2

Response of the GnRH neuronal network to inhibition by progesterone. The purpose of this study was to determine if exposure to testosterone during gestation altered the ability of another ovarian hormone, progesterone, to inhibit the pulsatile secretion of GnRH/LH. Progesterone is an extremely important regulator of events in the estrous cycle (as well as the human menstrual cycle) as it inhibits both the pulsatile mode of GnRH secretion during the luteal phase of the cycle and, if present during the follicular phase, blocks the estrogen-stimulated surge (Goodman, 1994). The current study was performed on the same animals that were used in Experiment 1 (aged about 24 months). Samples were collected at 10 min intervals for 6 hr on two occasions, once before and a second time 2 days after progesterone administration (1 CIDR device, InterAG NZ, subcutaneous). Progesterone concentrations were determined in samples of jugular blood, one collected before and one 2 days after the implantation of P.

Experiment 3

Detection of ovarian cycles. To determine whether in utero androgen exposure disrupted reproductive cyclicity, we monitored whether ovary-intact androgenized and control ewes (seven in each group) were having regular estrous cycles. This was determined in ewe lambs from approximately 9 weeks of age until the animals were about 24 months of age. Samples of jugular blood were collected twice per week and the concentration of progesterone in each sample determined. An ovarian cycle was defined as one in which progesterone was elevated to concentrations above 1ng/ml for three consecutive samples (i.e., 10–11 days: normal ovine luteal phase is approximately 12 days of a 16 day cycle). The time of onset of the first estrous cycle was considered the time of puberty. Plasma concentrations of progesterone were determined in single 100 μl samples using a commercially available kit (Coat-a Count, Diagnostic Products Corporation, U.S.A.).

Statistical analyses: Comparisons among groups were made using a one-way analysis of variance (ANOVA) with Tukey's post hoc test. Where two groups were compared, this was done using a Student's unpaired t test. Significance was considered as $p < .05$.

RESULTS

Experiment 1

Response of the GnRH neuronal network to stimulation by estrogen. The response of the control female

