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Developmental reprogramming of reproductive and metabolic dysfunction in sheep: native steroids vs. environmental steroid receptor modulators

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Abstract

The inappropriate programming of developing organ systems by exposure to excess native or environmental steroids, particularly the contamination of our environment and our food sources with synthetic endocrine disrupting chemicals that can interact with steroid receptors, is a major concern. Studies with native steroids have found that *in utero* exposure of sheep to excess testosterone, an estrogen precursor, results in low birth weight offspring and leads to an array of adult reproductive / metabolic deficits manifested as cycle defects, functional hyperandrogenism, neuroendocrine / ovarian defects, insulin resistance, and hypertension. Furthermore, the severity of reproductive dysfunction is amplified by excess postnatal weight gain. The constellation of adult reproductive and metabolic dysfunction in prenatal testosterone-treated sheep is similar to features seen in women with polycystic ovary syndrome. Prenatal dihydrotestosterone treatment failed to result in similar phenotype suggesting that many effects of prenatal testosterone excess are likely facilitated via aromatization to estradiol. Similarly, exposure to environmental steroid imposters such as bisphenol A (BPA) and methoxychlor (MXC) from days 30-90 of gestation had long-term but differential effects. Exposure of sheep to BPA, which resulted in maternal levels of 30-50 ng/ml BPA, culminated in low birth-weight offspring. These female offspring were hypergonadotropic during early postnatal life and characterized by severely dampened preovulatory LH surges. Prenatal MXC-treated females had normal birth weight and manifested delayed but normal amplitude LH surges. Importantly, the effects of BPA were evident at levels, which approximated twice the highest levels found in human maternal circulation of industrialized nations. These findings provide evidence in support of developmental origin of adult reproductive and metabolic diseases and highlight the risk posed by exposure to environmental endocrine disrupting chemicals.

Keywords

fetal programming; infertility; neuroendocrine; ovary; insulin resistance; endocrine disrupting chemicals; bisphenol A; methoxychlor; metabolic programming

Introduction

The developing fetus, in response to changes in the in utero environment develops compensatory strategies to overcome insults that they experience. Such compensations could be adaptive, if they support survival, or disruptive, if they compromise postnatal survival. *Developmental plasticity*, the ability of the developing fetus to change structure / function in response to physiological cues from the mother, underlies the developmental origin of disease or Barker hypothesis (Barker, 1994). The increased prevalence of some common diseases may be related to exposure during development to environmental pollutants,

lifestyle choices of the mother, and medical interventions, all of which can adversely influence developmental trajectory of target tissue differentiation. This review addresses the reproductive and metabolic disruptions resulting from exposure to excess native steroids and environmental steroid receptor modulators with specific focus on those that signal through estrogen and androgen receptors.

Developmental programming of reproductive / metabolic dysfunction with native steroids

Steroid hormones play a major role during development in setting the trajectory of developing organ systems. Because, differentiation of organ systems depend upon precise exposure to steroid hormones at specific times during development, exposure to low doses of endocrine disrupting compounds (EDCs) that can signal through steroid receptors during these hormone sensitive, critical periods of development can lead to long term deleterious effects on the adult organism. It is well established that inappropriate exposure to excess testosterone (T) during fetal life leads to phenotypic virilization and behavioral masculinization in the female offspring (Jost *et al.* 1973; Gorski, 1986; Wood & Foster, 1998). The amount as well as the timing of T exposure dictates the degree of masculinization of external genitalia in the female (Wood & Foster, 1998). Inappropriate perinatal exposure to excess T during early development also disrupts reproductive cyclicity in several species (Abbott *et al.* 2006).

For the remainder of the review, focus is on the reproductive and metabolic disruptions resulting from inappropriate developmental exposure of sheep to native steroids or environmental steroid mimics. Sheep are exceptionally well suited for investigating developmental programming of adult disorders. They have long been used as model systems to study fetal physiology (Harding & Bloomfield, 2004). Their developmental time line (gestation length: 147 days, puberty in female: ~ 28 weeks) is ideally suited for integrative studies that address progression of reproductive / metabolic disruption from the initial developmental insult to manifestation of adult consequences, especially those that involve detailed hormonal profiling or sequential monitoring of ovarian follicular dynamics. Importantly, they can be studied in natural social settings thus reducing level of stress. From a reproductive perspective, ovarian differentiation in sheep is similar to humans with full follicular differentiation occurring by birth (Fig.1, panel A) (Padmanabhan *et al.* 2007). Neuroendocrine aspects of reproductive cyclicity are also similar to human (Goodman & Inskeep, 2006; McNeilly, 1991).

