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High Fetal Estrogen Concentrations: Correlation with Increased Adult Sexual Activity and Decreased Aggression in Male Mice

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Abstract. *In the house mouse* (Mus musculus), *fetuses may develop in utero next to siblings of the same or opposite sex. The amniotic fluid of the female fetuses contains higher concentrations of estradiol than that of male fetuses. Male fetuses that developed in utero between female fetuses had higher concentrations of estradiol in their amniotic fluid than males that were located between other male fetuses during intrauterine development. They were also more sexually active as adults, less aggressive, and had smaller seminal vesicles than males that had developed between other male fetuses in utero. These findings raise the possibility that during fetal life circulating estrogens may interact with circulating androgens both in regulating the development of sex differences between males and females and in producing variation in phenotype among males and among females.*

Sexual differentiation begins during early fetal life in mammals. If the gonads are removed surgically before the onset of sexual differentiation, mammals develop into phenotypic females regardless of their genetic sex (*1*). During sexual differentiation in males, therefore, masculine traits are induced (masculinization) and, in some species, feminine traits are suppressed (defeminization) (*2*). Androgens, primarily testosterone or its metabolites, are secreted at a high rate by the testes of males during fetal life (*3*) and are thought to induce most of the prenatal changes in morphology, physiology, and behavior potential.

In the house mouse (*Mus musculus*), fetuses may develop in utero next to (and possibly be influenced by the hormonal secretions of) siblings of the same or opposite sex. Offspring from known intrauterine positions can be obtained by time-mating female mice and delivering the offspring by cesarean section shortly before normal parturition. Intrauterine position influences morphology, physiology, and behavior in female mice and rats (*4*). For consistency, the classification scheme that has previously been used to identify female fetuses from known intrauterine positions is also used for males (*5*). Males that develop between two other male fetuses are referred to as 2M males, males that develop between a male and a female fetus are referred to as 1M males, and males that develop between two female fetuses are referred to as 0M males. In the experiments described here we used CF-1 mice to test whether the intrauterine proximity of a male fetus to other male or female fetuses is correlated with its adult phenotype.

Male CF-1 mice were castrated within 1 hour of cesarean delivery and injected with hormones in adulthood. The objectives were (i) to eliminate possible differences between 0M and 2M males in the concentrations of gonadal hormones that they would have been exposed to during postnatal life and (ii) to assess the sensitivity of the neural substrates mediating reproductive behaviors to the activating effects of a known amount of hormone in adulthood. Differences between 0M and 2M males could thus be related to prena-

tal rather than postnatal exposure to gonadal hormones.

The sensitivity of the castrated 0M (N = 20) and 2M (N = 20) male mice to testosterone was compared by assessing their aggressiveness toward another male before and during treatment with testosterone (*6*). The 2M males were significantly more sensitive to the aggression-inducing action of testosterone than were the 0M males (see Fig. 1C).

We then examined other castrated 0M and 2M males for their sexual attractiveness to stud males and their capacity to exhibit lordosis (female-typical sexual behavior) when mounted. They were placed individually into the cage (56 by 32 by 20 cm) of an intact stud 1M male, tested for 30 minutes, and then injected with hormones and tested for 30 minutes at weekly intervals for 4 weeks. No mounting by the stud males was observed in the test before hormone administration and little mounting occurred during the first 2 weeks of hormone treatment (0 to 20 percent per group were mounted). During the third and fourth weeks of hormone treatment, the stud male mounted significantly more of the 0M males that had been injected with estradiol benzoate and progesterone than 2M males that had received the same hormones (see Fig. 2A). The stud males mounted few of the 0M and 2M males that had received estradiol benzoate and then oil. All of the males that were mounted exhibited lordosis that reflected a low level of receptivity (standing still during a mount but with little head elevation), but too few 2M males elicited mounting to permit quantitative comparisons. Thus 2M males are more defeminized than 0M males during prenatal life with regard to the capacity to elicit mounting by a stud male, and this difference appears to reflect an inability to respond to progesterone in 2M but not 0M males.