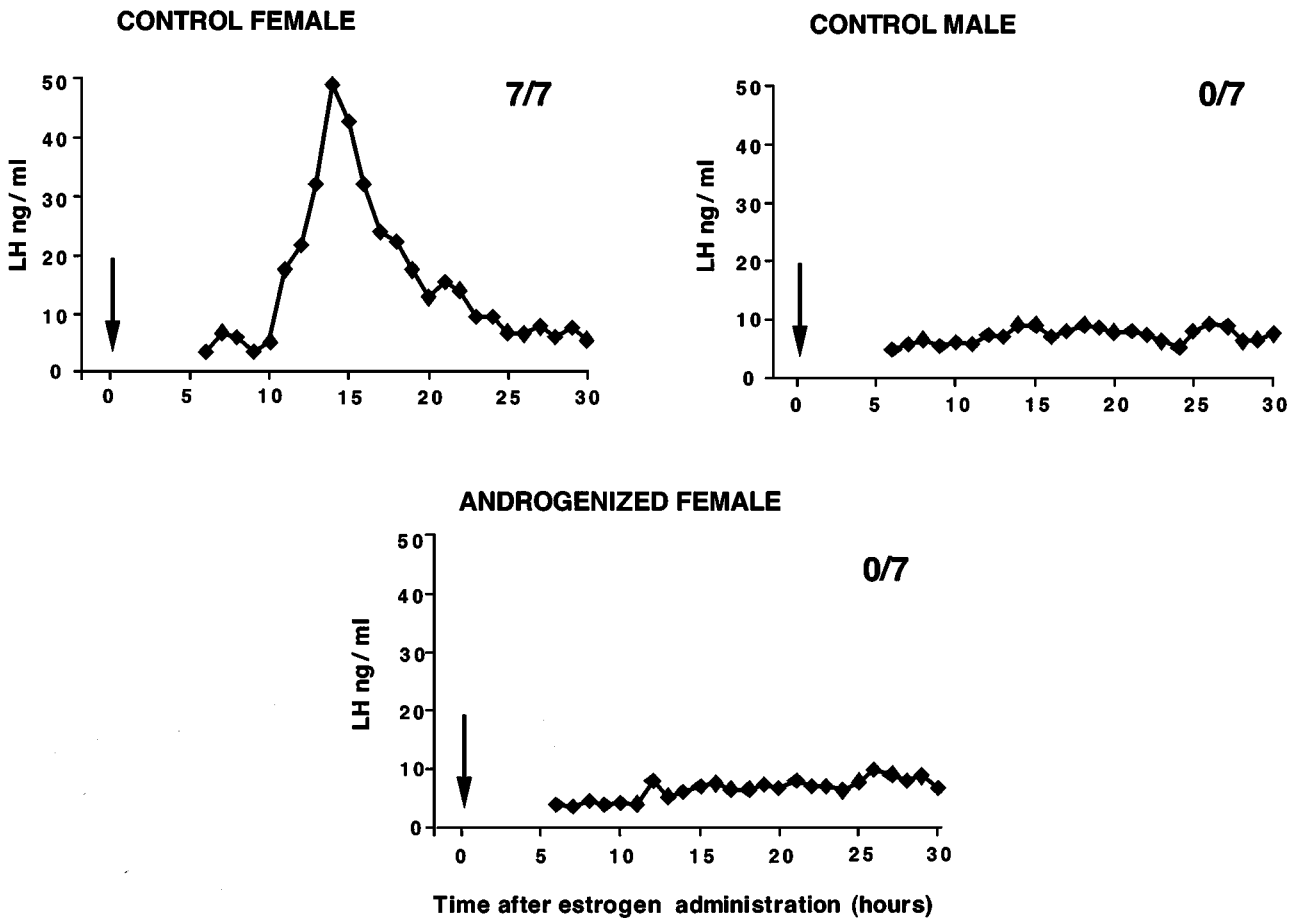

Fig. 1. Results of Experiment 1. Gonadectomized animals were given implants of estrogen at time 0 (arrow) and concentrations of circulating luteinizing hormone monitored hourly from 6 to 30 hr after implantation. Estrogen stimulated a LH surge in all the control females (top left) but in none of the males (top right) or females androgenized in utero (bottom).

and male sheep and the androgenized females to exogenous estrogen are depicted in Fig. 1. Following estrogen administration, mean LH concentrations in control ewes remained basal for about 10 hr after which the steroid-triggered GnRH/LH surge began. All of the control ewes exhibited a rise in LH that reached a peak some 15 hr after estrogen administration. In marked contrast, the same steroid treatment failed to elicit a surge in any of the male sheep or in the ewes that had been exposed to androgens for 60 days in early pregnancy.

Experiment 2

Response of the GnRH neuronal network to inhibition by progesterone. The mean concentrations of circulating progesterone did not differ among the groups either before, or after, the implantation of the single CIDR (before P: female, 0.12 ± 0.06; male, 0.11 ± 0.05; androgenized female, 0.30 ± 0.11; after P: female, 1.6 ± 0.15; male, 1.3 ± 0.35; androgenized female, 1.31 ± 0.26 ng/ml). These relatively low concentrations of progesterone inhibited episodic LH secretion in the control females as expected (Goodman & Karsch, 1980). Interestingly, progesterone had no effect on LH secretion in either the rams or the androgenized ewes. LH pulse profiles are shown in Fig. 2 (left) for a representative female, male, and androgenized female both before and after the implantation of P. Analysis of the data revealed that progesterone suppressed the frequency of LH pulses ($p <$ .01: from 9.6 ± 0.8 to 6.3 ± 1.0 pulses/6 hr) in the females (Fig. 2, right) although it did not alter either mean LH concentrations or the amplitude of the LH episodes.