Comparison of sheep treated with T (aromatizable androgen) from days 30 to 90 of gestation (T30-90 females) with those treated from days 60-90 of gestation (T60-90 females) has helped address critical period of programming of reproductive and metabolic disruptions. Comparison of prenatal testosterone (T), prenatal dihydrotestosterone (non aromatizable androgen, DHT) and T plus flutamide (an androgen antagonist) treatments has helped address the quality of steroid (androgen or estrogen) responsible for programming adult dysfunctions (Fig. 1, panel B). Earlier studies with the Dorset breed of sheep found T30-90 females showed progressive deterioration of cyclicity culminating in absent cycles during the second breeding season (Fig. 2, panel A) (Birch *et al.* 2003). Studies with other breed of sheep also found progressive loss of cyclicity (Clarke *et al.* 1977; Manikkam *et al.* 2006), the severity of which differing between breeds. In contrast, majority of the T60-90 females cycled during the second breeding season (Birch *et al.* 2003, Savabieasfahani *et al.* 2005).

Comparison of cycle dynamics of T30-90 and DHT30-90 females during the estrous cycle found that T30-90 females were characterized by increased preovulatory levels of estradiol, as well as delayed and severely dampened LH surges (Fig. 2, panel B) (Veiga-Lopez *et al.*

2009). Detailed characterization of circulating LH dynamics during the follicular phase found T30-90 and T60-90 females were characterized by excess LH release (Manikkam *et al.* 2008; Savabieasfahani *et al.* 2005). Studies testing the fertility status of T60-90 females found that 100% of the T60-90 females (T30-90 females are phenotypically virilized and natural mating is not possible (Wood and Foster, 1998) were mated by the ram. However, fecundity was reduced with only 40% of those mated becoming pregnant as opposed to the 90% pregnancy rate in the control herd (Fig. 2, panel C) (Steckler *et al.* 2007b). Even more importantly, recent studies found that excess postnatal weight gain amplifies the reproductive disruptions in T30-90 females (Fig. 3, panel A) (Steckler *et al.* 2009). These findings are supportive of the two-step process (Tang *et al.* 2008), the first involving early life epigenetic reprogramming of susceptible organ systems and a later event influencing the severity of the pathologic phenotype (Fig. 3, panel B).

Neuroendocrine disruptions

At the neuroendocrine level, prenatal T treatment reduces hypothalamic sensitivity to all three major feedback systems involved in the control of cyclic changes in GnRH / gonadotropin secretion; estradiol (E) negative feedback (Wood & Foster, 1998; Sarma *et al.* 2005), E positive feedback (Wood & Foster, 1998; Sharma *et al.* 2002, Unsworth *et al.* 2005) and progesterone negative feedback (Robinson *et al.* 1999; Veiga-Lopez *et al.* 2009) (Fig. 4). Further investigations have pointed to disruptions of E negative feedback being programmed by androgenic action of T, with both, T and DHT and not T + flutamide reducing sensitivity to E (Wood & Foster, 1998, Veiga-Lopez *et al.* 2009, Jackson *et al.* 2008). E positive feedback disruptions were found in T30-90 but not DHT30-90 females suggesting that this disruption is likely programmed via estrogenic actions of prenatal T (Wood & Foster, 1998, Veiga-Lopez *et al.* 2009). Studies testing pituitary sensitivity also found that both T30-90 and DHT30-90 females have enhanced sensitivity to GnRH suggesting that this aspect is programmed likely via androgenic actions of T (Manikkam *et al.* 2008).

Ovarian disruptions

In addition to reproductive neuroendocrine disruptions, prenatal T treatment resulted in larger ovaries with a multifollicular morphology (Fig. 5, panel A) (West *et al.* 2001). These effects appear not to be facilitated by the androgenic actions of T as prenatal DHT treatment failed to create a multifollicular ovarian phenotype (West *et al.* 2001; Steckler *et al.* 2007a). Detailed morphometric analyses found prenatal T and DHT treatment enhanced follicular recruitment with only prenatal T treatment reducing ovarian follicular reserve to ~ 50% by the end of the first breeding season (Smith *et al.* 2009) (Fig. 5, panel B). Similarly, detailed daily ultrasonographic evaluation found that follicles persist longer in prenatal T-treated female (Manikkam *et al.* 2006) (Fig. 5, panel C) and this appears to be programmed by estrogenic actions of prenatal T (Steckler *et al.* 2007a). As such, the multifollicular phenotype of prenatal T females appears to be the consequence of both enhanced follicular recruitment and failure to regress. Immunohistochemical studies found that prenatal T treatment increases androgen receptor expression in the stroma and granulosa cells during fetal life and culminates in increased granulosa cell androgen receptor expression in antral follicles of adult females (Ortega *et al.* 2009). Taken together these studies document that excess exposure to T disrupts the ovarian trajectory with some aspects programmed by androgenic and others estrogenic actions of T.

