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The Effect of Aromatase Inhibition on the Sexual Differentiation of the Sheep Brain

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This study tested the hypothesis that aromatization of testosterone to estradiol is necessary for sexual differentiation of the sheep brain. Pregnant ewes (*n* = 10) were treated with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) during the period of gestation when the sheep brain is maximally sensitive to the behavior-modifying effects of exogenous testosterone (embryonic d 50–80; 147 d is term). Control (*n* = 10) ewes received vehicle injections. Fifteen control lambs (7 males and 8 females) and 17 ATD-exposed lambs (7 males and 10 females) were evaluated for sexually dimorphic behavioral and neuroendocrine traits as adults. Prenatal ATD exposure had no significant effect on serum concentrations of androgen at birth, growth rates, expression of juvenile play behaviors, or the onset of puberty in male and female lambs. Rams exposed to ATD prenatally exhibited a modest, but significant, decrease in mounting behavior at 18 mo of age. However, prenatal ATD exposure did not interfere with defeminization of adult sexual partner preferences, receptive behavior, or the LH surge mechanism. In summary, our results indicate that aromatization is necessary for complete behavioral masculinization in sheep. However, before we can conclude that aromatization does not play a role in defeminization of the sheep brain, it will be necessary to evaluate whether intrauterine exposure of male fetuses to higher doses of ATD for a more extended period of time can disrupt normal neuroendocrine and behavioral development.

Key Words: Sexual maturation; sexual partner preference; copulatory behavior; puberty; aromatase inhibitor; ATD; sheep; LH surge; fertility.

Introduction

Sexual differentiation of the CNS depends in large part on the steroid hormone milieu during a critical period in perinatal development *(1)*. In several vertebrate species, perinatal administration of testosterone (T) to females defeminizes their ability to show female-typical receptive behaviors and enhances their capacity to show male-typical copulatory behaviors including sexual partner preferences. Conversely, neonatal castration of males enhances their capacity to show receptive behavior in response to ovarian hormones and attenuates their ability to show ejaculatory behavior. Similar to effects on behavior, perinatal treatment of females with T defeminizes neural mechanisms that control cyclic gonadotropin secretion by decreasing their ability to respond to estradiol (E2) with an LH surge, whereas neonatal castration makes it possible for males to show estrogen-induced LH surges *(2,3)*.

The effect of T on the brain mechanisms that control sexual behavior and gonadotropin secretion appears to require that T be converted to E2 by aromatase in neural tissues such as the hypothalamus. In rodents, neonatal administration of estrogen effectively defeminizes lordosis behavior and suppresses the LH surge mechanism *(4,5)*. Direct evidence of a normal role for neural aromatization in male brain differentiation was first provided by the demonstration that perinatal treatment with an aromatase inhibitor enhanced the capacity of male rats to show lordosis after castration and treatment with E2 and progesterone *(6,7)*. Subsequent studies, using estrogen receptor antagonists, antisense oligonucleotides to the estrogen receptor, and transgenic mouse models deficient in aromatase or estrogen receptor, have confirmed this observation in rodents *(8–11)*. Recently, it has been suggested that estrogen receptor beta may play a role in male-typical brain differentiation because estrogen receptor beta knockout male mice show high levels of lordosis when castrated and primed with estrogen and progesterone *(12)*.

Another feature of male-typical sexual differentiation is the development of a preference among males to seek out and attempt to mate with an estrous female. The capacity for males to display a sexual partner preference for conspecific females is organized by perinatal T exposure in several

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species *(1,13)*. In rats, the organizing action of T on sexual partner preference acting postnatally in the male results from the aromatization of T to E2 *(8,14)*.

Research on sexual differentiation is far less extensive in sheep than in rats. Gestation in sheep lasts approx 147 d with gonadal differentiation occurring between embryonic day (E) 30 and E35 *(15,16)*. The fetal testis secretes elevated levels of T starting around E35 with a peak at E70 *(17, 18)*. Fetal males have higher circulating levels of T at E35–E70 than do fetal females *(18,19)*. Concentrations of T in males decline between E70 and E90 and are not significantly different in males and females until late gestation *(18)*.

Early studies on sexual differentiation of the brain in sheep by Short *(15)* and Clarke and colleagues *(20,21)* determined that behavioral masculinization and defeminization is due to the organizing action of T during prenatal development. Fetal ewes exposed to exogenous T between approx E30 and E80 fail to show sexual receptivity and display increased mounting and aggressive activity as adults. The greatest degree of behavioral masculinization and defeminization was observed in ewes exposed to testosterone over either E50–E100 or E70–E120. Ewes exposed to testosterone from E90–E140 exhibited regular estrous receptivity, with only a slight nonsignificant enhancement of masculine behavior compared to control ewes. From these data it can be deduced that the period of maximal sensitivity to the behavioral effects of T fall between E50–E80.

Sexual partner preferences in sheep might also be influenced by exposure to gonadal steroids during prenatal development. The ovine sexually dimorphic nucleus is twice as large in female-oriented rams as in male-oriented rams and ewes and contains significantly more aromatase *(22)*. In several other species, this brain area is essential for the expression of heterosexual preferences in males *(23)* and is sexually differentiated through perinatal exposure to androgen-derived estrogens *(24)*.

Prenatal exposure of ewes to exogenous T also interferes with normal ovulation, blunts the LH surge mechanism in response to exogenous E2, and advances the onset of puberty to the time in normal males *(15,25–27)*. Full defeminization of the LH surge mechanism requires androgen exposure throughout E30–E90, whereas puberty can be advanced in females with only 20 d of exposure at either the beginning or end of this period *(28)*. In addition, the mechanisms organizing the surge system are more sensitive than pubertal timing to the amount of T exposure *in utero (28)*. Finally, aromatization appears to be required for sexual differentiation in sheep because prenatal exposure to T, but not the nonaromatizable androgen dihydrotestosterone, effectively suppresses the ability of E2 to induce sexual receptivity and LH surges in adult female sheep *(29)*.

As indicated, existing evidence supports the hypothesis that aromatization plays an important role in the process of brain sexual differentiation in sheep. Moreover, aromatase is present in the fetal sheep brain during midgestation and is especially enriched in hypothalamic and limbic areas involved in sexually dimorphic reproductive functions *(30)*. However, to-date no study has directly tested whether inhibition of neural aromatase interferes with normal organization of the male sheep brain. Thus, a study was conducted to determine the role of *in situ* estrogen formation for the sexual differentiation of the sheep brain. Pregnant female sheep (ewes) were treated with an aromatase inhibitor, 1,4, 6-androstatriene-3,17-dione (ATD), during E50–E80, a time period that represents the period of maximum behavioral sensitivity to the developmental effects of exogenous T. Both male and female fetuses exposed to ATD and their controls were assessed for effects on sexual maturation, reproductive behaviors, and gonadotropin release.

