Developmental programming: postnatal estradiol modulation of prenatally organized reproductive neuroendocrine function in sheep

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Abstract

Gestational testosterone (TS) excess, acting via both the androgenic and estrogenic pathways, advances puberty and disrupts the neuroendocrine estradiol (E₂) feedback and periovulatory hormonal dynamics in female sheep. These prenatally programmed defects may be subject to postnatal modifications by continued organizational and/or activational effects of steroids. This study investigated (1) the organizational contribution of prenatal estrogen excess and (2) the impact of postnatal exposure to E₂ in modulating the effects of prenatal androgen excess (TS and dihydrotestosterone (DHT)) on puberty, neuroendocrine feedback mechanisms, and periovulatory hormonal dynamics in sheep. Pregnant Suffolk sheep were treated with TS, DHT, E₂, or E₂ plus DHT (ED) from days 30 to 90 of gestation. A subset of the control (C), TS, and DHT female offspring received a constant-release E₂ implant postnatally. Findings revealed that (1) prenatal E₂-treatment failed to reproduce the neuroendocrine disruptions predicted to be programmed by the estrogenic pathway and (2) prenatal E₂D-treatment did not adequately replicate the reproductive neuroendocrine defects induced by prenatal TS excess. More importantly, continuous postnatal E₂-treatment, while delaying the onset of puberty and reducing the inhibitory effects of E₂ on tonic luteinizing hormone (LH) release, failed to amplify the E₂-positive feedback and periovulatory defects induced by prenatal TS-treatment. Our results indicate that disruptions in E₂-positive feedback mechanisms and periovulatory gonadotropin secretion induced by prenatal TS-treatment are programmed predominantly during the prenatal life with postnatal exposure to E₂ excess not contributing further to these disruptions.

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Introduction

Many diseases in adults, such as obesity, metabolic syndrome, and polycystic ovary syndrome (PCOS), may have their origins during fetal life. As proposed by Barker's developmental origins of adult disease hypothesis (Barker 2004), adverse prenatal insults can cause permanent changes in the physiology of the developing fetus leading to pathology during adulthood. These insults include inadvertent exposure to hormonal, nutritional, and environmental agents (Rhind *et al.* 2001, Tang & Ho 2007, Dumesic *et al.* 2014). The effects of increased exposure to native steroids in disease states (New 2006, Rosenfield 2007) and to environmental endocrine-disrupting chemicals (EDC) that can bind to steroid receptors (Vaiserman 2014) have been the focus of intense research. Due to the high rate of tissue differentiation and metabolism, a developing fetus is extremely sensitive to EDC exposures even at concentrations far below those

detrimental to adults (Diamanti-Kandarakis *et al.* 2009, Schug *et al.* 2011).

Congenital adrenal hyperplasia (CAH) (New 2006) and PCOS (Sir-Petermann *et al.* 2002) are disease states characterized by elevated testosterone levels, among other endocrine imbalances. Experimentally, prenatal TS-treatment in various species, including sheep, leads to dysfunctions during adult life that resemble those seen in women with PCOS (Padmanabhan & Veiga-Lopez 2013a,b, Dumesic *et al.* 2014). For instance, prenatal TS-treated sheep show progressive loss of cyclicity (Clarke *et al.* 1977, Birch *et al.* 2003, Manikkam *et al.* 2006) and disrupted periovulatory hormonal dynamics (Veiga-Lopez *et al.* 2008), with defects evident at both the neuroendocrine and ovarian levels (Padmanabhan & Veiga-Lopez 2013b). The neuroendocrine disruptions induced by prenatal TS excess include reduced sensitivity to estradiol (E₂) and progesterone (P) negative feedback (Wood & Foster 1998, Robinson *et al.* 2002,

Sarma *et al.* 2005), dampened or absent E_2 -positive feedback, and disrupted periovulatory hormonal dynamics (Sharma *et al.* 2002, Unsworth *et al.* 2005, Veiga-Lopez *et al.* 2009). Comparative studies with dihydrotestosterone (DHT), a nonaromatizable androgen (Veiga-Lopez *et al.* 2009), as well as TS+ androgen antagonist-treated sheep (Jackson *et al.* 2008) provided evidence that E_2 -negative, but not E_2 -positive, feedback perturbations are programmed by androgenic actions of TS. This led to the premise that E_2 -positive feedback and periovulatory hormonal dynamics are likely programmed by the estrogenic actions of TS (Foster *et al.* 2006).

Although the organizational period may extend beyond birth (Jackson *et al.* 2013), evidence suggests that activation effects of the postnatal environment can modify the impact of such organizational changes. For instance, the organizational effects of excess prenatal steroid hormone exposure in masculinizing the behavioral circuits in the males become only apparent during puberty when gonadal steroid output determines the expression of sex-typical behavior (Schulz *et al.* 2009). This can be explained by the two-hit hypothesis, which states that an early-life adverse event ("first-hit") programs a pathological condition that may be revealed only later in life by a subsequent exposure to an adverse influence or the so-called "second hit" (Bayer *et al.* 1999, Tang *et al.* 2008, Puttabyatappa *et al.* 2015). From an adverse exposure standpoint, humans are exposed throughout their lifespan to steroids involuntarily and sometimes unknowingly through food consumption (phytoestrogens), industrial byproducts (bisphenol A), diseases (CAH, PCOS), and/or voluntarily (contraception and anabolic steroids) (Bahrke *et al.* 1998, Jefferson *et al.* 2012, Pignatelli 2013, Peretz *et al.* 2014, Jensen *et al.* 2015). Such exposure(s) can be deleterious to fertility and may serve to mask/unmask or reduce/amplify prenatally programmed functions. For example, postnatal overfeeding (second hit) of prenatally TS-treated (first hit) sheep exacerbated the reproductive cyclicity defects, with a majority of animals ending their breeding season early (Steckler *et al.* 2009). Similarly, postnatal exposure to E_2 (second hit) amplified the ovarian defects induced by prenatal TS excess (first hit) (Veiga-Lopez *et al.* 2014).

