Novel concepts about normal sexual differentiation of reproductive neuroendocrine function and the developmental origins of female reproductive dysfunction: the sheep model

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The neuroendocrine regulation of GnRH secretion plays a central role in timing gamete release in both sexes. This regulation is more complex in the female because the discontinuous release of ova is more complex than the continuous release of spermatozoa. This review provides an evolving understanding of the sex differences in reproductive neuroendocrine controls and how these differences arise. The rules for sexual differentiation of steroid feedback control of GnRH secretion conceptually parallel the well-established principles that underlie the sexual differentiation of the internal and external genitalia. In the context of the neuroendocrine regulation of the ovarian cycle, and using the sheep as a model, four steroid feedback controls for GnRH secretion are inherent (default). They require no ovarian developmental input to function appropriately during adulthood. Two steroid feedback controls regulate the preovulatory surge mode of GnRH secretion, and two regulate the pulsatile mode. If the individual is a male, three steroid feedback controls of GnRH secretion become unnecessary or irrelevant, and these are abolished or become functionally inoperative through programmed reductions in hypothalamic sensitivity. This central programming occurs through exposure of presynaptic GnRH neurons in the developing male brain to the androgenic and estrogenic actions of testicular steroids. In precocial species such as ruminants, this programming begins well before birth. Understanding how GnRH secretion normally becomes sexually differentiated is of practical importance to determining how inappropriate hormonal environments during development can variously malprogram the neuroendocrine system to produce a variety of reproductive dysfunctions relating to patterning of gonadotropin secretion.

Introduction

Why should male and female neuroendocrine controls be different?

As issues of sex differences assume an expanding role in society, science is looked to for

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explanations regarding their basis. Of recent concern is the issue of ever-increasing synthetic compounds in the environment that have the potential to produce sex-specific fertility problems. Differences in neuroendocrine controls of male and female mammals contribute to sex differences in susceptibility to reproductive disruption by exogenous substances. In this review, we note that indeed, there are several important differences between the sexes in the control of the secretion of gonadotropin releasing hormone (GnRH), the hypophysiotropic hormone central to reproduction. These differences are organized during development and become manifest to affect the timing of reproductive activity beginning as early as puberty. However, signs of sex differences in brain function are evident well before this time as evidenced by differences in patterns of hormone secretion and behavior during early postnatal life. Sex-specific reproductive neuroendocrine controls between males and females are linked to differences in the pattern of gamete production and release. Males have a relatively simple system in which sperm are made continuously in great numbers. Females, however, are born with a fixed number of eggs and these are parceled out over a prolonged period. Given a sufficiently long life, the ovary will run out of eggs, as is the case for the modern human. To return to the question posed at the beginning, the suggestion emerges that simple reproductive systems require simple controls and complex systems require more complicated ones. Added to these differences in how sperm and eggs are made and released are differences in sexual and social behaviors. How an individual responds towards another with respect to aggressive actions, territoriality, rank *et cetera* is also sex-specific.

In this review, we provide emerging concepts that account for the marked differences in reproductive controls in male and female sheep. Drawing upon an area of research that has a rich history from rodent studies, we hope to show the utility of using the sheep as a model that can contribute both conceptual and practical information to understand animal and human reproductive processes. As intimated above and expanded below, an understanding of male-female differences will be increasingly important to understand the various impacts of synthetic endocrine imposters on reproductive function. These compounds have the potential to misdirect developmental processes, primarily in the female to alter the complex timing mechanisms underlying the ovulatory cycle.

Hypothesis for sexual differentiation of GnRH feedback controls

The hypothesis in perspective - sexual differentiation of genitalia

At the outset, it is instructive to review highlights of the classical hypotheses for other aspects of sexual differentiation. Rather than invent a new set of principles for sexual differentiation of the reproductive neuroendocrine system, we are guided initially by those well-known tenets for sexual differentiation of internal and external genitalia, and we are interested in assessing how they might apply to the neuroendocrine system. It is important to emphasize that ours is yet a working hypothesis whose purpose is to stimulate additional work that will either add to the evolving story or modify it with new information (Fig. 1).

The first principle learned from the sexual differentiation of internal genitalia is that one sex serves as the default phenotype and the other phenotype requires both regression of the default structures and further development of others. Regardless of genotypic sex, female internal genitalia develop in the absence of gonadal hormone action while the masculine phenotype requires the presence of testicular secretions. The testes produce a systemic hormone to stimulate the development of male internal genitalia (masculinization) and a local substance to repress development of female internal genitalia (defeminization). A second principle is that although the systemic hormone (testosterone) is secreted to differentiate the bipotential exter-

nal genitalia, it must be converted locally to a more potent androgen, dihydrotestosterone (DHT), to produce the normal male phenotype.

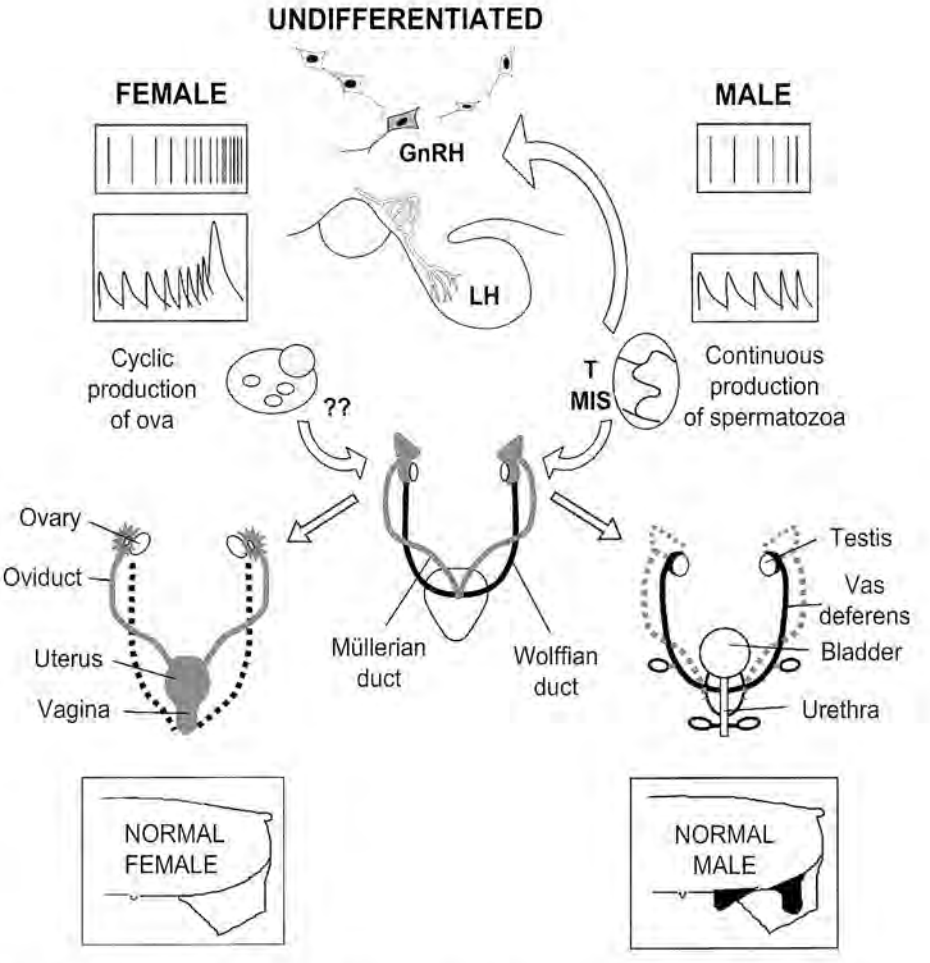

Fig. 1. Sexual differentiation of the reproductive axis (hypothalamo-hypophyseal system (top); gonads and internal genitalia (middle); external genitalia (bottom)). At all three levels of the axis, the female is the default condition. Due to the organizational effects of testosterone (T) secreted by the developing testis, feedback regulation of GnRH and LH secretion is defeminized, the undifferentiated Wolffian duct develops into male internal genitalia and the external genitalia are masculinized. A second testicular secretion, Müllerian inhibiting substance (MIS), causes regression of the Müllerian ducts which, in the female, develop into the internal genitalia. In the absence of testicular androgens the Wolffian ducts regress.

Hypothesis for sexual differentiation of reproductive neuroendocrine functions

Adapting the above well-known principles for somatic sexual structures to our system leads to the consideration that one sex has the default neuroendocrine phenotype. Logically this would be the female because of the complexity of the timing of the ovulatory cycle in comparison to spermatogenesis. Four steroid feedback mechanisms are necessary for the neuroendocrine

regulation of the ovarian cycle, but only one is used for regulation in the male. Either more feedbacks are added in the female (addition hypothesis) or alternatively, unnecessary ones are eliminated in the male (subtraction hypothesis). We believe that the latter mechanism is used. We propose that all four feedback controls are inherent in the female, and they require no further developmental input to function appropriately after birth. Two feedbacks regulate the surge mode of GnRH secretion, and two feedbacks regulate the pulsatile or tonic mode. However, three of these feedback controls are not required if the individual is a male, and only a single negative feedback system is required to regulate his much simpler, noncyclic reproductive system.