Results

Maternal Serum Steroids

Concentrations of ATD in the maternal sera were significantly elevated 4 h after daily drug injection (Fig. 1A). The elimination half-life of ATD calculated using a noncompartmental model was 8.7 ± 1.2 h (Fig. 1B). ATD treatment did not alter concentrations of E2 or progesterone in maternal serum (data not shown).

Serum Concentrations of Testosterone in Newborn Lambs

The serum concentrations of T (Fig. 2) were significantly greater in males than in females during the first 24 h after birth ($p < 0.01$). Prenatal exposure to ATD did not significantly affect concentrations of serum T or alter the sex difference observed in newborn lambs.

Play Behavior

Spontaneous play behaviors were observed in 100% of control and ATD-exposed ram lambs during the first 10 wk of life. In contrast, play behaviors were observed in 71.4% of control ewe lambs and 55.5% of ATD-exposed ewe lambs. Mounting represented approx 60% of all play behaviors in both ram and ewes lambs. Two-way ANOVA revealed a significant gender difference, in play behaviors [$F(1,28) = 21.6$; $p < 0.0001$], represented by the combined frequency of all behaviors displayed, and in mounting behavior alone [$F(1,28) = 22.6$; $p < 0.0001$], Fig.3. Ram lambs exhibited significantly greater frequencies of behavior than ewe lambs. Prenatal exposure to ATD did not significantly affect juvenile play behaviors or mounting behavior.

Growth Rate

Weights of lambs from birth until 9 mo of age are depicted in Fig. 4. Lambs born to ewes that were treated with ATD during pregnancy weighed significantly more at birth than control lambs [$F(1,28) = 23.4$; $p < 0.01$]. However, no significant weight differences were apparent between treatment groups after 1 mo of age. A significant gender difference in weight emerged by 4 mo of age, when ram lambs began gaining weight more rapidly than ewe lambs.

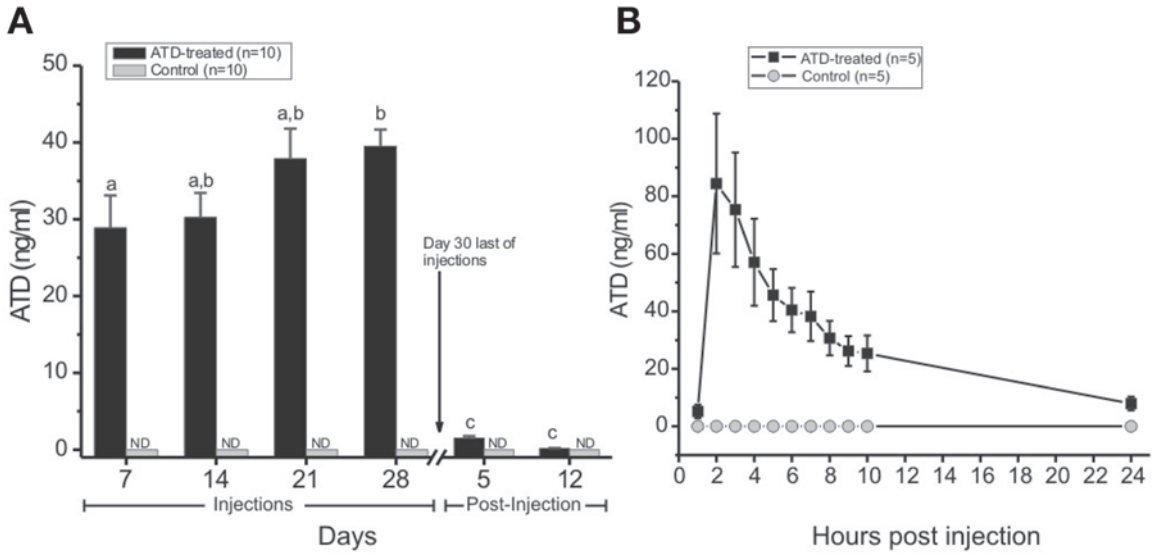

Fig. 1. (**A**) Weekly concentrations (mean ± SEM) of ATD in serum from control (n = 10) and ATD-exposed (n = 10) pregnant ewes measured 4 h after injection ($p < 0.05$). Bars with different letters differ significantly. (**B**) Hourly profile of serum ATD concentrations (mean ± SEM) on wk 3 of treatment (n = 4 in each treatment group). ND, nondetectable.

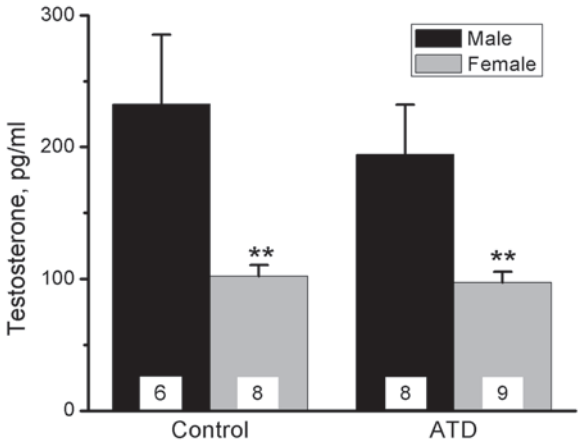

Fig. 2. Concentrations (mean ± SEM) of T in serum from control and ATD-exposed newborn lambs. *$p < 0.01$, male vs female.

Sexual Maturation in Ram Lambs

Control and prenatal ATD-exposed ram lambs had similar age-dependent increases in serum concentrations of T (Fig. 5). The first significant increase in systemic T occurred by 10 wk of age (June). This was followed by a steady seasonal increase in serum concentrations of T, which peaked by 29 wk of age in October. There was no significant difference between the maximum serum concentrations of T between control (15.3 ± 2.8 ng/mL) and ATD-exposed (13.4 ± 1.6 ng/mL) ram lambs. Scrotal circumference increased from August (20 wk of age) through October (28 wk) when the maximal size was attained in both groups of ram lambs (data not shown).

Sexual Maturation in Ewe Lambs

The onset of puberty as reflected by a sustained increase in serum concentrations of progesterone occurred at approximately the same time in control and ATD-exposed ewe lambs (27 ± 0.5 wk). Similarly, there were no differences in the total number of estrous cycles or the peak progesterone concentrations achieved between treatment groups, Table 1 and Fig. 6.

Male-Typical Sexual Behavior and Partner Preference

Each ram was given four preference tests in the first breeding season (8 mo of age) and two preference tests in the following breeding season (18 mo of age). With the exception that rams exposed to ATD prenatally showed significantly lower female-directed mounts in the second year of testing, there was no difference in sexual behaviors between control and ATD-exposed rams (Fig. 7). Prenatal ATD exposure did not significantly affect partner preferences. We observed that four of six control rams were female-oriented and two of six exhibited bisexual mounting. In contrast, three of eight ATD-exposed rams were female-oriented and five of eight were bisexual. No rams in these groups exhibited exclusively male-oriented sexual partner preference.

