The Interaction of Circulating Oestrogens and Androgens in Regulating Mammalian Sexual Differentiation

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1. Introduction

In mammals males are the heterogametic sex, and the Y chromosome codes for differentiation of the gonads into testes. With the exception of the primary sexual characteristic, gonadal sex, all other differences between males and females are thought to be mediated hormonally via secretions from the testes. In the absence of gonads, mammalian foetuses develop into phenotypic females (Jost 1972). The ovaries of female foetuses differentiate significantly later in development than do the testes, and it appears that the ovaries of female foetuses are not steroidogenic (cf., Gibori and Sridaran 1981). In mammalian species in which the period of gestation is relatively long, sexual differentiation is usually completed by birth. In short-gestation species, such as rats and mice, however, sexual differentiation commences during the last third of pregnancy and continues during the first week to 10 days of postnatal life. Previously, it was erroneously assumed that the differentiation of sexual behaviour in mice and rats began shortly after birth (Barraclough and Leathem 1954, Young et al. 1964). There is a critical coupling of the timing of the secretion of testosterone, the primary androgen secreted by the testes of foetuses and adults, and the development of both neural and peripheral androgen-target tissues. Extrinsic factors that interfere with this normal coupling (such as maternal stress or exposure to exogenous hormones) can radically alter the course of sexual differentiation.

Two processes have been identified as occurring during the normal development of behaviours that are influenced by sex steroids: masculinization, the development of male-typical behaviours, and defeminization, the loss of the potential to behave in a female-typical fashion (in this review complete physiological defeminization will refer to both the loss of the capacity of specific brain areas to respond to rising oestrogen titers by triggering periodic surges in luteinizing hormone that result in ovulation, and the loss of the production of olfactory cues that stimulate mating behaviour in males). There is now considerable evidence that behavioural masculination and behavioural defeminization are independent processes that occur during different periods of sexual differentiation in some species (Whalen 1974, Baum 1979).

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2. The Intrauterine Position Phenomenon: Hormone-Mediated Variation in Phenotype

During the course of evolution, selection has resulted in a broad range of morphological, physiological and behavioural traits being regulated by sex steroids during both the period of sexual differentiation in early life and in adulthood. One of the consequences of having hormones regulate sexual differentiation is that variation in phenotype is guaranteed, regardless of the amount of genetic variation within a population. Both the environment of the mother and intrauterine environment of a foetus are sources of variation in terms of the titers of hormones that a foetus is exposed to in utero. The titers of hormones that foetuses are exposed are modulated by the environment of the mother via the pituitary-adrenal axis of the mother. The intrauterine positioning of male and female foetuses has also proven to be an important variable which influences prenatal hormone titers and thus phenotype in both rats and mice. The intrauterine position phenomenon refers to the fact that during foetal life, foetuses are positioned randomly next to other foetuses of the same or opposite sex. Male and female foetuses secrete different titers of steroid hormones during the prenatal period of sexual differentiation in mice as well as every other mammal that has been studied (cf., vom Saal and Bronson 1980a). Steroids secreted by one foetus are somehow transmitted to contiguous foetuses, with the result that variation among males and among females in a broad range of characteristics is due to an individual's intrauterine proximity to foetuses of the same or opposite sex.

The mechanism by which hormonal communication between foetuses occurs is unknown. The report by Meisel and Ward (1981) that the uterine vasculature might be involved in transmitting hormones between foetuses was based on an erroneous interpretation of an anatomical study (cf., Del Campo and Ginther 1972), which did not address the issue of the direction of uterine blood flow in the rat as Meisel and Ward stated. Uterine blood flow in the rat and mouse is actually quite complex: both arterial and venus flow are bi-directional and in opposite directions (blood enters the uterine loop artery feeding each separate uterine horn rostrally from the descending aorta and caudally from the internal iliac; blood leaves the placentae of foetuses located near the cervix and flows caudally in the uterine vein, which drains into the iliac vein, while blood from the placentae of foetuses located near the ovary flows rostrally in the uterine vein, which leads into the ascending cava). The anatomical proximity of the uterine loop arteries and veins in rats and mice, and the fact that at virtually all points blood flow in a uterine loop artery and vein is in an opposite direction (McLaren and Michie 1960, vom Saal, unpubl.), is interesting in that it is suggestive of a counter-current exchange system.

The mechanism by which steroids are transmitted between foetuses could involve passage of steroids through the amniotic fluid, lymphatic system in the uterus, or maternal vasculature. In the studies that will be described in this paper, the scheme that is utilized to designate the intrauterine position of animals of both sexes involves utilizing male foetuses as the reference for labelling foetuses: 2M = between two males, 1M = between a male and a female, and 0M = not next to a male (between two females; see Fig. 1). The use of male foetuses as the reference occurred at a time when the intrauterine position phenomenon was assumed to be mediated solely by

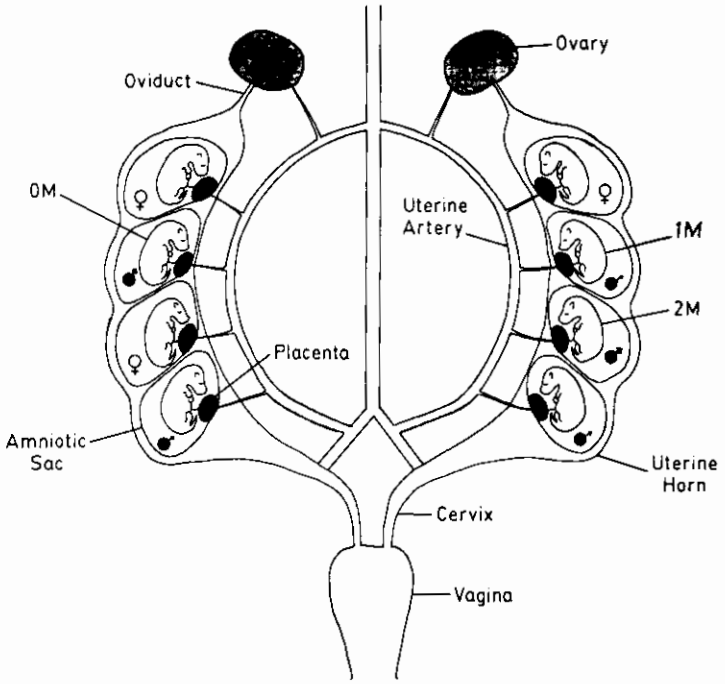

Fig. 1. Schematic diagram of the uterine horns and uterine loop arteries of a pregnant mouse at term. The intrauterine position of foetuses is determined at Caesarean delivery. *0M*, *1M*, and *2M* refers to the number of male foetuses to which an individual is contiguous (*2M* between 2 males; *1M* next to 1 male; *0M* between 2 females). This scheme is used to identify both male and female foetuses, but only males are labelled in this diagram

secretions from male foetuses rather than also by secretions from female foetuses. This assumption is now thought to be incorrect. The continued use of the 2M, 1M, 0M scheme thus does not imply that proximity to females is unimportant.

