SEXUAL DIFFERENTIATION IN LITTER-BEARING MAMMALS: INFLUENCE OF SEX OF ADJACENT FETUSES IN UTERO[1]

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ABSTRACT

In rodents and swine, individual differences in a broad range of characteristics correlate with intrauterine position during fetal life. By identifying the intrauterine position of mice at cesarean delivery, we can predict reliably postnatal reproductive traits such as genital morphology, timing of puberty, length of estrous cycles, timing of reproductive senescence, sexual attractiveness, sexual behavior, aggressiveness, daily activity level, body weight and tissue enzyme activity in females; in males we can predict genital and brain morphology, sexual behavior, aggressiveness, daily activity level, body weight, and tissue enzyme activity. In mice, as in all mammals, male fetuses have greater concentrations of testosterone than do females. In addition, female mouse fetuses have greater circulating concentrations of estradiol than do male fetuses, a condition not found in all mammals. A mouse fetus positioned between males has greater concentrations of testosterone than does a fetus of the same sex positioned between females, and a fetus positioned between females has greater concentrations of estradiol than does a fetus of the same sex positioned between males. Gonadal steroids regulate differentiation of secondary sexual characteristics. Studies in which the effects of intrauterine position have been eliminated by exposing fetuses to steroid receptor blockers reveal the critical role of steroids in mediating this phenomenon. The intrauterine position phenomenon provides the only mammalian model for relating postnatal traits to concentrations of endogenous hormones to which individuals are exposed during fetal life. Results from studies using this naturally occurring experimental system in litter-bearing species have given insights concerning the consequences of individual differences in steroid concentrations during sexual differentiation that likely apply to all mammals. One specific hypothesis is that circulating estradiol may interact with testosterone in mediating some aspects of sexual differentiation in rodents and, thus, possibly in other mammals.

(Key Words: Sex Differentiation, Masculinizing Effect, Sexual Behavior, Aggression, Puberty, Estrous Cycle.)

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Introduction

Most research concerning effects of hormones on development of the masculine and feminine phenotype has been conducted with rodents, which are polytocous (litter-bearing) mammals (Baum, 1979; Booth, 1979; vom Saal, 1983a). Less is known about sexual development in other mammals, including domestic species such as pigs, goats, sheep and cattle. The basic process of hormonal regulation of differentiation of secondary sexual characteristics generally has been presumed to be similar in all mammals. However, the

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specific hormones (testosterone, androstenedione, 5α-dihydrotestosterone, estradiol-17β, progesterone) that influence development of various tissues, including the brain and thus behavior, vary greatly between species. There also are marked species differences in the timing (prenatal vs postnatal) of various developmental events (vom Saal, 1983a, 1989a; Baum, 1979; Dohler et al., 1984; Ford and D'Occhio, 1989).

Significant advances are being made in understanding the regulation of primary (gonadal) sexual differentiation. Evidence is accumulating that a segment of the short arm of the Y chromosome may code for a testis determining factor, which is not H-Y antigen (McLaren et al., 1984; Page et al., 1987). The development of secondary sex characteristics that distinguish males from females begins subsequent to gonadal differentiation. The subject of this paper will be the process by which secondary sexual characteristics in mammals are induced by steroids. Steroids are secreted by the gonads and adrenals (both maternal and fetal) and the placenta. I will present evidence that small differences in the concentrations of steroids during fetal life lead to marked differences in secondary sexual characteristics in both males and females.

Models of regulation of sexual differentiation in rodents and other mammals have emphasized the critical role of testosterone in mediating both masculinization (induction of traits characteristic of males) and defeminization (loss of traits such as the capacity to exhibit the sexually receptive, lordosis posture or a surge in LH secretion in response to estradiol). But the timing of the organization of various aspects of the masculine and feminine phenotype differs dramatically between species. In ferrets, defeminization in males appears to occur primarily during fetal life (Baum, 1979), whereas in rats, steroids have the greatest effect on defeminization in males shortly after birth (Davis et al., 1979; Nordeen and Yahr, 1983). In pigs, defeminization may occur as late as 3 mo after birth (Ford, 1982; Ford and Christenson, 1987; reviewed in Ford and D'Occhio, 1989). In this paper, findings from experiments conducted with mice and rats will be described showing that development of some aspects of the masculine phenotype is correlated with circulating concentrations of estradiol, rather than of testosterone, during fetal life. Preliminary findings from studies with pigs reveal some interesting similarities between sexual development in pigs and rodents and suggest that some common developmental phenomena might occur in all litter-bearing species.

Prenatal Sexual Differentiation

Around the time of the 2nd wk of gestation in rats, mice and hamsters, the testes in male embryos differentiate and begin secreting testosterone (Block et al., 1971; Feldman and Bloch, 1978; Pointis et al., 1979; Vomachka and Lisk, 1986). In swine, testosterone in males reaches high concentrations between d 30 to 35 of fetal life but then decreases to low concentrations by d 50 (Ford et al., 1980).

In female mammals, organization of primordial follicles in the ovaries occurs later in fetal life than does organization of the spermatic cords in the testes of males. In rodents, organization of the primordial follicles begins shortly before birth (reviewed by vom Saal and Finch, 1988). This does not mean that the ovaries are quiescent in terms of steroid secretion. In human fetuses, the ovaries have the capacity to synthesize estradiol from C_{19} substrates prior to follicular organization (George and Wilson, 1978; Wilson et al., 1981). Ovaries in developing rats and mice may not secrete estradiol until after birth, but, as in the human, capacity to synthesize estrogen prior to birth has been demonstrated in vitro (Terada et al., 1984; Weniger et al., 1985). In fetal mouse ovaries cultured in the presence of FSH, aromatase activity is enhanced markedly prior to the time that organization of the primordial follicles occurs (Terada et al., 1984). Interestingly, female mouse fetuses have very high circulating concentrations of FSH during late fetal and early postnatal life (Stiff et al., 1974). However, whether ovaries in human or rodent female fetuses secrete estradiol in situ remains unanswered.