LH Responsiveness in Males

The effects of prenatal ATD exposure on feedback mechanisms that control LH release are presented in Fig. 8. Serum concentrations of LH were significantly lower in the ATD-exposed rams than in controls both during the control bleeding period and after injection of estradiol. E2 treatment significantly inhibited LH secretion between 8 and 20 h after injection ($p < 0.05$). The concentrations returned to pre-injection values by 36 h after injection in both groups; however, neither group exhibited a LH response to E2 injection that reached the criterion for a surge, i.e., two times pre-injection baseline LH for six consecutive hours.

The LH secretion of control and ATD-exposed rams given GnRH is shown in Fig. 9. Basal levels of LH in ATD-

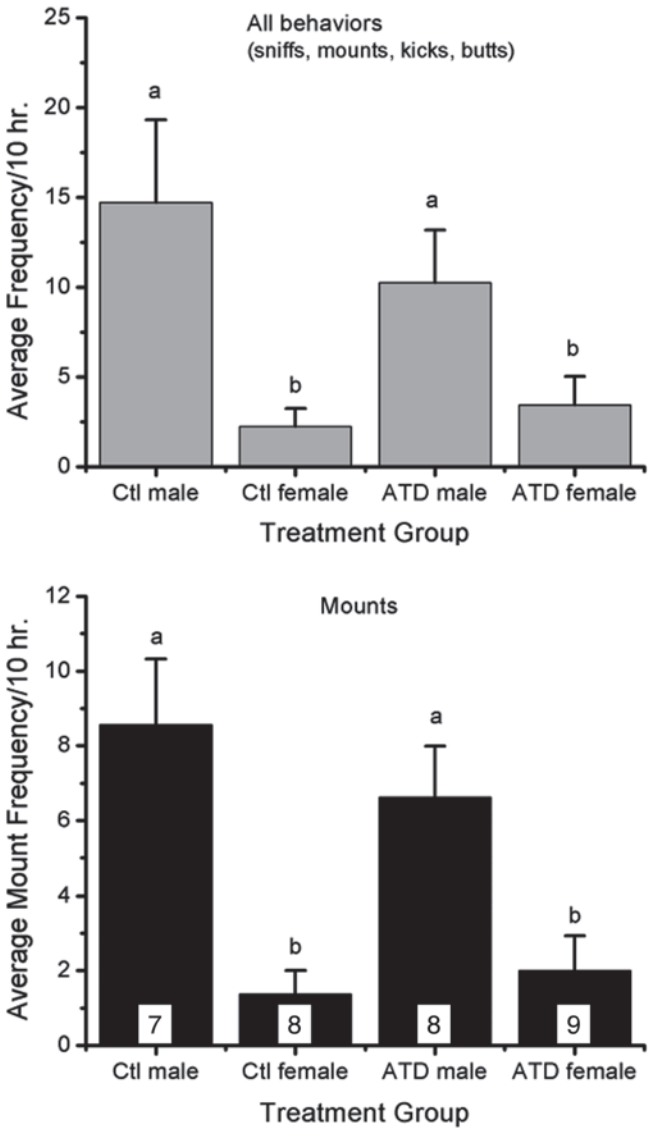

Fig. 3. Average frequency (± SEM) of male-like sexual play behavior patterns displayed during the first 10 wk following birth by control (Ctl) and ATD-exposed (ATD) male and female lambs. (**A**) Average frequency of all behaviors including sniffs, foreleg kicks, mounts, and head butts. (**B**) Average frequency of mounting behavior. Bars with different letters differ significantly ($p < 0.05$).

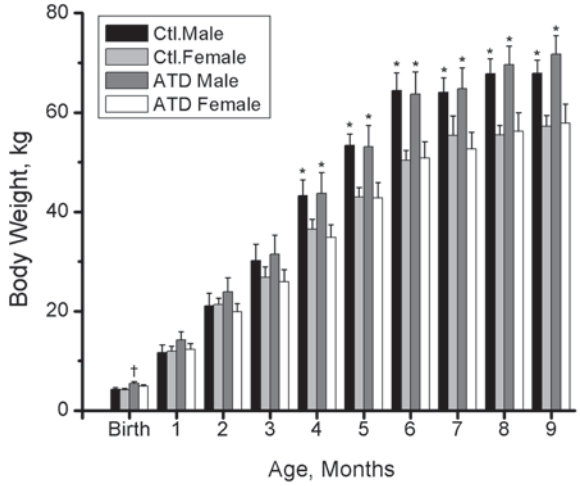

Fig. 4. Differences in mean body weights (± SEM) of control (Ctl) and ATD-exposed (ATD) male and female lambs. $^{\dagger}p < 0.05$ ATD male vs all other groups at birth. $^{*}p < 0.05$, male vs female between 4 and 9 mo of age.

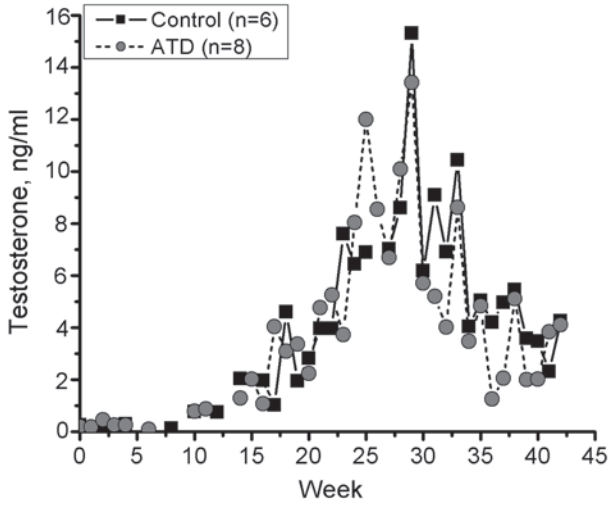

Fig. 5. Mean (± SEM) serum concentrations of T in control and ATD-exposed male lambs from birth through February of the first breeding season.

exposed rams were significantly lower than in controls ($p < 0.05$). Injection of GnRH caused a significant increase in LH secretion in both control and ATD-exposed rams, but the surge release of LH did not differ ($p > 0.05$). These data suggest that the lower basal LH levels in ATD-exposed rams represent a difference in the hypothalamic function rather than anterior pituitary responsiveness to E2.