The objective of this study was to investigate the contributions of prenatal and postnatal E_2 in programming and amplifying reproductive neuroendocrine defects. Specifically, this study tested the following hypotheses: (1) prenatal E_2 -treatment alone induces neuroendocrine defects that have been postulated to be programmed by the estrogenic effects of prenatal TS-treatment, (2) prenatal ED-treatment replicates the effects of prenatal TS-treatment, and (3) continuous postnatal E_2 exposure amplifies the effects of prenatal androgen excess.

Materials and methods

All procedures used in this study were approved by the University of Michigan Animal Care and Use Committee and are consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Generation of experimental animals

The study was conducted at the University of Michigan Sheep Research Facility, Ann Arbor, MI, USA. Adult Suffolk ewes purchased from local farmers were mated with Suffolk rams of proven fertility. General husbandry and nutrition have been described previously (Manikkam *et al.* 2004). Starting at 2–3 weeks before breeding, ewes were group-fed daily with 0.5 kg shelled corn and 1.0–1.5 kg alfalfa hay/ewe. Once mated, females were assigned randomly to the different treatment groups and housed under a natural photoperiod in the pasture and group-fed with a daily maintenance diet of 1.25 kg alfalfa/brome mix hay/ewe. Lambs born to these ewes were fed a pelleted diet (Shur-Gain, Strykersville, NY, USA) consisting of 3.6 MCal/kg digestible energy and 18% crude protein. At ~8 weeks of age, lambs were weaned and maintained outdoors. They were fed *ad libitum* until they attained ~40 kg of body weight, at which point they were switched to a diet consisting of 2.3 MCal/kg digestible energy and 11.3% crude protein. The treatment groups used in the study and the various studies performed are summarized in Fig. 1.

The lambs used in the study received either no treatment prenatally (control; C) or were treated prenatally from gestational days 30 to 90 with TS (TS-treated), DHT (D-treated), estradiol (E_2 -treated), or ED (ED-treated) (Fig. 1A). The number of female offspring born in each treatment group was C=13, E_2 =11, ED=6 and 7 each for TS and D prenatal treatment groups. A subset of prenatal C, TS-, and D-treated females received E_2 implants postnatally starting at ~12 weeks of age. Sample size of postnatal groups was 7 for C+ E_2 and TS+ E_2 , and 6 for the D+E group (Fig. 1A). There were insufficient animals to generate postnatal treatment groups from prenatal E_2 - and ED-treated animals. Changes in ovarian follicular dynamics in this cohort of animals have been reported previously (Veiga-Lopez *et al.* 2014).

Prenatal groups

Prenatal TS- and D-treated female sheep were generated as reported previously (Wood *et al.* 1991). Briefly, pregnant Suffolk ewes were injected (intramuscular) twice weekly from 30 to 90 days of gestation with either 100 mg TS propionate (1.2 mg/kg; Sigma-Aldrich) or 100 mg DHT propionate (Stereoids, Newport, RI, USA) suspended in 2 mL cottonseed oil. Females in the C group did not receive vehicle because no differences in reproductive parameters were found between vehicle-treated and untreated controls in our previous study (Veiga-Lopez *et al.* 2008). Prenatal E_2 -treated animals were generated by placing a 30 mm SILASTIC implant (Dow Corning, Midland, MI, USA) filled with crystalline E_2 subcutaneously in the axillary region for the same duration as described before

Figure 1 Schematic showing the study design. (A) Prenatal and postnatal treatment groups. (B) Timeline of studies carried out. (C) Timeline of the E₂-negative and E₂-positive feedback studies. Periods 1, 2, and 3 indicate pulse bleed at the end of the GnRH-A treatment, 72 h after cessation of the GnRH-A (Pre-E₂) treatment and 72 h after the E₂-treatment (Post-E₂) respectively. Hatched bar indicates timing of E₂-positive feedback. (D) Timeline of the assessment of the periovulatory hormonal dynamics carried out during the first and second breeding seasons. Arrows indicate the time of estrous synchronization with PGF_{2α} injections and gray boxes show periods of 2-hourly samples and daily sample collection.

(Jackson *et al.* 2008). Control females did not receive sham implants. The efficacy of this approach in suppressing LH secretion in sheep has been well established (Karsch *et al.* 1993). The 30 mm implant produces circulating E₂ concentrations of ~3 pg/mL, similar to those seen during the follicular phase in the female sheep (Goodman *et al.* 1980, 1981, Jackson *et al.* 2013). The prenatal ED-treated group received E₂ implants along with twice weekly injections of DHT.