The testes are not the only source of testosterone during fetal life in rodents. Placentas in rats and mice (but not in hamsters; Soares and Talamantes, 1982a) secrete both androstenedione and testosterone (Soares and Talamantes, 1982b, 1983; Vreeburg et al., 1983; Jackson and Albrecht, 1985). The placenta in primates does not contain the appropriate enzymes (17α-hydroxylase, C17,20-lyase) to synthesize androgen. Instead,

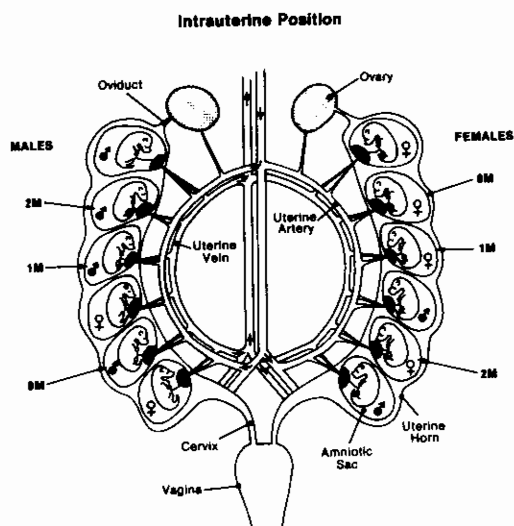

Figure 1. Schematic diagram of the uterine horns and uterine loop arteries and veins of a pregnant mouse at term. The intrauterine position of fetuses was determined at cesarean delivery. The labels 0M, 1M and 2M refer to the number of contiguous male fetuses. These labels are still used, even though fetuses are now classified based on proximity to both male and female fetuses (2M = between two males, 1M = between a male and a female, and 0M = between two females). Arrows within the loop artery and vein feeding each uterine horn indicate the direction of blood flow as revealed by injecting carbon dye into the maternal heart (for arterial flow) and individual placentas (for venous flow; unpublished observation).

progesterone is secreted and C_{19} substrates (primarily dehydroepiandrosterone secreted by the adrenals) are metabolized to estrogen due to the presence of the enzyme aromatase (Siiteri and Thompson, 1975).

In rats and mice, both male and female fetuses are exposed to supplemental androgen of placental origin, but the activity of enzymes involved in synthesis of testosterone (17α-hydroxylase and C17,20-lyase) was significantly greater in placentas collected from female than from male fetuses on d 18 of pregnancy in CF-1 mice (vom Saal et al., 1987). This likely accounts for the finding that female mouse fetuses have fairly high concentrations of circulating testosterone during the last 4 d of pregnancy. However, male mouse fetuses have about 2.5 times greater circulating concentrations of testosterone than do female fetuses and pregnant females due to secretion of testosterone by the testes (vom Saal and Bronson, 1980a). Similar sex differences in testosterone exposure during prenatal sexual differentiation also have been reported in rats (Weisz and Ward, 1980), hamsters (Vomachka and Lisk, 1986), monkeys (Resko, 1975), humans (Reyes et al., 1974), pigs (Ford et al., 1980), sheep (Pomerantz and Nalbandov, 1975) and cattle (Challis et al., 1974).

The Intrauterine Position Phenomenon

The intrauterine position phenomenon provides a unique method for examining the relationship between exposure to sex steroids during fetal life and postnatal characteristics in litter-bearing species such as mice, rats and pigs. Implantation of embryos relative to sex of adjacent fetuses is a random event (vom Saal, 1981), so there should be no systematic difference in genotype between animals from different intrauterine positions.

The scheme used to classify animals from different intrauterine positions is shown in Figure 1. Individual differences in circulating concentrations of testosterone in both male and female mouse fetuses are correlated with sex of the fetuses next to the animal being examined (Figure 2). Thus, male fetuses have higher blood concentrations of testosterone than do females, and fetuses positioned in utero between male fetuses (referred to as 2M males or 2M females) have significantly greater blood concentrations of testosterone than do animals of the same sex positioned between female fetuses (0M males and 0M females). Animals situated between a male and a female (1M males and 1M females) represent 50% of the population of males and females and are intermediate between 0M and 2M animals of the same sex in their blood testosterone concentrations (vom Saal and Bronson, 1980a; vom Saal et al., 1988). Exactly the opposite relationship is observed for blood concentrations of estradiol-17β; females have significantly greater concentrations than males, and 0M fetuses have significantly greater concentrations than 2M fetuses of the same sex (vom Saal et al., 1983, 1988). In humans (Reyes et al., 1974), monkeys (Resko et al., 1975) and cattle (Challis et al., 1974), female fetuses also have greater circulating concentrations of estradiol than do male fetuses.

Findings concerning the effects of prior intrauterine position on postnatal reproductive characteristics in rats and mice, as well as preliminary findings from experiments with pigs, will be described. In these experiments,

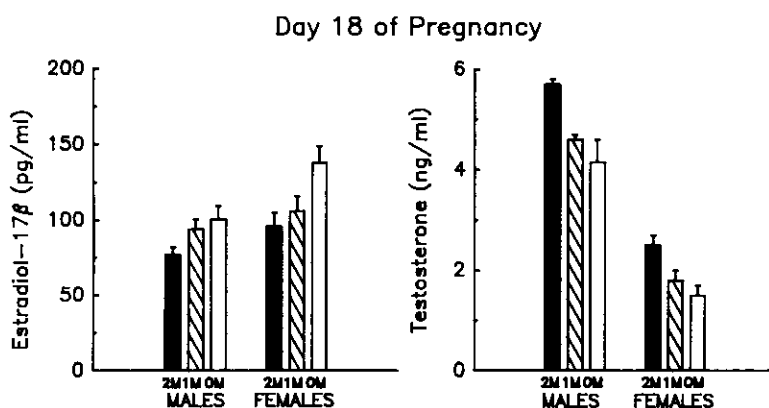

Figure 2. Serum concentrations of testosterone and estradiol in fetuses from different intrauterine positions (0M, 1M and 2M) on d 18 of pregnancy in CF-1 mice (day of mating is d 0 of pregnancy). For both testosterone and estradiol, males vs females, and within each sex, intrauterine position differences were significant ($P < .05$; vom Saal et al., 1988).

females were time-mated, and young were delivered by cesarean section shortly before normal parturition. The intrauterine position of each fetus was identified by toe-clipping or ear-clipping, and litters were fostered to mothers that had delivered within the preceding 24 h. In most studies in which comparisons of animals from different intrauterine positions have been conducted, only animals from the extreme positions (0M and 2M animals) have been tested. In all studies in which 1M males and 1M females have also been examined, they have been intermediate between 0M and 2M animals of the same sex in their characteristics. The approach in most of these experiments was to compare 0M and 2M males and 0M and 2M females in terms of characteristics that potentially could influence reproductive performance.

The Freemartin in Cattle. The intrauterine position phenomenon should not be confused with the freemartin in cattle. The freemartin refers to situations in which female cattle have ovotestes and are sterile due to developing in utero with a male co-twin (Marcum, 1974). Lower fertility also has been reported in bulls born co-twin with a freemartin (Dunn et al., 1979). An important feature of the freemartin syndrome is that masculinization of the external genitalia often is not found. Jost (1972) demonstrated that the freemartin was not due to androgen exposure in utero when he injected pregnant cattle with various androgens; treatment of pregnant cattle with androgen led to masculinization of the external genitalia in female offspring but did not produce ovotestes. The ovotestes found in freemartins are presumed to occur due to vascular anastomoses found between the chorionic membranes of twins, although this has yet to be proven; both the male and female fetuses thus share blood cells and are chimeras. Elucidation of the freemartin syndrome will first require an understanding of the factors regulating gonadal differentiation.