Female-Typical Sexual Behavior in Rams

When control and prenatal ATD-exposed rams were treated with E2 and paired individually with a sexually vigorous teaser ram 24 h later, all of the experimental rams investigated (sniffed) the teaser and were, in turn, investigated by the teaser. The teaser did not attempt to mount any of the experimental rams and performed leg kicks on only one prenatal ATD-exposed ram. Neither control nor prenatal ATD-exposed rams exhibited any definitive proceptive or receptive behaviors. Agonistic behavior (butting) was displayed by one control and one ATD-exposed ram.

Effect of Prenatal ATD Exposure on LH Surge Mechanism and Fertility in Ewes

Four control and four prenatal ATD-exposed ewes were tested for an intact LH surge mechanism in response to E2 injection. Before E2 stimulation, circulating concentrations of LH were similar in control and prenatal ATD-exposed ewes (1.3 ± 0.05 ng/mL). Regardless of their prenatal treatment, all of the ewes produced a surge of LH beginning 10 h after estrogen exposure, which lasted 8 ± 0.5 h and reached a peak height of 216.6 ± 13.6 ng/mL (Fig. 10). The prenatal

Table 1
Comparison of Age at Puberty, Number of Estrous Cycles, and Peak Levels of Progesterone Achieved During the First Breeding Season Between Control and Prenatal ATD-Exposed Ewes[a]

Treatment Group	Age at first cycle (wk)	Number of cycles	Peak levels of progesterone (ng/mL)
Controls ($n = 8$)	26.9 ± 0.9	6.1 ± 0.4	6.5 ± 0.5
ATD-exposed ($n = 9$)	27.3 ± 0.8	6.7 ± 0.4	7.2 ± 0.5

[a]Mean ± SEM.

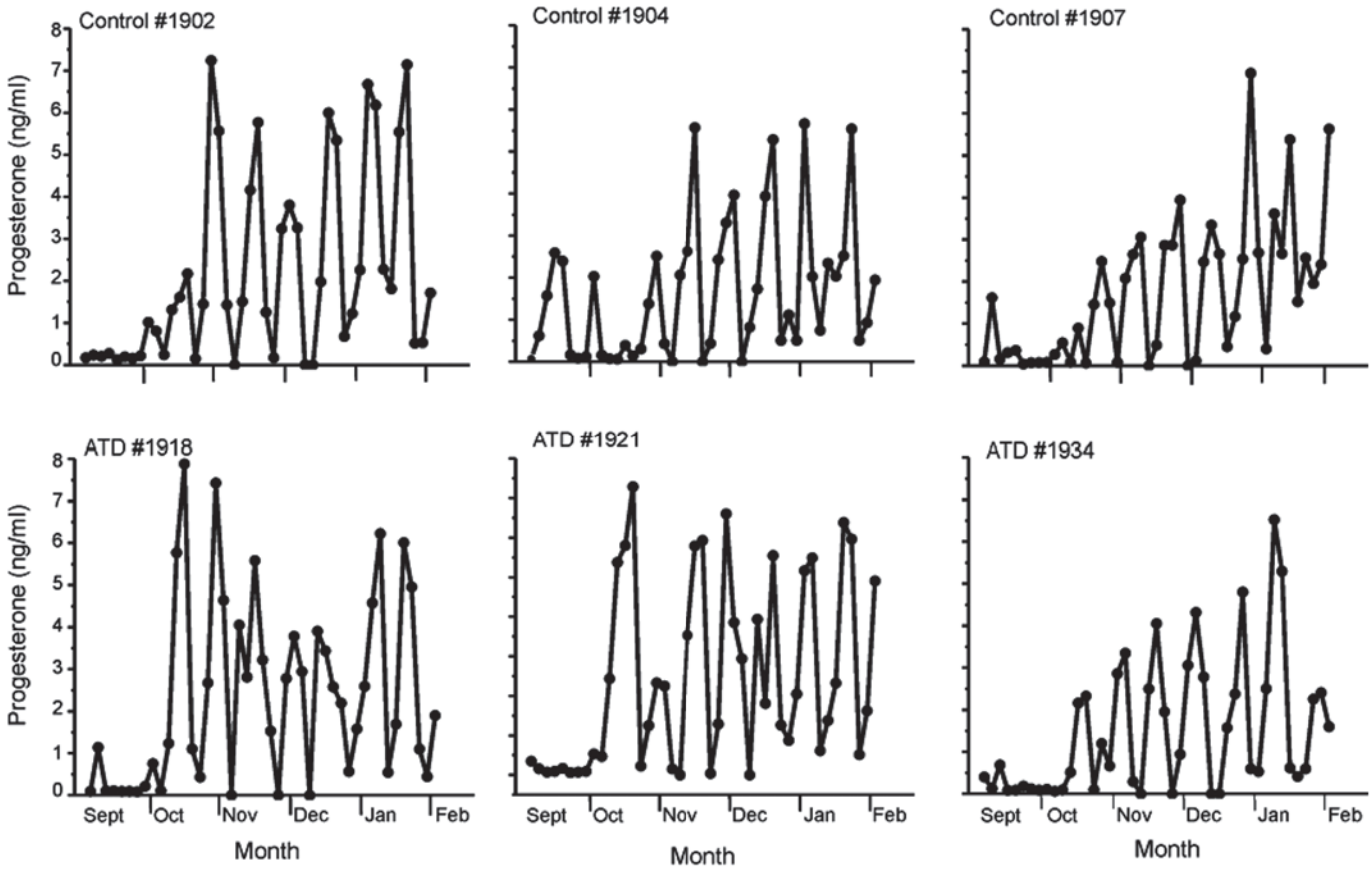

Fig. 6. Serum progesterone profiles from representative control and prenatal ATD-exposed ewes. Blood samples were taken twice weekly beginning just before the expected onset of the first breeding season (September) until the expected end of the breeding season (February).

exposure of ewes to ATD did not significantly affect measures of fertility and fecundity assessed when the sheep were 2 yr old (Table 2). All control and prenatal ATD-exposed ewes cycled in their 2nd year and all carried pregnancies to term (Table 2). There were no differences between treatments in the average cycle length, number of offspring born, lamb weights, lamb vigor, lambing difficultly, or maternal attentiveness.