In addition to accounting for the sex differences in regulation of adult reproductive function, we must also explain the sex difference in the timing of puberty that is evident in most mammals. Puberty is controlled by the single feedback control that persists in both sexes, but males and females exhibit quantitative differences in sensitivity to this feedback. We propose that for the timing of puberty, the female system is also the default, and that *when* the transition to adulthood occurs in the male depends on the rate of maturation and species-specific reproductive strategies including seasonality of reproduction and social systems. For example, in slowly maturing, non-seasonal primate species (humans) puberty generally occurs earlier in the female. In more rapidly maturing, seasonally-constrained species that reach puberty within the same year as their birth, the transition to adulthood in the male may begin well ahead of that in the female. This second strategy, used by the sheep, maximizes the likelihood that a young male will reach sexual maturity and successfully compete for females during his first breeding season. Thus, according to our working hypothesis, changing any of the feedback controls of GnRH from the female default to the male type is, by definition, a process of defeminization. Exposure to testosterone secreted by the testes and its androgenic and estrogenic metabolites alters or eliminates female GnRH control systems. As is the case for differentiation of genitalia, the organizational actions of testosterone are exerted during critical periods of development. However, multiple critical periods may exist and may even be interdependent such that the presence of organizing steroids during the early critical period(s) increases susceptibility to further organizing actions of steroids in later development.

Historical perspective

Much of our present understanding of sexual differentiation of the control of gonadotropin secretion stems from investigations in the rat. However, important observations in the sheep over the past 30 years have led to the consideration that sexual differentiation of reproductive neuroendocrine function has broad application with potential practical usefulness.

Rodent studies

In the early 1900s, Sand (as described in Pfieffer 1936) was the first to recognize that in the guinea pig, ovarian grafts functioned differently in females and males. A few years later, Pfieffer began to focus on the pituitary and the importance of the developing gonad in regulating later sex-specific differences in hypophyseal function. From a landmark study comprising 495 rats under 18 experimental conditions, he concluded that... "The hypophysis in the rat at birth is bipotential and capable of being differentiated as either male or female, depending upon whether an ovary or testis is present." With an expanded understanding of the importance of the brain's control over pituitary function, later investigators would substitute the "hypothalamus" for "hypophysis". Moreover, the ovaries would be found to be unnecessary in determining the sex of

the control mechanism, this being the exclusive role of testicular testosterone. These pioneering studies inspired the elegant work in the rat by Gorski (1979, for review) to develop the concept of sexual differentiation of reproductive neuroendocrine function. He and his colleagues focused on the LH surge system for largely practical reasons, namely that it was present in the female, but absent in the male, and its assessment was relatively easy because of the massive release of LH. The eventual understanding that emerged from this large body of work is that the surge system is inherent, and becomes operational unless given information *not* to develop. The active organizing steroid that renders the surge system insensitive is an estrogen that is formed in the brain from the testosterone produced by the testes. This programming only occurs during a limited time of development, a critical period, and involves changes in a "surge center" in the anterior hypothalamus. These major findings have provided the conceptual underpinnings of all other work in this area, including the work in the sheep as discussed below. The location of controls for the GnRH surge mechanism and how they are rendered insensitive to the positive feedback action of estrogen remain under intensive study at the anatomical, electrophysiological, cellular and molecular levels in rodent models (Sullivan & Moenter 2004; Foecking *et al.* 2005).

Sheep studies

The earliest report of the influence of prenatal testosterone on neuroendocrine function was with a small number of sheep of various sexes and physiological states: two anestrous ewes, two ovariectomized ewes, two intact rams, two castrated rams and three freemartins (ovotestes). In addition to examining differences among the above phenotypes, the study included an experiment to probe for a critical period in females that had been treated before birth with a testosterone pellet (1g) beginning at various prenatal ages during the 147-day gestation (Days 20 n = 2, 40 n = 2, 60 n = 5, 80 n = 1) (Short 1974). From his studies, Short concluded that ..."the essential difference between the male and female hypothalamus of the sheep lies in its inability to initiate a LH discharge from the pituitary in response to oestrogen feedback. The presence of testosterone up to the 60th day of gestation is able to masculinize the hypothalamus in this respect, whereas testosterone given after day 60 is without effect." Although the number of animals in each treatment group was small, the results were variable, and the conclusion was too restrictive with respect to the timing, the observations were nevertheless very important. The findings raised the possibility that prenatal testosterone might abolish the GnRH surge and suggested the presence of an early critical period for this differentiation. Importantly, this initial report by Short led to additional detailed studies of the sexual differentiation of the GnRH surge mechanism and, of equal importance, of the differentiation of the inhibitory feedback controls of GnRH secretion that control the transitions between states of hypo-, eu- and hyper-gonadotropism, and hence, gonadal activity.

Sex differences in steroid feedback controls of GnRH

Studies of stimulatory feedback versus inhibitory feedback

Virtually all of the above work in the rat and sheep focused on the stimulatory feedback action of estrogen because the preovulatory gonadotropin surge is so clearly differentiated in those species. Moreover, systematic studies of the negative feedback control system have been few in small animals such as rodents for largely practical reasons relating to size. Evaluation of the pulsatile mode of LH secretion requires precise measurement of the frequency and amplitude

of LH release, and this is difficult because of the limited blood supply in a small animal. This is made more problematic for studies of the developing rodent. Although rapid blood sampling has been conducted in the rat by several investigators in other areas of reproductive research, none have used this approach in systematic studies of sexual differentiation of the neuroendocrine system. Another factor limiting interest in negative feedback controls in the rodent model is that the corpus luteum is not functional during a luteal phase when mating does not occur, unlike several other species including the primate and sheep as noted below. In these latter species, the inhibitory feedback action of progesterone serves as a major regulator timing the ovarian cycle. The current general lack of understanding of the sex differences in the control of negative feedback is unfortunate as changes in sensitivity to estrogen feedback inhibition of GnRH secretion also time many long-term aspects of reproductive activities that are different between males and females; including puberty, transitions between non-breeding and breeding seasons, reproductive response to nutrition and inhibition of reproduction by stress. In contrast to these multiple timing responsibilities of negative feedback controls, the GnRH surge system is highly specialized. It is reflexively activated to cause ovulation in response to the presence of high circulating concentrations of estradiol, the timing of which results from a quantitative reduction in amount of negative feedback control of GnRH secretion. Thus, whereas inhibitory feedback systems are responsible for long and short-term timing, the stimulatory feedback is responsible for immediate action.

Critical periods for sexual differentiation

The timing of the critical period deserves special mention. In the rat, this has been determined to occur during the first few days before and after birth. Outside the perinatal period the organizing actions of steroids are ineffective. Birth occurs somewhat arbitrarily during development in mammals, and species are born in differing stages of somatic and neurobiological maturation. This has given rise to classifying them as altricial, those born helpless and highly dependent on parental care, and precocial, those born at an advanced stage of development and relatively independent. With this in mind, it would be predicted that the critical period for sexual differentiation of GnRH secretion analogous to that which occurs perinatally for the altricial rat should be well before birth in the precocial sheep. Perhaps the term "critical period" will need to be redefined as it may be too restrictive to further concepts about programming several GnRH feedback controls. Within a broad critical period, there are likely multiple critical periods, each defined for a given programming function that may or may not overlap with one another. Conversely, there may even be multiple, sequential critical periods programming a given function. Finally, other complex developmental relationships may emerge; for example, the presence of steroids in an early critical period may organize the development of a later critical period.

Differentiation of multiple feedback controls

According to our working hypothesis, at least four steroid feedback controls of GnRH secretion are sexually differentiated (Fig. 2). These feedback mechanisms are inherent in the female and are either abolished in the male or their sensitivities are reduced markedly. The first and most dominant feedback control is active in both sexes (*Control 1: Estrogen negative feedback).* In both females and males, high sensitivity to estrogen feedback inhibition of GnRH secretion during prepubertal development maintains hypogonadotropism to keep the gonad relatively quiescent. This sensitivity decreases during puberty to increase gonadal activity. However,

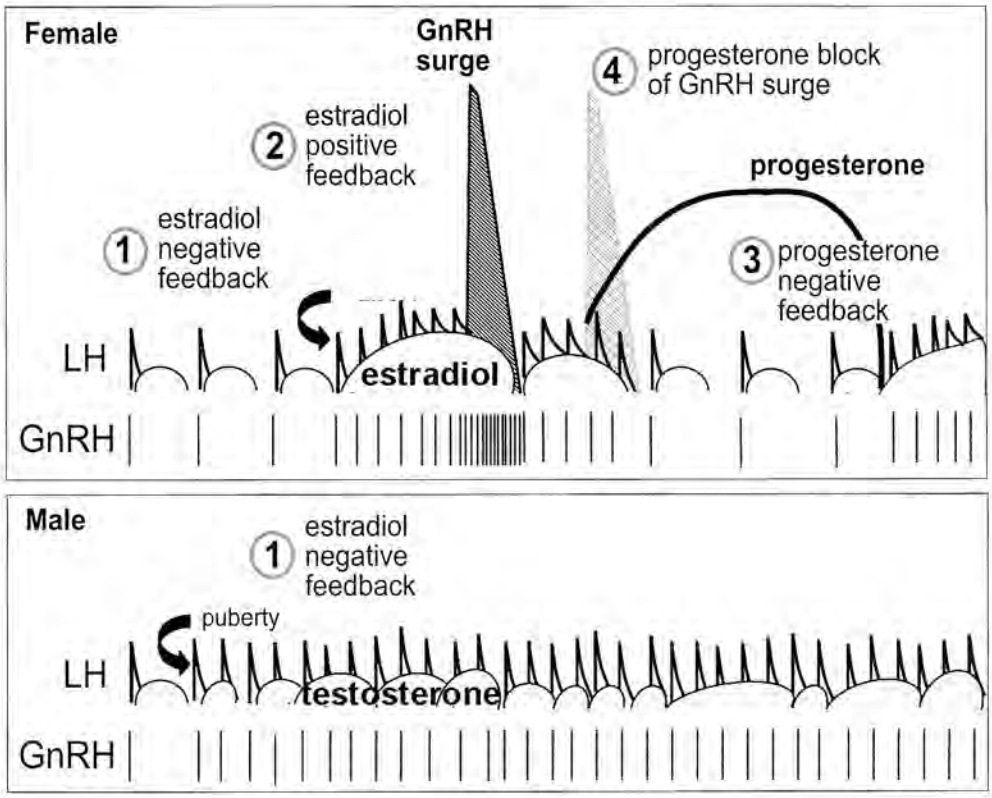

Fig. 2. Feedback controls of GnRH secretion in the sheep. In the female (upper panel), estradiol (feedback 1) and progesterone (feedback 3) have inhibitory actions on pulsatile GnRH secretion; the GnRH surge system is regulated by the stimulatory feedback action of estradiol (feedback 2) and the progesterone blockade of a GnRH surge (feedback 4). In the male (lower panel), only the estradiol inhibitory feedback mechanism is required to maintain spermatogenesis. (Redrawn from Foster *et al.* 2006).