In contrast to the freemartin in cattle, I have examined fertility in two female CF-1 mice that appeared to have their placenta fused with the placenta of an adjacent fetuses of opposite sex; this occurs approximately once per 100 litters in CF-1 mice. Both females produced normal litters of young (F. vom Saal, unpublished observation). McLaren and Michie (1959) reported that in the rare cases in which they found placental fusion in mice, there was a connective tissue band separating the placentas. In litter-bearing species such as mice, protective mechanisms against phenomena such as the freemartin thus appear to have evolved. Females from all intrauterine positions do not differ in their fundamental capacity to produce and raise young in optimum laboratory conditions, although there are numerous differences due to intrauterine position that may alter the likelihood that females will mate successfully and produce young in natural environments (vom Saal and Bronson, 1978, 1980b; vom Saal and Moyer,

1985). The differences in phenotype in mice due to intrauterine position described below all are consistent with the hypothesis that this phenomenon is mediated by differential exposure to steroids, which Jost (1972) found did not mediate the freemartin syndrome. In summary, the intrauterine position phenomenon appears to be mediated by mechanisms entirely different from those that mediate the freemartin syndrome in cattle.

Genital Morphology and Prenatal Androgen Exposure. In both rats (Clemens et al., 1978) and mice (vom Saal and Bronson, 1978), length of the tissue separating the anus and genital papilla (this tissue becomes the scrotum in males) is longer at birth in 2M females than in 0M females (1M females are intermediate between 0M and 2M females). The relationship of this finding to differences between 0M, 1M and 2M female mice in circulating testosterone concentrations was revealed by an experiment in which pregnant female mice were treated with the antiandrogen cyproterone acetate. Anogenital distance at birth was reduced in 2M and 1M females, and they were both similar to 0M females on this measure (vom Saal, 1976). Administration of the antiandrogen Flutamide to pregnant rats similarly decreased anogenital distance in female offspring (Clemens et al., 1978).

Activity Level and Body Weight. It is well recognized that female mice have higher activity levels than do males (Perrigo and Bronson, 1985). Variation among male and among female mice in daily activity is due to prior intrauterine position. The direction of differences in activity due to intrauterine position is exactly what would be predicted based on the assumption that fetal testosterone concentration is the mediator of this phenomenon. Specifically, Kinsley et al. (1986b) reported that in R-S mice, 2M males had the lowest daily activity levels, determined by photobeam interruptions in an activity cage, whereas 0M males and 2M females had similar activity levels. 0M females had almost twice the daily activity levels of 2M females. This finding may explain why BW differences exist due to intrauterine position in R-S mice in the absence of differences in food intake: males are heavier than females, and postweaning BW is greater in 2M males and 2M females relative to 0M animals of the same sex (Kinsley et al., 1986c).

Timing of Puberty, Estrous Cycles and Reproductive Senescence. Adult 0M female mice and rats have shorter estrous cycles (mostly 4 d in length) than do 2M females (5 to 7 d in length; vom Saal and Bronson, 1980b; vom Saal, 1981). However, no difference in length of estrous cycles between 0M and 2M female pigs was observed (Rohde Parfet et al., 1988). First estrus occurs at a significantly younger age in 0M than in 2M female rats (vom Saal, 1981), but again no difference was observed between 0M and 2M female pigs (Rohde Parfet et al., 1988). It is uncertain that intrauterine position alters hormone concentrations in pigs. Yet, differentiation of the neuroendocrine-ovarian axis may occur enough later so as to be unaffected by potential hormone differences. This is consistent with the finding that defeminization in pigs occurs well after birth (Ford and D'Occhio, 1989), whereas in rodents, defeminization begins shortly before birth and continued into the early postnatal period (Davis et al., 1979; Yahr, 1988; vom Saal, 1989a).

Ovulation in female mice is regulated by pheromones excreted in urine by both males and females. 2M females are less responsive than 0M females to the effects of pheromones produced by other females that inhibit ovulation (vom Saal, 1981, 1989b; vom Saal et al., 1981). In an environment with a high density of other females, 2M female mice ovulate and mate at a younger age than do 0M females, and the first postpubertal estrous cycle of 0M females is longer than that of 2M females. Exactly the opposite occurs if female mice are housed near or with a male but without other females around: 0M females enter puberty earlier and postpubertal estrous cycles are shorter for 0M females than for 2M females (vom Saal and Bronson, 1978; vom Saal et al., 1981; vom Saal, 1989b). An important aspect of the above findings is that adolescent 0M female mice have estrous cycles that are short if density of females in the environment is low. The advantage conferred on adolescent 0M females of entering puberty early and exhibiting short estrous cycles (i.e., more frequent ovulations) thus operates only when population density is low.

The decline in reproductive capacity during aging was compared in 0M and 2M female mice. Females were repeatedly mated beginning at puberty, and the age-related decline in production of young was assessed. 2M females ceased producing live young at a younger age

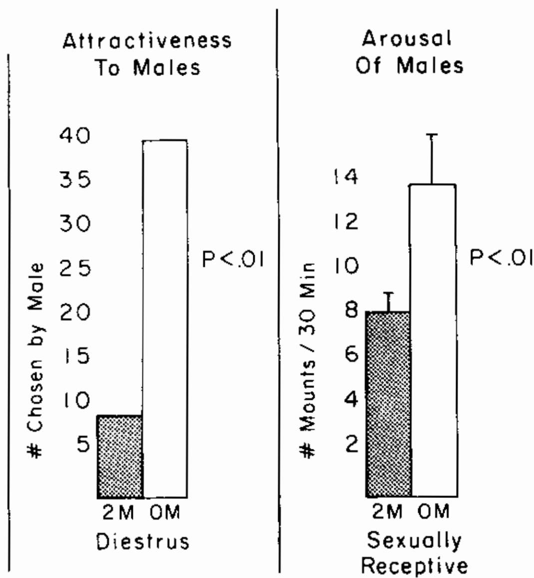

Figure 3. Sexual attractiveness of 0M and 2M female mice as assessed by the number of males (n = 49) that chose either a 0M or 2M female. 0M and 2M females were placed in adjacent chambers, and a male was placed onto a ramp from which he could jump into the chamber containing a 0M or a 2M female (vom Saal and Bronson, 1978, 1980a). Arousal of males was assessed in 0M and 2M females after ovariectomy. Regardless of the presence or absence of estradiol and progesterone (or the dose administered), 0M females were mounted more than 2M females (vom Saal and Bronson, 1978).

and after fewer litters than 0M females. The initial loss of reproductive capacity was not associated with an inability to become pregnant. Instead, 2M females became pregnant, but all young were born dead, perhaps due to accelerated breakdown in 2M females of the mechanisms mediating parturition (vom Saal and Moyer, 1985). Further experiments are needed to test this hypothesis and to determine whether neuroendocrine, ovarian or uterine changes are involved.