Discussion

The results of the present study demonstrate that exposure of the male sheep fetus to the aromatase inhibitor ATD during E50–E80 of gestation partially disrupts normal masculinization of adult copulatory behavior. A prenatal reduction in estrogenic stimulation caused a significant decrease in adult mounting behavior, but had no effect on courtship behavior. Similar but more dramatic effects of prenatal ATD treatments on mounting have been described in ferrets *(31)*; however, there are conflicting reports of deficient mounting capacity after prenatal ATD treatment of rats *(32,33)* and guinea pigs *(34,35)*. Gonad-intact aromatase knockout (ArKO) mice show pronounced deficits in mounting, intromissions, and ejaculations that were largely, but not entirely, corrected when ArKO males were castrated and treated as adults with E2 or E2 + DHT *(36)*, further highlighting that species differences exist in the extent to which aromatization

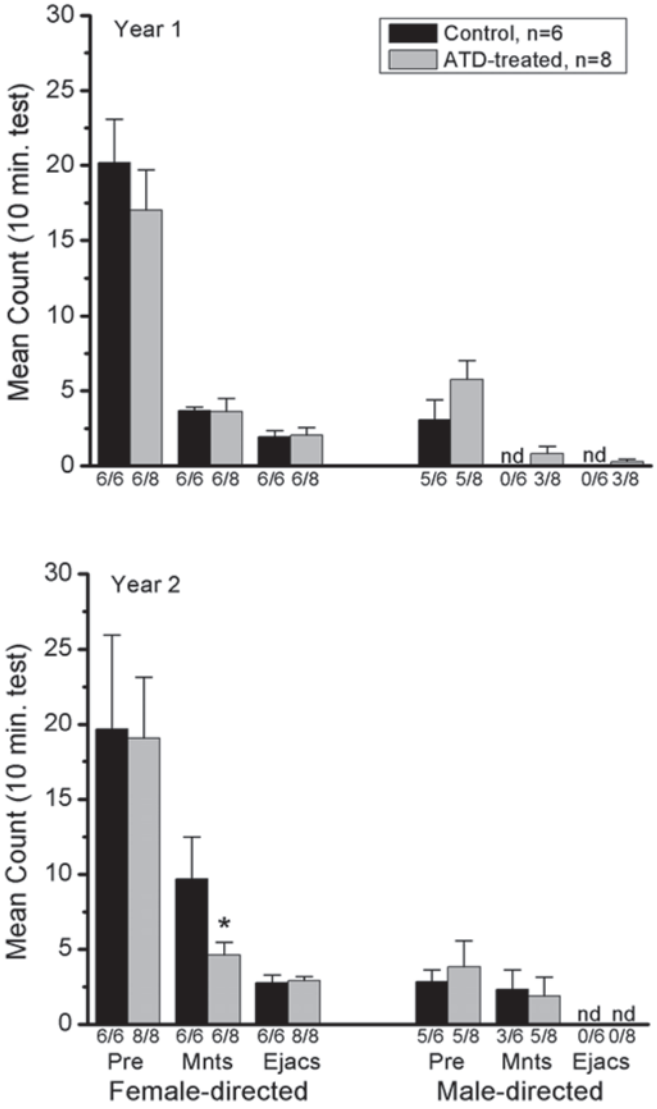

Fig. 7. Reproductive behaviors of control and prenatal ATD-exposed rams. Each male was given four preference tests (see *Methods* for details) in the first breeding season at 8 mo of age (yr 1) and two tests in the second season at 18 mo of age (yr 2). The frequencies of precopulatory (Pre) behaviors (anogenital sniffs, foreleg kicks, flehmen, and vocalizations), mounts (Mnts), and ejaculations (Ejacs) directed at either estrous female stimulus animals (Female-directed) or male stimulus animals (Male-directed) were recorded. Values are mean ± SEM. *$p < 0.05$ control vs prenatal ATD-exposed. The fraction under each bar represents the proportion of rams exhibiting each behavior.

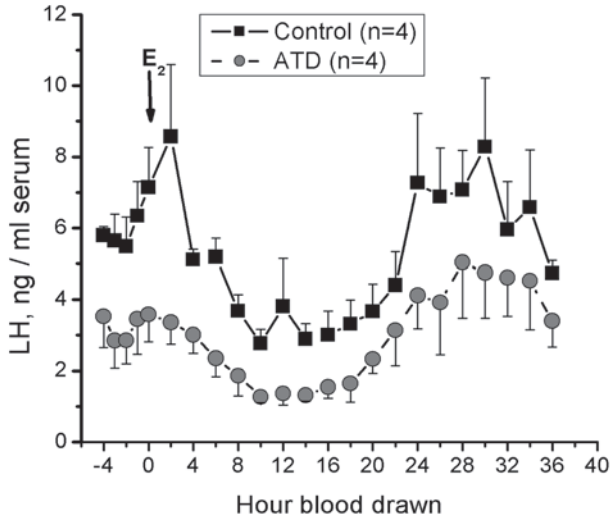

Fig. 8. LH secretion in response to a surge-inducing dose of estradiol (50 μg E2/animal) in gonadectomized control and prenatal ATD-exposed rams. Blood samples were collected hourly until E2 was injected and then every 2 h thereafter for another 36 h. Values are mean ± SEM. No animal met the criterion for a surge defined as LH values exceeding twice the average pre-E2 baseline for a minimum of 6 h *(29)*.

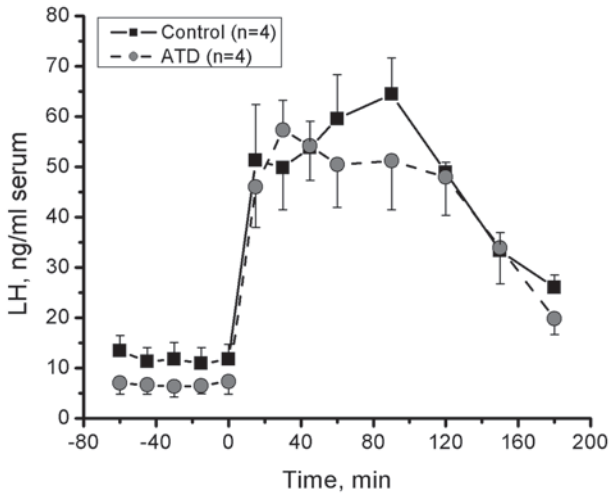

Fig. 9. LH secretion in response to an iv injection of GnRH (100 μg/animal) in gonadectomized control and prenatal ATD-exposed rams. Blood samples were collected every 15 min before and after GnRH administration. Time 0 = time of GnRH injection. Values are mean ± SEM.

is required to organize the expression of male coital behaviors. Research with ferrets suggests that behavioral masculinization could depend on an initial exposure to locally formed estrogen *in utero* that, in turn, sensitizes the developing brain to the masculinizing action of T during postnatal development *(31)*. There is some suggestion that a postnatal phase of sexual differentiation may exist in male sheep *(27, 37)*. Our observation that serum concentrations of T are significantly higher in newborn male lambs than in females supports the possibility that the critical period is not restricted to fetal life in sheep. However, a more rigorous assessment of the effect that castration and hormone manipulations in early postnatal life has not yet been performed on sheep.