Two months later, we tested for aggression the same groups of 0M (N = 30) and 2M (N = 30) males that had been tested for female sexual behavior. These males were each implanted with a Silastic capsule containing testosterone and tested for aggression by the same procedure as described above. Again, more 2M than 0M males showed aggression within a 16-day test period although, overall, fewer of these males (now about 200 days old) fought than did the 90-day-old males. (A decrease in responsiveness to testosterone as a function of time since castration and aging is commonly observed.) When autopsied after 35 days of testosterone treatment, the 2M males also had heavier seminal vesicles than did the 0M males (*7*). Seminal vesical growth in response to testosterone treatment is used as a morphological index of sensitivity to testosterone, while time to induce aggression provides a behavioral index of sensitivity of the neural substrate mediating aggression to testosterone. Thus both behavioral and morphological data indicated that 2M male mice are more sensitive to testosterone than 0M males.

We also examined the behavior of normal 0M and 2M males that had not been surgically or hormonally manipulated. The sexual behavior of adult 0M (N = 20) and 2M (N = 20) males was compared during tests with ovariectomized 1M females that had been made sexually receptive by injections of estradiol benzoate and progesterone. A female was placed into a male's cage for 30 minutes. The 0M males showed significantly more mounts and intromissions than did the 2M males (Fig. 1B). In a related experiment, intact 0M male Sprague-Dawley rats showed significantly more ejaculations to satiety (30 minutes without a mount) when paired with a 1M female than did 2M males (*8*).

That sexual performance was enhanced in 0M males compared with 2M males is interesting given that 0M female mice exhibit higher levels of sexual receptivity (higher lordosis quotients) when paired with a male and are more sexually attractive to males than are 2M females (*3*, *9*). Thus 2M female mice are more defeminized than 0M females. In addition, 2M female mice are highly ag-