Experiment 3

Detection of ovarian cycles: The first breeding season. Patterns of progesterone secretion, indicative of ovarian cycles, in a representative control and androgenized

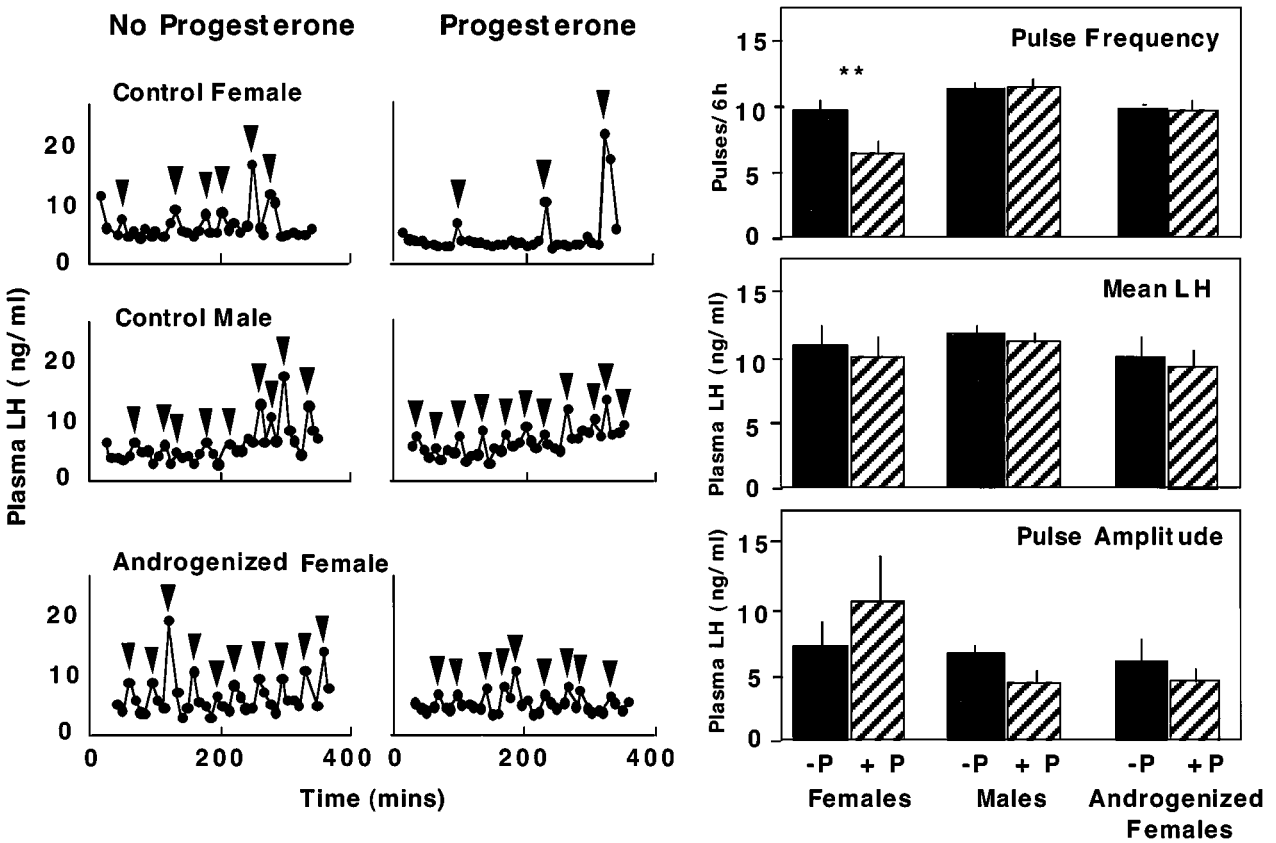

Fig. 2. Results of Experiment 2. (Left panels); Patterns of episodic LH release in an individual gonadectomized female, male, and androgenized female in the absence of progesterone and 2 days following the administration of this steroid. The arrows indicate statistically identified pulses of LH. (Right panel); Characteristics of LH secretion in the separate groups of animals ($n = 7$ each) in the absence (−P) and presence (+P) of progesterone. Note that progesterone inhibited LH pulse frequency only in the control females. Mean LH release and pulse amplitude were unaffected.

ewe are shown in Fig. 3. All of the control ewes had regular estrous cycles during the first breeding season (see Fig. 4). In these ewes, the time of onset of the first cycles in progesterone, that is, puberty, occurred on average on 11 October ± 4 days (about 30 weeks of age) and continued until 12 January ± 12 days. Two of the androgenized ewes failed to generate any normal estrous cycles during this period. Of the remaining animals, cycles began on 11 October ± 9 days and finished on 22 January ± 6 days. These dates do not differ significantly from controls. Further, the average peak progesterone concentration attained in cyclic animals did not differ between the groups (Control ewes, 3.9 ± 0.3; Androgenized ewes 4.9 ± 0.4 ng/ml).