It should be noted that, as we reported previously *(19)*, treatment with ATD did not significantly suppress maternal serum estrogen levels. However, very low concentrations of circulating E2 are detected normally in pregnant sheep until late in gestation, i.e., after 140 d; parturition approx 147 d *(38)*. Thus, it seems unlikely that the low levels of E2 circulating in maternal blood could compensate for that inhi-

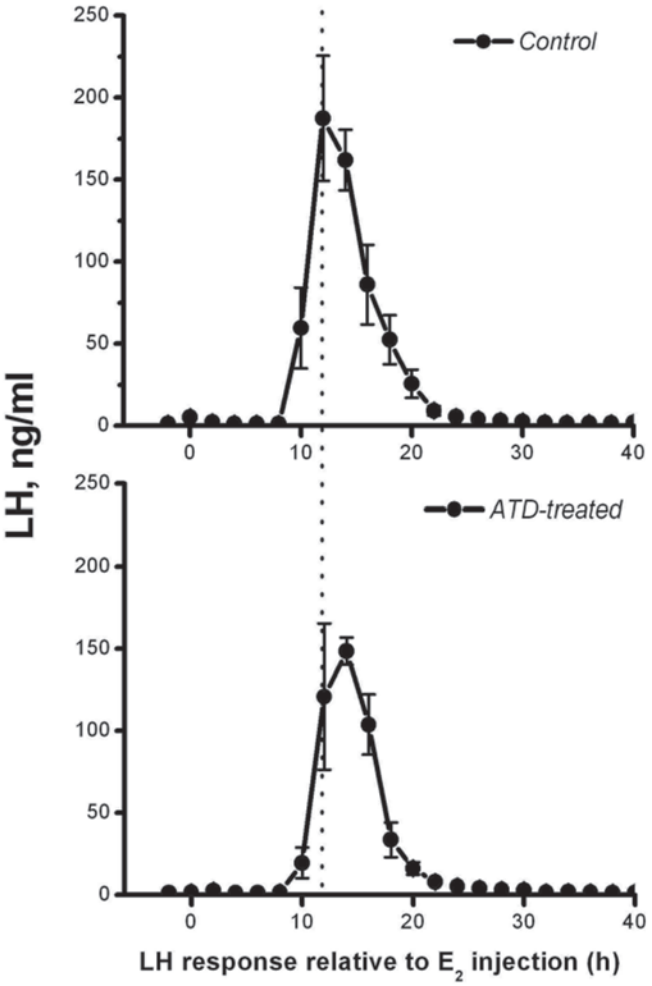

Fig. 10. LH secretion in response to a surge-inducing dose of estradiol (50 μg E2/animal) in control and prenatal ATD-exposed ewes during the nonbreeding season. Blood samples were collected every 2 h beginning 2 h before the E2 injection (time 0) until 40 h after the injection. The dashed vertical line is aligned to the peak of the LH surge in control ewes. Values are mean ± SEM.

bition of estrogen synthesis that occurred within discrete brain regions of the fetus.

Prenatal inhibition of aromatase activity in fetal sheep had no significant effect on adult sexual partner preferences given the conditions used in the current study. These results differ with findings in rats that show that prenatal antiestrogen treatment and postnatal aromatase inhibition cause significant reductions in the sexual preference of adult males for estrous females *(8,39)*. However, recent studies using transgenic mice suggest that, although perinatal estrogen exposure is necessary for the full development of male sexual preference, males lacking the ability to aromatize androgens (ARKO) or bind estrogen (ERαKO) do not express female-typical sexual partner preferences *(11,40)*. Thus, it is possible that other factors, such as nonendocrine experiential factors, can interact with gonadal hormone exposure or genetic background to determine sexual preferences in animals *(41)*.

Current evidence suggests that aromatization of androgens to estrogens is obligatory to produce defeminization of the LH surge mechanism and behavioral receptivity in rats. Neonatal treatments with T or E2 block adult ovarian cyclicity and lordosis behavior in genetic females, whereas treatments that reduce neural estrogen stimulation in males have been shown to support ovarian cyclicity and enhance the display of female-typical sexual behavior *(7)*. Moreover, recent data from knockout mice indicate that ERβ is essential for behavioral defeminization in males *(12)*. In long gestation animals such as guinea pigs, prenatal ATD exposure blocks behavioral defeminization, but does not affect the lack of capacity for cyclic gonadotropin secretion in males *(34)*.

Aromatization is presumed to be obligatory for neuroendocrine and behavioral defeminization in sheep because prenatal treatment with T, but not DHT, blocks development of the LH surge mechanism and decreases the capacity of females to show cyclic or steroid-induced receptive behavior *(29,42)*. However, in the current study, prenatal exposure to ATD, at a dose that strongly inhibits aromatase activity in the fetal hypothalamus, did not block behavioral defeminization or prevent inactivation of the LH surge mechanism from occurring normally in genetic males. These results cannot be attributed to counteracting androgenic actions of ATD or its metabolites that have been reported previously *(43,44)*, because E2-induced surges showed no qualitative or quantitative differences between control and ATD-exposed ewes. However, there are other possible reasons for why the prenatal ATD exposure used in the current study did not defeminize male sheep. First, the treatment period of ATD exposure may have been initiated too late to block all of the effects of endogenous T secretion in males. While the period of maximal behavioral masculinization appears to fall between E50 and E80 *(20,21)*, complete defeminization of the LH surge, and possibly of behavioral receptivity, requires a longer exposure to T *(28)*. Second, given that the dose of ATD used in this study inhibits aromatase activity in the fetal sheep brain by approx 85% *(19)* and (and *Methods*), the low levels of aromatase activity (≤15% of normal) remaining may provide enough local estrogen to partially masculinize and fully defeminize neuroendocrine function and behavior. In fact, previous evidence indicates that the developmental mechanisms organizing the surge system are sensitive to low levels of steroids *(45)*. Third, it is possible that after aromatase is inhibited, redundant hormonal systems and/or feedback mechanisms are able to compensate and overcome large, but nonetheless transient, interruptions in hormone signals. Finally, our results could indicate that prenatal aromatization of T to E2 is not critical for defeminization of the sheep brain. Instead T, and not its estrogenic metabolite, could be the agent responsible for organizing this aspect of the male sheep brain in a manner similar to what has been suggested for nonhuman primates *(1)*. Studies are currently being conducted

Table 2
Comparison of Mean (± SEM) Cycle Length and Fertility Measures Between Control and Prenatal ATD-Exposed Ewes

Treatment Group	Second year cycle length (d)	Average number of lambs born	Lamb weight (kg)	Lamb vigor score	Lambing difficulty score	Mother attentiveness score
Controls (n = 8)	17 ± 0.2	1.6 ± 0.2	8.4 ± 0.4	2.9 ± 0.7	2.0 ± 0.0	5.0 ± 0.0
ATD-exposed (n = 9)	17 ± 0.3	1.7 ± 0.2	8.0 ± 0.5	2.9 ± 0.8	2.0 ± 0.0	5.0 ± 0.0

to distinguish between these possibilities by evaluating the effect of higher doses of ATD given over a more extended period of time.