In female Mongolian gerbils, age at sexual maturation correlates with fecundity: early maturing females produce twice as many young during their reproductive lifetime as do late maturing females. Early maturing females also produce large, female-biased litters, and most female offspring in these litters mature early. In contrast, late maturing females produce small, male-biased litters, and most female offspring in these litters mature late (Clark et al., 1986). Clark and Galef (1988) have shown that virtually all 0M female gerbils mature early (mean = 18 d), whereas most 2M females mature late (mean = 29 d). The effects of intrauterine position on sexual maturation in female gerbils, rats and mice thus are identical. However, the dramatic effects of prior intrauterine position on litter size and sex ratio observed by Clark and Galef (1988) in gerbils have not been found in mice (vom Saal and Bronson, 1978; vom Saal and Moyer, 1985).

Taken together, these findings confirm predictions concerning the effects of exposure of females to elevated testosterone concentrations during the developmental period when testosterone exerts a defeminizing action on the brain-pituitary-ovarian axis. Exposure to elevated concentrations of testosterone (either of endogenous or exogenous origin) can delay puberty, lengthen estrous cycles and accelerate the decline in reproductive performance (vom Saal and Finch, 1988). 2M females thus appear defeminized relative to 0M females (1M females are intermediate on all characteristics examined). The site of action of testosterone underlying these differences due to intrauterine position is not yet determined. Gorski (1979) reviewed evidence showing that early androgen exposure influences defeminization of neuroendocrine function. But, the fetal period during which 2M females are exposed to elevated concentrations of testosterone is coincident with organization of primordial follicles. There is evidence that sensitivity of follicles to gonadotropin stimulation may differ in female rats with short estrous cycles (0M females) and females with prolonged cycles (2M females; Nequin et al., 1979; vom Saal and Finch, 1988).

Sexual Behavior and Sexual Attractiveness in Females. In comparisons of attractiveness of 0M and 2M female mice to males, most (82%) males entered a chamber containing a 0M female rather than a 2M female (Figure 3). In another experiment, arousal of male mice was assessed by how sexually active they were when housed with either a sexually receptive 0M or 2M female mouse: 0M females were mounted almost twice as many times per session as 2M females (vom Saal and Bronson, 1978, 1980a). When placed together with a 0M and a 2M female that both were in estrus, most male mice inseminated the 0M female prior to

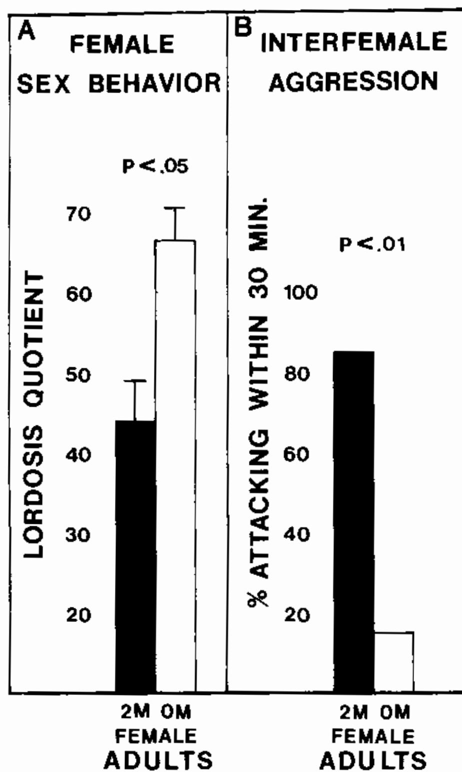

Figure 4. (A). Lordosis quotient (no. of lordoses/no. of mounts × 100) for adult 0M and 2M female mice that were ovariectomized and injected with estradiol benzoate 48 h prior to testing and progesterone 3 h prior to being placed with a stud male (Rines and vom Saal, 1984). (B) The percent of 0M and 2M females (placed together when in diestrus and matched for age and weight) that attacked and established dominance over the opponent (based on 20 pairs that fought; vom Saal and Bronson, 1978).

inseminating the 2M female (Rines and vom Saal, 1985).

Differences in attractiveness have been proposed to be related to differences between 0M and 2M females in preputial gland secretions: activity of preputial gland beta-glucuronidase was greater in 2M female mice than in 0M females (1M females were intermediate; Wechman et al., 1985). Secretions from the preputial gland in urine are involved in aggressive interactions in mice (Mugford and Nowell, 1971), and 2M females urine-mark their environment at greater rates than do 0M females (vom Saal and Bronson, 1978). Intrauterine position effects on the emission of pheromones thus are implicated in studies showing differences between 0M and 2M females in pheromonal modulation of the timing of puberty and subsequent estrous cycles, attractiveness to males, urine marking behavior and preputial gland beta-glucuronidase activity.

Sexual behavior has been compared in 0M and 2M female mice, rats and pigs. 0M female mice had higher lordosis quotients (number of lordoses/number of mounts), an index of sexual receptivity, than did 2M females (Rines and vom Saal, 1984) (Figure 4) 0M and 2M female rats were compared for their intensity of lordosis (degree of arching of the back) when the flanks were palpated, and 0M females had a significantly higher lordosis index than did 2M females (F. vom Saal, A. Coquelin, A. Schoonmaker, J. Shryne and R. Gorski, unpublished observation). Intact, cycling 0M and 2M female pigs were tested for duration of estrus (exhibition of the standing response when their flanks were palpated every 12 h), and 0M females were in estrus significantly longer than 2M females (Rohde Parfet et al., 1988).