To obtain male and female foetuses from known intrauterine positions, female mice are time-mated. The young are removed from the uterus by Caesarean delivery shortly before parturition on Day 19 of pregnancy. The young are then raised in foster-litter groups by mothers that had delivered a litter within the preceding 24 h (Fig. 1).

2.1 The Intrauterine Position Phenomenon in Female Mice and Rats

The testes of male mice differentiate and begin secreting testosterone during early foetal life (about Day 13 of gestation; Block et al. 1971), thus marking the onset of sexual differentiation. Male CF-1 mouse foetuses *(Mus musculus)* have about three times more testosterone circulating in their blood than do female foetuses on Day 17 of gestation, which is during the time that sexual differentiation is occurring (vom Saal and Bronson 1980a). In addition, the intrauterine positioning of female foetuses

relative to males influences testosterone titers in female foetuses: females that develp between 2 male foetuses (2M females) have higher amniotic fluid and blood titres of testosterone than do female fetuses that do not develop next to a male (0M females; see Fig. 2). Females located in utero between a male and a female fetus (1M females), the third possible intrauterine position, have not been examined for foetal hormone titres, but after birth 1M females are intermediate in phenotype between 0M and 2M females. 0M female foetuses also tended to have higher amniotic fluid titres of oestradiol than 2M female foetuses, although the difference was not statistically significant (vom Saal and Bronson 1980a). Intrauterine position thus results in a complex pattern of hormonal differences in female foetuses during the prenatal period of sexual differentiation of both the brain and peripheral organs in mice. It is presumed that differences in hormone titres between 0M and 2M females occurs during prenatal but not early postnatal life due to the transmssion of steroids from one foetus to another. By adulthood, 0M and 2M female mice do not differ in their blood titres of testosterone during the dioestrous phase of the oestrous cycle (vom Saal and Bronson 1980a). It is likely, however, that 0M and 2M females will be found to differ in the pattern of gonadal and pituitary hormones secreted during different stages of the oestrous cycle, since 0M and 2M females differ in the length of their oestrous cycles (vom Saal and Bronson 1980b).

Numerous comparisons of female mice and rats from different intrauterine positions have been conducted. By way of a brief summary, 0M, 1M and 2M female mice have been found to differ: (1) in their morphology – the space separating the anus and genital papilla at birth is longest in 2M females and shortest in 0M females (vom Saal and Bronson 1978), which has been interpreted as indicating that 2M females are masculinized due to their proximity to male foetuses (this measure is a sensitive bioassay for prenatal testosterone exposure in rodents); (2) in their physiology – 0M, 1M and 2M females differ in the timing of puberty (vom Saal and Bronson 1978, vom Saal 1981, vom Saal et al. 1981) and in the length of subsequent oestrous cycles (2M females have longer and more irregular oestrous cycles than do 0M females; vom Saal and Bronson 1980b); 0M females are also more sexually attractive and sexually arousing to males than are 2M females (males choose 0M females over 2M females, males exhibit more mounts and intromissions toward 0M than toward 2M females, and males ejaculate into 0M females prior to ejaculating into 2M females when given a choice; vom Saal and Bronson 1978, 1980a, vom Saal et al. 1982); 2M females also cease producing live offspring at a younger age than do 0M females when they are allowed to repeatedly mate and produce litters (vom Saal et al. 1982).

Intrauterine position effects have also been found in studies involving Sprague Dawley rats: 2M females have larger anogenital spaces at birth than do 0M females (Clemens et al. 1978). This difference is eliminated by treating pregnant females with antiandrogens in both rats (Clemens et al. 1978) and mice (vom Saal 1978). 2M female rats also enter puberty (first vaginal oestrus) significantly later and have longer adult oestrous cycles (5.7 ± 0.03 days) than do 0M female rats (4.7 ± 0.01 days; $p < 0.01$; unpubl.). Most of these studies involving comparisons of 0M and 2M females have been previously reviewed (vom Saal 1981).

The emphasis of this section will be on behavioural comparisons of 0M and 2M female mice. Studies that will be described will thus involve comparisons of animals

from these two extreme intrauterine positions. 0M and 2M females were compared for their aggressiveness toward a female opponent. Intact 0M and 2M female mice (in the dioestrous phase of the oestrous cycle) were paired for 30 min after being matched for age and weight. Aggression was observed in 20 of 28 pairs, and in 17 cases, the 2M female was the aggressor. 2M females are thus significantly more aggressive toward another female than are 0M females. In contrast, neither 0M or 2M females are aggressive toward male mice. Aggression between females appears to result in the establishement of dominance hierarchies among females, and dominance status has been observed to correlate with reproductive success in female mice (Lloyd and Christian 1969) as well as male mice (DeFries and McClearn 1970).

