Defeminization of the Reproductive Response to Photoperiod Occurs Early in Prenatal Development in the Sheep

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ABSTRACT

Photoperiod times the transition to sexual maturity in many seasonal breeders. In male and female sheep, photoperiod influences the timing of puberty differentially. Whereas in females, age at sexual maturity is highly dependent on photoperiod, puberty in males begins at the same age regardless of day length. We have determined that this sex difference is due to the organizing action of androgens during prenatal development. In the present investigation, we studied when during gestation (term: ~150 days) androgens defeminize the reproductive response to photoperiod. We compared the age at sexual maturity in female lambs treated with testosterone prenatally from Days 30 to 76 (Early, \(n = 7\)) or 89 to 135 (Late, \(n = 8\)) to that of normal males \((n = 8)\) and normal females \((n = 7)\). To reveal differential responsiveness to photoperiod, all lambs were maintained from birth under constant long days \((16L:8D)\), a treatment that inhibits puberty in normal females. The age at the pubertal LH rise was determined in a standardized experimental model (lambs gonadectomized and treated with estradiol). As expected in the long day photoperiod, only 1 of 7 normal females had a pubertal rise in LH. In contrast, all males increased LH secretion by 6.7 ± 0.6 wk. Similarly, in the Early group, a sustained increase in LH occurred in all females, but this was delayed relative to the increase in the males \((16.8 ± 1.7\) wk; \(p < 0.001)\). The Late group had LH patterns similar to those of the normal females, with only 3 of 8 females having sustained elevations in LH. These data suggest that a “critical period” for the defeminization of the reproductive response to photoperiod occurs early in prenatal development. In addition, it appears that this critical period and the period for defeminization of the surge mode of gonadotropin secretion occur at similar stages in development. When challenged with an acute increase in estradiol, all normal and Late androgenized females responded with an LH surge. In contrast, none of the males and only 1 of 7 Early females produced a robust response to estradiol.

INTRODUCTION

In a variety of mammalian and avian species, sexual differentiation of neuroendocrine function underlies many differences in hormonal and behavioral patterns between males and females (see [1] for review). The classic studies in the rat nearly 60 yr ago by Pfeiffer [2], and those by Barraclough, Gorski, and co-workers [3–6], have paved the way for our current understanding of the influence of gonadal steroids during early development on reproductive neuroendocrine function. Relative to the time of birth, there exists substantial species-specificity with respect to the stage in development at which the brain is sensitive to the organizing action of gonadal steroids (see [1] for review). For instance, in the guinea pig, sheep, and rhesus monkey, this period occurs prenatally. For most rodents, the ferret, and the dog, the “critical period” for brain sexual differentiation may encompass the time immediately before and after birth [1].

Sexually distinct characteristics are either masculinized (i.e., assume a male trait) or defeminized (i.e., abolish a female trait). Several studies in the sheep have suggested that masculinization or defeminization of specific traits may have different “critical periods” before birth [7–11]. For example, masculinization of the external genitalia appears to occur early and briefly in fetal development (between Days 30 and 50 [7, 8, 11]). In contrast, masculinization of the posture and pattern of urination occurs during a broader period in gestation (between Days 30 and 80 [7, 8]). During this same period in development, defeminization of estrous behavior and the acquisition of masculine sexual behavior also occur [9, 10].

We have determined that in sheep, the age at the pubertal increase in tonic LH secretion is markedly different between the sexes (males, 10 wk; females, 30 wk), and that this is due to prenatal exposure to androgens [12]. Interestingly, the etiology of this sex difference lies in the differential responsiveness of males and females to photoperiod cues [13]. Whereas the timing of the pubertal LH rise in females is highly influenced by photoperiod [13–16], in males it is not [13, 17–19]. When females are treated with testosterone before birth, their response to photoperiod is similar to that in males. They virtually ignore day length cues that are inhibitory to sexual maturation in normal females and attain puberty at the same time as males [13].