Sexual Behavior, Prostate Weight, and SDN-POA Volume of 0M and 2M Males. Sexual behavior and brain morphology were examined in 0M and 2M male rats. 0M and 2M male rats were tested to sexual satiety (30 min without a mount when paired with a sexually receptive female), and 0M males had higher scores on all measures of sexual behavior. For example, 0M males exhibited significantly more ejaculations to satiety than did 2M males (F. vom Saal, A. Coquelin, A. Schoonmaker, J. Shryne and R. Gorski, unpublished observation). Gorski and colleagues have shown that the volume of the sexually dimorphic nucleus of the preoptic area (**SDN-POA**) in rats is two to three times greater in males than in females (Dohler et al., 1984). In the same male rats tested for sexual behavior, 0M males had a significantly larger volume of SDN-POA than did 2M males (F. vom Saal, A. Coquelin, A. Schoonmaker, J. Shryne and R. Gorski, unpublished observation). 0M male mice also exhibited more mounts and intromissions than did 2M males when paired with a sexually receptive female (vom Saal et al., 1983) (Figure 5).

0M and 2M male mice that had been castrated at birth to eliminate defeminizing effects of postnatal exposure to gonadal steroids were treated with estradiol and progesterone and placed with a stud male. More 0M

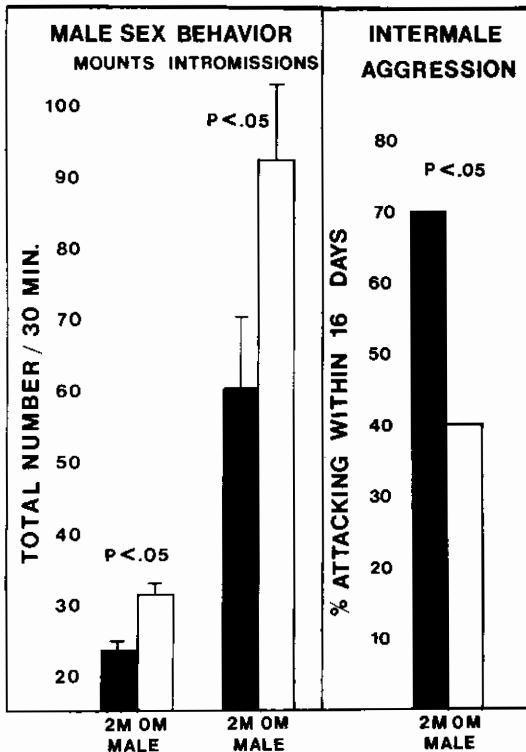

Figure 5. Sexual behavior (number of mounts and intromissions during a 30-min test) in adult, gonadally intact 0M and 2M males when paired with a sexually receptive female. Intermale aggression exhibited by adult 0M and 2M male mice toward a male intruder within 16 d of being implanted with a silastic capsule containing testosterone (tests for aggression occurred every other day); these males were gonadectomized within 30 min of birth (vom Saal et al., 1983).

males were mounted than 2M males, and 0M males exhibited lordosis when they were mounted; too few 2M males were mounted to assess their lordosis behavior (vom Saal et al., 1983). Adult 0M and 2M gonadally intact male mice were examined for prostate weights (after dissection away from the urethra). Prostates of 0M males were significantly heavier than those of 2M males (D. Nonneman, W. Welshons, V. Ganjam and F. Vom Saal, unpublished observation).

Findings concerning the effects of prior intrauterine position on sexual behavior in adult males were unexpected. Males with the lowest concentrations of testosterone during fetal sexual differentiation (0M males) were the most sexually active in adulthood (which is typically used as an index of masculinization). A hypothesis proposing that circulating estradiol mediates differences due to intrauterine position in adult sexual behavior in male mice is presented below. As predicted, however, 2M males were more defeminized than 0M male mice in terms of eliciting mounting by a stud male, 2M females also were more defeminized than 0M females.

Prior Intrauterine Position and Aggression

Males. The response to treatment with testosterone in castrated 0M and 2M male mice has been assessed in terms of the duration of treatment required to induce intermale aggression. Adult 2M males were more responsive to testosterone than were 0M males: more 2M males exhibited aggression within 16 d of testosterone treatment (Figure 5). Whereas 2M male mice were more aggressive toward an adult male mouse than were 0M males, 2M males were less likely to attack a newborn mouse pup than were 0M males. In fact, the behavior of most 2M males toward young was similar to that of lactating females (vom Saal, 1983c). Being located in utero between male fetuses thus does not lead to an enhancement of all types of aggression in male mice.

Females. When adult 2M and 0M female mice in diestrus were paired, 2M females were more aggressive, and most 2M females (85%) became dominant over the 0M female opponent (Figure 4). The level of aggression between nonlactating female mice is not so intense as that observed in males of most domestic stocks bred for generations in the laboratory, but biting, chasing and clear submissive behavior by the defeated female were observed (vom Saal and Bronson, 1978). However, recent experiments conducted with wild female house mice (*Mus domesticus*) trapped in Alberta, Canada reveal that these females are as aggressive toward other females as are males toward other males (P. Franks, F. vom Saal and S. Parmigiani, unpublished observation). Studies examining intrauterine position effects on aggression and other reproductive traits in wild mice are underway. In a study using pigs, 0M, 1M and 2M females were placed into a pen and observed for 15 min. The 2M females exhibited significantly more biting and mounting of other females than did 0M females, whereas 1M females were intermediate between 0M and 2M females in their aggressiveness (Rohde Parfet et al., 1988). The effect of intrauterine position

on behavior in female mice and pigs thus appears remarkably similar.

In an experiment examining postpartum (maternal) aggression, 2M female mice were more aggressive toward an intruder than were 0M females (vom Saal and Bronson, 1978). 2M female mice also were more aggressive than 0M females during late pregnancy (Kinsley et al., 1982a). These findings have been related causally to differences in prenatal exposure to testosterone by the experiment of Mann and Svare (1983); pregnant female mice were treated with a low dose of testosterone so that normal development of the internal and external genitalia occurred in female offspring. Prenatally treated females were raised, and in adulthood they were mated; all females delivered live young. While nursing, the females prenatally exposed to testosterone were more aggressive toward an intruder than were nontreated females in defense of their young.

The Concept of Aggression as a Masculine Trait. For both males and females, exposure to elevated concentrations of testosterone during fetal life, due to developing between male fetuses, enhances aggression. However, characteristics of aggressive behavior (target site attacked: head vs flanks) and both stimulus and hormonal control differ markedly between males and females in both mice and rats (DeBold and Miczek, 1981; Parmigiani et al., 1988). Thus it is incorrect to consider the enhanced aggressiveness of 2M females toward other adult females or in defense of their young to be a masculine trait, and aggressive females should not be labeled as masculinized (vom Saal, 1983b).