the timing of when the sensitivity to estrogen negative feedback decreases is different between the two sexes and this accounts for the sex difference in timing of puberty in males and females of many species. Estrogen negative feedback is the only relevant feedback control of GnRH in the male. In the female beginning during and continuing after puberty, a complex interplay between the ovaries, hypothalamus, and pituitary is required to initiate and sustain repeated and regular ovarian cycles. During the pubertal transition, when the sensitivity to estradiol negative feedback is being reduced, high frequency GnRH secretion results in high frequency LH secretion which, in turn, drives the preovulatory follicle to increase its production of estradiol to high levels. The increasing concentrations of circulating estradiol from the preovulatory follicle exert a stimulatory feedback to produce a massive discharge of GnRH (*Control 2: Estrogen positive feedback)* to cause ovulation and formation of a corpus luteum. With a decrease in sensitivity to estrogen negative feedback at puberty, GnRH pulse frequency remains high, and a new negative feedback control emerges to regulate pulsatile GnRH secretion (*Control 3: Progesterone negative feedback*). After first ovulation, during the luteal phase, progesterone is secreted in high amounts for the first time to inhibit pulsatile GnRH

secretion. As a consequence, GnRH pulse frequencies are too low to stimulate LH secretion to levels that promote further development of follicles to the preovulatory stage and produce high circulating concentrations of estrogen. Withdrawal of progesterone negative feedback, as the corpus luteum regresses, then permits the high frequency GnRH secretion to initiate the next follicular phase. A second action of progesterone serves as a back-up mechanism to prevent a preovulatory gonadotropin surge during the early luteal phase when concentrations of progesterone are low (*Control 4: Progesterone blockade of the GnRH surge*). Immediately after ovulation, when negative feedback is entirely absent, high frequency LH pulses persist from the follicular phase and can begin to stimulate the secretion of estradiol from developing follicles in an early luteal phase follicular wave. As the corpus luteum simultaneously forms, the resultant low circulating concentrations of progesterone prevent the GnRH surge mechanism from responding to any possible estrogen positive feedback.

An essential part of our hypothesis is that the four feedback controls of GnRH secretion are differentially organized by testosterone and its androgenic and estrogenic metabolites. We propose that the inhibitory feedback controls of GnRH secretion are developmentally organized by the androgenic actions; the stimulatory feedback controls are organized by estrogenic actions. More specifically, the negative feedback actions of both estradiol and progesterone on pulsatile GnRH secretion are developmentally programmed by androgens. By contrast, the GnRH surge mechanism consists of two parts, one of which is the stimulatory feedback action of estradiol with the other being the blockade of this action by progesterone, yet both components are developmentally programmed by estrogens. This hypothesis regarding the role of progesterone in the GnRH surge being distinct from its negative feedback effects is guided by the concept that the tonic and surge modes of GnRH release have distinctly different controls. However, it is possible that there is just one inhibitory or blocking feedback effect of progesterone, and that this is developmentally programmed by a single steroid, either an androgen or an estrogen.

Developmental programming of sex differences in feedback controls

Models to study sex differences in steroid feedback controls of GnRH

Two models have been used to study the sexual differentiation of feedback controls of GnRH secretion. The first is a neuroendocrine model in which the ovaries are removed neonatally and an estradiol implant is inserted subcutaneously to maintain chronic low concentrations of estradiol (Ovx+E). The advantage of this model is that neuroendocrine feedback controls can be studied in the presence of constant steroid conditions. In this review, we will focus on this neuroendocrine model, the one with which we have the most experience, and then provide necessary commentary about the results being obtained in the second model which is ovarian-intact. Both models are treated the same before birth, but after birth their treatments differ with respect to steroid exposure. Sexual differentiation of the feedback mechanisms will be discussed in the order in which they become relevant to an individual, beginning with the estradiol negative feedback mechanism timing the pubertal increase in GnRH (Fig. 2).

Estrogen inhibitory feedback

A sex difference in feedback inhibition of GnRH secretion is readily apparent in the developing sheep (Fig. 3). In the neuroendocrine model, the developing female remains hypersensitive to low, constant levels of circulating estradiol (implant) for several months, and LH secre-

tion remains suppressed. By contrast, the developing male is initially hypersensitive to estradiol negative feedback but within a few weeks after birth this is no longer the case and LH rises progressively. This increase in circulating LH reflects an increase in GnRH pulse frequency (see Foster & Jackson 2006). That this decrease in estradiol negative feedback is causal to activation of the gonads is evident by the close associations of the pubertal LH rise with the increase in testicular activity or the onset of ovarian cycles. Thus, while there are obvious differences between the ovary and testis that could contribute to their differential activation during puberty, when the increase in their tropic stimulus occurs is paramount.

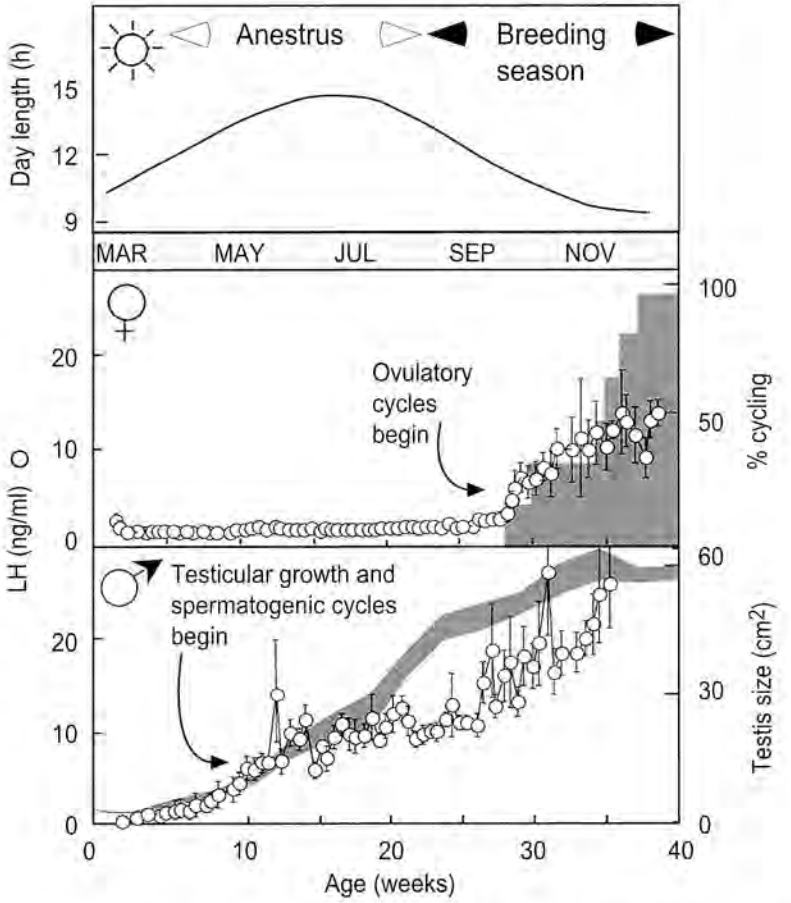

Fig. 3. Neuroendocrine model. Sex differences in the negative feedback control of GnRH secretion by estrogen in developing sheep. LH concentrations in semiweekly blood samples collected from neonatally gonadectomized and estradiol-replaced (3-5 pg/ml by sc implant) lambs. Note that the pubertal increase in LH secretion occurs earlier in the male compared to the female, and puberty in the female does not occur until the breeding season. The rise in LH concentrations coincides with the onset of progestagenic cycles in intact females and increased testicular volume in intact males (shaded areas). (Redrawn from Foster *et al.* 2006).

What times the decrease in sensitivity of the mechanisms controlling tonic/pulsatile GnRH secretion to the inhibitory feedback action of estrogen is complex (Foster & Jackson 2006). The developing individual must have achieved a minimum stage of development and a sufficiently positive energy balance and size to begin reproductive activity (Sisk & Foster 2004). Moreover, reproduction is seasonal in the sheep, so one would predict that these indices of maturity would only be expressed during the breeding season. In the adult sheep, the major environmental cue timing seasonal reproduction is photoperiod, and reproductive activity begins during decreasing day lengths (Karsch *et al.* 1984). In the female lamb, there is a strict photoperiod requirement for the synchronous timing of puberty, and the pubertal increase in GnRH begins during decreasing daylengths (Fig. 3 top). Reversing the photoperiod delays puberty well beyond the normal age (25-30 weeks) and prevents it from occurring synchronously among females of the same cohort (Fig. 4). Puberty cannot be delayed indefinitely and occurs both at older ages and asynchronously (Foster *et al.* 1986).