Ovarian cycles in the second breeding cycle: After an anestrous season that lasted several months, cycles resumed in the control animals on 27 July ± 10 days and continued until 27 March ± 8 days (see Fig. 4). In marked contrast, none of the androgenized ewes had a single ovarian cycle.

DISCUSSION

These studies showed that the response of the GnRH neural network to the ovarian steroid hormones estrogen and progesterone was sexually differentiated in the adult sheep and that this was a consequence of exposure of the fetus to testosterone (Robinson, Forsdike, & Taylor, 1999; Wood, Robinson, Forsdike, Padmanabhan, & Foster, 2000). These hormones play key roles in the control of the mammalian reproductive cycle, including that of humans. Specifically, elevated concentrations of estrogen during the follicular phase of the cycle trigger the GnRH surge that is responsible for successful ovulation. During the luteal phase, when progesterone concentrations are elevated, GnRH secretion is inhibited and estrogen is unable to stimulate the GnRH surge.

These results were obtained in the gonadectomized sheep given implants of estrogen or progesterone. The data led us to hypothesize that reproductive cycles in ovary-intact animals would be severely disrupted by early

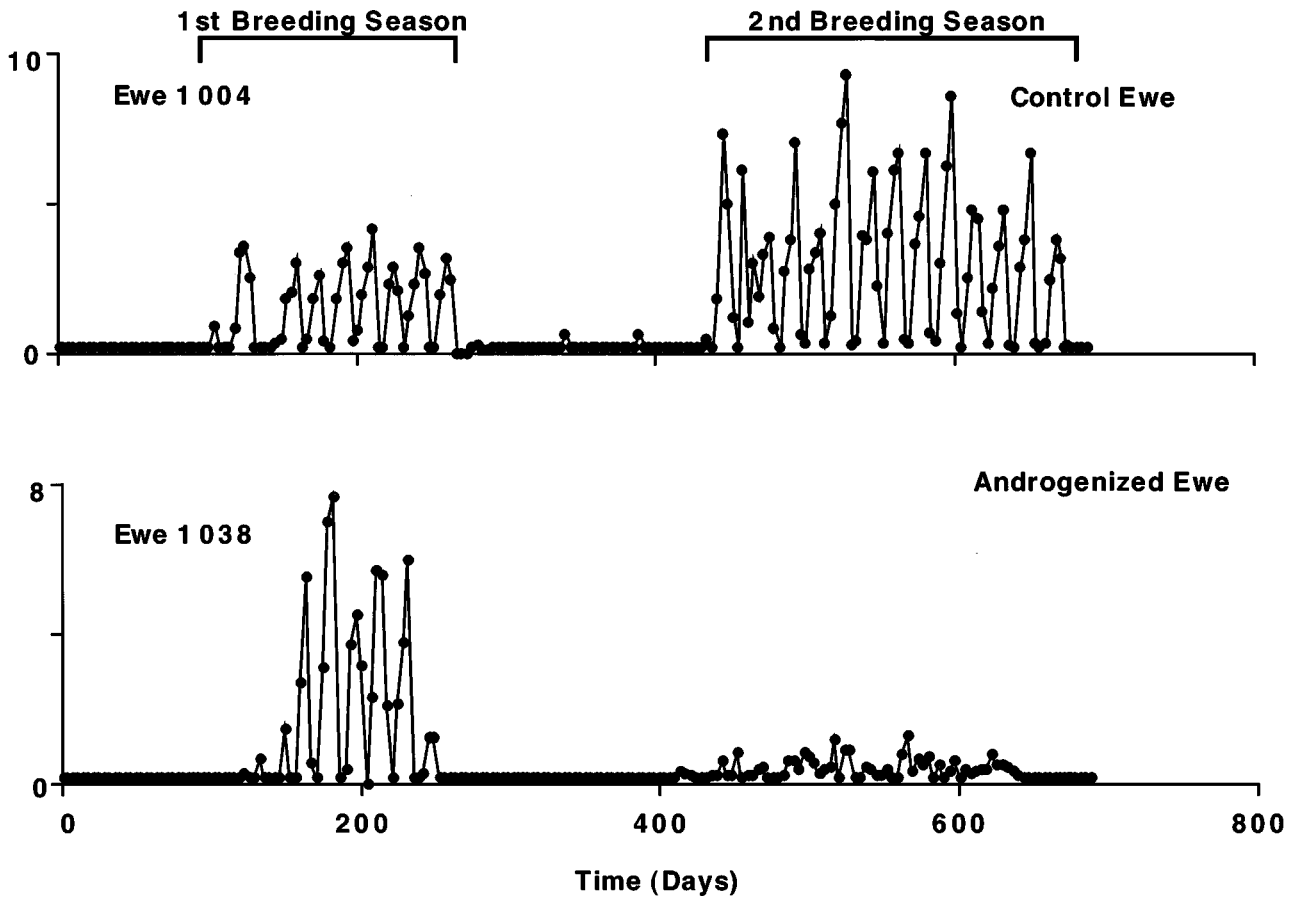

Fig. 3. Cyclic pattern of progesterone release in a control (top) and in utero androgenized ewe (bottom) from 9 weeks of age to the end of the second breeding season. Note the lack of repeated ovarian cycles during the second breeding season in the androgenized animal.

androgen exposure. We were surprised, therefore, to find that the majority of androgenized animals had apparently normal estrous cycles during the first breeding season. Specifically, the first cycle in androgenized ewes began at the same time as controls. Further, the peak progesterone concentrations