Female mice can be induced to exhibit "male-typical" behaviors by treatment with testosterone in adulthood. When ovariectomized and treated with testosterone in adulthood, 2M female mice respond to a greater degree than do 0M females: 2M females exhibit more attacks toward a male and more mounting of a sexually receptive female than do 0M females (vom Saal and Bronson, 1978; Rines and vom Saal, 1984). These findings suggest that elevated concentrations of testosterone during fetal life in 2M females lead to an enhanced sensitivity to testosterone in adulthood. Further support for this hypothesis is provided by the finding that serum concentrations of testosterone did not differ in gonadally intact, adult 0M and 2M female CF-1 mice (vom Saal and Bronson, 1980a). But, the concentration of epidermal growth factor in the submandibular gland (a protein induced by testosterone) was greater in 2M female CF-1 mice than in 0M females (Brown et al., 1984), which may reflect a differential sensitivity to endogenous testosterone. Again, these findings should not be interpreted as indicating that 2M females normally are more masculinized than 0M females in their behavior; gonadally intact 2M females in estrus mate with, but do not attack, males.

Results of these experiments raise questions concerning the assumption that testosterone induces masculinization, whereas "normal" females develop in the absence of effects of testosterone. A unique aspect of the model that testosterone (or other androgens) influences "normal" female development is the proposition that feminization is a complex (rather than purely passive) process, with differences among females in feminine traits being mediated by testosterone during sexual differentiation.

Differences in adult aggressiveness among females (and among males) due to variation in testosterone during fetal life may influence the likelihood of successfully competing with members of the same sex for territories and mates. Support for this hypothesis has been provided by studies of freely growing populations of mice: when the environment became crowded, only a few aggressive females produced and weaned young successfully (Lloyd and Christian, 1969; reviewed in vom Saal, 1983b, 1984, 1989a). Thus, aggressiveness, rather than being a masculine trait, may be an adaptive behavior in females as well as in males.

Differences between 0M and 2M female mice are summarized in Table 1. One prediction from studies of the intrauterine position phenomenon is that in a natural habitat, when population density is low, 0M females would have a reproductive advantage over 2M females due to accelerated puberty, shorter estrous cycles, and greater attractiveness and sexual responsiveness to males. In contrast, exposure to elevated concentrations of testosterone during fetal life by 2M females leads to enhanced aggressiveness as well as insensitivity to density-development pheromonal cues, characteristics that could render 2M females the most likely to produce and raise offspring successfully in high-density populations.

TABLE 1. DIFFERENCES THAT HAVE BEEN IDENTIFIED IN COMPARISONS OF 0M AND 2M FEMALE MICE.[a]

Point of comparison	0M Mice	2M Mice
Blood steroid concentrations during fetal life		
Elevated testosterone		XX
Elevated estradiol	XX	
Morphology		
Largest anogenital separation at birth		XX
Greatest postweaning body weight		XX
Stimulus characteristics		
Most attractive to males	XX	
Sexual arousal of males: elicits most mounts	XX	
Behavior		
Most sexually receptive	XX	
Highest daily activity level	XX	
Most aggressive		
Toward other females while in diestrus		XX
During late pregnancy		XX
Postpartum nest defense		XX
Most territorial marking		XX
Reproductive performance		
Enter puberty earliest		
Individually housed + male	XX	
Grouped with other females + male		XX
Regular (4-d) estrous cycles	XX	
Reproductive aging: produce live young longest	XX	
Enzyme activity		
High preputial gland beta-glucuronidase activity		XX
Response to adult testosterone treatment		
Most aggressive toward a male opponent		XX
Most mounting of a sexually receptive female		XX

[a]References for specific comparisons are included in the text.

The Interaction of Testosterone and Estradiol in Mediating Differences due to Intrauterine Position

Circulating testosterone regulates differentiation of tissues by 1) binding to androgen receptors without being metabolized (for example, differentiation of Wolffian ducts during fetal life; Jost, 1972; Siiteri and Wilson, 1974), 2) binding to androgen receptors after intracellular reduction to 5α-dihydrotestosterone by the enzyme 5α-reductase (for example, in urogenital sinus during fetal life; Siiteri and Wilson, 1974; Wilson et al., 1981) and 3) after intracellular aromatization to estradiol, which then binds to estrogen receptors (for example, the prostate [Mawhinney and Neubauer, 1979; Bashirelahi and Sidh, 1980; Jung-Testas et al., 1981; Cunha et al., 1987; Marts et al., 1987], neural areas involved in male sexual behavior, and in SDN-POA [Naftolin et al., 1976; MacLusky and Naftolin, 1981; Dohler et al., 1984, 1986; Yahr, 1988]).

In mice and rats, circulating estradiol is bound to the plasma protein alphafetoprotein (**AFP**), an alpha-globulin that binds estrogen but not androgen or other steroids (MacLusky and Naftolin, 1981; Westphal, 1986). Steroids bound to plasma proteins (other than albumin) are unable to enter cells via diffusion, whereas free steroids in the blood passively enter all cells (Pardridge, 1981). Circulating estradiol has been presumed to be inhibited from entering cells and "interfering" with the normal development of female phenotype in rat and mouse fetuses. If there were no mechanism for selectively inhibiting estrogen from entering cells, then the specific components of masculinization and defeminization that have been demonstrated to be mediated by estradiol binding to intracellular estrogen receptors should occur (Gorski, 1979; Yahr, 1988). Virtually all estrogen available within cells thus has been thought previously to result from the intracellular aromatization of testosterone (the aromatization hypothesis of sexual

TABLE 2. DIFFERENCES THAT HAVE BEEN IDENTIFIED IN COMPARISONS OF 0M AND 2M MALE MICE.[a]

Point of comparison	0M Mice	2M Mice
Blood steroid concentrations during fetal life		
Elevated testosterone		XX
Elevated estradiol	XX	
Morphology		
Greatest postweaning body weight		XX
Heaviest seminal vesicles		XX
Heaviest prostate	XX	
Behavior		
Most sexually active	XX	
Highest daily activity level	XX	
Most aggressive		
Toward an adult male		XX
Toward young (infanticide)	XX	
Most parental toward young		XX
Enzyme activity		
Highest seminal vesicle 5α-reductase activity		XX

[a]References for specific comparisons are included in the text.

differentiation; MacLusky and Naftolin, 1981). In mice, most testosterone in the circulation is not bound to a high-affinity plasma protein, and thus, unlike estrogen, can enter cells without special transport mechanisms (Stupnicki and Bartke, 1976).

A perplexing problem with the model that estrogen must be inhibited from entering cells during sexual differentiation is that AFP binds estrogen only in rodents, but not in other species that have been examined (MacLusky and Naftolin, 1981; Westphal, 1986). Whether circulating estrogen influences development of estrogen-sensitive tissues in species other than rodents still is unknown. Whereas AFP may inhibit estradiol from ubiquitously entering all cells in rats and mice, estradiol bound to AFP can enter neurons in selected areas of the brain in mice and rats, most likely via receptor-mediated endocytosis (Toran-Allerand, 1984). Within cells containing estrogen receptors, estradiol should dissociate from AFP and bind to the intracellular receptor, which has a higher association constant. Circulating estradiol bound to AFP thus should influence differentiation of these cells.