Interestingly, the earlier pubertal increase in GnRH begins in male lambs during increasing photoperiod which raises the possibility that either the developing male has a different photoperiod requirement or is not photoperiodic with respect to the timing of puberty. Other neuroendocrine responses are influenced by photoperiod in the developing male, including the patterns of melatonin and prolactin secretion (Foster *et al.* 1989), and photoresponsiveness begins before birth (Ebling *et al.* 1989). However, this does not seem to be the case for reproductive neuroendocrine system. Unlike the developing female, the male can initiate its pubertal increase in GnRH at the same age in increasing and decreasing photoperiods (Fig 4).

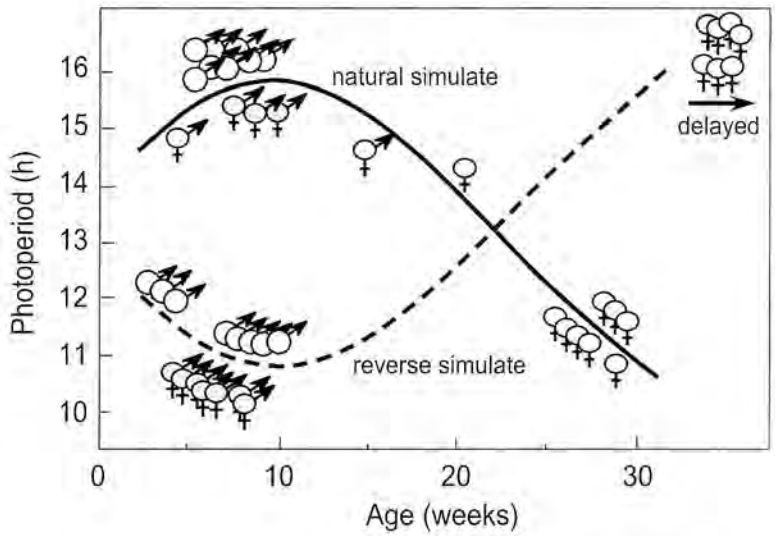

Fig. 4. Neuroendocrine model. Sex differences in the photoneuroendocrine control of the timing of the pubertal LH rise in the gonadectomized, chronically estrogen-treated sheep. The time of the increase is designated by the placement of the symbol (male, female, prenatally testosterone-treated female) in relation to age and photoperiod. Note that females are highly responsive to photoperiod and begin puberty under decreasing day lengths, but males and prenatally testosterone-treated females are not photosensitive, and they begin their pubertal rises in LH at a young age under both decreasing and increasing day lengths. (Redrawn from Foster & Jackson 2006).

The finding that the photoperiodic control of the mechanism timing the pubertal rise in GnRH is sexually differentiated leads to the hypothesis this is due to prenatal exposure to testosterone from the developing testes. This hypothesis is supported by the results in female lambs whose mothers were injected with testosterone from Days 30-90 of gestation (Wood *et al.* 1991). Female lambs exhibited masculinized internal and external genitalia, and in the neuroendocrine model (Ovx+E), they exhibited resistance to estradiol negative feedback beginning at an early age (Fig. 5), much like the developing male. This allows the pubertal rise to occur under an inhibitory photoperiod (long days) when untreated females typically remain hypersensitive to negative feedback and hypogonadotropic (Fig. 5). Thus, we conclude that the default control for photoperiodic regulation of the timing of puberty is defeminized (removal of a female trait) by testosterone in the developing male lamb. It is of interest that by the second year, the reproductive system of the young male also becomes regulated by photoperiodic cues, much like that for the female to result in seasonal changes in sensitivity to negative feedback of GnRH secretion. The reason for the apparent absence of photoperiodic control of reproduction in the male during the first year is speculative, but it could be necessary to increase the GnRH/LH drive to the testes early to develop fully the steroid-dependent behaviors necessary to begin competition with older males for females.

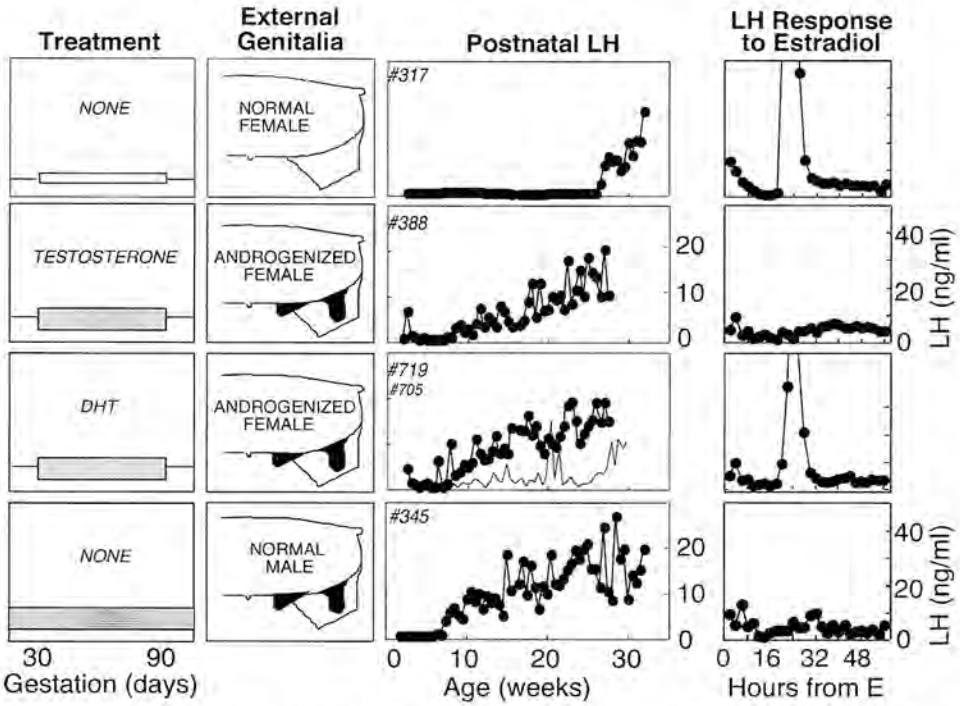

Fig. 5. Neuroendocrine model. Prenatal sexual differentiation of the inhibitory and stimulatory feedback actions of estrogen in the gonadectomized chronically estrogen treated sheep. Left column: Prenatal treatment. Second column: Schematics of postnatal external genitalia. Third column: Circulating concentrations of LH in the presence of chronic estradiol (3-5 pg/ml). Right column: Circulating concentrations of LH after administration of a late follicular phase increment in estradiol (10-15 pg/ml) designed to induce a GnRH surge. Note the advancement of the pubertal LH rise in the female with prenatal exposure to testosterone or DHT and the lack of the LH surge in testosterone-treated females (exposed to both androgen and estrogen actions) but not in females exposed to DHT (androgen only). (Redrawn from Foster & Jackson 2006).

The timing, duration, amount and type of steroid exposure all contribute to the degree of defeminization of control of pulsatile GnRH secretion. The *time of testosterone* exposure is important. A broad critical period has been identified early in development; exposure of the unborn female to testosterone between Days 30 and 90 of gestation (147 days term) advances the timing of the pubertal GnRH rise, whereas this same treatment later is without effect (Herbosa & Foster 1996). Lesser exposures (for example, Days 30-60 or Days 60-90) to testosterone are effective, but the timing of the pubertal LH rise is advanced less (Wood *et al.* 1995). The *amount of testosterone* exposure is important with the greater amounts producing the earliest decreases in response to steroid feedback, and hence, the earliest increase in LH secretion (Kosut *et al.* 1997). The *type of steroid* is important. Testosterone can be metabolized by the brain to a more potent androgen or to an estrogen, and the type of steroid action cannot be assessed by administration of only testosterone. Administration of dihydrotestosterone (DHT), the androgenic metabolite that cannot be converted to an estrogen, has been used to begin to differentiate these actions of testosterone. DHT advanced the time of the pubertal rise in LH suggesting that androgenic action is involved in this programming (Masek *et al.* 1999). However, the results were not identical to those for testosterone raising the possibility that estrogens may have some responsibility in organizing the timing of the pubertal increase in GnRH secretion. The role of estrogens is difficult to evaluate for the technical reason that administration of these steroids can act on the maternal uterus to compromise the pregnancy. Thus, other approaches must be developed to test hypotheses relating to the relative contribution of prenatal estrogenic action, if any, in the early androgen programming of the inhibitory feedback control of estrogen on pulsatile GnRH secretion.

Estrogen stimulatory feedback

Early work in the sheep indicated that the mechanism governing the stimulatory feedback action of estrogen is sexually differentiated because high amounts of estradiol were incapable of inducing a LH surge in males (Karsch & Foster 1975). A later study, directly measuring GnRH in the pituitary portal vasculature, offered the first direct proof that the absence of the LH surge was due to the failure of the male to release the massive amount of GnRH as in the female in response to follicular phase levels of estradiol (Fig. 6). This study also revealed that exposure to testosterone *in utero* renders the GnRH surge mechanism inoperative, as the large estrogen-induced surge of GnRH did not occur in female lambs treated prenatally with testosterone (Herbosa *et al.* 1996). While these observations provided the necessary direct evidence for the earlier inference of Short (1974) about prenatal testosterone and the surge mechanism, there is an important conceptual difference emerging based on the current working hypothesis. Rather than testosterone "masculinizing" the hypothalamus, we now may use different terminology in view of the consideration that the default reproductive neuroendocrine control is the female system. To distinguish removal of an innate female characteristic as opposed to the addition of a male trait the term "defeminized" is more appropriate.