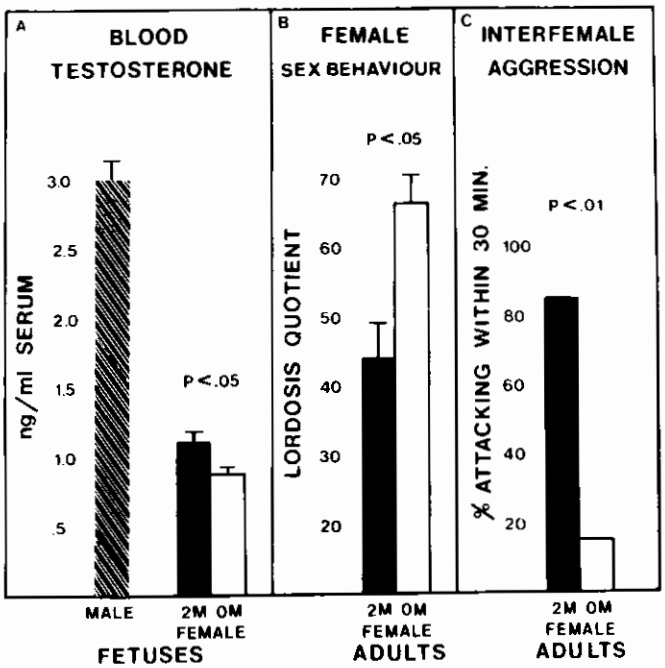

Fig. 2. A Mean (± SEM) blood titres of testosterone (expressed as nanograms/millilitre of serum) for male (10 pools; 25 foetuses/pool) and for 0M and 2M female (5 pools; 25 foetuses/pool) mouse foetuses on Day 17 of gestation. B mean (± SEM) lordosis quotient (number of lordoses/number of mounts × 100) for 0M and 2M female mice (10/group). All females were ovariectomized when 90 days old and tested for their sexual receptivity when 150 days old. Forty-eight h before testing, the females were injected with 5 μg oestradiol benzoate, and 4 h before testing with 200 μg progesterone. A 0M and a 2M female were then matched for age and weight and placed into the cage of a 1M stud male for 30 min. C The percent of 0M and 2M female mice that exhibited aggression (tail rattling, chasing, and biting) during a 30-min test. Twenty-eight matched pairs of 0M and 2M females were observed for aggression when both females were in the dioestrous phase of the oestrous cycle. In 8 pairs no aggression was observed. Results are based on the 20 pairs in which aggression was observed. Significance levels are for A and B t-test and C Chi Square comparisons

Aggression toward a female is not a masculine trait, and it cannot be concluded that 2M females are behaviourally masculinized relative to 0M females based on this finding. Rather than label 2M females as masculinized, it is more appropriate to state that a significant component of the variance in interfemale aggression (a feminine rather than masculine trait) is due to intrauterine position. 2M females have also been found to urine mark their environment at a higher rate than do 0M females (vom Saal and Bronson 1978). Rates of urine marking in male mice are correlated with dominance status (Desjardins et al. 1973), and urine marking also serves to delineate territorial boundaries (Harrington 1976).

The fact that 0M and 2M female mice differ in the length of their oestrous cycles makes studying sex behaviour in intact 0M and 2M females very difficult. Thus, 0M and 2M females (20/group) were ovariectomized, and 2 months later they were treated with oestradiol benzoate and progesterone to induce sexual receptivity (the frequency of lordosis responses when mounted by a male). When placed with a 1M male for 30 min, 0M females had significantly higher lordosis quotients (number of lordoses/number of mounts × 100) than did the 2M females (see Fig. 2). It can thus be concluded that 2M females are defeminized in terms of their sexual behaviour, capacity to exhibit regular oestrous cycles, and the production of sex-attracting cues (most likely pheromonal) relative to 0M females (vom Saal et al. 1982). What is readily apparent from the data presented in Fig. 2 is that 2M females are more aggressive toward other females than are 0M females, but 2M females exhibit a decrement in sexual performance relative to 0M females. Intrasex aggression and sexual performance are thus negatively correlated in female mice.

2.2 The Intrauterine Position Phenomenon in Male Mice and Rats

Based on the finding that 2M female mouse foetuses had higher amniotic fluid and blood titres of testosterone than did 0M female foetuses, it was predicted that 2M male mouse foetuses (that develop in utero between two male foetuses) would also have higher amniotic fluid and blood titres of testosterone than 0M male mouse foetuses (that develop in utero between two female foetuses). However, 0M and 2M male mouse foetuses were not found to have significantly different amniotic fluid or blood titres of testosterone, at least on Day 17 of gestation (when male foetuses are secreting high titres of testosterone). Instead, female mouse foetuses were found to have about twice the amniotic fluid titres of oestradiol-17β as did male foetuses on Day 17 of gestation, and 0M males were found to have about 50% higher titres of oestradiol in their amniotic fluid than did 2M male foetuses (vom Saal et al. 1983; see Fig. 3). In humans, female foetuses also have high amniotic fluod and blood titres of oestradiol relative to male foetuses (foetal blood and amniotic fluid concentrations of steroids are presumed to be in equilibrium; Belisle and Tulchinsky 1980).

The data presented in Fig. 3 reveal the same relationship of intrauterine position to aggressiveness and sexual performance in 0M and 2M male mice that was observed in comparisons of 0M and 2M female mice: 2M males appear to be more aggressive toward other males but less sexually active than 0M males, The sex behaviour study consisted of placing a sexually receptive 1M female into the cage of a gonadally intact