Postnatal treatment

Approximately 12 weeks after birth, half of the prenatal C-, TS-, and D-treated female lambs received 10 mm E₂ implants, which produces circulating concentrations of E₂ at ~1 pg/mL (Goodman *et al.* 1980, 1981, Jackson *et al.* 2013). Figure 1 summarizes the treatment groups and the type and sequence of studies conducted.

Assessment of puberty

Beginning at ~21 weeks of age, P concentrations in blood samples collected twice a week were assessed in all animals. Age at puberty was defined as the age at the first sample having circulating concentrations of P greater than 0.5 ng/mL.

Testing E₂-negative feedback responses

This test was conducted during the prepubertal period (~18 weeks of age; Fig. 1B), when female lambs are extremely

sensitive to the E₂-negative feedback (Foster & Jackson 2006). Details of the E₂-negative feedback test are summarized in Fig. 1C. The gonadotropin-releasing hormone antagonist (GnRH-A), acyline (Contraception and Reproductive Health Branch, National Institutes of Health, Bethesda, MD, USA), was administered subcutaneously (10 µg/kg) every 12 h for 72 h to abolish LH pulsatility and reduce endogenous E₂ concentrations (Sarma *et al.* 2005, Steckler *et al.* 2008). Blood samples were collected at 20 min intervals for 6 h after the last GnRH-A injection to determine the GnRH-A ablation of LH pulsatility (Period 1; Fig. 1C). To establish the impact of reduced endogenous E₂ on LH pulse frequency, blood samples were collected for 6 h beginning 72 h after cessation of the GnRH-A treatment (Period 2; Pre E₂; Fig. 1C). The E₂-negative feedback was then evaluated by inserting a single 3 mm SILASTIC E₂ implant (subcutaneously) to produce circulating concentrations of <1 pg/mL (Goodman *et al.* 1980, 1981, Jackson *et al.* 2013). Blood samples were collected beginning 72 h after insertion of the E₂ implant for 6 h at 20 min intervals (Period 3; Post E₂; Fig. 1C).

Testing E₂-positive feedback responses

This test was carried out following the E₂-negative feedback testing (Fig. 1C) in prepubertal females at ~19 weeks of age in the absence of P priming, as reported previously (Sharma *et al.* 2002). All animals received four 30 mm SILASTIC E₂

implants (subcutaneously) to provide late follicular phase concentrations of E_2 (Goodman *et al.* 1980, 1981, Jackson *et al.* 2013). Blood samples were collected every 2 h for 72 h starting 1 h before insertion of E_2 implants.

Periovulatory hormonal dynamics

The impact of experimental treatments on periovulatory hormonal dynamics was studied during the first and second breeding season at ~8 and ~20 months of age respectively (Fig. 1B). Studies were carried out during two breeding seasons to determine whether these animals show a progressive loss in cyclicity as observed previously in prenatal TS-treated animals (Manikkam *et al.* 2006). Estrous was synchronized with two injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 mg, intramuscular; Lutalyse, Pfizer Animal Health, Florham Park, NJ, USA) administered 11 days apart (Fig. 1D). Following the second $PGF_{2\alpha}$ injection, blood samples were collected every 2 h for 192 h during the first breeding season and for 120 h during the second breeding season to assess the periovulatory hormonal changes. This was followed by daily blood sampling for 19 additional days to assess luteal P secretion. LH and FSH were assayed every 2 h, E_2 every 4 h, and P in daily samples.

Hormone measurements

Plasma LH, FSH and P concentrations were measured in duplicate using validated radioimmunoassays (RIA) (Niswender *et al.* 1969, Padmanabhan *et al.* 1995, 1997). The sensitivity of LH RIA was 0.1 ± 0.01 ng/mL ($n=46$ assays; mean $\pm$ S.E.M.). Mean intra-assay coefficient of variation (CV) based on four quality control pools measuring 3.1 ± 0.1 , 7.2 ± 0.1 , 13.4 ± 0.1 , and 23.8 ± 0.01 ng/mL were 10.0, 6.0, 6.8, and 6.0% respectively. The corresponding interassay CVs averaged 20.5, 7.2, 5.6, and 4.5%. The sensitivity of FSH RIA was 0.06 ± 0.01 ng/mL ($n=31$ assays). The intra-assay CVs based on two quality control pools measuring 5.0 ± 0.1 and 11.2 ± 0.2 ng/mL were 9.9 and 4.8% respectively. The corresponding interassay CVs averaged 13.9 and 8.7%. Plasma E_2 for the E_2 -negative feedback study was measured using the commercially available MAIA kit (Polymedco Inc., Courtlandt Manor, NY) and for the periovulatory hormonal dynamics assessment was measured using a validated RIA (Rozell & Keisler 1990). For the MAIA kit sensitivity of E_2 assay was 0.1 ± 0.02 pg/mL ($n=6$ assays). Mean intra-assay CVs based on two quality control pools measuring 1.09 ± 0.08 and 5.30 ± 0.054 pg/mL were 14.22 and 13.76% and the corresponding inter-assay CVs averaged 17.02 & 24.59%. For the periovulatory hormonal dynamics study sensitivity of E_2 RIA was 0.3 ± 0.1 pg/mL ($n=36$ assays). Mean intra-assay CVs based on two quality control pools measuring 0.53 ± 0.02 and 3.32 ± 0.15 pg/mL were 12.8 and 12.6%, respectively. The corresponding inter-assay CVs averaged 18.36 and 25.38%. The sensitivity of P RIA (Coat-a-Count, DPC/Siemens) was 0.001 ± 0.0002 ng/mL ($n=8$ assays). Mean intra-assay CVs based on three quality control pools measuring 0.2 ± 0.006 , 1.7 ± 0.02 , and 14.3 ± 0.2 ng/mL were 4.8, 5.1, and 2.4% respectively. The corresponding interassay CVs averaged 12.2, 3.6, and 3.8%. Sample sets from experimental groups were randomly distributed such that each assay included samples from all treatment groups.