The popular term "androgenization" of the LH surge mechanism is also inappropriate as this implies that the androgenic action of testosterone organizes the GnRH surge mechanism to reduce its response to the stimulatory feedback action of estrogen. This does not appear to be the case because when DHT, the potent androgenic metabolite of testosterone, was administered to the developing female lamb from Days 30-90 of gestation, the GnRH surge mechanism remained highly responsive to estrogen positive feedback when tested after birth (Masek *et al.* 1999) (Fig. 5). These same amounts of DHT were capable of advancing the pubertal increase in GnRH secretion attesting to the efficacy of the doses of androgen used to produce

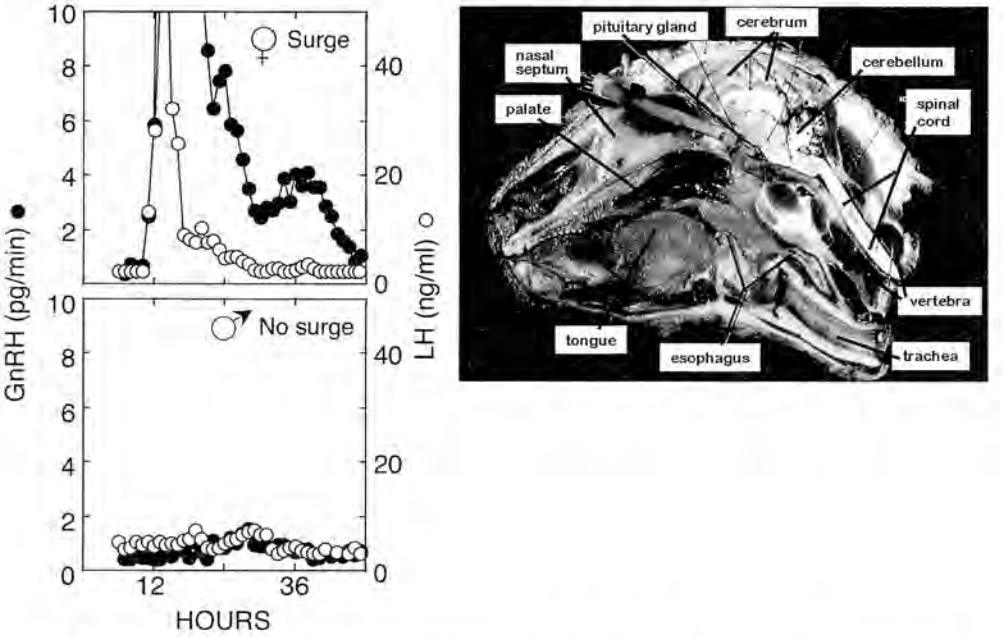

Fig. 6. Sex differences in patterns of GnRH and LH release after exposure (Hour 0) to late follicular phase concentrations of estradiol (10-15 pg/ml by implant). GnRH concentrations were assayed in blood collected from the pituitary portal vasculature. The sagittal section of the sheep's head presented in the right panel illustrates the placement of the collection device for pituitary portal sampling. In the female (top left panel), a GnRH surge occurs and is accompanied by a LH surge. The GnRH and LH surges are absent in the male (bottom left panel). (Data redrawn from Herbosa *et al.* 1996).

organizational actions on feedback control, albeit in the inhibitory feedback system (Fig. 5). Much the same as with the organizational actions of prenatal androgens on programming the sensitivity to negative feedback, the amount and timing of prenatal estrogen exposure are important in programming reduced sensitivity to positive feedback. Exposure during the early part of gestation is critical for complete defeminization of the GnRH surge system as are higher amounts of testosterone. Lesser amounts of testosterone and shorter exposure periods result in estrogen-induced LH surges that are of lower amplitude or that are delayed (Kosut *et al.* 1997; Wood *et al.* 1995). Interestingly, in most studies of the rat model (Gorski 1979), the LH surge system is rendered unresponsive by the organizational actions of estrogen. More recently, there is evidence for an androgenic action as well in the rat leading to the suggestion that either both androgens and estrogens are normally needed to inactivate the GnRH surge system or that the two actions are redundant (Foecking *et al.* 2005). To date there is no evidence for this in the sheep.

Progesterone feedback controls

Progesterone feedback regulation of GnRH secretion assumes importance only after puberty and only in the female. Its major role is to serve to inhibit feedback control of pulsatile GnRH secretion after the sensitivity to estradiol negative feedback becomes reduced during puberty,

and estradiol no longer inhibits GnRH secretion. Progesterone from the newly formed corpus luteum reduces GnRH pulse frequency during the luteal phase. If no conception occurs, progesterone declines and allows GnRH pulse frequency to increase and begin the next follicular phase. In the male, this feedback hormone is not present in any appreciable amounts, and has no known physiological role in the regulation of GnRH secretion. Thus, progesterone action in the male has been generally ignored. One study has determined that the male sheep is relatively insensitive to progesterone negative feedback, and prenatal testosterone exposure of the female lamb reduces its postnatal sensitivity to progesterone feedback inhibition of LH secretion (Fig. 7) (Robinson *et al.* 1999). Whereas it appears that estrogen negative feedback is largely organized by the prenatal androgenic action of testosterone, whether this second inhibitory feedback control system is similarly sexually differentiated remains to be determined.

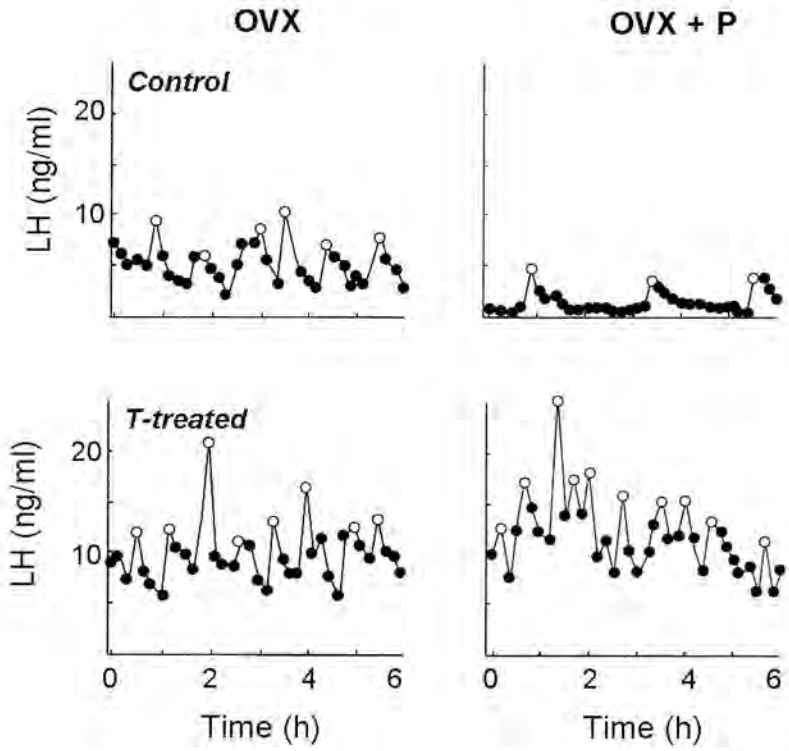

Fig. 7. Neuroendocrine model. Sex differences in the inhibitory feedback action of progesterone in the ovariectomized sheep. Whereas exogenous progesterone readily inhibits pulsatile LH secretion in control females (top), it is virtually ineffective in females exposed prenatally to testosterone (bottom), much like in the male (data not shown). (Data redrawn from Robinson *et al.* 1999).

Progesterone has a second important action, namely it blocks the stimulatory feedback action of estrogen on the GnRH surge mechanism during the luteal phase (Fig. 8) (Kasa-Vubu *et al.* 1992). We do not know if this action is also sexually differentiated by estrogen as part of the surge system. The alternative possibility, that it is differentiated by androgen, would not be expected as we propose that androgen actions primarily organize negative feedback controls.

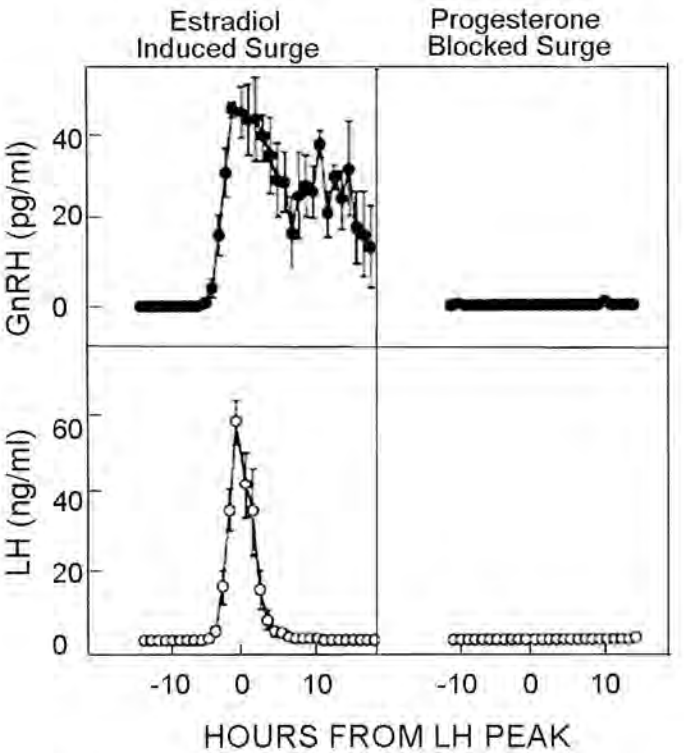

Fig. 8. Neuroendocrine model. Patterns of GnRH and LH release after exposure to high follicular phase concentrations of estradiol (10-15 pg/ml by implant). Left panels: Estradiol only. Right panels: Chronic treatment with progesterone beginning seven days before the estradiol treatment prevented both the GnRH and LH surges. (Data redrawn from Kasa-Vubu *et al.* 1992).