It is not known when during development the reproductive response to photoperiod is sensitive to the organizing action of androgens. One hypothesis is that the responsiv-
ness to day length cues is abolished during approximately the same broad period in prenatal development when other sexually differentiated traits are established (i.e., Days 30–90 in gestation). On the other hand, the responsiveness to photoperiod could be susceptible to androgens throughout a much longer duration in gestation. In the present study, we tested these competing hypotheses by exposing lambs to testosterone either early or late in gestation and then maintaining them after birth under a constant, long day photoperiod. This photoperiod inhibits puberty in normal females [14], and should reveal differential responsiveness to photoperiod as a function of prenatal androgen exposure. The age at the pubertal LH rise in these androgenized females was compared with that in males and normal females maintained under the same conditions. In addition, we determined whether the organization of the reproductive response to photoperiod occurs during the same time as the ability to respond to the stimulatory feedback action of estradiol. Using exogenous estradiol, we attempted to induce the LH surge in early- and late-androgenized females and compared their responses with those of gonadectomized, estradiol-treated males and normal females.

MATERIALS AND METHODS

Animals

Spring-born lambs of predominantly Suffolk breeding were born at a commercial sheep farm (Wolf Creek Farms, Hubbard Lake, MI). Males (n = 8), females (n = 7), and two groups of androgenized females (Early group, n = 7; Late group, n = 8) were used (see below). Their mean (± SEM) birth date was April 11 ± 0.8 days (range, April 4–18). At approximately 8 wk of age, lambs were weaned and fed ad libitum with alfalfa hay and a commercial pellet ration (Lamb 18; Kent Feed, Inc., Muscatine, IA) containing 18% protein, supplemented with vitamins and minerals. Weights were determined every 2 wk (from birth to 15 wk) and then every 3 wk until the lambs reached 30 wk of age (Fig. 1).

Female lambs were exposed to testosterone early and late in prenatal development to determine when during gestation the reproductive response to photoperiod is modified by fetal testosterone (Fig. 1, inset). To produce the Early androgenized females, pregnant ewes (n = 4) received injections of testosterone cypionate (200 mg in 0.5 ml chloroform, i.m.; Sigma Chemical Company, St. Louis, MO) weekly for 7 wk, from Days 30 to 76 in gestation (term: ~150 days). To produce the Late androgenized females, another group of pregnant ewes (n = 6) were similarly treated with testosterone for 7 wk, but during a later stage in gestation (from Days 89 to 135). We have used the same dose of testosterone in past studies [11, 13], but shortened the duration of exposure to androgens in the present study to produce a 2-wk interval between the Early and Late treatments and thus two discrete treatment periods. As determined in a separate study, after a single injection of testosterone to pregnant sheep, daily testosterone values decreased steadily to approximately half-maximal concentrations by the end of the week after the injection (B.G. England, R.I. Wood, and D.L. Foster, unpublished data). On the basis of these observations, we expected that testosterone would have reached low concentrations by the end of the second week and thus that any overlap between the Early and Late treatment periods in the present study should be minimal. Also, in the present study, testosterone was not administered earlier than 30 days in gestation because androgens are not detected in the fetal testes before that time in development [20]. Treatments were not carried out later than the 140th day in gestation to avoid the dystocia and lactational failure observed when testosterone is administered close to parturition [7].

Photoperiod Treatment

Ewes were bred in November and maintained under natural conditions. Within a few weeks after birth, all lambs
were moved into light-sealed, controlled photoperiod rooms at the Reproductive Sciences Program Program Sheep Research Facility at the University of Michigan, Ann Arbor (42°18'N), where they were maintained under a constant long photoperiod (16L:8D). The ewes remained with their lambs until weaning at 8 wk of age. In the rooms, photoperiod was controlled electronically by timers (System X10 appliance model; BSR, Miami, FL). During the day, fluorescent light bulbs provided a light intensity of approximately 200 lux; in addition, a single red bulb (< 2 lux) illuminated the rooms continuously. The rooms were ventilated through a light baffle but were not temperature controlled; thus, temperature changed seasonally.