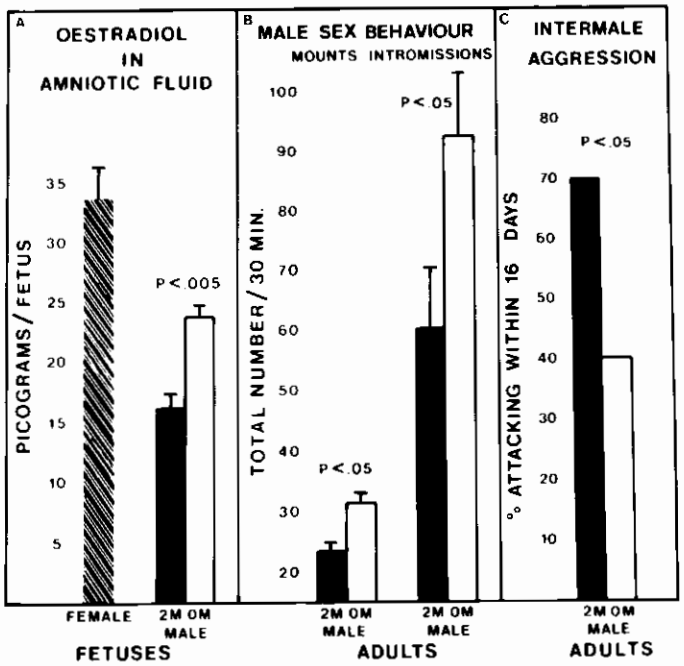

Fig. 3. A Mean (± SEM) amniotic fluid titres of oestradiol (expressed as picograms/foetus) for female (10 pools; 25 foetuses/pool) and 0M and 2M male (5 pools; 25 foetuses/pool) mouse foetuses on Day 17 of gestation. B mean (± SEM) number of mounts and intromissions (thrusting movements) during a 30-min test with a sexually receptive 1M female for gonadally intact, 90-day-old 0M and 2M male mice (20/group). C the percent of neonatally castrated, 90-day-old 0M and 2M male mice (20/group) that exhibited a 5-s sustained biting attack toward a 1M male intruder within 16 days after the 0M and 2M males were implanted with a silastic capsule containing testosteone. Ten-min tests for aggression were conducted every other day after implantation of testosterone. Significance levels are for A and B t-test and C Chi Square comparisons

0M or 2M male (20 males/group) for 30 min and recording the number of mounts and intromissions by the males. 0M males exhibited significantly more mounts and intromittive thrusts than did 2M males (vom Saal et al. 1983). In a similar experiment, gonadally intact, adult male Sprague Dawley rats were tested to sexual satiety (30 min without a mount) when paired with a sexually receptive 1M female. The 0M males exhibited significantly more ejaculations to satiety (4.5 ± 0.37) than did the 2M males (3.1 ± 0.37) (unpubl. observ.). Thus, in both rats and mice, sexual performance is enhanced in 0M males relative to 2M males.

Intermale aggression was examined in 0M and 2M male mice that had been gonadectomized within 1 h of Caesarean delivery. The main objectives of this experiment were to: (1) eliminate possible differences between 0M and 2M males in the titres of gonadal steroids that they were exposed to during postnatal life by castrating the males at birth, and (2) to assess the sensitivity of the neural substrate mediating intermale aggression to the aggression-inducing action of a known amount of testosterone administered in adulthood via silastic capsules. When implanted with a silastic capsule

containing testosterone and tested every other day for 10 min for aggression against a 1M male mouse that had had its olfactory bulbs removed, significantly more 2M than 0M males exhibited a 5-s sustained attack within 16 days after implantation of testoserone (see Fig. 3). This finding was replicated with another group of 0M and 2M male mice, and in addition, after both 2 and 5 weeks of testosterone treatment, 2 M males were found to have significantly heavier seminal vesicles (vom Saal et al. 1983). Thus, 2M males are more sensitive to testosterone than 0M males, both in terms of the tropic action of testosterone on seminal vesicle growth and the induction of intermale aggression.

0M and 2M male mice that had been castrated at birth were also examined for the capacity to elicit mounting from a 1M stud male and to exhibit the female sexually receptive posture (lordosis) when mounted during a 30-min test. The 0M and 2M males were treated with only oestradiol benzoate or both oestradiol benzoate and progesterone prior to being tested with a 1M male. Little mounting was observed toward either the 0M or 2M males that were only treated with oestradiol benzoate, but significantly more 0M than 2M males that were treated with oestradiol benzoate and progesterone elicited mounting by the 1M male (see Fig. 4). Too few 2M males elicited mounting to allow a comparison of the lordosis quotients of the 0M and 2M males (vom Saal et al. 1983). These results reveal that 2M males are more defeminized than 0M males during foetal life, at least in terms of the loss of the cues involved in eliciting mounting by a stud male. This difference between 0M and 2M males appears to reflect a loss in the capacity of 2M males to respond to progesterone. As reviewed above, 2M female mice also elicit fewer mounts by stud males than do 0M females, indicating that 2M females are more defeminized than are 0M females.

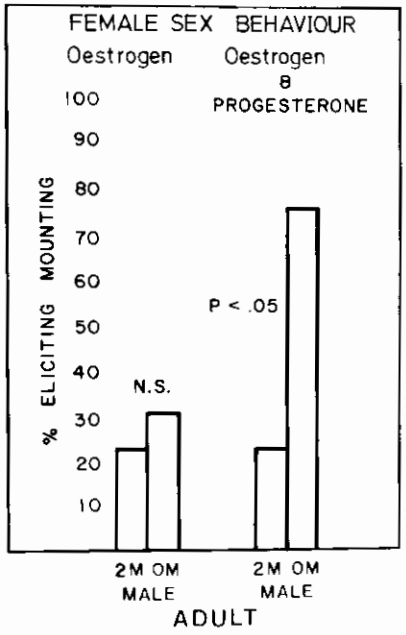

Fig. 4. The percent of neonatally castrated, 90-day-old 0M and 2M male mice that elicited mounting by a 1M stud male during a 30-min test. All 0M and 2M males received injections of oestradiol benzoate 48 h before testing. The 0M and 2M males were then divided into two groups (15 males/treatment conditions): 4 h before testing one-half of the males received an injection of progesterone, while the remaining males received an injection only of oil. The animals were administrered hormones and tested at weekly intervals for 4 weeks. Data are for the percent of 0M and 2M males in each group that were mounted during both the third and fourth weeks of testing. Significance levels are for Chi Square comparisons (*N.S.* not statistically significant)

What is most interesting about these findings is the fact that differences between 0M and 2M female mice appear to be related to differences in exposure to testosterone based on intrauterine proximity to male foetuses, while differences between 0M and 2M male mice appear to be related to differences in exposure to oestradiol based on intrauterine proximity to female foetuses. Yet the data presented in Figs. 2 and 3 reveal that the effect of intrauterine position on both sex-typical aggressiveness and sexual performance in males and females is remarkably similar.