Postnatal programming - clues from other models

The foregoing studies in the neuroendocrine model, in which the postnatal steroid milieu remains constant (Ovx + E), provide the basis for our working hypothesis for sexual differentiation of the feedback control of GnRH. To test the working hypothesis in more physiologically relevant conditions requires a complete reproductive system, one in which the ovaries remain *in situ* and the female is not exposed chronically to exogenous steroid. A second important reason for returning to the intact model is to study the effects of prenatal programming on ovarian function. Interestingly, studies in the intact model, although identical with respect to prenatal treatments, often produce results that appear dissimilar. A conceptually interesting explanation is that such differences point to yet unrecognized facets of sexual differentiation. One such possibility that arises is that *postnatal* sexual differentiation of neuroendocrine function may be occurring, a surprising option for such a precocial species.

The differences between the two models become apparent when one predicts the expected ovarian activity in the intact model based on the results from those of the neuroendocrine model. Recall that in the neuroendocrine model prenatal testosterone programs both inhibitory and stimulatory feedback controls of GnRH to result in an early pubertal GnRH rise and a failed GnRH surge

system. In the intact model, the identical prenatal exposure should have two main consequences: 1) early hypergonadotropism (androgenic action to prematurely decrease sensitivity to negative feedback) resulting in a polyfollicular ovary; and 2) puberty does not occur (GnRH surge system rendered inoperative therefore no ovulations). Certain results for prenatally treated intact females were as predicted; females became less sensitive to estrogen negative feedback and were hypergonadotropic (Sarma *et al.* 2005). As a consequence, the ovary became polyfollicular at an early age (West *et al.* 2001) (Fig. 9). However, the most unexpected finding was that puberty did occur in most prenatally testosterone-treated lambs as evidence by repeated increases in circulating progesterone (Sharma *et al.* 2002; Birch *et al.* 2003) (Fig. 10). Although some progestagenic cycles were abnormal in this model, the fact that they even occurred was not predicted because the GnRH surge system had been demonstrated to be nonfunctional in several similar studies using the neuroendocrine model. When the positive feedback action of estradiol was tested in the ovarian-intact prenatally testosterone treated female, the results were mixed. In one study, a LH surge could be induced by exogenous estradiol but the amplitude was markedly reduced (Sharma *et al.* 2002). In another study, the surge mechanism was completely nonfunctional (Unsworth *et al.* 2005). Studying prenatally testosterone-treated females for a second year revealed that the ovarian cycle deteriorated and most females became anovulatory (Birch *et al.* 2003). Although not rigorously studied, the negative feedback of progesterone on GnRH secretion in the intact model seems to be compromised because LH pulse frequency during the luteal phase in prenatally testosterone exposed females tends to be greater compared to controls (Savabieasfahani *et al.* 2005).

That ovulatory cycles were possible in the prenatally testosterone treated female is not without precedent as this had been observed in the earliest studies of Short (1974) that preceded the development of the neuroendocrine model. Thus, the difference between the two models can no longer be ignored. An interesting possibility is that in the neuroendocrine model programming is more complete and hence is easier to demonstrate. Perhaps the chronic presence of small amounts of estradiol (2-4 pg/ml by implant) used to study activational (inhibitory and stimulatory) actions on GnRH also may have continuing *organizational* actions to program GnRH controls. According to this extended hypothesis, postnatal estradiol may be necessary to complete the organizing action of prenatal androgens and estrogens. Alternatively, prenatal steroids may increase the susceptibility of the hypothalamus to the postnatal actions of estradiol. In this context, in the absence of chronic estradiol in the intact model, incomplete defeminization or programming occurs and the prenatally testosterone-treated female is able to initiate ovarian cycles. The continued deterioration and cessation of reproductive cycles by the second year in the prenatally testosterone-treated females supports this notion as these females would be repeatedly exposed to estradiol during multiple follicular phases. Under normal physiological conditions, the male is first exposed to testosterone and its metabolites, including estrogens, from the beginning of the critical period through birth and continuing with increasing intensity during postnatal testicular development. By contrast, the postnatal ovary makes only small amounts of estradiol, and most likely, in a cyclic manner (Ryan *et al.* 1991). These multiple hypotheses are currently being tested to address the possibility of postnatal programming of GnRH feedback controls by estradiol (Malcolm *et al.* 2006). Finally, a completely different explanation is that the ovary produces one or more substances that modify the manifestation of the effects of prenatal steroids on postnatal GnRH secretion. These substances could be other steroids, peptides, or proteins that could modulate either ovarian or neuroendocrine function or both. However, a protective role of the ovary in controlling sexual differentiation of extraovarian tissues is currently without precedent.

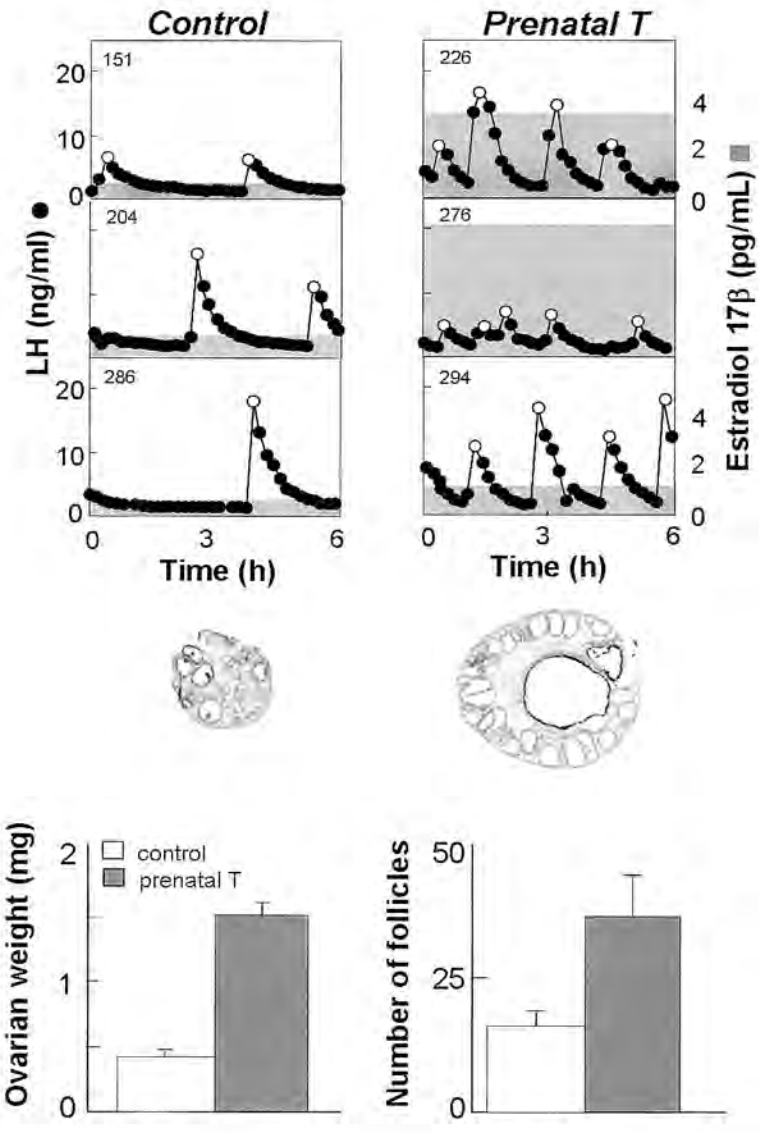

Fig. 9. Intact model. Patterns of LH release, appearance of ovaries, ovarian weight and number of preantral and antral follicles in control female lambs (left) or female lambs that had been treated prenatally with testosterone (right). Female sheep exposed to testosterone prenatally become hypergonadotropic and have large, multifollicular ovaries. (Data redrawn from Sarma *et al.* 2005 and West *et al.* 2001).

Neurobiological mechanisms underlying sexual differentiation of GnRH controls

Programming of the feedback controls of GnRH could occur either at the level of the GnRH neuron or its afferents. It is unlikely that this is within the neuron itself as GnRH neurons are generally devoid of classic estradiol-α and progesterone receptors (reviewed by Herbison 1998).