**Neuroendocrine Sexual Maturity**

The sustained rise in LH secretion, reflecting reduced responsiveness to steroid negative feedback, was used as the index of neuroendocrine sexual maturity as in past studies [11, 13, 19, 21]. This increase in LH that drives gonadal development is robust, and it correlates well with indices of sexual maturation in gonad-intact males (initiation of testicular growth [22]) and females (initiation of ovarian cycles [23]). To determine the time of the pubertal LH rise, the model used in this study was the gonadectomized lamb treated chronically with estradiol. Estradiol is the major feedback hormone regulating the secretion of LH before puberty in the female (for review see [24]), and is an effective feedback hormone in the immature male as well [12, 13, 19, 22]. Before sexual maturity, the secretion of LH is low because the hypothalamus is highly responsive to the inhibitory effects of estradiol [24]. At puberty, responsiveness to the negative feedback effect of estradiol diminishes dramatically, and the secretion of the gonadotropins, particularly LH [24], increases. The increase in LH secretion drives spermatogenesis and the development of the pre-ovulatory follicle. To monitor changes in circulating LH concentrations throughout development, blood samples (5 ml) were collected twice per week from each lamb by jugular venipuncture after estradiol implants were in place (see below). Samples were left to clot overnight at 4°C; serum was decanted after centrifugation and stored frozen until analyzed for LH. Blood sample collection continued until lambs were ~40 wk of age, when monitoring of tonic LH secretion was terminated. This is sufficient time for determining whether puberty is delayed in the normal females.

Testes were removed under local lidocaine anesthesia when males were approximately 1 wk of age. Ovaries were removed under acepromazine/ketamine anesthesia (0.2 mg/kg and 20 mg/kg BW, respectively, i.m.) through a small, midline abdominal incision when females were about 3 wk of age. Immediately after gonadectomy, a Silastic capsule containing estradiol was implanted s.c. in the axillary region of each lamb to provide chronic physiologic concentrations of the hormone (3–5 pg/ml [21, 23]). The Silastic capsules (o.d. 0.46 cm, i.d. 0.34 cm; Dow Corning, Midland, MI) contained a 30-mm column of packed crystalline estradiol 17-β (Sigma) and were sealed with Silastic adhesive type A (Dow Corning). To prevent a postimplantation peak in steroid release, estradiol implants were preincubated in water overnight before insertion [25]. The implants were left in place until the termination of the study of tonic LH secretion, when the lambs were ~40 wk of age. Implants were then removed in preparation for the study on surge secretion (see below).

**Induction of Stimulatory Feedback Response**

Our past studies [11, 12, 26, 27] and those of others [7, 9, 10] have found that males and prenatally androgenized females fail to produce an LH surge in response to estradiol. In the present study, we examined the possibility that the critical period for fememinization of the surge mechanism is concurrent with that for the fememinization of the reproductive response to photoperiod. The ability to produce a positive feedback response to high concentrations of estradiol was evaluated in the Early and Late androgenized females by the same paradigm used in our previous studies [27, 28]. When lambs were ~40 wk of age, the single subcutaneous estradiol implant that provided constant steroid feedback throughout development (see above) was removed. Approximately 2 wk later, four similarly sized Silastic capsules containing estradiol were inserted s.c. to produce high physiologic concentrations of the hormone (~12 pg/ml). To characterize LH patterns, samples were collected every 2 h beginning 6 h before and ending 60 h after the insertion of implants.

**LH Assay**

LH was measured in duplicate 25–200-μl aliquots of serum by a modification [29, 30] of an RIA developed by Niswender et al. [31, 32]. Assay sensitivity, defined as two standard deviations from maximum binding, averaged 0.74 ± 0.03 ng/ml (n = 17 assays) for 200 μl of serum, expressed relative to NIH-LH-S12. Intraassay coefficient of variation (CV), determined from a serum pool that bound at 20% (n = 16 assays), averaged 6.60%; interassay CV averaged 9.32%. For a serum pool that bound at 80% (n = 14 assays), intraassay CV averaged 12.32% and interassay CV averaged 13.29%.