3. The Evolution of Variance Due to Intrauterine Position in Polytocous Mammals

I have proposed that prior intrauterine position influences individual reproductive success in both male and female mice (vom Saal 1981). For example, 2M males and 2M females appear to be more aggressive than 0M males and 0M females. This enhanced aggressiveness should confer a reproductive advantage upon 2M males and 2M females when population density is high. 0M females are more sexually attractive and arousing to males and at low population densities, 0M females enter puberty earlier and have shorter and more regular oestrous cycles than 2M females. 0M females thus have a phenotype that may confer a reproductive advantage upon them when population density is low. Whether 0M male mice will be found to be more sexually attractive to females remains to be examined. Based on these observations, it is proposed that there is no absolute advantage or penalty to developing between male or female foetuses in utero. It is interesting in this regard that the traditional concept that in small mammals all fertile females are either pregnant or lactating (Conoway 1971) does not appear to be true. In fact, as described above, dominance status influences reproductive success in female mice, and when population densities are high, only a small proportion (10%–15%) of female mice may actually be able to reproduce (Lloyd and Christian 1969). Christian (1971) has related this finding to the disruptive effects of stress due to crowding on reproductive capacity in subordinate mice, since mice that live in high density populations exhibit signs of hightened adrenal activity.

House mice are opportunistic colonizers that are almost global in their distribution, mice having spread throughout the world as commensals of man (Bronson 1979). In addition, in mice as well as many other small mammals, population densities increase and decrease dramatically as a function of food availability and climatic conditions, and also other as yet unexplained factors that periodically lead to dramatic "crashes" in population size (Christian 1971). The reason that this point is raised is that it is proposed that the capacity of foetuses to communicate with each other hormonally evolved in response to positive selection pressure due to the adaptiveness of this phenomenon, not to the species (which would imply group selection), but to an individual pregnant female. The presence of variance in phenotype due to the intrauterine position phenomenon may increase the likelihood that at least some of the offspring produced by an individual female may have a phenotype that renders them highly competitive to reproduce in whatever type of environment that they

happen to be born into. Any factor, whether genetic or environmental, that increases variance in phenotype increases the capacity of some animals in a population to survive and eventually reproduce. In terms of the issue of genetic versus environmental influences on phenotype, it is important to emphasize that intrauterine positioning by sex is a random developmental event, and random events are highly predictable with large sample sizes. Males and females from particular intrauterine positions thus do not differ systematically in genotype (vom Saal 1981). In conclusion, the intrauterine position phenomenon may have evolved since it provides a predictable degree of variance in phenotype, regardless of the amount of underlying variance in genotype (the CF-1 mice utilized in the experiments described above are maintained as an outbred strain in a closed colony, but are presumed to have very little underlying genetic variation; the CF-1 strain is derived from a single brother-sister mating after 20 generations of inbreeding).

In contrast to the intrauterine position phenomenon, which appears to be adaptive, other clearly maladaptive developmental events are also found to occur. For example, the freemartin syndrome in cattle (a single-birth species), which is most likely due to the formation of vascular anastomoses between contiguous foetuses and the sharing of tissue, results in both male and female offspring being chimeras and sterile in virtually all situations in which dizygotic male and female twins develop in utero together (Marcum 1974, vom Saal 1981). There appears to have been intense selection against this possibility in polytocous species. In house mice, for example, even in situations in which the placentae of contiguous foetuses are in contact (a rare event), a connective tissue band separates the placentae of individual foetuses, thus providing a barrier to the potential formation of vascular anastomoses between the chorionic membranes of foetuses of the opposite sex (McLaren and Michie 1959).

The argument that the intrauterine position phenomenon has evolved not simply as a correlate of the overwhelming need of a prey species to produce many young at once and thus to have multiple uterine residence, but due to the adaptiveness of this phenomenon, also suggests that intrauterine position differences may only be highly adaptive in colonizing species, such as house mice, in which the density of populations is found to vary widely. In contrast, in litter-bearing species in which population size tends to be stable (K-selected species; Wilson 1975), variance in phenotype due to intrauterine position may not be found to occur.

4. The Interaction of Oestrogens and Androgens in Mediating Differences in Phenotype Due to Intrauterine Position

While it has been known for some time that there are sex differences in foetal oestrogen titres in some species, foetuses have been assumed to be protected from circulating oestrogens by blood proteins that bind and thus inhibit oestrogens from entering cells (bound or conjugated steroids are presumed to be inactive while free steroids are presumed to be active). In mice and rats, foetuses have high circulating concentrations of alpha-foetoprotein, which binds oestrogens but not androgens with high capacity. Thus, the high concentrations of oestradiol that are present during the

latter stages of pregnancy were perviously not believed to influence sexual differentiation (at least in mice and rats, since it is unclear whether circulating oestrogen-binding proteins occur in high concentrations during foetal life in other species; MacLusky and Naftolin 1981).

It has been proposed that in neurons in the brain that respond to testosterone, testosterone acts on the genome to influence neural development after being converted to oestradiol (i.e., aromatized) by aromatase enzymes. Oestradiol produced by intracellular aromatization of testosterone is presumed to be translocated into the cell nucleus by oestrogen receptors; oestrogen receptors have been found to be present in the brains of foetal mice and rats (Vito and Fox 1982). In other tissues such as the prostate, testosterone acts during foetal life to influence development after being reduced to dihydrotestosterone (Kelch et al. 1971), which is then translocated into the nucleus by androgen receptors.

The proposition that oestradiol is actually the hormone that induces masculinization and defeminization, thus making testosterone a prohormone, is referred to as the aromatization hypothesis (MacLusky and Naftolin 1981). Testosterone was thought to be needed as a prohormone due to the fact that circulating oestrogens would be inhibited from entering cells by alpha-foetoprotein. There is evidence from studies with rats that the development of male sex behaviour involves the aromatization of testosterone to oestradiol. For example, aromatase inhibitors interfere with the development of male sex behaviour (Gladue and Clemens 1980), while administration of oestrogens to neonatally castrated male rats can facilitate the development of male sex behaviour (Booth 1977). The finding that 0M males are exposed to higher titres of oestradiol than 2M males durinng foetal life, and in adulthood 0M males are more sexually active than 2M males, is consistent with the aromatization hypothesis. Thus, in neural tissues mediating male copulatory behaviour (mounting, intromitting and ejaculating), testosterone may serve as a prohormone that is aromatized to oestradiol prior to interacting with cytoplasmic oestrogen receptors and translocation into the cell nucleus. Within the nucleus, the oestrogen-receptor complex is thought to exert an organizing or sensitizing effect on the genome. The elevated titres of oestradiol observed in the amniotic fluid of 0M male mice may lead to a greater intracellular pool of oestradiol and an increase in the number of activated oestrogen receptors that are translocated into the nucleus of target cells, thus producing an enhancement of sexual performance during later life (see Fig. 5).