attained in a cycle and the length and the number of cycles in the first breeding season did not differ from controls. It was clear, however, that fetal testosterone exposure severely disrupted the generation of estrous cycles in the second breeding season. Specifically, none of the ewes that had been androgenized had a single estrous cycle. Our observations that the androgenized ovary-intact animals can exhibit normal repetitive estrous cycles is not an isolated finding, but is supported by the results of a preliminary study performed on a different breed of sheep by Herkimer, West, Robinson, Foster, and Padmanabhan (2000). Further, earlier studies in ewes that were androgenized from day 30 to 80 or 50 to 100 of gestation indicated that some ewes in these experiments were capable of ovulation and the production of corpora lutea, although reproductive cycles were irregular (Clarke, Scaramuzzi, & Short, 1977). Although it has never been tested, these data strongly suggest that the ovary-intact androgenized ewe, unlike her ovariectomized steroid-implanted sister, is able to generate a normal preovulatory surge of LH. The reason why is currently unknown.

The most obvious difference between the two ewe models that we used to explore the mechanisms by which the GnRH neural network is masculinized by androgens in utero is the pattern of steroid hormones experienced in the early postnatal period. In the case of the ovariectomized model, estrogen concentrations are clamped at about 1–2 pg/ml by removal of the ovaries and implantation of a silastic capsule of steroid. In contrast, the ovary-intact ewe is exposed to the fluctuations in ovarian steroid hormones produced by the developing ovary. Our results would be explained if the postnatal E treatment induced further sexual differentiation of the GnRH neuronal network. This raises the possibility that a second “critical