**Data Analysis**

The age at neuroendocrine sexual maturity was determined from the pattern of LH in blood samples collected twice per week. By a criterion previously established in our laboratory [33], a consistent elevation in LH above 1 ng/ml for at least 3 wk (6 biweekly samples) defines the onset of the pubertal LH rise. For the males and Early androgenized
females, which all had robust increases in LH secretion. Student's t-test was used to compare the ages at neuroendocrine sexual maturity. On the other hand, because not all of the normal and Late androgenized females exhibited a pubertal LH rise, the average age at puberty in these groups was not determined. Instead, only the proportion of lambs attaining puberty among the normal and Late androgenized females was compared through use of Fisher's Exact test. In addition, another method was used to compare the degree of reproductive neuroendocrine activity in the normal and Late androgenized females. The number of samples and cumulative amount of LH in excess of 1 ng/ml were determined for each lamb, averaged in each group, and compared by Mann-Whitney U test; this nonparametric test was used because initial analysis of the data revealed unequal variances in the groups. In all groups, the limit of detection of the assay was recorded for samples in which LH levels were below assay sensitivity.

LH surges induced by the acute increase in circulating estradiol concentrations were identified through use of a modification of the criterion by Legan et al. [34, 35]. A surge was present if LH increased for at least 8 h (four samples) above the average concentration secreted in the absence of steroid feedback (i.e., before implants were inserted). In addition, the peak LH secreted had to exceed at least twice the average pre-estradiol concentration. To estimate the time of the surge, the time interval from initiation of estradiol treatment to the peak of the surge was determined. This method was used rather than the onset of a marked elevation above baseline (e.g., as used in [36]) because LH concentrations before the surge were highly variable. LH was elevated in the absence of steroid feedback before estradiol treatment and then suppressed after the insertion of estradiol, thus precluding a meaningful assessment of baseline values.

RESULTS

Neuroendocrine Sexual Maturity

Males (Fig. 2). All males maintained under the constant long photoperiod exhibited a robust pubertal increase in LH secretion, the average age being 6.7 ± 0.6 wk (range: 5.0–10.0 wk, n = 8). Circulating concentrations of LH in this group remained high after the pubertal increase until the termination of the experiment at ∼40 wk.

Normal females (Fig. 3). Only 1 of 7 females exhibited a sustained rise in LH under the constant long photoperiod; this occurred at 21 wk of age (lamb #439). The remaining females did not exhibit the robust and sustained increases in LH secretion observed in the males. In those females, elevations in LH were mostly short-lived and of low amplitude.

Androgenized females (Figs. 4 and 5). The Early androgenized females exhibited uniform, completely masculinized external genitalia, consistent with our past observations with similar androgenization paradigms [11, 13]. The genitalia consisted of a penis situated close to the navel, and a scrotum (albeit empty) caudal to the penis about halfway between the anus and navel, as for a typical male. The urination posture and pattern of this group of androgenized females were also like those of males; i.e., they stood while urinating in spurts. Examination of circulating LH (Fig. 4)
revealed robust increases in LH secretion beginning at 16.8 ± 1.7 wk (range: 9.5–22.0 wk, n = 7). The pubertal rise in LH was initiated at a later age than in the males (p < 0.001). Circulating LH concentrations in this group remained high after the pubertal increase until the termination of the experiment at ~40 wk, except in one lamb (#459), in which LH fell below assay sensitivity at 33 wk of age.