The finding that 0M males are less sensitive to the effects of testosterone on intermale aggression and seminal vesicle growth suggests that exposure to high titres of oestradiol during foetal life interferes with the organizational effects of testosterone in the neural substrate mediating aggression and in the seminal vesicles. Oestradiol may bind to (and inhibit the translocation into the nucleus of) androgen receptors that are present in some hypothalamic neurons and in the seminal vesicles during prenatal life in both mice and rats. During foetal life (but not necessarily in adulthood) oestradiol may thus act as an antiandrogen in these tissues. Comparisons of 0M and 2M males suggest that the neural areas mediating intermale aggression in mice are organized perinatally as a result of testosterone (or possibly its reduced metabolite, dihydrotestosterone; cf., Lieberburg and McEwen 1980) interacting with cytoplasmic androgen receptors rather than as a result of testosterone being aromatized to oestradiol

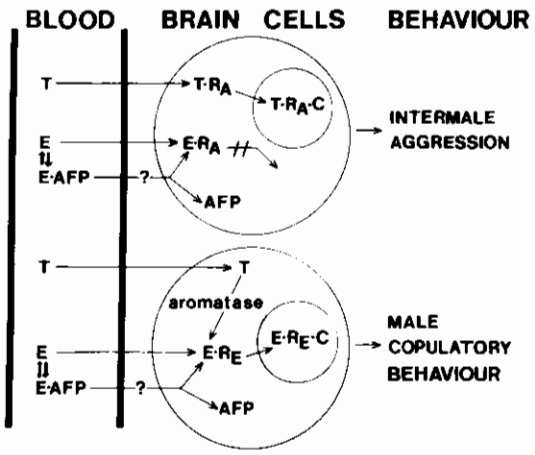

Fig. 5. Schematic diagram of the proposed interaction of oestrogens *(E)* circulating in the foetal blood stream, that are either free or are bound to alpha-foetoprotein *(AFP)*, with putative androgen receptors (R_A) in the cytoplasm of cells in brain areas mediating intermale aggression or putative oestrogen receptors (R_E) in brain areas mediating male copulatory behaviour. Brain areas mediating intermale aggression may contain only androgen receptors in foetuses, and high blood concentrations of oestrogen may thus act to block masculinization in these tissues by competitively binding to cytoplasmic androgen receptors. The testosterone *(T)*-androgen receptor complex is thought to regulate the functioning of specific genes in chromosomes *(C)* after translocation from the cytoplasm into the nucleus, thus leading to an increase in aggressiveness in adulthood. The occupation of an androgen receptor by oestrogen might inhibit translocation of the occupied androgen receptor into the nucleus. There is thus a correlation between high concentrations of oestradiol in 0M male foetuses and low aggressiveness in adulthood. In contrast, during prenatal life brain cells that mediate male copulatory behaviour are hypothesized to contain only oestrogen receptors and aromatase enzymes, which convert testosterone to oestradiol. Since testosterone is hypothesized to influence the differentiation of these cells after being aromatized to oestradiol, high titers of oestradiol in the circulation of 0M males are predicted to lead to an increase in the number of oestrogen receptors that are translocated into the nuclei of these cells. This would thus explain the enhanced sexual performance of 0M males relative to 2M males in both mice and rats

(which would then interact with cytoplasmic oestrogen receptors). Thus, as circulating oestradiol titres increase in foetuses, there is a decrease in the response to testosterone in the neural areas mediating intermale aggression, which results in a decrease in the sensitivity of these neurons to the aggression-inducing action of testosterone during later life (see Fig. 5).

The obvious question that arises is how could oestradiol titres influence sexual differentiation if alpha-foetoprotein really does inactivate all circulating oestradiol during fetal life? One possible answer to this question may be that alpha-foetoprotein may actually pass into brain cells (possibly into selected cells due to active endocytotic mechanisms). The evidence for this is that alpha-foetoprotein has been found in brain cells in rats (McEwen et al. 1975, Benno and Williams 1978). Whether alpha-foetoprotein actually transports oestrogen from the circulation into brain cells is

unknown. But, alpha-foetoprotein does not appear to be synthesized in the brain (Schachter and Toran-Allerand 1982).

It is important that the effects of steroids during early life (referred to as organizational effects, which are permanent) be distinguished from the effects of steroids during adult life (referred to as activational effects, which are usually transient). During postnatal life tissues may acquire different enzymes and receptor systems, and it is possible that oestradiol may serve to inhibit development of a tissue during foetal life, while the same steroid might have a stimulating effect on the same tissue at a later time in life.

5. Sex Differences in Foetal Adrenal Physiology and the Intrauterine Position Phenomenon

The previous findings demonstrate that there are sex differences in oestradiol titres during foetal development in mice. In other mammals, including humans, female foetuses also have higher titres of oestradiol than do male foetuses (Reyes et al. 1974, Belisle and Tulchinsky 1980). One hypothesis that has been developed from comparisons of 0M and 2M males is that differences in foetal oestradiol titers due to intrauterine proximity to females lead to differences in phenotype in 0M, 1M and 2M male mice (and also contribute to differences in phenotype between 0M, 1M and 2M female mice). At present, however, this hypothesis is only based on correlational studies. This hypothesis does not discount the importance of individual differences in either the rate of secretion of or tissue sensitivity to testosterone in mediating variation in phenotype in males. But if future research confirms that variation in phenotype among male mice is mediated by at least in part by differences in foetal oestradiol titres due to intrauterine proximity to female foetuses, then differences between males and females should also be mediated in part by the known sex differences in oestradiol titres during foetal life.