Males. Differences between 0M and 2M male mice are summarized in Table 2. It is interesting to contrast findings from comparisons of seminal vesicle weight and onset of intermale aggression in 0M and 2M males, on the one hand, with prostate weight, sexual activity and SDN-POA volume, on the other. In tissues with high-affinity estrogen-binding proteins, even small changes in circulating estradiol might influence differentiation. We have verified that, as in many other species, the prostate in CF-1 mice has estrogen receptors. Prostatic estrogen receptors are found at lower concentrations but have the same association constant as uterine receptors (D. Nonneman, W. Welshons, V. Ganjam and F. vom Saal, unpublished observation). A question being investigated is whether all estradiol available to bind to estrogen receptors in these cells during fetal life is derived from intracellular aromatization of testosterone, or whether estradiol in blood can enter these, and possibly other, cells.

If differences in fetal testosterone concentrations were the only mediator of differences between 0M and 2M males in prostate weight, sexual behavior and SDN-POA volume after intracellular aromatization, then 2M males would be expected to have larger prostates and SDN-POA volume and higher rates of sexual behavior than 0M males. An alternative possibility, therefore, is that during fetal life, circulating estradiol (either the free AFP or albumin-bound fractions) may enter cells in the prostate, neurons mediating sexual behavior, and neurons in the SDN-POA and regulate differentiation after binding to estrogen receptors. Neurons in the SDN-POA may have only estrogen receptors, whereas neurons involved in the regulation of male sexual behavior and cells in the prostate also have androgen receptors (Jung-Testas et al., 1981,

Dohler et al., 1984; Cunha et al., 1987). Elevated estradiol concentrations in 0M males thus could interact with testosterone within these tissues and enhance their organization in 0M males relative to 2M males, even though background concentrations of circulating testosterone are lower in 0M males than in 2M males. One possibility being examined is that activity of aromatase in these tissues may be low enough to damp out the effects of differences in circulating testosterone.

In contrast to the finding that prostate weight, SDN volume and sexual behavior are elevated in 0M males relative to 2M males, intermale aggression and seminal vesicle weight are both elevated in 2M males relative to 0M males (vom Saal et al., 1983). When adult male CF-1 mice were either gonadally intact or castrated and implanted with a silastic capsule containing testosterone, seminal vesicle 5α-reductase concentrations were significantly greater in 2M males than in 0M males (D. Nonneman, W. Welshons, V. Ganjam and F. vom Saal, unpublished observation). The higher concentrations of 5α-reductase in 2M males relative to 0M males result in elevated intracellular concentrations of dihydrotestosterone, which binds to androgen receptors and leads to mRNA synthesis. An interesting feature of fetal differentiation vs adult function of seminal vesicles is that 5α-reductase is not synthesized until after Wolffian duct differentiation is completed (Wilson et al., 1981). Differences between adult 0M and 2M male CF-1 mice in seminal vesicle weight thus appear to be due to the induction of greater concentrations of 5α-reductase by elevated concentrations of testosterone in 2M male fetuses.

We were unable to detect specific estrogen-binding proteins in the seminal vesicles of adult CF-1 mice (D. Nonneman, W. Welshons, V. Ganjam and F. vom Saal, unpublished observation). In other species, such as rats, guinea pigs and cattle, high-affinity estrogen-binding proteins have been detected in seminal vesicles (Robinette et al., 1978; Mawhinney and Neubauer, 1979; Bashirelahi and Sidh, 1980). Although the neural areas mediating intermale aggression and the steroid-metabolizing enzymes and steroid receptors that these neurons contain are not well understood, differentiation of the seminal vesicles from Wolffian ducts is known to be mediated by testosterone binding to androgen receptors. In an organ that has androgen receptors, but not estrogen receptors, such as the seminal vesicles in CF-1 mice, differences in estradiol concentrations (within a physiological range) during sexual differentiation may not influence development. Because pharmacological doses of estrogen influence seminal vesicle development (Rajfer and Coffey, 1978, 1979; Lung and Cunha, 1981), possibly via binding of estrogen to androgen receptors (Fox, 1975), effects of endogenous estrogen on development of seminal vesicles cannot be ruled out. It appears, however, that differentiation of seminal vesicles (and possibly also intermale aggression) in CF-1 mice is not mediated by intracellular aromatization of testosterone to estradiol and activation of estrogen receptors.

In summary, the organization of tissues that have androgen receptors, but not estrogen receptors, is enhanced in 2M male fetuses, most likely due to exposure of 2M male fetuses to elevated concentrations of testosterone. In contrast, in tissues with estrogen receptors, fetal organization is correlated positively with circulating estradiol concentrations (0M > 2M), suggesting that the concentration of circulating estradiol is an important factor in differentiation during fetal life. An important point to emphasize is that estradiol alone does not lead to masculinization of tissues that have both androgen and estrogen receptors. These tissues required binding of both androgen and estrogen to specific receptors to show normal development (Dohler et al., 1986; Yahr, 1988).

Females. In comparisons of 0M and 2M female rats, mice and pigs, 0M females were more sexually receptive than were 2M females. Thus it is possible that exposure to high concentrations of circulating estradiol by 0M female fetuses relative to 1M or 2M female fetuses enhances sexual behavior in adulthood. A contrasting hypothesis is that elevated concentrations of testosterone during fetal life defeminizes 2M females by actively interfering with the development of female-typical traits (emission of cues that attract males, sexual receptivity when mounted by a male, and exhibition of regular estrous cycles). This latter model is the most parsimonious explanation of differences between females due to the intrauterine position phenomenon.

Possible Mechanisms of Steroid Transport Between Fetuses

We have examined mechanisms by which steroids might be transported between fetuses in rats and mice. One mechanism we examined is that steroids pass via the amniotic fluid across the fetal membranes surrounding each fetus. This could occur in rodents because the fetuses are packed together, and the chorionic membranes surrounding adjacent fetuses are pressed against each other. In contrast, this type of transport might be less likely in swine, because fetuses are spaced quite far apart. To examine this possibility in rats, we placed capsules containing [^{3}H]testosterone into the amniotic fluid of one fetus and examined the amniotic fluid of adjacent fetuses for the presence of tritium. There was tritium in the amniotic fluid of fetuses on both sides of the fetus with the [^{3}H]testosterone implant, although there was a threefold greater recovery of tritium from amniotic fluid of fetuses located on the cervical side of the implanted fetus than from fluid of those located on the ovarian side (F. vom Saal, M. Dhar and M. Even, unpublished observation).