Statistical analysis

Age at puberty was assessed in all animals. Results from twin females were averaged for assessment of puberty before analysis to ensure mother was the experimental unit. Only a subset of animals was used for E_2 -negative and E_2 -positive feedback studies ($n=7$ each for C, TS, D, E_2 , C + E_2 , and TS + E_2 , and $n=6$ each for ED and D + E) and for assessment of periovulatory hormonal dynamics ($n=6$ each for C, TS, D, ED, C + E_2 , and D + E groups, $n=7$ for E_2 and $n=5$ for TS + E_2). During the second breeding season, only animals from C, TS, and E_2 for prenatal treatment groups and C + E_2 and TS + E_2 for postnatal groups were available for this study. The number of animals in each treatment group during their second year were C = 9, TS = 5, E_2 = 9, C + E_2 = 7, and TS + E_2 = 4. For the feedback and periovulatory dynamic studies, when twin female births were involved, only one offspring (selected randomly) was used.

For E_2 -negative feedback, LH values from serially collected samples were subjected to pulse analysis using the cluster algorithm (Veldhuis & Johnson 1986). For cluster analysis, the minimum number of data points to identify either a peak (the highest concentration reached during a pulse) or a nadir (basal level) for the 20 min sampling frequency was set at one. The Student's *t* statistic values used to identify significant increases from preceding nadirs and decreases to following nadirs were set at 1.0. Increases in LH concentrations more than two times the assay sensitivity over the preceding nadir were considered as a pulse. The number of pulses was assessed over a 6 h period. For both E_2 -positive feedback and periovulatory hormonal characterization, the LH and FSH surges were defined based on the circulating gonadotropin concentrations being above the baseline plus two times the assay sensitivity and remaining high for at least 8 h (Padmanabhan *et al.* 2015). For synchronization of the estrous cycle following $PGF_{2\alpha}$ injection, daily P concentrations had to fall below 0.5 ng/mL. For all statistical analysis, $P < 0.05$ was considered significantly different.

Age at puberty

The age at puberty was analyzed by one-way ANOVA for prenatal treatment group comparisons and a two-way ANOVA for testing postnatal E_2 modulation. In view of the large number of comparisons that reduces power, the magnitude of treatment effects on age at puberty was also examined by effect size analysis (Cohen 1992, Nakagawa & Cuthill 2007, Padmanabhan *et al.* 2015). This analysis allows comparison of the means between two treatments with respect to the magnitude of difference between them. The computed statistic is Cohen's *d* value, and values above 0.2, 0.5, and 0.8 were considered as small, medium, and large effect sizes, respectively (Cohen 1992, Nakagawa & Cuthill 2007).

E_2 -negative feedback

Variables compared were E_2 concentrations and LH pulse frequency. Number of pulses during the 6 h period was square-root-transformed before analysis. For comparison among prenatal groups, ANOVA was used followed by *post hoc* analyses adjusting for multiple comparisons. For postnatal

Figure 2 Results (mean $\pm$ S.E.M.) of the E₂-negative feedback test in prenatal groups are shown in panels A and B and age of puberty in panel C. Panel A shows the circulating concentrations of E₂ pre- and post-E₂ administration. Panel B shows the number of LH pulses over a 6-h period pre- and post-E₂ administration. Asterisks indicate significant difference ($P < 0.05$) within the respective prenatal treatments. Bars with different superscripts in the post-E₂ period in panel B are significantly different. Panel C shows age of puberty in weeks. Cohen's d values as determined by effect size analysis are shown in panels A (Pre E₂ vs Post E₂) and C (prenatal treatment vs C).

comparisons, paired t -test was used to compare postnatal groups with corresponding prenatal-only treated groups. To test whether number of pulses changed significantly between pre-E₂ (72 h post-GnRH-A) and post-E₂ period in each treatment groups, Wilcoxon signed-rank test was used. In addition, Cohen's effect size analysis was used as a secondary analysis to relate the magnitude of differences.

E₂-positive feedback and periovulatory dynamics

Comparisons involved peak time, peak hormone concentration, total hormone released during the surge, the duration of surge, and time from E₂ peak to LH peak. All continuous variables were log-transformed before analysis. For comparing categorical variables (% synchronized and

% showing LH surges in the periovulatory dynamics study and % responded in the positive feedback study) between prenatal groups and each of the postnatal pairs, Fisher's exact test was used. For comparison of LH and FSH surge dynamics among only prenatal treatment groups, ANOVA was used followed by *post hoc* tests after adjusting for multiple comparisons. For variables with only one TS subject providing data in E₂-positive feedback test or two TS subjects in periovulatory hormonal dynamics examination, given all other prenatal groups are not significantly different, the mean and standard deviation (SD) of the outcome using combined data from the C, D, E₂, and ED groups were first calculated. Then, the probability of observing a value larger or equal to the value observed in the single TS subject assuming it is drawn from a normal distribution with the above derived mean and SD was calculated. A smaller P -value indicates that TS subject was less likely to have the same distribution as other groups. For prenatal vs postnatal treatment comparisons, unpaired t -test was used.