The above hypotheses lead to an important question, namely, where do oestrogens in the foetal and maternal circulation come from? Considerably more is known about the source of oestrogens during pregnancy in humans than in rodents. In humans, most of the oestrogens in both the fetal and maternal circulation are of foetal origin. Circulating oestrogens are derived from aromatizable androgens (principly dehydroepiandrosterone sulfate, DHEA-S) which are secreted from the foetal, and to a lesser degree the maternal, adrenals. These androgens are then aromatized to oestrogen in the placenta after they are desulphated (Kime et al. 1980). The fact that adrenal androgens are sulphated prior to secretion has been considered to be quite important, since sulphated steroids are hydrophilic and have been thought to have little ability to enter cells and thus influence sexual differentiation. This is most likely untrue, however (cf., Vignon et al. 1980).

The available evidence suggests that the ovaries of female foetuses do not secrete oestrogens in measurable quantities, and in rats as in humans, oestrogens in the maternal blood stream are of foetal-placental origin (cf., Gibori and Sridaran 1981). In mice, the data suggest that oestrogens are also of foetal-placental origin, since female foetuses have over two times the blood concentrations of oestradiol as do their

mothers (vom Saal and Bronson 1980a). Unfortunately, the source of this oestradiol within the foetal-placental unit in rodetns is unknown. In rats, for example, the placenta appears to have little or no capacity to aromatize testosterone to oestradiol, and the adrenal cortex of rats appears to secrete low concentrations of androgens relative to humans (Kime et al. 1980).

These is considerable evidence that the adrenal cortex of males and females in mice and other mammals is sexually dimorphic after birth (Christian 1971, Kime et al. 1980). It is proposed that the adrenals of mice are also sexually dimorphic during foetal life. Secretions from the foetal adrenals may thus be contributing to the sex difference in amniotic fluid oestradiol concentrations in mice, either directly in terms of the secretion of androgens which are then aromatized, perhaps within the liver which contains aromatase (Gibori and Sridaran 1981), or indirectly through effects of glucocorticoids on both aromatase enzymes or rates of clearance of substances in the liver or placenta. The evidence is that the secretion of steroids by the foetal adrenal is primarily under the control of ACTH, which is secreted by the foetal pituitary, and possibly also chorionic gonadotropin or some other substance secreted by the placenta. In adult mice testosterone inhibits ACTH secretion while oestradiol enhances ACTH secretion (Kime et al. 1980), thus accounting for the known sex differences in adrenal morphology and physiology. It is possible that testosterone and oestradiol also influence the secretion of ACTH during foetal life in mice. The secretion of high titres of testosterone by the testes of male foetuses may inhibit ACTH secretion by the males' pituitary, which would result in a decrease in the secretion of steroids by the adrenals. It would also be expected that the adrenal cortex of foetal males would be hypotrophic relative to that of females, which is characteristic of adult male and female mice (Kime et al. 1980). In addition, since 2M female mouse foetuses have higher amniotic fluid and blood titres of testosterone than 0M female foetuses, it would also be expected that 2M female foetuses should have lower titres of oestradiol than 0M females due to inhibition of ACTH, which is suggested by the available evidence (vom Saal and Bronson 1980a).

6. The Effects of Prenatal Stress on Differences in Phenotype Due to Intrauterine Position

Corticosterone, but not ACTH, can pass between the maternal and foetal circulation across the placenta. Thus, corticosterone released by the adrenals of pregnant female mice that are subjected to stress should enter the foetal circulation and inhibit ACTH secretion by the foetal pituitary (Kime et al. 1980). This should result in a decrease in the secretion of adrenal steroids in the foetuses of stressed mothers. The fact that the pituitary-adrenal axis is functional in foetuses during the period of sexual differentiation, when steroids are regulating the development of sex phenotype, suggests that the functioning of this system should be sensitive to maternal stress, such as stress due to high population density in mice.

While the previous discussion is highly speculative, it is proposed that adrenal secretions interact with testicular secretions in regulating normal sexual differentia-

tion. Thus, it is possible that stressing pregnant mice might inhibit secretions from the foetal adrenal, and possibly attenuate or eliminate sex differences in foetal adrenal secretions. The result would be an attenuation or elimination of intrauterine-position related differences in phenotype. To test this hypothesis, forty-five mice were time-mated and stressed by being placed under 150 W flood lights (350 fc; 38°C) three times per day from Day 13 through 18 of pregnancy. An equal number of control females remained undisturbed. Ward and Weisz (1980) have reported that this procedure advances the timing of the maximum relase of testosterone in male rat foetuses. In adulthood, prenatally stressed male rats are less masculinized (exhibit lower levels of male copulatory behaviour) and less defeminized (exhibit higher levels of female copulatory behaviour) than are normal males (Ward 1972).

At Caesarean delivery all offspring of stressed and control mice were weighed. Regardless of intrauterine position or sex, the offspring of stressed mothers weighed significantly (8%) less (1.25 g) that did the offspring of control mothers (1.36 g). No difference in body weight between stressed and control males or females was found when the animals were 24 days old. The data presented in Fig. 6 reveal that all of the female offspring of stressed mothers resembled 2M females in the length of the anogenital space at birth. The previously reported difference in anogenital distance of control 0M, 1M, and 2M female mice was again found. In adulthood, the control 0M females had shorter oestrous cycles than did the control 2M females, again confirming previous reports (vom Saal and Bronson 1980b, vom Saal et al. 1981). But the prenatally stressed 0M females had oestrous cycles that were characteristic of control 2M females (unpubl. observ.). It is interesting that the 2M female offspring of stressed mothers exhibited significantly more pseudopregnant cycles than did any of the other females. This finding is consistent with the report by Herrenkohl (1979) that prenatally stressed female rats exhibited prolonged oestrous cycles and a high incidence of pseudopregnancies relative to control females. If maternal stress had only disrupted the secretion of testosterone by the testes of male foetuses as the findings of Ward and Weisz (1980) suggest, then the loss of females with a 2M phenotype would have been expected. Unfortunately, Ward and Weisz (1980) did not measure foetal oestradiol titres.