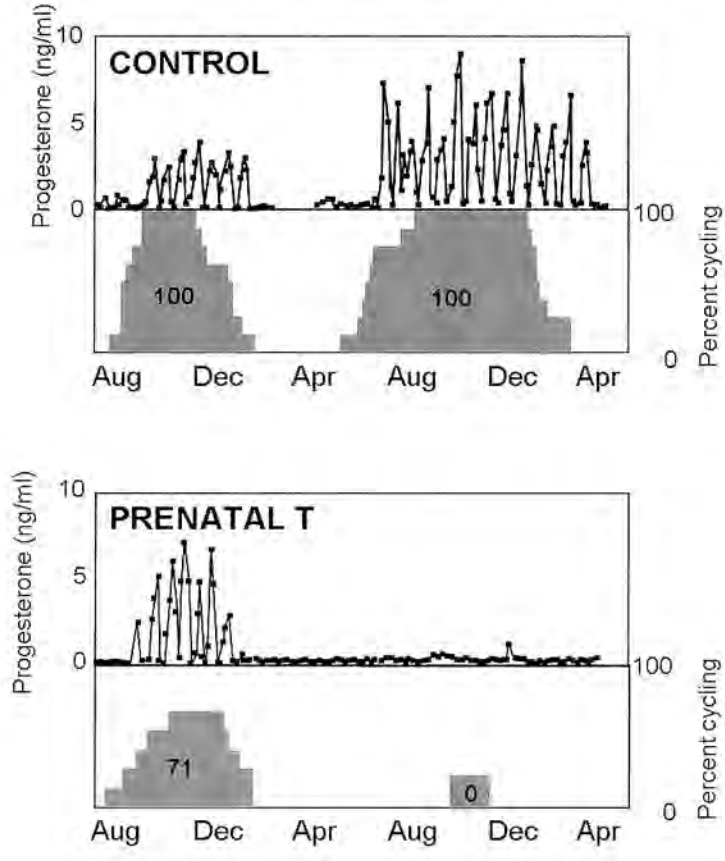

Fig. 10. Intact model. Patterns of circulating progesterone concentrations in individual female sheep during the first two breeding seasons and number of females cycling: controls (top panel) compared with female sheep prenatally treated with testosterone (bottom panel). Female sheep exposed to testosterone prenatally become anovulatory during the second year. (Data redrawn from Birch *et al.* 2003).

Furthermore, several studies found no sex differences with respect to GnRH number, morphology or location. GnRH neurons in the sheep brain have been detected as early as 43 days of gestation (Caldani 1986) and examining their anatomy in the preoptic area/hypothalamus at 85 days of gestation reveals no difference between the sexes (Wood *et al.* 1992) (Fig. 11). The total number of GnRH neurons, their relative distribution and the percentages of different morphological subtypes (unipolar, bipolar, multipolar) are the same in males and females. Moreover, because all of these characteristics were similar to those in the adult ewe (Lehman *et al.* 1986; Caldani *et al.* 1988), there appears to be no gross postnatal alteration of GnRH neurons. In a further study at the electron microscopic level, ultrastructural differences have been observed. There are more synaptic contacts on GnRH neurons of adult female sheep than male sheep, and prenatal exposure of female lambs to testosterone decreases the number of synapses in females (Kim *et al.* 1999). It is not yet known if this is due to the organizing action of androgen or estrogen on the surge or tonic mode of GnRH secretion. Finally, it is of interest that a reduction in GnRH neuronal activity in response to estradiol has been found in females

exposed to testosterone *in utero* (Wood *et al.* 1996). This was determined by comparison of Fos co-localization with GnRH in neurons located in the preoptic area, anterior and mediobasal hypothalamus in normal and androgenized females during a surge-inducing dose of estradiol.

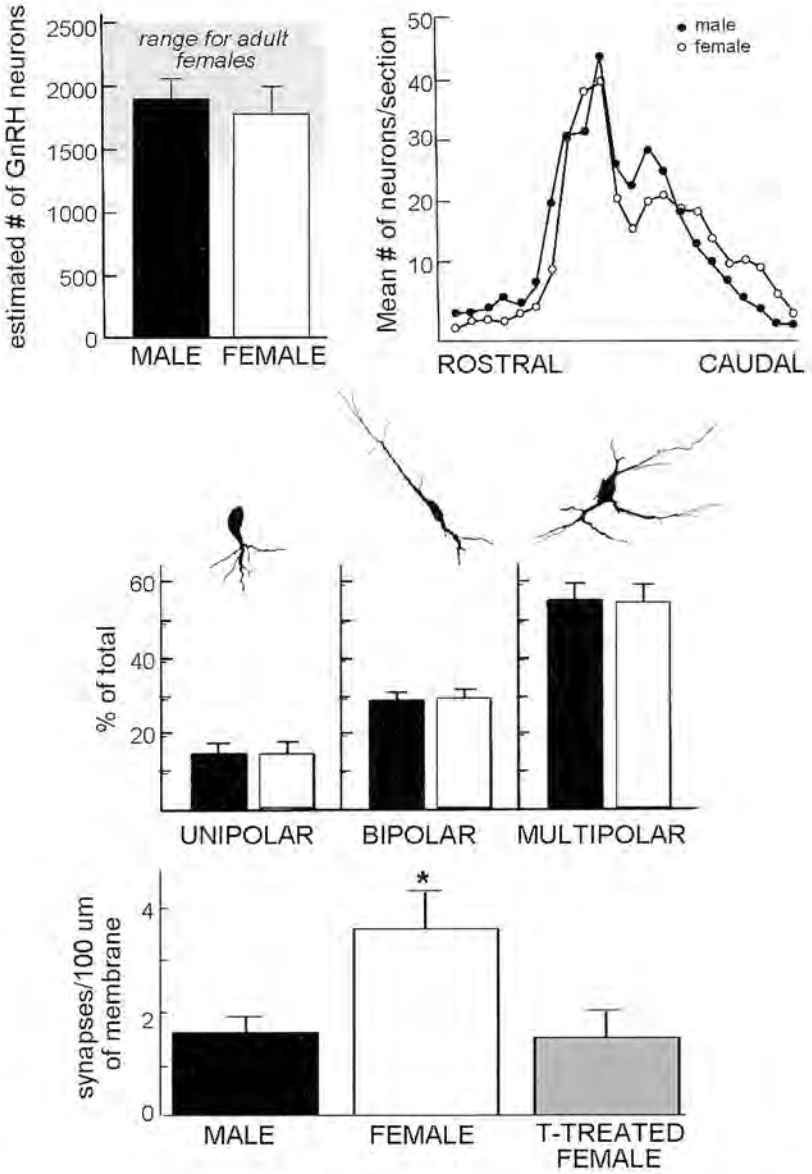

Fig. 11. Neuroendocrine model. Number of GnRH neurons in fetal male and female sheep (top left panel); the shaded area is the range for GnRH neuron counts in adults. Rostral to caudal distribution of GnRH neurons in male and female lambs (top right panel). Types of GnRH neurons in male and female lambs (middle panel). Number of synapses on GnRH neurons in male and female lambs, and in prenatally testosterone-treated female lambs (bottom panel). Although the type and location of GnRH neurons are not sexually differentiated, the number of inputs (synapses) is reduced by early testosterone exposure. (Data redrawn from Wood *et al.* 1992 and Kim *et al.* 1999).

Other considerations about sexual differentiation of the reproductive neuroendocrine control of the brain

Physiological versus pathological programming

When administering sex steroids to a developing individual one must be concerned about whether the effects are physiological or pathological. If the goal is to document the pharmacologic effects of high native sex steroids or endocrine disruptors, then higher peripheral doses of compounds that would be present in the environment can be administered to the mother. However, if the goal is to test hypotheses about normal physiology including sexual differentiation, then small doses of naturally occurring sex steroids delivered to the target within the conceptus should be the ideal approach. At present, relatively high doses of steroids are necessarily administered to the mother in an attempt to adequately expose the conceptus, given that the compounds must cross the placenta. Targeting the conceptus by a more direct route is technically difficult given its friability during known critical periods that typically occur during early development. Thus, the only present alternative is to use minimally effective doses of steroid. The general problem of embryonic/fetal accessibility is compounded for estrogens. While testosterone can cross the placenta to affect developmental functions directly or through its androgenic and estrogenic metabolites, broad administration of estrogens via the mother is problematic as they increase uterine activity during a time when it must be quiescent. Other novel strategies must be used such as administration of testosterone along with an anti-androgen so that the conceptus is functionally exposed to the estradiol metabolite. Even with the use of such strategies, experimentally administered steroids that attempt to model physiological programming of reproductive neuroendocrine functions often produce unintended consequences. These exogenous steroids not only affect reproductive tissues and their controls, but they also alter non-reproductive systems; for example, prenatal testosterone can produce metabolic alterations, such as insulin resistance and growth retardation (reviewed by Padmanabhan *et al.* 2006). Interestingly, with high doses of testosterone males show many of these non-reproductive effects suggesting that pathologies can be induced with steroid exposure that is above normal. Thus, some healthy degree of caution must be exercised in interpreting present physiological studies that broadly expose the fetus to relatively large amounts of sex steroids. Nevertheless, we believe that with our present approaches, some physiologic principals are emerging.

Prenatal programming versus sexual differentiation

Whereas most developmental programs use no external information for their execution, some do. Both prenatal programming of some metabolic systems and sexual differentiation use such external information. Arguments can be made that fetal programming and sexual differentiation are different processes conceptually with the former having the potential for some type of adaptive role and the latter being more resistant to novel adaptations. However, while we can provide a historical distinction between concepts about prenatal programming and sexual differentiation, one quickly realizes that the working distinction fades the more we learn. We now find that we must use encompassing phrases such as "prenatal programming of sex differences"; in doing so, we recognize the futility of such distinctions and relegate them to semantics.