Figure 3 Gonadotropin surge characteristics (mean $\pm$ S.E.M.) during the E₂-positive feedback test in prenatal-only-treated groups. (A) The percentage of animals that responded to E₂-positive feedback test, (B) total LH secreted during the surge, (C) time of LH surge peak after E₂, (D) peak LH concentration, (E) duration of LH surge, (F) total FSH secreted during the surge, (G) time of FSH surge peak after E₂, (H) peak FSH concentration and (I) duration of FSH surge. Observations in the TS-treated group are from a single animal that responded to the E₂-positive feedback challenge. Asterisks indicate significant difference from C and # indicates a significant difference between the TS-animal and the composite of all treatment groups (see statistical analysis for modeling).

Figure 4 Perioviatory hormonal dynamics in prenatal treatment groups. (A) Results of the estrous synchronization, (B) total LH secreted during the surge, (C) time of LH surge peak, (D) peak LH concentration, (E) duration of LH surge, (F) percentage of synchronized animals showing a definable LH surge, (G) total FSH secreted during the surge, (H) time of FSH surge peak, (I) peak FSH concentration and (J) duration of FSH surge from perioviatory hormonal studies during the first and second breeding seasons. Observations in the TS-treated group are mean from two animals that showed definable LH surge. *Indicates a significant difference between the TS-animal and the composite of all treatment groups. ND, no data available, as the animals in this group did not exhibit both primary gonadotropin surge.

Results

Effects of prenatal treatments

E₂-negative feedback responses

The results of the *E₂*-negative feedback study in animals that received only prenatal treatments are summarized in Fig. 2A and B, with representative circulating LH profiles shown in Supplementary Fig. 1, see section on supplementary data given at the end of this article. Administration of GnRH-A reduced *E₂* concentrations to ~0.3 pg/mL in all groups (data not shown). Approximately 72h after the last injection of GnRH-A, endogenous *E₂* concentrations had increased slightly but were not statistically different among prenatal treatment groups (Fig. 2A, Pre E). Insertion of a 3 mm *E₂* implant caused the concentration of *E₂* to increase to ~0.6 pg/mL across all prenatal treatment groups (Fig. 2A, Post E). Effect size analysis found a large size effect of *E₂* implants in C, D, and ED groups, but not in TS and *E₂* groups. Administration of GnRH-A reduced the LH pulsatility in all prenatal treatment groups (data not shown).

Seventy-two hours after cessation of GnRH-A injection, LH pulsatility increased in all prenatal treatment groups, and LH pulse frequency did not differ among groups (Fig. 2B, Pre *E₂*). *E₂*-treatment ablated LH pulses in C animals (Fig. 2B, Post *E₂*). Prenatal TS-treated animals had significantly more LH pulses during the *E₂*-treatment period than those of C females (Fig. 2B, Post *E₂*). A reduction in LH pulses was evident in TS-, D-, and *E₂*-treated animals relative to pre *E₂* period, albeit to a lesser degree than in C animals (Fig. 2B, Post *E₂*). While not reaching statistical significance, five of six animals showed suppression following *E₂*-treatment in the ED group (Fig. 2B, Post *E₂*).

Age at puberty

The large number of treatment comparisons relative to the sample size precluded identification of statistical differences among prenatal groups (Fig. 2C). However, Cohen's analysis found a large size effect between C and prenatal TS, *E₂*, and ED groups, but not D treatment.

E₂-positive feedback responses

Results of the *E₂*-positive feedback test in prenatal groups are summarized in Fig. 3 and representative hormonal profiles are depicted in Supplementary Fig. 2. While all females in the C and prenatal D-, *E₂*- and ED-treated groups demonstrated gonadotropin surges, only one prenatal TS-treated female responded to the *E₂*-positive feedback challenge (Fig. 3A). Mean total release (Fig. 3B and F), peak time (Fig. 3C and G), peak concentrations (Fig. 3D and H), and duration (Fig. 3E and I) of the LH/FSH surges did not differ among C, D, *E₂*, and ED groups. The only prenatal TS-treated female that responded to the *E₂*-positive feedback showed a delayed and reduced gonadotropin surge.

Perioviatory hormonal dynamics

Results of the perioviatory hormonal dynamics during the first and second breeding season in prenatal groups are summarized in Fig. 4 and representative hormonal profiles are depicted in Supplementary Figs 3 and 4 respectively. During the first breeding season, 100% of the animals were synchronized in the C and *E₂* groups, while 66.7, 83.3, and 83.3% of the animals showed estrous synchronization following PGF_{2α} administration in TS, D, and ED groups respectively (Fig. 4A). Of the female sheep that synchronized, 100% of the animals showed LH surges in C, D, and *E₂* groups, while only two of four and four of five animals showed definable LH surges in the TS and ED groups respectively (Fig. 4F). No differences in mean total release (Fig. 4B and G), peak time (Fig. 4C and H), peak gonadotropin concentration (Fig. 4D and I), and duration (Fig. 4E and J) were observed between C, *E₂*, D, and ED groups. Of the two

prenatal TS-treated animals that met the criteria of an LH surge (sustained increase in LH for over 8 h), total LH was lower than that of the control animals, but no difference was observed with other surge parameters.