The Late androgenized females were not masculinized in either appearance or behavior. Only 3 of 8 lambs had robust increases in LH; these occurred at 23.2 ± 1.7 wk of age (lambs #466, #467, #468). The other Late androgenized females had sporadic elevations in LH that were of low amplitude (Fig. 5), similar to the elevations in normal females. Fisher’s Exact test did not reveal a significant difference in the proportion of
CRITICAL PERIOD DEMENTIZATION OF PHOTOPERIOD RESPONSE

Induction of Stimulatory Feedback Response (Fig. 6)

A stimulatory feedback response to an acute increase in estradiol occurred in all normal females (n = 7), with LH rising well above pretreatment concentrations and peaking at 16 ± 1.1 h after estradiol treatment. In 3 of 5 males, an increase in LH secretion occurred beginning at ~24 h after estradiol treatment; however, LH concentrations never exceeded those in the absence of steroid feedback. In the Early androgenized females, an unequivocal LH surge occurred in 1 lamb 40 h after initiation of estradiol treatment (#459). In 3 of the remaining 6 individuals, increases in LH were present, but these never exceeded pretreatment concentrations, as was the case in the males. In contrast, all lambs in the Late group (n = 8) exhibited LH responses rising well above pretreatment concentrations. Five of eight lambs produced surges that were similar in timing to surges in normal females (peak concentration occurred between 16 and 20 h). However, the others produced surges that peaked 36 h after estradiol treatment. This latent period was much longer than the latest surges in the normal females (peak at 20 h).

DISCUSSION

The results of the present study strengthen the concept that photoperiod has minimal influence on the timing of sexual maturity in the male sheep. The age at the pubertal LH rise in the males of this study was similar to that observed previously in males raised under natural [17] or simulated natural photoperiods [13, 19]. Past studies have shown that neuroendocrine sexual maturity in males raised under seasonally reversed changes in day length is normal [13] or only slightly delayed [19] when day length changes have been amplified and accelerated. In addition, exposure to a constant short photoperiod does not delay testicular growth [17], nor does treatment with melatonin implants suppress the secretion of reproductive hormones [18]. The present study demonstrates that a constant long photoperiod also does not inhibit the time of the pubertal LH rise in males.

In contrast, the age at puberty in the female sheep is highly influenced by photoperiod, as evidenced by this and numerous studies [13–16, 33, 37]. In the present study, exposure to a constant long photoperiod inhibited high levels of gonadotropin secretion in all females but one. It may be considered that with regard to this single female, exposure to its male twin during prenatal development diminished its normal reproductive response to constant long days. However, this explanation is unlikely, because most of the other females in the group had at least one male sibling and their reproductive responses were quite different. In those females, there were sporadic episodes of increased LH secretion, but they were short-lived. Circulating estradiol concentrations in the females were not determined, so we were unable to establish whether or not the sustained increase in...
FIG. 6. Representative patterns of circulating LH during the attempt to induce a stimulatory feedback response to an acute increase in estradiol. Four estradiol implants were inserted at Hour 0 to produce circulating concentrations of ~12 pg/ml. In response to estradiol, all females (n = 7, first column), but none of the males (n = 5, second column) exhibited increased LH that exceeded concentrations in the absence of steroid feedback. One lamb (#459) from the Early group (n = 7, third column) produced a robust positive feedback response; in contrast, all the lambs in the Late group (n = 8, fourth column) produced responses rising well above pretreatment concentrations. Among the Late androgenized females, three lambs had a longer latency to peak LH values than others (e.g., #457, #471). Shading indicates average LH concentrations before estradiol treatment for each lamb.
LH occurred in the one female because of a less effective steroid feedback. It is of interest to note that on rare occasion, lambs maintained under constant photoperiods beginning shortly after birth exhibit ovulatory cycles at about 30 wk of age [14]. It is likely that this past observation was paralleled by our present observation of increased LH secretion in one female.

That the reproductive response to photoperiod is a sexually differentiated trait established during prenatal development has only recently been determined [13]. The earlier studies of Short [7], Clarke et al. [8, 9], and Clarke and Scaramuzzi [10] examined the effects of testosterone administration at various stages in prenatal development on reproductive behavior, ovarian function, or urination posture, but not the timing of sexual maturity. It is possible that in those studies, the age at puberty was modified to various degrees by androgen treatment at different stages in gestation and, further, that the variation in the age at puberty may have occurred because of differential organization of the photoperiod response. Indeed, our recent studies have determined that a sex difference in the pubertal response to photoperiod is established by testosterone before birth [13]. In the present study, we addressed the possibility that the photoperiod response mechanism is susceptible to androgens only during a discrete period in development.