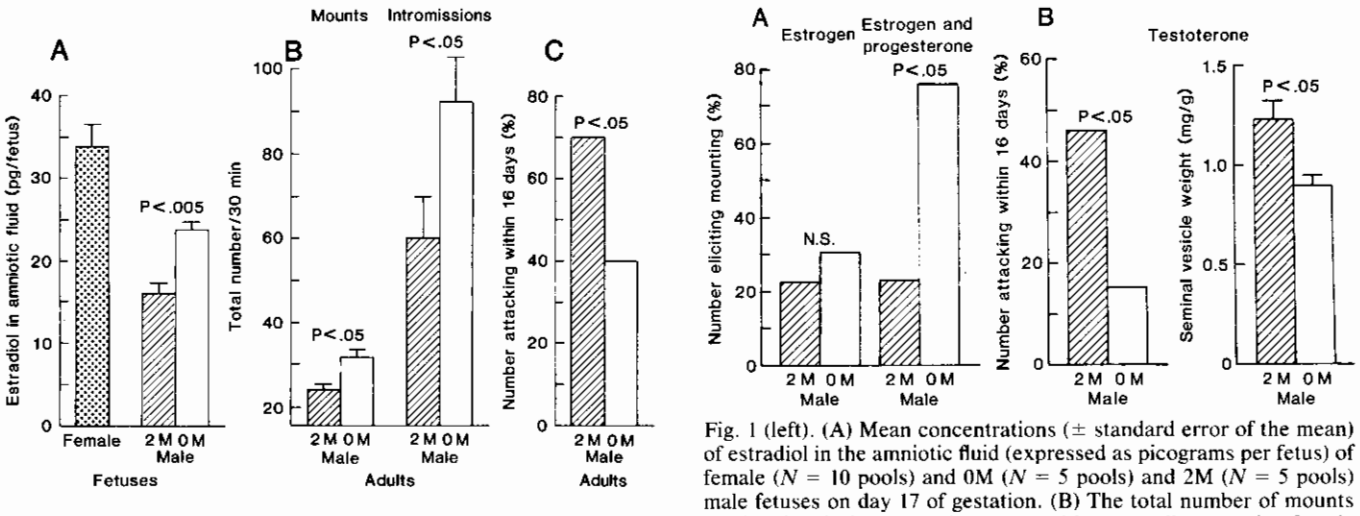

Fig. 1 (left). (A) Mean concentrations (± standard error of the mean) of estradiol in the amniotic fluid (expressed as picograms per fetus) of female (N = 10 pools) and 0M (N = 5 pools) and 2M (N = 5 pools) male fetuses on day 17 of gestation. (B) The total number of mounts and intromissions (thrusting movements) made by 90-day-old 0M and 2M male mice during a 30-minute test with a sexually receptive female (mean ± standard error of the mean; 20 animals per group). (C) The percentage of neonatally castrated, 90-day-old 0M and 2M males (20 per group) that showed a 5-second sustained biting attack toward a 1M male intruder. The mice were tested for 10 minutes on alternate days for 16 days after a 10-mm Silastic capsule (Dow 602-285) containing 5 mg of testosterone in 0.02 ml of oil was implanted in the neck region. Significance levels are for (A) and (B) t-test and (C) χ^2 comparisons. Fig. 2 (right). (A) The percentage of neonatally castrated, 90-day-old 0M and 2M male mice that elicited mounting by a stud 1M male during a 30-minute test during the third and fourth weeks of hormone treatment. All males received weekly injections of 25 μg of estradiol benzoate in oil 48 hours before being tested. One-half of the 0M and 2M males were injected with 200 μg of progesterone in oil 4 hours before being tested, while the other males received only oil (15 males per treatment condition). (*N.S.*, not statistically significant). (B) The percentage of neonatally castrated, 200-day-old 0M and 2M males (30 per group), previously tested for female sexual behavior, that showed a 5-second sustained biting attack toward a 1M male intruder. The mice were tested for 16 days after a Silastic capsule containing testosterone was implanted in the neck region. After the 0M and 2M males had been exposed to testosterone for 35 days, all the animals were weighed and the seminal vesicles were weighed after the fluid was removed by blotting. Seminal vesicle weights (mean ± standard error of the mean) are expressed as milligrams of tissue per gram of body weight. Significance levels are for χ^2 or t-test comparisons.

gressive toward other females compared with 0M female mice (*4*). Since male mouse fetuses have higher blood concentrations of testosterone than females, and since 2M female fetuses have higher concentrations of testosterone in their amniotic fluid and blood than 0M females (*3*), we hypothesized that 2M male fetuses would have higher concentrations of testosterone in their blood and amniotic fluid than 0M males.

Female mice were killed by cervical dislocation on day 17 of pregnancy (insemination on day 0), and amniotic fluid and blood were collected from each fetus. The fluids from fetuses in equivalent intrauterine positions were pooled. The concentrations of testosterone, 17β-estradiol, and progesterone in the amniotic fluid and of testosterone in the blood were determined by radioimmunoassay as described (*3*). Female fetuses had significantly higher concentrations of estradiol than any of the males, and 0M males had significantly higher concentrations of estradiol than 2M males (Fig. 1A). In previous experiments, 0M females had higher concentrations of estradiol in the amniotic fluid than 2M females, although the difference was not statistically significant (*3*). The amniotic fluid circulates through the gut of fetuses, and estradiol in the amniotic fluid is presumed to be in equilibrium with estradiol in the fetal blood (*7*). There were no significant differences between male and female mouse fetuses or between 0M and 2M males in the concentrations of either testosterone or progesterone in the amniotic fluid. Also, 0M and 2M males did not differ in the concentrations of testosterone in their blood (*10*).

According to the aromatization hypothesis, testosterone is converted (aromatized) to estradiol within some androgen target cells, including certain areas of the hypothalamus (*11*). Testosterone is thus thought to be a prohormone, and estradiol the hormone that induces masculinization and defeminization during early life. It was previously thought that circulating estrogens could not enter cells in fetuses because of the presence in the blood of high concentrations of an estrogen-binding protein, α-fetoprotein. For masculinization and defeminization to occur, a prohormone not bound and inactivated by α-fetoprotein (that is, testosterone) was thought to be required (*11*). However, there is now evidence that α-fetoprotein that is bound to estradiol (or other estrogens) may enter target cells in the brain (*12*), suggesting that circulating estradiol might directly influence sexual differentiation and that both circulating estradiol and estradiol derived from intracellular aromatization of testosterone might act on cytoplasmic estrogen receptors to regulate differentiation of the neural tissues mediating male sexual performance and aggression. According to the aromatization hypothesis, fetuses exposed to increased concentrations of estradiol should be more aggressive and sexually active (that is, masculinized) than fetuses exposed to low concentrations.