During the second breeding season, 88.9, 60.0, and 66.7% of the animals from C-, TS-, and E₂-treatment groups synchronized following PGF_{2α} administration respectively (Fig. 4A). Of these, 100% of the animals in C and E₂ groups while none of the animals in the TS group showed definable LH surges (Fig. 4F). No differences in the gonadotropin surge characteristics were observed between C and E₂ groups (Fig. 4B, C, D, E, G, H, I and J).

Effects of postnatal E₂ exposure

E₂ negative feedback responses

The impact of postnatal E₂-treatment on modulating the effects of prenatal steroid treatment on

E₂-negative feedback is summarized in Fig. 5A and B and representative circulating LH profiles in Supplementary Fig. 1. GnRH-A markedly reduced E₂ concentrations in the postnatal groups (C + E₂, TS + E₂, and D + E) to ~0.3 pg/mL (data not shown), and E₂ implant insertion increased concentrations of E₂ to ~0.6 pg/mL (Fig. 5A, Pre E₂). Effect size analysis comparing postnatal E₂-treatment groups with corresponding prenatal-only treated groups showed large effects following E₂ implants. Suppression of LH pulsatility following GnRH-A treatment and an increase in the number of LH pulses following cessation of GnRH-A treatment in the postnatal groups were similar to changes described for the prenatal treatment-only groups. There were no differences in LH pulse frequency among treatment groups during pre-E₂ period (Fig. 5B, Pre E₂). While E₂-treatment completely ablated LH pulsatility in the C group, this complete suppression was not achieved in four out of seven animals in the C + E₂ group, which had pulses during the pre-E₂ period

Figure 5 Results (mean $\pm$ S.E.M.) of the E₂-negative feedback test in postnatal groups are shown in panels A and B and age of puberty in panel C. Panel A shows the circulating concentrations of E₂ pre- and post-E₂ administration. Panel B shows the number of LH pulses over a 6-h period pre- and post-E₂ administration. Asterisks indicate significant difference ($P < 0.05$) between pre- and post-E₂ periods within the respective treatment groups. Note the prenatal treatment-only groups C, TS, and D are repeated from respective panels from Fig. 2, to allow comparison between postnatal groups and prenatal-only groups. Cohen's d values as determined by effect size analysis are shown in panels A (Pre E₂ vs Post E₂) and C (prenatal treatment vs postnatal E₂).

Figure 6 Gonadotropin surge characteristics (mean $\pm$ S.E.M.) during the E₂-positive feedback test in postnatal groups. (A) The percentage of animals that responded to E₂-positive feedback test, (B) total LH secreted during the surge, (C) time of LH surge peak after E₂, (D) peak LH concentration, (E) duration of LH surge, (F) total FSH secreted during the surge, (G) time of FSH surge peak after E₂, (H) peak FSH concentration and (I) duration of FSH surge. Data from postnatal groups were compared with the respective prenatal groups (data are repeated from Fig. 3 for comparison purposes). Observations in the TS-treated group are from a single animal, while TS + E₂ group is the mean of two animals that responded to the E₂-positive feedback challenge. Asterisks indicate significant differences ($P < 0.05$) between postnatal and prenatal groups.

(Fig. 5B, Post E₂). The remaining three animals had no LH pulses during the pre-E₂ period, but showed low pulse frequency (1–2 pulses/6 h) during E₂-treatment period. Postnatal E₂ failed to reduce LH pulsatility in TS+E₂ and D+E groups (Fig. 5B, Post E₂) as opposed to suppressive effects being evident in prenatal TS- and D-only females.

Age at puberty

ANOVA revealed a significant postnatal E₂-treatment effect in delaying age at puberty (Fig. 5C). Similarly, effect size analysis showed robust effects of postnatal E₂-treatment in delaying the age at puberty in all treated groups.

E₂-positive feedback responses

Results of the E₂-positive feedback test in the postnatal groups are summarized in Fig. 6 and Supplementary

Figure 7 Effects of postnatal E₂-treatment on periovulatory hormonal dynamics. (A) Results of the estrous synchronization, (B) total LH secreted during the surge, (C) time of LH surge peak, (D) peak LH concentration, (E) duration of LH surge, (F) percentage of synchronized animals showing a definable LH surge, (G) total FSH secreted during the surge, (H) time of FSH surge peak, (I) peak FSH concentration and (J) duration of FSH surge from periovulatory hormonal studies in prenatal and postnatal treatment groups during the first and second breeding cycles are shown. Data from postnatal groups were compared with the respective prenatal groups (data are repeated from Fig. 4 for comparison purposes). Observations in the TS-treated group are mean from two animals and one animal in the TS+E₂ group that showed definable LH surge. ND, no data available, as none of the animals in this group had both primary gonadotropin surge.