Metabolic dysfunctions

In addition to the neuroendocrine and ovarian disruptions, prenatal T treatment leads to intrauterine growth restriction (IUGR), low birth weight and postnatal catch-up growth

(Manikkam *et al.* 2004), risk factors for adult well being (Boney *et al.* 2005; Dulloo, 2008). Developmental changes in the insulin-like growth factor (IGF) / IGF binding protein (IGFBP) system in the prenatal T-treated sheep were consistent with changes in growth trajectory with a reduction in IGF bioavailability evident during IUGR and an increase during postnatal catch-up growth (Crespi *et al.* 2006, Manikkam *et al.* 2004). Prenatal T-treatment also culminated in insulin resistance (DeHaan *et al.* 1990; Hansen *et al.* 1995; Recabarren *et al.* 2005; Padmanabhan *et al.* 2009) with programming of insulin resistance facilitated via androgenic actions of prenatal T (Padmanabhan *et al.* 2009). Importantly, the window of susceptibility for developing insulin resistance was found to be confined to a shorter programming window, namely 60-90 days of gestation (Padmanabhan *et al.* 2009). Recent studies assessing the impact of prenatal T excess revealed tissue specific regulation of members of the insulin-signaling cascade (Nada *et al.*, 2009). At the hepatic level, there was a general downregulation of many members of the insulin signaling cascade consistent with liver being insulin resistant. In contrast, prenatal T excess upregulated many members of the insulin signaling cascade at the level of the adipose tissue supportive of increased insulin sensitivity (Nada *et al.*, 2009). Our unpublished observations also indicate increased visceral adiposity in the prenatal T-treated females. Radiotelemetric studies found that the T30-90 females are also hypertensive (King *et al.* 2007). Metabolic disruptions have also been reported in other prenatal T treated animal models (Abbott *et al.* 2006, Demissie *et al*. 2008).

Male reproduction

In contrast to several studies addressing the impact of prenatal T excess in female sheep, limited information is available addressing the impact of prenatal T excess on male reproduction / metabolism in sheep. Prenatal T treatment increased ano-genital distance in the male offspring compared to controls (Manikkam *et al.* 2004), altered the developmental trajectory of gonadal responsiveness to GnRH in prepubertal males (Recabarren *et al.* 2007) and culminated in reduced sperm count and motility (Recabarren *et al.* 2008). Prenatal T treatment also increased the volume of the sexually dimorphic nucleus in the males (Roselli, 2007), a complex of aromatase-expressing neurons, whose size has been correlated with sexual attraction in rams. Exposure to an aromatase inhibitor prenatally (days 50-80 of gestation) has also been correlated with decreased adult mounting behavior (Roselli, 2006). Information is lacking as to whether prenatal T excess disrupts the metabolic axis in the ovine male.

Translational significance

The reproductive phenotype of T30-90 sheep parallels features seen in women with polycystic ovarian disease (PCOS) (Table 1). PCOS is one of the most common reproductive disorder affecting >100 million women worldwide with the economic burden exceeding several billion dollars annually in the U.S. women with PCOS are characterized by oligo- / anovulation, hyperandrogenism, polycystic ovaries, LH hypersecretion and reduced fecundity with most manifesting insulin resistance (Franks, 1995). Some view PCOS as a clinical phenotype of the metabolic syndrome (Essah & Nestler 2006; Sam & Dunaif, 2003). Because the reproductive and metabolic phenotype of prenatal T-treated sheep recapitulates characteristics of women with PCOS, they provide a valuable cost-effective resource for addressing the mechanisms underlying the etiology of development of PCOS phenotype. The constellation of reduced insulin sensitivity, hypertension and visceral adiposity found in prenatal T treatment suggest that these animals may also be suitable for understanding the developmental origin of the metabolic syndrome phenotype (Mikhail, 2009).

Developmental programming by endocrine disruption chemicals

The inappropriate exposure to steroids is becoming a major concern in the context of development of adult pathologies. The fetus is exposed to exogenous steroids via failed contraception, use of anabolic steroids or inadvertent exposure to environmental compounds with estrogenic or anti-androgenic activity. Public concern has been mounting over harmful effects of environmental EDC, which can interfere with hormone signaling by acting as agonists or antagonists (Damstra *et al.* 2002; Hotchkiss *et al.* 2008). Of particular concern is the contamination of our environment and our food sources with the synthetic androgenic and estrogenic EDCs, which have the potential to disrupt normal androgen and estrogen signaling. This review focuses predominantly on two such EDCs namely, bisphenol-A (BPA) a widely used industrial plasticizer and methoxychlor (MXC), a pesticide. BPA is widely used in the manufacture of epoxy resins and polycarbonate plastics and accounts for most estrogenic activity in landfill leachates (Vandenberg *et al.* 2009; Ranjit *et al.* 2009). It has been detected in river water and sediments, and more recently, in indoor air and dust (Vandenberg *et al*. 2009; Ranjit *et al.* 2009). MXC, was used to control pests in agricultural, dairy, and domestic settings and found to persist in the environment (National Research Council, 1999). Both these EDCs have been shown to possess estrogenic and anti-androgenic properties (Vandenberg *et al.* 2009, Staub *et al.* 2002).