The results of the sex behavior but not the aggression experiment are consistent with the aromatization hypothesis. We propose that the increased estradiol in 0M male fetuses may lead to a greater intracellular pool of estradiol within developing brain areas that mediate sexual behavior and thus enhance the adult sexual performance of 0M males. In contrast, that 0M males were less aggressive than 2M males in response to testosterone suggests that in the neural areas mediating aggression (and in the seminal vesicles), increased estrogen in 0M male fetuses may interfere with the action of testosterone, perhaps by estradiol competitively binding to androgen receptors which are found in some hypothalamic neurons as well as in the seminal vesicles of mice and rats (*1*, *13*). The suggestion that estrogen acts directly during perinatal life to facilitate the organization of female sexual behavior in rodents (*14*) leads to the hypothesis that adult sexual performance (whether masculine in response to circulating testosterone or feminine in response to circulating estradiol and progesterone) might be enhanced in 0M males because of their exposure to high concentrations of estradiol.

Most of the estrogens in the fetal and maternal circulation in humans and rodents appear to be of fetal origin (*15*). In humans, female fetuses have higher concentrations of estradiol in their amniotic fluid than male fetuses (*7*), and both fetal and maternal estrogens are derived from androgens secreted from the fetal, and to a lesser degree maternal, adrenals; these androgens are aromatized to estrogens in the placenta. Our findings suggest that circulating estrogens influence sexual differentiation in mice and, in humans, sex differences in fetal adrenal secretions lead to sex differences in circulating estrogen titers. We propose, therefore, that during intrauterine development, sex differences in fetal adrenal secretions interact with sex differences in gonadal secretions in regulating sexual differentiation.

Our data indicate that a male or a female mouse that develops in utero between two male fetuses (2M phenotype) is more aggressive and less sexually active than animals with the 0M phenotype. [Male and female mice that develop between a male and a female fetus (1M phenotype) are intermediate between 0M and 2M animals in phenotype (*4*, *16*)]. If 0M and 2M males had been tested only with females for their sexual behavior, 0M males would have been labeled as more masculinized than 2M males. However, if aggression only toward a male opponent had been examined, 2M males would have been labeled as more masculinized than 0M males. Even though 0M males are less likely than 2M males to be aggressive toward another adult male, 0M males are significantly more likely than 2M males to be aggressive toward the newborn young of another male [that is, to commit infanticide (*16*)]. Comparing 0M and 2M male mice on a scale of masculinization is therefore meaningless. Our data suggest that conclusions concerning the general process of masculinization drawn from previous studies in which only one behavior was studied should be reexamined.

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5. A classification scheme based on the direction of uterine blood flow [as suggested by R. L. Meisel and I. L. Ward, *Science* **213**, 239 (1981)] does not appear feasible, since blood flow in the uterine arteries and veins (which each form a continuous loop in mice and rats) is bidirectional [A. McLaren and D. Michie, *Nature (London)* **187**, 363 (1960)]. Meisel and Ward did not report data on the direction of uterine blood flow, and their premise that blood flows in one direction in the uterine arteries and veins in rats does not appear to be supported by the anatomical study [C. Del Campo and O. Ginther, *Am. J. Vet. Res.* **33**, 2561 (1972)] that they cited.
6. No aggression was observed in the test given before the administration of hormones. The olfactory bulbs of the stimulus 1M male mice were removed. The number of days of testosterone exposure to induce aggression (but not the latency to attack, number of attacks, or duration of attacks) is a measure that is sensitive to hormonal manipulations of fetuses in mice [F. vom Saal, *Horm. Behav.* **12**, 1 (1979)].
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10. Fluids from 25 fetuses were pooled for radioimmunoassay. Group means (± standard error of the mean) are based on ten pools for females and five pools each for 0M and 2M males. The data for females were reported previously (*3*). Concentrations in the amniotic fluid (picograms per fetus) were, for testosterone: all females, 115.1 ± 4.8; 0M males, 115.1 ± 8.5; 2M males, 110.2 ± 6.5; for progesterone: all females, 259.3 ± 14.0; 0M males, 253.4 ± 6.2; 2M males, 253.1 ± 18.9; concentrations of testosterone in the blood were, for all females (nanograms per milliter), 0.98 ± .05; 0M males, 2.89 ± .15; 2M males, 3.13 ± .20.
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