Fig. 2. While five of seven C+E₂ and two of seven TS+E₂ animals responded to the positive feedback challenge, all D+E females demonstrated a response (Fig. 6A). Except for a reduction in total LH and FSH released in response to postnatal E₂-treatment in the C+E₂ group (Fig. 6B and F), and surge duration in the D+E group relative to corresponding prenatal control groups (Fig. 6E), postnatal E₂-treatment had no effect on other LH and FSH surge parameters.

Periovulatory hormonal dynamics

The impact of postnatal E₂-treatment on periovulatory hormonal dynamics during the first and second breeding season is summarized in Fig. 7, and representative hormonal profiles are depicted in Supplementary Figs 3 and 4 respectively. Among the animals that received postnatal E₂-treatment, 71.4% of C+E₂, 60.0% of TS+E₂, and 66.7% of the D+E animals showed estrous synchronization following PGF_{2α} administration during the first breeding season (Fig. 7A). Of those that synchronized, 100% of the animals showed definable LH surges in C+E₂ and D+E groups, while only 1 of the 3 animals that synchronized had an LH surge in the TS+E₂ group (Fig. 7F). Postnatal E₂-treatment had no effect on any of the attributes of the preovulatory surge dynamics (Fig. 7). Because only one TS+E₂ animal had a definable LH surge, comparison between TS and TS+E₂ animals was not possible.

During the second breeding season, 85.7 and 75.0% of the animals in C+E₂ and TS+E₂ treatment groups synchronized following PGF_{2α} administration respectively (Fig. 7A). Of these, 100% of the C+E₂ and none of the TS+E₂ animals showed definable LH surges (Fig. 7F). Postnatal E₂-treatment had no effect on any of the parameters of preovulatory surge dynamics in C animals, and since TS+E₂ animals did not surge, such comparisons were not possible (Fig. 7).

Discussion

The results from this study confirm our previous findings of neuroendocrine feedback, pubertal timing, and periovulatory disruptions in prenatal TS-treated female sheep. However, the data fail to support our hypotheses that (1) prenatal E₂-treatment replicates the neuroendocrine disruptions attributed to estrogenic programming of E₂-positive feedback and periovulatory hormonal dynamics in prenatal TS-treated females and (2) concomitant prenatal treatment with D and E₂ reproduces the E₂ neuroendocrine feedback and periovulatory defects induced by prenatal TS excess. Furthermore, our results indicate that postnatal E₂-treatment (1) reduces the E₂ inhibitory effects on tonic LH release in prenatal TS and D groups, (2) delays the onset of puberty in all groups, and (3) fails to amplify the dampening of LH/FSH surges during the E₂-positive feedback challenge in prenatal TS- and D-treated animals. The significance of these

findings and their relevance to the understanding of the steroid component in programming these neuroendocrine feedback disruptions are discussed below.

Estrogenic regulation of prenatal TS-induced disruptions

Our previous findings showing lack of disruptions in E_2 -positive feedback and periovulatory hormonal dynamics in prenatal D-treated sheep (Veiga-Lopez *et al.* 2009) and failure of androgen antagonist cotreatment to prevent E_2 -positive feedback disruptions induced by prenatal TS-treatment (Abi Salloum *et al.* 2012) are consistent with the premise that these defects are mediated by estrogenic programming stemming from aromatization of TS to E_2 . Paradoxically, the present observations that prenatal E_2 -treatment did not result in E_2 -positive feedback and periovulatory disruptions fail to support this premise. One possibility for this discrepancy is that not sufficient fetal E_2 concentrations were achieved. Alternatively, these findings, in concert with previous findings of partial restoration of preovulatory LH surges in animals cotreated with TS and androgen antagonist (Padmanabhan *et al.* 2015), raise the possibility that both androgens and estrogens synergize in programming the prenatal TS-induced disruptions in E_2 feedback and periovulatory hormonal dynamics.

Similarly, the failure of concomitant treatment with E_2 and D to replicate the reproductive neuroendocrine defects might also be a function of inadequate estrogen levels achieved and consequent imbalance in androgen to estrogen ratio at the fetal level relative to what is attained with TS-treatment alone. An altered androgen to estrogen ratio could arise from insufficient estrogen levels being achieved due to placental metabolism of the peripherally administered E_2 to lesser active metabolites as seen in monkeys (Slikker *et al.* 1982) or from increased androgen actions, since D has higher potency than TS (Grino *et al.* 1990). The ratio could also be altered, as D can be metabolized to 3β -diol, an estrogen receptor β -agonist (Handa *et al.* 2008). Therefore, additional studies involving the use of estrogen antagonist alone or in combination with an androgen antagonist are required to clearly address the relative role of androgens and estrogens in mediating these effects.

Effects of postnatal E_2 exposure

The lack of response to the E_2 -negative feedback challenge in control animals postnatally treated with E_2 may be attributed to the downregulation of the estrogen receptor in the hypothalamus and pituitary gland due to continuous exposure to E_2 . In fact, E_2 -induced downregulation of estrogen receptor expression has been observed in other tissues, such as the uterus (Medlock *et al.* 1991, Farnell & Ing 2003). Therefore, the interval

between the removal of the postnatal E_2 implants and the E_2 -negative feedback challenge (7 days) may have been insufficient to allow recovery of estrogen receptor expression in the hypothalamus and pituitary to facilitate responsiveness to the E_2 -negative feedback challenge. Considering that puberty was delayed in postnatal E_2 -treated animals, this lack of E_2 -negative feedback response was surprising. To what extent this relates to the short interval between the removal of postnatal E_2 implant and the E_2 -negative feedback testing remains to be determined. One possibility is that the delay in puberty could be a function of reduced gonadotropic support to the ovary (Foster *et al.* 1986).