Targeting critical periods established by treating with native steroids, our recent studies found that prenatal BPA and MXC treatment had differential effects on the reproductive axis (Savabieasfahani *et al.* 2006). Prenatal BPA treatment, like prenatal T treatment, resulted in low birth weight offspring, early hypergonadotropism and severely dampened or absent preovulatory LH surges (Fig. 6, panels A, B, C). In contrast, MXC had no effect on somatic growth (Fig. 6, panel A) but delayed the onset of LH surges (Fig. 5, panel C). The levels of BPA achieved in maternal circulation following administration of 5 mg / kg body weight of BPA (Fig. 6, panel D) were 2-3 fold higher than the highest levels observed in the maternal circulation of U.S. women (Fig. 6, panel E) (Padmanabhan *et al.* 2008) and other industrialized nations (Vandenberg *et al.* 2009, Schonfelder *et al.* 2002). MXC levels in abdominal fat (Savabieasfahani *et al.* 2006) were several-fold higher than that found in human population (Botella *et al.* 2004). Comparison of reproductive defects in BPA and MXC treated females with prenatal T-treated model reveal considerable similarities between prenatal BPA and T treated models (Table 2). Both groups of animals showed reduced birth weight, LH excess and severely dampened preovulatory LH surges (Savabieasfahani *et al.* 2006). Others studies in sheep found administration from days110 to 115 days of gestation of octylphenol, a alkylphenol polyethoxylate used in detergents and pesticides with estrogenic properties, suppressed FSH levels in both female and male offspring (Sweeney *et al.* 2000). Administration of octylphenol starting from day 70 of gestation to birth advanced the time of puberty in female offspring (Wright *et al.* 2000).

Studies testing the effects of BPA and MXC in male sheep are not available. The only available information testing effects of EDC in male sheep comes from studies testing the effects of octylphenol. Prenatal octylphenol treatment from days 70 of gestation to birth reduced testis weight and Sertoli cell number in newborns (Sweeney *et al.* 2000) but not semen volume / concentration and motility in adult males (Sweeney *et al.* 2007). As opposed to the limited information available in sheep, a large volume of literature already exists relative to impact of BPA on the male offspring using rodent models. These rodent studies provide evidence that prenatal BPA exposure leads to disruptions in the male reproductive system, which include constricted urethra and prostate hyperplasia and cancer (Talsness *et al.* 2009; Diamanti-Kandarakis *et al.* 2009). A recent study found exposure to BPA from gestational day 12 to postnatal day 21 reduced sperm count and motility leading to subfertility in the offspring with effects persisting in F2 and F3 generations (Salian *et al.*

2009). Similarly exposure to MXC orvinclozolin during gestation resulted in reduced spermatogenic capacity and increasedincidence of male infertility with effects transferred to subsequent generations (Anway *et al*. 2005).

Conclusions

Studies discussed in this review centering on sheep as a model system enforce that the organizational program involved in establishing the adult phenotype is the result of the interplay between genetic susceptibility and developmental insults (Fig. 7). These findings reinforce the concern that inappropriate exposure to steroid hormones / steroid mimics pose to the well being of the developing offspring. The pathology programmed in sheep by BPA and MXC provides further support for the deleterious effects of EDCs on developing organ systems. Clearly, an understanding of mechanisms underlying developmental reprogramming following exposure to EDCs is essential for developing interventions to prevent development or reduce severity of pathology in adults. Several recent studies point to restoration of function via methylation by dietary supplements (Waterland, 2006; Pennisi, 2005; Burdge *et al.* 2009; Dolinoy *et al.* 2007), providing hope that dietary interventions may be beneficial in improving human health. Environmental exposures are modifiable risk factors and can be effectively regulated at the personal, behavioral as well as the regulatory policy level. For instance, exposure to BPA through sources such as over consumption of fast food and canned food and overuse of baby bottles, can be addressed through public health education campaigns and by health care providers, including physicians, nurses, social workers, and dentists. At the policy level, environmental justice advocates can mobilize efforts to protect poor neighborhoods from exposures to EDCs.