Recent studies show that female aromatase knockout (ArKO) mice exhibit reduced levels of lordosis behavior, suggesting that E2 is required for the development of the neural mechanisms controlling receptive behaviors in females *(46)*. We did not test the effect of prenatal ATD treatment on female sheep sexual behavior, but we surmise that any effect that the dose of ATD used in this experiment had on normal female development was subtle because no effects on fertility and fecundity were observed.

Sexually differentiated control of gonadotropin secretion extends beyond whether or not gonadotropins are released cyclically. Detailed studies conducted using the gonadectomized, E2-treated lamb model have demonstrated that neuroendocrine puberty occurs much earlier in the male than in the female *(27)*. Treatment of female lambs with T or DHT during the critical period markedly advances the time of neuroendocrine puberty to an age that is virtually the same for the normal male. In gonad-intact sheep, neuroendocrine puberty is coincident with the onset of progesterone cycles in females and the initiation of testicular growth and T secretion in males. In the present study we observed the first significant increase in T secretion at 10 wk of age, which signaled the start of neuroendocrine puberty in males, while in females the onset of puberty was evidenced by the initiation of progesterone cycles at approx 27 wk of age. As expected, prenatal exposure to ATD did not affect the timing of puberty in either sex, nor did it have any discernible impact on reproductive development as evidenced by the seasonal pattern of T secretion in males and the number and duration of estrous cycles in females.

Rams exposed to ATD *in utero* had lower systemic levels of LH after castration than did controls, but showed equivalent LH responses to a bolus injection of GnRH. These results suggest that tonic release of LH is organized in males by prenatal estrogen exposure. This is reminiscent of the sexually dimorphic response observed in the serum LH response to gonadectomy in rats. Serum concentrations of LH in adult male rats rise rapidly after gonadectomy, whereas the rise of LH in gonadectomized females lags several days behind *(47)*. Neonatal treatment with low levels of T propionate was shown to masculinize the LH response to gonadectomy in females suggesting that this aspect of LH regulation is sexually differentiated. More research is needed to understand the significance of these observations for adult fertility in sheep.

In summary, we found that prenatal exposure to ATD during the developmental period defined by maximal behavioral sensitivity to T partially disrupted normal masculinization of adult copulatory behavior suggesting that aromatization is necessary for complete male-typical brain differentiation. In contrast, prenatal exposure to ATD did not interfere with defeminization of adult sexual receptive behaviors, partner preferences, or the LH surge mechanism in adult male sheep. However, before we can conclude that aromatization does not play a role in defeminization of the sheep brain, it will be necessary to evaluate whether exposure of male fetuses to higher doses of ATD for a more extended period of gestation can disrupt normal neuroendocrine and behavioral development.

Materials and Methods

Animals

Twenty Polypay ewes were bred to Suffolk rams and maintained under normal husbandry conditions at the Sheep Research Facility at Oregon State University in Corvallis, OR. Animal husbandry and experimental protocols were conducted in accordance with the principles and procedures specified by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees at Oregon State University and the Oregon Health and Sciences University.

Prenatal Treatments

Pregnant ewes with known conception dates received daily sc injections of ATD (100 mg/d in 3 mL propylene glycol, Sigma, St. Louis, MO; n = 10) or propylene glycol vehicle (3 mL; n = 10) from E50 to E80 of gestation (term = 147 d). The treatment period spanned the period of gestation when the sheep brain is maximally sensitive to the behavioral effects of exogenous testosterone as delineated by Clarke *(21)*.

We demonstrated previously that ATD crosses the sheep placenta and inhibits aromatase activity in the fetal brain *(19)*. Results of a preliminary experiment in which 100 mg/

mL of ATD was injected into a pregnant ewe for 2 wk (gestational day 50–64) indicated that aromatase activity in the hypothalamus and amygdala was inhibited >85% by this treatment (data not shown).

Jugular blood samples were collected weekly from all dams during treatment and for 2 wk after treatment ended. During the 3rd week of treatment hourly blood samples were drawn from five treated and five control dams to obtain a profile of serum ATD for pharmacokinetic analysis. Sera, harvested from blood clotted overnight at 4°C and centrifuged at 1000*g* for 15 min, were stored frozen (–20°C) until assayed for steroids.

Postnatal Treatment

Fifteen control lambs (male, $n = 7$; female, $n = 8$) and 17 ATD-exposed lambs (male, $n = 7$; female, $n = 10$) were born in April. Eight singlets, 11 sets of twins, and 1 set of triplets were born from dams in this experiment. No adverse effects of treatments were noted for lambing difficulty, dam attentiveness, or lamb vigor. The lambs were weighed within the first 24 h after birth and at monthly intervals thereafter until they were 9 mo old. Jugular blood samples were drawn in the first 24 h of life and then weekly for 2 mo. From September through February (approx 5–10 mo of age), weekly blood samples were collected from males to measure T concentrations, while twice weekly blood samples were collected from ewes to measure progesterone concentrations and monitor the initiation and frequency of ovulatory cycles during the first breeding season. Serum was harvested as described above and stored at –20°C until assayed for hormones. ATD-treated and control lambs were raised together and weaned at 90 d of age when they were placed into the same sex groups. Ram and ewe lambs were isolated in separate non-adjoining pastures during the spring, summer, and fall. During the winter, they were fed alfalfa hay supplemented with barley-based concentrate. Lambs had *ad libitum* access to water and minerals and were regularly treated with antihilminetics to reduce parasite infestation.

Play Behavior

Play behavior in lambs consists primarily of male-like sexual behavior patterns, which occurs during the first postnatal months of life in both males and females *(48,49)*. This behavior is considered play behavior because it occurs in what appears to be spontaneous episodes that are not organized in any particular sequence *(50)*. The frequency of play behavior in lambs is higher in males than in females and is, therefore, considered sexually dimorphic *(48,49)*. The sexual dimorphism was shown to depend on prenatal androgen exposure, which masculinizes the behavior *(51)*. To determine whether prenatal ATD exposure altered juvenile play behavior, spontaneous interactions between lambs were recorded by observing the subjects for 1 h per week during wk 1–10 following birth. The following components of play behavior were recorded: anogenital sniffs, mounts, foreleg kicks, flehmen (i.e., curling of the upper lip with mouth open, which is thought to facilitate the detection of nonvolatile pheromones from conspecifics), and head butts.