Prenatal treatment with androgens, when provided early in gestation, diminished the inhibitory effect of a constant long photoperiod. In the Early androgenized females, neuroendocrine sexual maturity occurred as evidenced by a robust increase in LH secretion. However, this was much later than in the males. We hypothesize that the duration of androgen exposure in this study (7 weekly injections) was not sufficiently long to fully defeminize the photoperiod response. In our earlier study, when we administered testosterone weekly for 9 wk (i.e., prenatal Days 30–90), the timing of neuroendocrine sexual maturity in androgenized females was identical to that in the males [13]. We believe that extending the duration of testosterone treatment by 2 wk in the present study would have fully diminished the inhibitory influence of a constant long photoperiod.

Later exposure to androgens during prenatal development was much less effective in defeminizing the response to photoperiod. In most of the Late androgenized females, LH secretion was not maintained above baseline concentrations. Visual inspection of the data initially suggested that there might be greater gonadotropin secretion in this group than in the normal females, but this was not borne out by statistical analysis of the number of samples with high LH. However, overall LH tended to be greater in the Late androgenized females than in the normal females. These results suggest that the reproductive response to photoperiod is largely organized early in prenatal development (most likely between Days 30 and 90); however, androgens later in gestation may also have some influence.

Overall results from this study suggest that there is considerable overlap between the critical periods for defeminization of the photoperiod response and that for the mechanisms governing the surge mode of gonadotropin secretion. In evaluating the LH response to the stimulatory feedback action of estradiol, we established the criterion that for the LH surge to be present, concentrations of the gonadotropin before estradiol treatment must be exceeded. Based on this criterion, the ability of estradiol to produce an LH surge was unequivocal in all of the lambs in the Late group. However, in the Early androgenized females, with the exception of one (#459), LH did not exceed pre-estradiol concentrations. This lamb (#459) produced a robust positive feedback response much later than the normal females and also had a later onset of the pubertal LH rise (22 wk) compared to others in the same group (see Fig. 4). We hypothesize that this female was the least masculinized of the group. Interestingly, its twin (#460) attained neuroendocrine sexual maturity at an early age (9.5 wk, Fig. 4) and did not produce a robust response to estradiol (data not shown). Such an observation supports our earlier hypothesis that there may be individual variations in androgen sensitivity or metabolism by the fetus [12].

Prenatal androgens later in gestation may have subtle effects on the surge mode of LH secretion. This is evidenced by the long latent period of response to an acute increase in estradiol among some females of the Late group. Unlike the effect on the surge early in gestation, which appears to largely diminish the positive feedback response to estradiol, the effect of androgens later in gestation may be to alter the timing and perhaps even the shape of the LH surge. It is possible that later exposure to androgens modifies the sensitivity of neuronal elements governing the LH surge to the stimulatory effects of estradiol. Such findings warrant a more detailed study of the influence of prenatal steroids on the timing of the gonadotropin surge as it has physiological relevance to normal ovulation and estrus behavior. Clarke and Scaramuzzi [10] reported that females exposed to androgens between Days 50 and 100 in gestation can be induced to exhibit estrous behavior, although the timing is delayed relative to estrus in the normal female. In another study, females exposed to androgens beginning from Day 50 in gestation were found to be capable of ovulation, but the quality of receptive behavior in these females was markedly reduced [38]. These studies reported either an attenuation [10, 38] or a longer latency [38] of the LH surge.

In general, the results of the present study suggest that defeminization of the photoperiod response mechanism largely occurs early in prenatal development. It is also likely that it occurs during a broader stage in development than does differentiation of the external genitalia [7, 8, 11]. This was evidenced in the Early androgenized females by a delayed attainment of neuroendocrine sexual maturity relative to that of males, despite highly masculinized external gen-
italia. However, it appears that defeminization of the surge mechanism, and masculinization of urination posture and reproductive behavior as shown by other studies [7–10], occur concurrently with the defeminization of photoperiod response.

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REFERENCES