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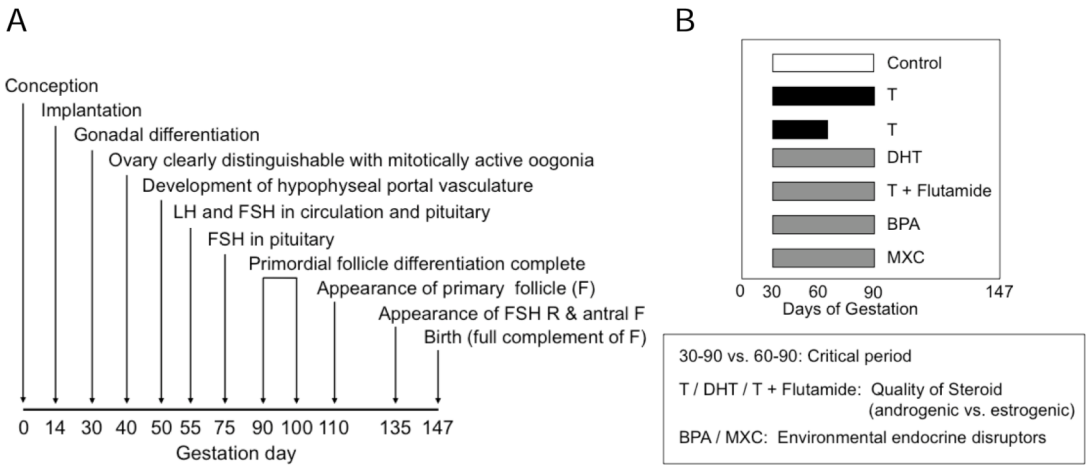

Fig. 1.

Panel A: Schematic showing the time of appearance of different classes of follicles in sheep, timing of establishment of hypophyseal portal vasculature to pituitary and timing of appearance of LH and FSH in circulation and pituitary during fetal life in sheep. Panel B: Schematic showing the timing and duration of the various steroid / EDC treatments used in studies discussed in this review.

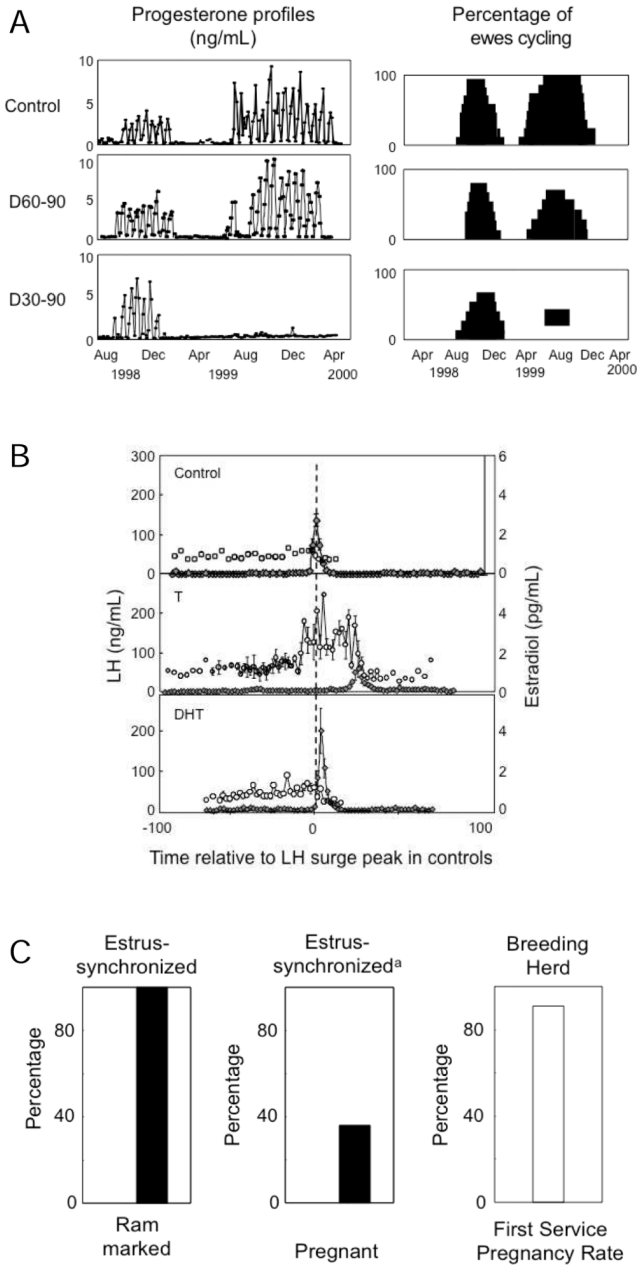

Fig. 2.