Diminished gonadotropin release in response to E_2 -positive feedback was observed in prepubertal female sheep chronically treated with E_2 in this study and in previous studies (Malcolm *et al.* 2006). However, a reduction in the periovulatory release of gonadotropins was not observed when examined during the first and second breeding season. The reason for this difference might relate to the heightened sensitivity of prepubertal females to E_2 -negative feedback compared with postpubertal females, resulting in reduced storage of LH in the pituitary that is available for release during the E_2 -positive feedback challenge. In contrast to normal periovulatory LH release in our study, postnatal exposure to E_2 was found to block endogenous LH surge in female sheep (Ozturk *et al.* 1998). The differences in LH surge responses between this study and our study may be a function of the circulating E_2 concentrations achieved by the treatment and/or the timing of treatment.

Postnatal E_2 modulation of prenatal steroid effects

Comparison of the effects of prenatal TS plus postnatal E_2 -treatment with prenatal TS-treatment alone found that postnatal E_2 -treatment does not amplify the effects of prenatal TS-treatment in reducing responsiveness to E_2 -negative feedback and advancing puberty, a finding consistent with our previous studies (Sarma *et al.* 2005, Veiga-Lopez *et al.* 2009, Padmanabhan *et al.* 2015). Instead, postnatal E_2 -treatment delayed puberty relative to controls, while failing to reduce gonadotropin levels in response to the E_2 -negative feedback challenge. Considering that an escape from E_2 -negative feedback is an important event controlling the onset of puberty, this dissociation between delayed puberty and reduced sensitivity to E_2 -negative feedback is paradoxical. As discussed above, this dissociation may be a function of the short interval between the removal of the E_2 implant and the testing for the E_2 -negative feedback.

Furthermore, the finding that E_2 -positive feedback and periovulatory hormonal dynamics were impaired in prenatal TS-treated sheep, which is in agreement with our previous findings (Sharma *et al.* 2002, Veiga-Lopez *et al.* 2009, Padmanabhan *et al.* 2015), coupled with failure of postnatal E_2 exposure to amplify these disruptions,

suggests these defects are organized primarily during the prenatal life, with little or no impact of continuous postnatal exposure to excess E_2 , at least at the dose tested. Alternatively, low concentrations of E_2 produced by the ovary during postnatal life may be sufficient to further differentiate (masculinize) the GnRH/gonadotropin surge mechanism in ovary-intact, prenatal TS-treated female sheep, with exogenous postnatal E_2 -treatment having no additional impact on the gonadotropin surges. This possibility is supported by the observation that neonatal ovariectomy restores the E_2 -induced LH surge in prenatal TS-treated sheep (Jackson *et al.* 2013).

Contrary to our expectation and in disagreement with our previous studies (Veiga-Lopez *et al.* 2009), prenatal D-treatment failed to disrupt E_2 -negative feedback. The reason for this discrepancy is unclear, but it may involve the different ages at which the E_2 -negative feedback tests were carried out, suggesting that the desensitization to the E_2 -negative feedback may occur only at a later developmental stage in D-treated compared with TS-treated females. As prenatal D-treated animals do not develop a multifollicular ovarian morphology (Smith *et al.* 2009), the delay in the desensitization of the neuroendocrine axis to the E_2 -negative feedback could be due to lower secretion of E_2 and/or other ovarian-derived factors unlike prenatal TS-treated females. Failure of postnatal E_2 to alter the E_2 -positive feedback or periovulatory hormonal dynamics in prenatal D-treated animals is again consistent with such defects being programmed primarily during the prenatal life.

Conclusions

In conclusion, the results from this study combined with our earlier findings indicate that disruptions in E_2 feedback mechanisms, timing of puberty, and periovulatory hormonal dynamics seen in prenatal TS-treated sheep require activation of both androgenic and estrogenic pathways. Additionally, the present findings indicate that these neuroendocrine disruptions are programmed primarily during the prenatal life and are not amplified or modified by postnatal exposure to E_2 excess, at least at the dose tested. Unfortunately, likely due to a putative metabolism of D into 3β -diol or insufficient fetal E_2 concentrations achieved, the relative role of androgens or estrogens in programming some of these reproductive neuroendocrine defects could not be completely discerned by this study. In the future, comparative experiments utilizing antagonists of androgen and estrogen receptors separately and in combination need to be undertaken in both the ovariectomized and ovary-intact models. Although postnatal E_2 exposure did not amplify the adverse effects of prenatal TS exposure, it did negatively affect the timing of puberty and preovulatory gonadotropin release in control animals. These data indicate that

postnatal exposure to EDC with estrogenic properties, through activational effects, may have the potential to adversely affect reproductive neuroendocrine function in females. Therefore, although extrapolation of findings in this sheep model to human pathology should be done cautiously, these observations highlight the potential for gestational and postnatal endocrine imbalances to negatively impact the offspring's reproductive health.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0065>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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