The second possibility is that steroids are transported somehow between fetuses via the uterine circulation of the mother. The uterine vasculature in mice and rats is interesting in that both the uterine artery and vein form a continuous loop (Figure 1; DelCampo and Ginther, 1974). We have examined blood flow in these vessels by infusing a dye or radiolabeled microspheres into the vessels via an intracardial cannula. Uterine loop arteries in both rats and mice have the capacity for blood to flow in both directions. For example, when a clamp is placed at any point on the uterine loop artery in pregnant rats, the dye moves through the artery up to the clamp from both directions. Also, the middle section of a uterine horn incorporates significantly fewer microspheres (and thus has lower blood flow) than does either end. Because blood flow in the uterine loop artery is bidirectional, whereas intrauterine movement of [3H]testosterone is greater toward the cervix than toward the ovary at both the ovarian and cervical ends of the uterus, uterine blood vessels do not appear to be involved in steroid transport between fetuses (M. Even, M. Dhar and F. vom Saal, unpublished observation).

The uterine vasculature in swine is completely different from that in rodents. Uterine arteries and veins feeding each uterine horn branch profusely (with numerous anastomoses) from a number of trunks (Oxenreider et al., 1965). Based on differences in both spacing of fetuses and uterine vasculature between rodents and swine, it is difficult to conceptualize a common mechanism of transport of steroids between fetuses, other than diffusion via the uterine lumen, that could account for differences due to intrauterine position in both rodents and swine.

Evolution of Differences in Reproductive Characteristics due to Intrauterine Position

Perhaps the intrauterine position phenomenon (that is, the possibility of movement of steroids from one fetus to another) evolved because it is adaptive for pregnant females to have offspring within a litter that vary in phenotype due to a random developmental event, regardless of the degree of underlying genetic variation (vom Saal, 1981, 1984). Even in highly inbred strains of mice, such as the C57BL/6J strain, there is substantial unexplained variability in numerous traits (Felicio et al., 1984; Svare et al., 1984; vom Saal and Finch, 1988). Variation among animals due to intrauterine positioning relative to fetuses of the same or opposite sex thus could increase the possibility that some offspring in a litter will have a phenotype that is well adapted for the environment in which they live, compete and reproduce. This would increase the fitness of the mother.

Conclusion

Studies using mice from different intrauterine positions have revealed unexpected relationships between serum concentrations of both testosterone and estradiol during fetal life and adult morphology, physiology and behavior. Males that develop between females fetuses (0M males) have greater estradiol concentrations during fetal life and, in adulthood, greater rates of sexual behavior and heavier prostates. In rats, we have not examined serum steroid concentrations during fetal life, but adult 0M males also have greater rates of sexual behavior as well as a large volume of the SDN-POA relative to 2M males. These findings were unexpected, because 0M male

mice have the lowest concentrations of testosterone during fetal life.

These studies are correlative, and the observation of a positive relationship between fetal estradiol concentrations (which is elevated on 0M relative to 2M fetuses) and adult morphology and behavior suggests that circulating estradiol may enter specific tissues and influence their differentiation during fetal life. In males, an important relationship between the prostate, SDN-POA, and the neural substrate regulating male sexual behavior (which all show evidence of enhanced organization during fetal differentiation in 0M males) is that these tissues all contain estrogen receptors. All of these tissues also contain the enzyme aromatase.

The most widely accepted model of regulation of differentiation of tissues with aromatase and estrogen receptors proposes that testosterone enters cells and is metabolized (aromatized) to estradiol, which then binds to estrogen receptors and directs cellular activities. The evidence is that mice and rats do not have a plasma protein that binds testosterone, such as sex steroid binding globulin (Corvol and Bardin, 1973; Renoir et al., 1980), and most testosterone is free to enter tissues (Stupnicki and Bartke, 1976). Because circulating estrogen is bound to a plasma protein (AFP) that binds estrogen, but not other steroids, during perinatal life in rats and mice, circulating estrogen is presumed to be unable to enter cells and influence sexual differentiation (Booth, 1979; vom Saal, 1983a; Toran-Allerand, 1984).

One problem with studies that have focused on rats and mice is that it appears that AFP binds estrogen only in rats and mice, but not in any other mammal so far examined. Thus, even if AFP inhibits estrogen from entering cells in rats and mice, there is no explanation of why circulating estrogen should not disrupt sexual differentiation in females in other species (for example, in cattle and humans, in which female fetuses have higher estradiol concentrations than do male fetuses; Challis et al., 1974; Reyes et al., 1974). Even in rodents, our comparisons of 0M and 2M male mice and rats suggest that estrogen might enter cells and influence sexual differentiation during fetal life. This could be due to the presence of receptors for AFP on a specific cell and active transport of the AFP-estrogen complex into the cell, where estrogen would dissociate from AFP and bind avidly to intracellular estrogen receptors (Booth, 1979; Westphal, 1986). We are also examining the possibility that a much higher percentage of total circulating estradiol than predicted by the aromatization hypothesis may diffuse into cells, because albumin-bound steroid is freely dialyzable (Pardridge, 1981).

Some tissues in which extradiol acts as a "masculinizing" hormone may also have androgen receptors. There are examples of cells that have both androgen and estrogen receptors (Horwitz et al., 1975). Alternatively, cells with androgen receptors and other cells with estrogen receptors might interact within a particular organ, such as in the prostate (Jung-Testas et al., 1981). In either case, a certain balance of estradiol and testosterone might be required for maximal differentiation in the masculine direction to occur (Fox, 1975). For example, either of the above hypotheses might apply to differentiation of the neural areas mediating male sexual behavior (vom Saal, 1989a).

Whereas the focus here has been on the interaction of testosterone and estradiol, there are sex differences in other hormones that have to be considered in models of the hormonal regulation of sexual differentiation as well as the hormonal basis of differences due to intrauterine position. We have found significantly (58%) greater serum concentrations of corticosterone in female fetuses relative to male fetuses during late pregnancy in CF-1 mice (M. Montano, M. Wang and F. vom Saal, unpublished observation). In contrast, placental content of placental lactogen is significantly (58%) greater in male relative to female CF-1 mouse fetuses (vom Saal et al., 1987). Both of these hormones interact with androgen and estrogen in regulating the function of organs such as the prostate (Tisell, 1971; Santi and Johansson, 1973; Mawhinney and Neubauer, 1979; Sandberg, 1981; Martikainen et al., 1987). One of the major challenges in the field of sexual differentiation is to develop a better understanding of the interaction between androgen, estrogen, and other hormones in regulating development of specific tissues in different species.

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