Panel A: Plasma progesterone profiles from representative control, T60-90, and T30-90 females during the first and second breeding seasons are shown on the left. On the right are shown percentages of sheep cycling during the first and second breeding seasons. (modified from Birch *et al.* 2003. Panel B: Patterns of LH (*closed circles*) and E (*open circles*) from control (*top*), T30-90 (*middle*), and DHT30-90 (*bottom*) following estrous synchronization with $PGF_{2\alpha}$ (Veiga-Lopez *et al.* 2009). Panel C: Percentage of T60-90 females mated and becoming pregnant following estrous synchronization. Estrus was synchronized with two injections of $PGF_{2\alpha}$ administered 11 days apart. Mating was determined by heavy rump markings left by a fertility-proven raddled ram (modified from Steckler *et al.* 2007b).

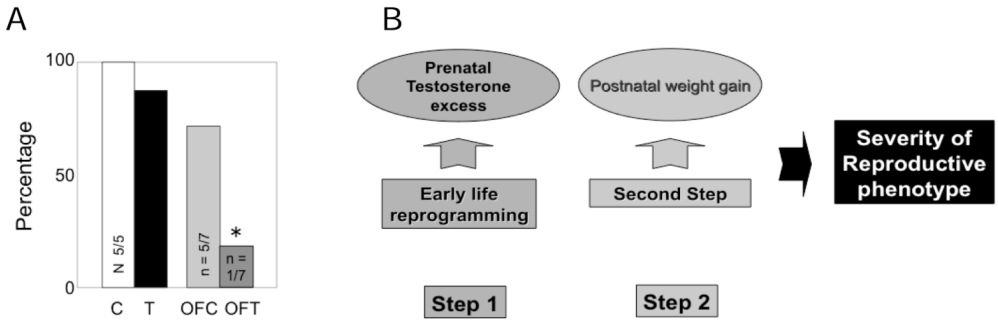

Fig. 3.

Panel A:Percent of control (C), over-fed control (OFC), T30-90 (T) and overfed T30-90 (OFT) females that showed a luteal progesterone increase following estrus synchronization with progesterone. Note that almost all of the overfed T30-90 females were anovulatory (modified from Steckler *et al.* 2009). Panel B: Schematic showing the two step model of programming severity of reproductive dysfunction with the first insult occurring from prenatal T excess during fetal life and the second metabolic insult stemming from overfeeding.

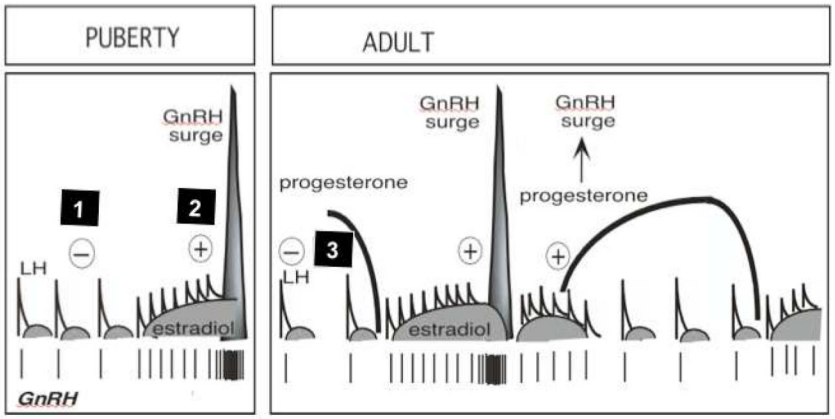

Fig. 4.

Neuroendocrine feedback systems involved in the control of GnRH / LH secretion that are reprogrammed by prenatal T excess. GnRH / LH release is under the control of negative feedback action of estradiol (E) which is predominant during the prepubertal and anestrus period (feedback 1), stimulatory feedback action of E responsible for generation of the preovulatory LH surge (feedback 2) and negative feedback action of progesterone, operational during the luteal phase (feedback 3) (modified from Foster *et al.* 2007).