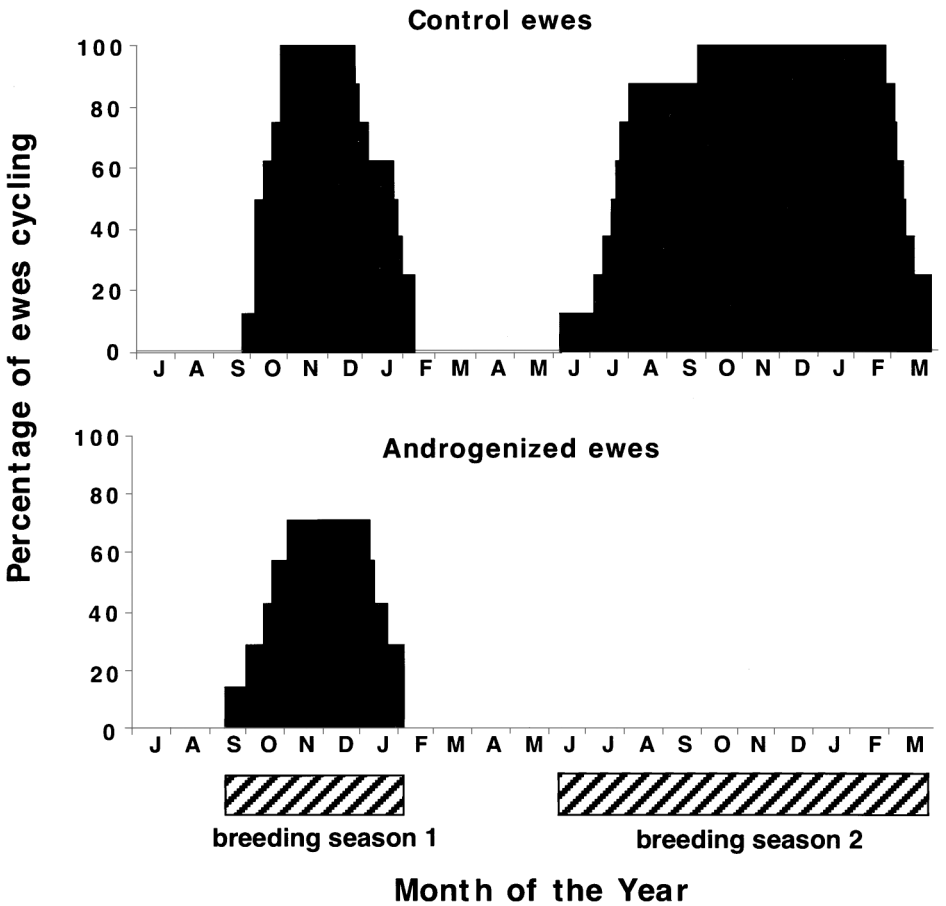

Fig. 4. Percentage of control (top) and androgenized (bottom) ewes exhibiting estrous cycles over a period of 21 months. None of the androgenized animals had a single estrous cycle during the second breeding season.

period" exists in the time after birth, during which the exogenous estrogen continues to masculinize gonadotropin secretion in the ovariectomized, steroid-implanted animal. In contrast, the patterns or concentrations (or both) of estrogen in the ovary-intact animal are not sufficient to exert this action. It is also possible that the ovary secretes a substance that in some way "protects" the hypothalamus from further masculinization during the postnatal period. Both these possibilities are worthy of further study.

It was of further surprise to us that there was a progressive loss with age of the ability of the androgenized ewes to generate normal repetitive estrous cycles. Similar findings were documented in rats that were treated with low concentrations of testosterone propionate late in the postnatal "critical period" (Gorski, 1968). Gorski termed this physiological phenomenon "delayed anovulatory syndrome (DAS)." Data that suggested that DAS might also occur in androgenized ewes was first published by Clarke et al. (1977). In this study, female lambs were born to mothers treated with testosterone implants from day 50 to 100 of pregnancy. The incidence of ovulation was lower in the second breeding season (assessed by laparotomy) than in the first season (assessed from the plasma concentrations of progesterone collected over a period of 25–35 days). Why there is a progressive loss of normal repetitive estrous cycles is unclear, as is whether the reason for the loss of fertility resides primarily at the level of the brain, pituitary gland, or ovary. Relative to the last, we have found that 3-week-old androgenized lambs have abnormal cystic ovaries (Wood et al., 2000). Although such ovaries may be capable of initiating cycles, they may not be able to maintain them. These ovaries may, in addition, continue to be damaged by sustained exposure to the elevated gonadotropin concentrations characteristic of androgenized ewes or the elevated concentration of other hormones including testosterone synthesized by the ovarian stroma (Birch & Robinson, unpublished data). Studies are currently underway to explore these possibilities.

Another explanation for our results that is currently under investigation is that androgenized animals progressively lose the ability to generate an LH surge in response to elevated concentrations of estrogen. This might be because the pituitary gland no longer responds appropriately to the GnRH or that oestrogen fails to trigger the GnRH surge.

In summary, exposure of the ovine foetus to testosterone for either the whole or part of the critical period for sexual differentiation leads to a progressive inability of the adult ewe to produce normal repetitive estrous cycles. Eventually, she becomes infertile. We believe that the reason for this resides in androgen driven developmental changes at several levels of the reproductive axis including the network of neurons that control the activity of the GnRH neuron.

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