Historically, the areas of prenatal programming and sexual differentiation have distinctly different conceptual origins. Prenatal programming, a more recent concept, considers that prevailing conditions outside the developing individual can alter its development in a graded

manner to optimize one or more body functions for extrauterine survival. These conditions are transmitted through hormonal, nutritional and metabolic signals, according to the Barker Hypothesis (Barker 1994), to permanently "program" the individual's physiology. This predictive process would take advantage of the inherent plasticity in developmental processes. In theory, this flexible developmental process would work well provided the extrauterine environment does not differ from what is predicted. In a short-lived species (day, weeks, months), this could be an important adaptive mechanism. In long-lived species (years, decades), such a mechanism would likely be burdensome at best, and pathologic at worst if conditions are not totally predictable. With respect to the latter, the Barker Hypothesis was derived to account for the fetal origins of adult disease in human populations. Barker based his hypothesis on epidemiological observations of a cohort of individuals born under World War II famine conditions whose mothers had been calorically restricted during pregnancy. He linked the current epidemic of adult coronary disease to conditions in fetal life 50-60 years earlier. The idea was that the wartime caloric deprivation during pregnancy created a "thrifty phenotype" prenatally that was programmed to capture efficiently after birth the meager available calories that were being predicted. This seemingly adaptive strategy failed once calories no longer remained at a premium after birth and returned to historic norms. In postwar conditions of caloric plenty, these prenatally programmed changes led to obesity, poor glucose tolerance, Type-2 diabetes and heart disease. This most notable and dramatic example of prenatal programming that produces a mismatch between the predicted and actual environments to result in disease is now spawning a plethora of investigations about the types of environmental information that might influence the developmental trajectory of the unborn and whether such physiological changes are adaptive or maladaptive.

In contrast to prenatal programming, there are no known influences from the environment outside the mother that *normally* modulate the degree or time course of sexual differentiation in mammals. In non-mammals, such as fish, this can be a useful strategy to even alter the entire sex of the individual to respond to environmental pressures (too many or few of one sex locally). In comparison to the novel evolving concept about prenatal programming and its adaptive role for prevailing environmental conditions, sexual differentiation is generally considered to be a normal process that allows evolved sex differences to be expressed. This differentiation occurs at the level of the gonad (ovary *versus* testis), internal genitalia (Müllerian and Wolffian duct systems), external genitalia (clitoris, labia; penis, scrotum), patterns of gonadotropin secretion (cyclic *versus* noncyclic) and numerous sex behaviors. In contrast to our understanding of prenatal programming, there are presently no known adaptive effects for altering the degree of sexual differentiation at these various levels. Optimally, this differentiation should be all or none, and intermediate forms are often pathologies that interfere or prevent normal reproductive functions. An ovotestis in either sex, or retained Müllerian system and hypospadias in the male, or an enlarged clitoris and extensive Wolffian duct derivatives in the female are all deviations from established sex-specific structures. However, the distinction between concepts of sexual differentiation and fetal programming are less rigid if one takes into consideration that *within* the developing individual several substances are used to "program" "sexual differences". The physiology of roughly half of all conceptuses (males) is influenced by steroid and protein hormones from the developing gonad, and it is unlikely that all developing males are exposed to exactly the same level or pattern of these factors. Furthermore, it is crucial that the developing reproductive systems of both males and females are appropriately matched to the endocrine milieu that will be present in adult life. As is the case for prenatal growth restriction and metabolic disorders later in life, deviations from the "predicted" postnatal steroid environment could result in varying degrees of reproductive dysfunc-

tion. As detailed below, we are learning that various steroid hormones can program in a dose-dependent manner several sex differences in reproductive functions, and the boundaries between normal and abnormal function are now being defined. Some steroid "imposters" from the environment can gain access to the conceptus to partially or wholly modify its sex-specific developmental trajectory. While this seemingly calls into question the issue of external environmental influence on sexual differentiation, this type of external modulation is largely maladaptive.

Genes and steroids in perspective

A long-held belief emanating from the behavior literature is that steroids program sex differences in brain controls. The role of genes was simply to direct the indifferent gonad to develop either as a testis, or by default if a female, as an ovary. The testicular tissue then produced the differentiating steroids. More recently, our concepts have changed. We are beginning to learn that sex specific genes appear before the testes are formed (Dewing *et al.* 2003) and that environmental information can be inserted to modulate expression of encoded genes (Sutherland & Costa 2003; Skinner & Anway 2005). Such findings will necessitate broadening our scope of how steroids and steroid-like compounds program sex differences. Perhaps steroids serve both to reinforce inherent genetic differences and to modify genetic differences. In the case of the sheep model, genetic differences are being noted and breed differences may account for the observed severity of the effects of prenatal testosterone exposure on the initiation and maintenance of progesterone cycles; for example, Dorset females (Birch *et al.* 2003; Unsworth *et al.* 2005) appear to be more sensitive to cycle disruption than Suffolk females (Sharma *et al.* 2002; Manikkam *et al.* 2006). Finally, whether the types of neuroendocrine defects programmed by excess prenatal testosterone exposure in the sheep persist in subsequent generations remains to be determined.

Conclusions

Studies of the sexual differentiation of reproductive neuroendocrine control in the sheep lead to the hypothesis that the four major steroid feedback controls for GnRH secretion are inherent (default) (Fig. 12). They require no steroidal developmental input to function appropriately during adulthood; by contrast, the organizational actions of specific sex steroids during development selectively reduce their postnatal functions by acting on presynaptic inputs to GnRH neurons. Two steroid feedback controls regulate the preovulatory surge mode of GnRH secretion and two regulate the pulsatile mode. The inhibitory feedback action of estrogen is required in both sexes to regulate the timing of the pubertal increase in GnRH that activates the gonads; the sensitivity to this feedback is set by the organizational action of androgens produced by the developing testes. The other three feedbacks are required for the complex control of follicular development. After the first ovulation at puberty progesterone assumes the inhibitory feedback role formerly ascribed to estrogen and as an inhibitory feedback, its sensitivity is set by the organizational action of androgens beginning before birth. The early organizational actions of estrogens set the sensitivities of the stimulatory feedbacks that facilitate ovulation, the positive feedback action of estrogen and the progesterone blockade of the GnRH surge. If the individual is a male, three steroid feedback controls of GnRH secretion become unnecessary, and these irrelevant feedbacks are abolished or become functionally inoperative through the foregoing programmed reductions in hypothalamic sensitivity. While this programming

begins before birth, it continues well after birth. In this respect, prenatal exposure to androgenic and estrogenic actions alters the susceptibility to the postnatal organizational actions of sex steroids.

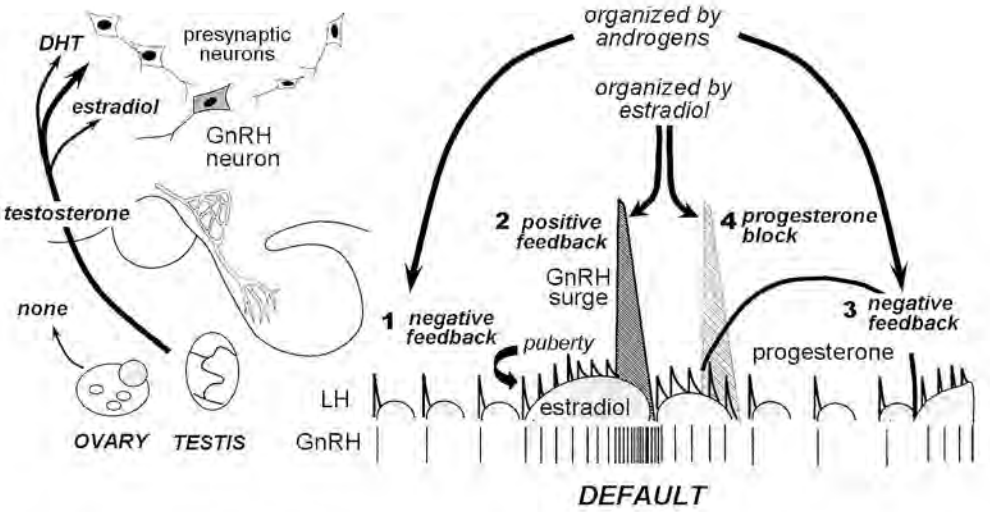

Fig. 12. Working hypothesis for sexual differentiation of steroid feedback controls of GnRH secretion in the sheep. Key elements of the hypothesis are that the four feedback controls of GnRH (right) exist in the undifferentiated (default) state and that androgens and estrogens by acting on GnRH presynaptic neurons (left) reduce, in part or totally, the sensitivity to steroid feedbacks

A major emphasis of presenting and testing hypotheses about the programming actions of testosterone and its metabolites on GnRH control systems is to develop the underlying concepts for normal sexual differentiation of reproductive functions. Once this is accomplished, understanding how programming mistakes originate should be simpler. However, in the types of studies being described, malprogramming is by necessity used to test hypotheses, and a variety of neuroendocrine phenotypes are being created with reproductive defects that become instructive. With a combination of at least four feedback controls for GnRH that can be malprogrammed, these phenotypes provide useful models to unravel the etiologies of many aspects of infertility such as premature or delayed puberty, primary anovulation, premature anovulation and a host of reproductive cycle defects. This understanding of underlying mechanisms assumes even greater urgency as it is likely that the prevalence of these defects will increase. There is a clear and increasing danger in modern societies that inadvertently produce and release into the environment numerous synthetic compounds that may act like steroids (endocrine disruptors, endocrine mimics, endocrine imposters). Compounds that can enter the conceptus during critical periods of development may program reproductive defects that will only become apparent months or years later, depending upon species. These compounds acting like estrogens or androgens or steroid antagonists may be the tragic mediators of a megafeedback loop between human populations and the environment that could ultimately impair fertility in both human and animal populations.

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