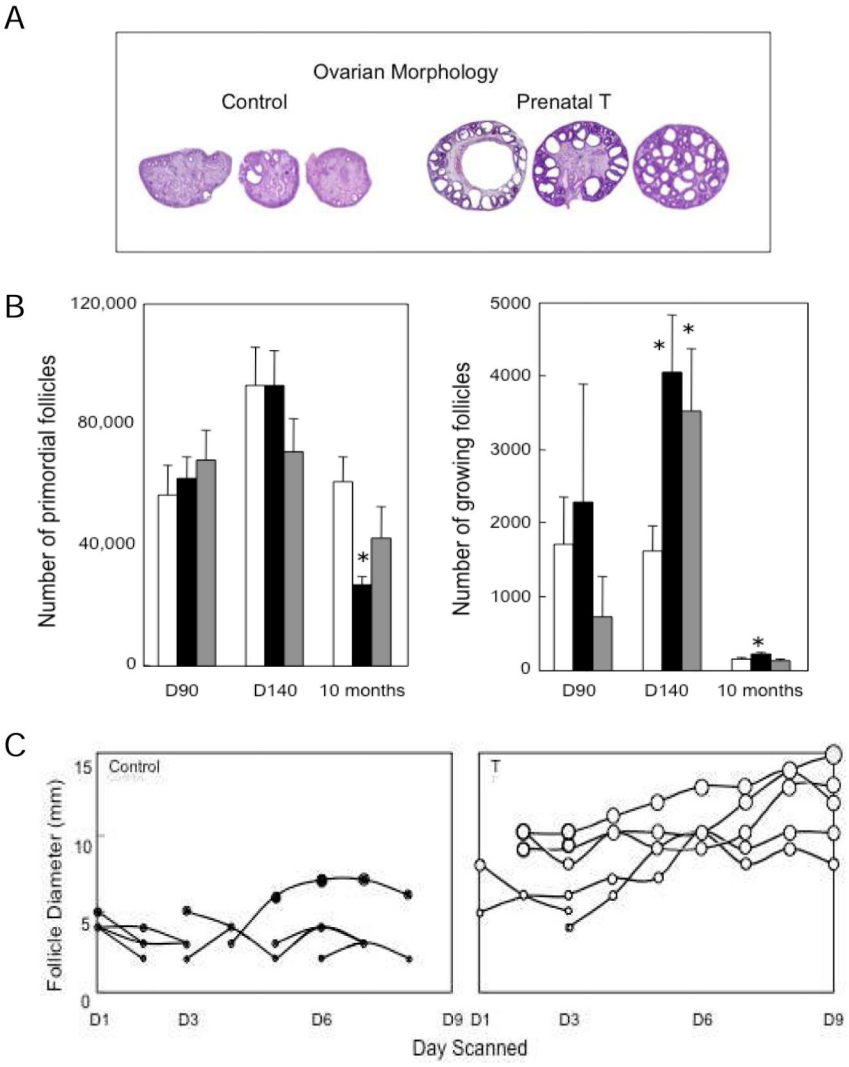

Fig. 5.

Panel A: Follicular morphology of ovary from control and T30-90 females. Note the disrupted nature of follicular development in T30-90 sheep (from West *et al.* 2001). Panel B: Mean (± SEM) number of primordial and growing follicles on fetal days 90 and 140 and 10 months of age in control (*open bars*), T30-90 (*closed bars*) and DHT30-90 (*gray bars*) ovaries (from Smith *et al.* 2009). Panel C: Ovarian follicular dynamics determined by ultrasonography for 8 days in both ovaries control and T30-90 sheep during the first breeding season (from Manikkam *et al.* 2006). Each line represents only one follicle and follicles from both ovaries are shown in the same panel. Only follicles that reached a size of 3 mm and persisted for at least 2 days are shown. Note the increase in maximum size and duration of the largest follicles on the ovary in T30-90 sheep compared to controls.

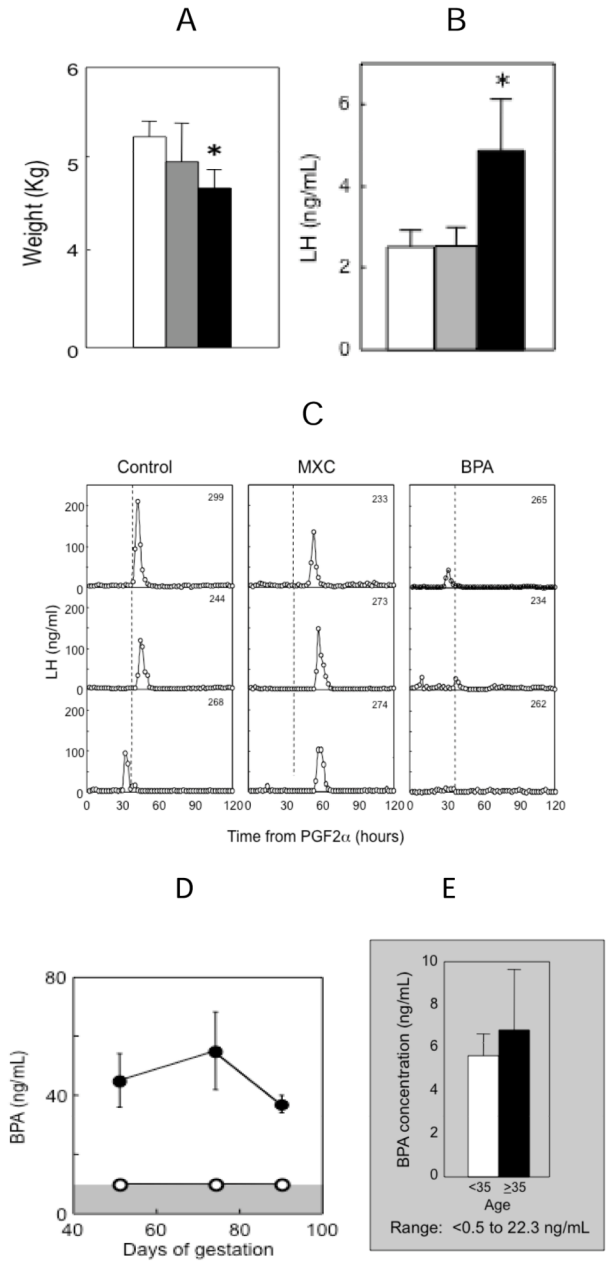

Fig. 6.