Sexual Partner Preference Tests

Sexual preference tests were conducted when the rams were approx 8 mo of age and repeated at 18 mo of age. Rams were housed in individual pens for 5 d prior to each test session. The sexual partner preference test was adapted from the method of Perkins and Fitzgerald *(52)* and consisted of exposing the subject to two estrous ewes and two unfamiliar rams in a 10 × 10 m arena. The four stimulus animals were restrained in a four-way stanchion that allowed approach of the test animals from the sides and rear. The rams being tested were free to choose among the four stimulus animals or remain neutral. Tests were 10 min in duration and repeated every 3 wk for a total of four consecutive tests in the first breeding season and an additional two tests in the second breeding season. The sex of the preferred stimulus animal and the frequency of anogenital sniffs, foreleg kicks, flehmen, vocalizations, mounts, and ejaculations were recorded.

LH Surge Secretion

Although female sheep release a large discharge of LH in response to an acute increase in systemic E2, the LH surge mechanism is not found in rams *(53)*. This difference between rams and ewes is believed to be due to prenatal exposure of the developing fetus to T or its estrogenic metabolites in males and not in females *(29)*. To assess whether aromatization of T to E2 by the fetus is required to suppress the development of the LH surge mechanism, control and prenatally ATD-exposed rams were castrated at 20 mo of age and 5 wk later challenged with E2. Castration was performed under Xylazine anesthesia (0.1 mg/lb BW) supplemented with 5 cc of 2% lidocaine administered into each spermatic cord. Following surgery, Tolazoline (3 cc iv/ram) was administered to reverse the effects of Xylazine.

To evaluate the responsiveness of the LH surge mechanism, castrated control and ATD-exposed rams were treated for 5 d with a progestin ear implant (6 mg norgestomet/implant; Rhone Merieux Inc., Athens, GA). Eighteen hours after implant removal, each sheep received an im injection of E2 (50 μg in 1 mL corn oil) into the upper thigh. LH was measured in blood samples collected every hour for 4 h before the E2 injection and at 2 h intervals thereafter for a total of 36 h. An LH surge was defined as LH values exceeding twice the average pre-E2 baseline for a minimum of three consecutive samples *(29)*.

Female-Typical Sexual Behavior

Adult rams and ewes exhibit dimorphic repertoires of sexual behavior. Ewes respond to sequential treatment with progesterone and E2 by displaying sexual receptivity, whereas this hormonal treatment is ineffective in rams *(37)*.

Analogous to the estrogen-stimulated LH surge mechanism, the hormone stimulated expression of female sexual behavior is suppressed in rams, i.e., defeminized, due to prenatal exposure to T or its estrogenic metabolites *(54)*. To determine whether aromatization of T to E2 by the fetus is required for behavioral masculinization/defeminization in sheep, control rams and rams exposed to ATD prenatally were tested for female sexual behavior during the evaluation of the LH surge system. Measures of female sexual behavior were based on the methods described by Clarke *(25)* and were evaluated after 24 h of E2 stimulation by pairing each animal with a sexually vigorous ram for 5 min. Proceptive (sniffs, head turns, and tail fanning), receptive (standing), and agnostic (butting) behaviors were recorded.

GnRH Challenge

To determine whether prenatal exposure to ATD altered hypothalamic or anterior pituitary function, rams were administered exogenous GnRH (i.e., GnRH challenge test). At 30 mo of age, the same castrated rams as described above (i.e., four control and four ATD-exposed) were given 100 μg GnRH (Cystorelin, Abbott Laboratories, North Chicago, IL) iv at 0 h. Blood samples were collected at 15 min intervals for 1 h prior to and after GnRH challenge, and then at 30 min intervals for the following 2 h. Serum was harvested and analyzed for LH concentrations.

Assessment of Ewe Fertility

To assess whether prenatal ATD exposure affected ewe fertility and fecundity, control and ATD-exposed ewe offspring were tested for an intact LH surge mechanism during the anestrous season and then bred during the following fall. Ovary-intact anestrous ewes were confined to individual pens for 5 d to acclimate and then at time 0 the sheep received an im injection of E2 (50 μg in 1 mL corn oil). Serum LH was measured in blood samples collected every 2 h starting 2 h before and 48 h after the E2 injection. An LH surge was defined as described above.

To determine whether prenatal ATD exposure had any adverse effect on estrous cyclicity in the second year, vasectomized rams with painted briskets were kept with the flock during the breeding season. When a ewe was marked on the rump with paint by the ram she was removed from the flock and tested for sexual receptivity with a second vasectomized ram. If the ewe was found to be in estrus, she was removed from the flock for 4 d before being reintroduced. After the ewe was marked a second time, the number of days between markings was calculated and used as an indication of estrous cycle duration.

Control ($n = 8$) and ATD-exposed ($n = 9$) ewes were bred when they were approx 18 mo of age. The number, weight, and sex of offspring were recorded and the lambing difficulty (1, assistance needed; 2, no assistance needed), dam attentiveness (1, does not clean off lamb—does not nurse; 2, does not clean off lamb—nurses; 3, does not clean off lamb—lamb dead or weak; 4, cleans off lamb—does not nurse; 5, cleans off lamb—nurses), lamb vigor (1, lamb dead; 2, lamb weak; 3, lamb alive and well) were scored.

Steroid RIAs

Specific RIAs that have been described previously *(54, 55)* were used to measure steroid hormones in aliquots of serum (500 μL) that were extracted with ether and fractionated by Sephadex LH-20 column chromatography. The mean percentages of recovery, water blanks, and intraassay coefficients of variability were as follows: T: 68.5%, 3.0 pg, 5.3%; DHT: 81.4%, 4.2 pg, 2.6%; progesterone: 90.9%, 7.4 pg, 7.1%; E2: 80.3%, 1.9 pg, 7.1%; estrone (E1): 83.7%, 4.2 pg, 6.7%; and ATD: 93.6%, 2.0 pg, 12.0%.

LH RIA

Concentrations of LH were determined in duplicate using NIH-anti-ovine-LH as primary antibody and NIH-ovine-LH-13 as standard according to previously published methods *(56)*. The mean detection limit of the assays (2 SD from the buffer controls) was 0.3 ng/mL. The intra- and interassay coefficients of variation were 13% and 15%, respectively.

Statistical Analysis

Noncompartmental pharmacokinetic analysis of ATD elimination half-life was performed with Winnonlin Analysis Program v. 4.1 (Pharsight Corporation, Mountain View, CA.). Data on body weight and hormone concentrations collected over time were analyzed by a one or two-way ANOVA for repeated measures followed by post-hoc Newman–Keuls test when appropriate. Serum concentrations of T at birth and behavioral measures were analyzed by Mann–Whitney *U* tests. Comparisons of the proportions of control and ATD-exposed rams exhibiting various behaviors were analyzed by a complex χ^2 test. Estrous cycle characteristics and fertility measures in ewes were compared with *t*-tests. In all tests $p < 0.05$ was considered significant.

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