A comparison of the behaviour toward young of 0M, 1M and 2M male mice from the stressed and control mothers has been conducted. In control 0M, 1M and 2M males, there are differences in the tendency to commit infanticide or behave parentally toward young: most 0M males commit infanticide while most 2M males behave parentally. Prenatal stress eliminated this difference, and all male offspring from stressed mothers resembled 2M males (most behaved parentally toward young) regardless of prior intrauterine position (vom Saal 1983). Thus, prenatal stress eliminated the effects of developing between female foetuses in male mice, while there appeared to be little or no effect of prenatal stress on the behaviour of 2M males. In summary, all offspring from stressed mothers have resembled 2M animals in phenotype in the studies that have been completed at this time.

2M male mice and rats have previously been found to be the least sexually active of all offspring. Thus, we proposed that the previous report (Ward 1972) that prenatal stress resulted in a decrement in male sexual behaviour in rats was explained by the fact that prenatal stress results in the elimination of variance due to intrauterine

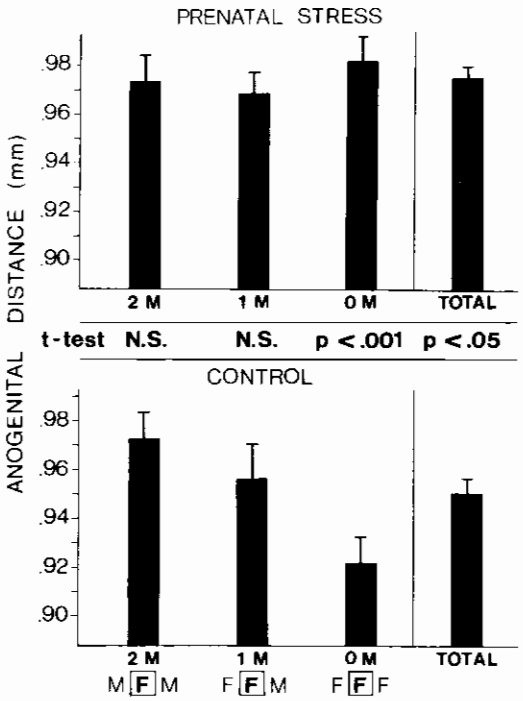

Fig. 6. The mean (± SEM) length of the space between the anus and the posterior aspect of the genital papilla at birth in the 0M, 1M and 2M female offspring of stressed and control mice (40 females/intrauterine position). Pregmant mice were stressed by being placed for 30 min under 150 W flood lights (350 fc; 38°C) 3 times per day from Day 13 through 18 of pregnancy (18 stress sessions). *TOTAL* combined mean for all 0M, 1M and 2M females measured. Significance levels are for t-test comparisons of prenatally stressed vs. control females from each intrauterine position

position in the offspring of stressed mothers and a shift in the population mean for sex behaviour toward that characteristic of 2M males. 0M and 2M male mice from stressed and control mothers were compared in terms of their sexual activity and this prediction was confirmed (Even and vom Saal 1983). Animals with a 0M and 1M phenotype thus do not appear to be produced by female mice or rats that are subjected to severe stress during the last third of pregnancy. It is proposed that the elimination of intrauterine position differences in the offspring of stressed mice is mediated by the suppression of steroid secretion by the foetal adrenal due to negative feedback inhibition of foetal ACTH by maternal corticosterone. If the enhanced titres of oestradiol in the amniotic fluid of female mouse foetuses derive from secretions from the foetal adrenal, then suppression of foetal ACTH might reduce oestradiol levels in female foetuses and eliminate the effects on siblings of developing between female foetuses. The findings from comparisons of the 0M and 2M offspring of stressed and control mice are thus consistent with the hypothesis that the high rate of secretion of oestradiol in female mice (and thus intrauterine proximity to female foetuses) plays an important role in mediating the intrauterine position phenomenon in both male and female mice.

7. Summary and Conclusions

During foetal life circulating oestrogens may interact with androgens secreted by the foetal testes in regulating both differences in phenotype between males and females and in producing variation in phenotype among males and among females (i.e., differences in phenotype due to intrauterine position). The evidence from studies involving human foetuses is that circulating oestrogens are derived from androgens (primarily DHEA-S) secreted by the foetal adrenals, and the adrenals of females secrete higher titres of steroids than the adrenals of males. It is thus also proposed that in mice, the observed sex difference in amniotic fluid titres of oestradiol reflects a sex difference in foetal adrenal physiology. The foetal adrenal may thus play a critical role in modulating normal sexual differentiation in mammals. The ability of secretions from the maternal adrenals to cross the placental barrier and depress the secretion of steroids from the foetal adrenals provides a mechanism via which the environment of the mother can influence foetal development. In addition, the intrauterine environment of foetuses, i.e., their intrauterine proximity to other foetuses of the same or opposite sex, provides another source of variation in phenotype in mice and rats, and possibly other mammals in which multiple uterine residence occurs.

The way in which oestradiol might interact with testosterone to regulate sexual differentiation is obviously complex, since elevated foetal titres of oestradiol are correlated with enhanced sexual performance and decreased aggression and decreased seminal vesicle size in male mice. The negative relationship between aggressiveness and sexual performance observed in comparisons of 0M and 2M male mice presents an interesting problem. Psychobiologists typically refer to males that exhibit a decrement in male sex behaviour (usually due to some hormonal manipulation or to prenatal stress) as being less masculinized than are males which exhibit more mounts, intromissions or ejaculations when paired with a female. Thus, if only sexual behaviour had been examined in 0M and 2M male mice, 0M males would have been labelled as more masculinized than 2M males. But the induction of aggression in response to testosterone treatment is also utilized by psychobiologists as an index of prenatal masculinization, and if only aggression had been examined in 0M and 2M male mice, 2M males would have been labelled as more masculinized than 0M males. Obviously, if two behaviours are commonly used as indices of masculinization and they are found to be negatively correlated, labelling one male as more or less masculinized than another male in an experiment in which only one of the behaviours is analyzed is meaningless. Thus, 0M, 1M and 2M males are all masculinized in that they are all capable of reproducing, but variance in both aggressiveness and sexual performance in male mice is due in part to prior intrauterine position.

In conclusion, a significant component of the variance in a broad range of morphological, physiological and behavioural characteristics in populations of mice and rats has been found to be due to prior intrauterine position. The intrauterine position phenomenon is thus a valuable model system for examining the relationship between foetal hormone titres and adult phenotype.

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