Panel A: Birth weight of control (*open bars*), prenatal MXC (*gray bars*) and BPA-treated (*closed bars*) female offspring (Savabieasfahani *et al.* 2006). Panel B: Mean circulating levels of LH in prepubertal control (*open bars*), prenatal MXC (*gray bars*) and BPA-treated (*closed bars*) female offspring (Savabieasfahani *et al.* 2006). Panel C: Circulating patterns of LH from 3 control, 3 prenatal MXC- and 3 BPA-treated females taken at 2 hourly intervals for 120 h, after induction of luteolysis with 2 injections of $PGF_{2\alpha}$ 11 days apart (Savabieasfahani *et al.* 2006). Panel D: Levels of circulating BPA achieved in control (*open circles*) and BPA treated (*closed circles*) pregnant sheep on day 50, 70 and 90 of gestation (days 20, 40 and 60 of treatment) following administration of 5 mg / kg / daily administration of BPA s.c. (Savabieasfahani *et al.* 2006). Panel E: Maternal levels of BPA

(mean ± SEM) in Southeastern Michigan relative to maternal age (Padmanabhan *et al.* 2008).

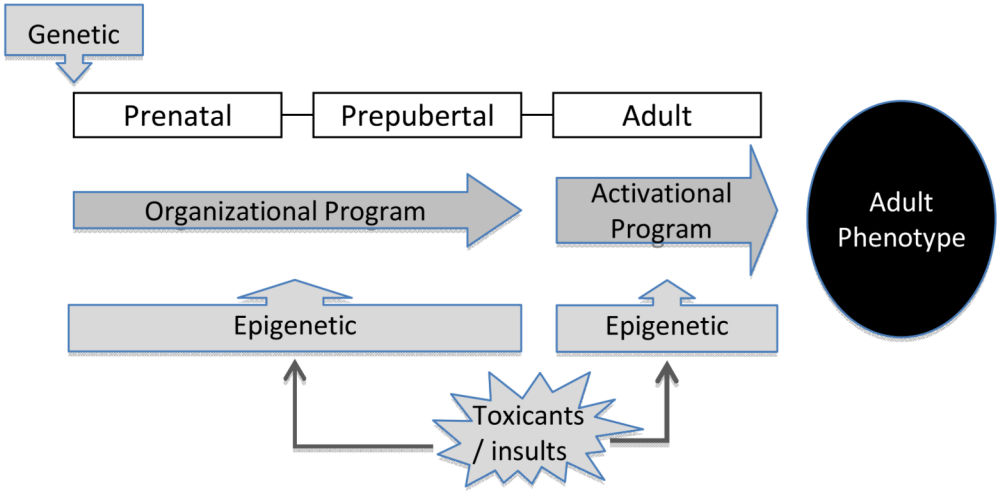

Fig. 7.
Schematic showing organizational palette of adult phenotype as influenced by genetic and epigenetic interactions.

Table 1

Characteristics of women with PCOS vs. prenatal T-treated sheep

Attributes	*Women with PCOS*	*Prenatal T-treated sheep*
Anovulation	Yes	Yes
Hyperandrogenism	Yes (functional)	Yes
Hypergonadotropism	Yes	Yes
Reduced sensitivity to steroids	Yes	Yes
Multifollicular ovaries	Yes	Yes
Increased follicular recruitment	Yes	Yes
Altered insulin sensitivity	Yes	Yes
Insulin resistance	Yes	Yes
Fetal growth retardation	Yes[A]	Yes
Altered behavior	Yes	Yes
Hypertension	Yes[B]	Yes
Visceral adiposity	Yes	Yes (observational)
Obesity amplification	Yes	Yes

[A] Spanish cohort

[B] Risk factor in PCOS

Table 2

Characteristics of prenatal T, BPA and MXC treated sheep

Attributes	*Prenatal T-treated*	*Prenatal BPA-treated*	*Prenatal MXC-treated*
Hypergonadotropism	Yes	Yes	No
Cycle disruption	Yes	Yes	Yes
Dampened LH surge	Yes	Yes	No
Increased amplitude of E_2	Yes	Yes	No
Delayed LH surge onset	Yes	Yes	No
Fetal growth retardation	Yes	Yes	No