PRENATAL UTERINE ENVIRONMENT AND SEXUAL DIFFERENTIATION OF RATS

PRENATAAL MILIEU EN SEKSUELE DIFFERENTIATIE VAN RATTEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR

AAN DE ERASMUS UNIVERSITEIT ROTTERDAM

OP GEZAG VAN DE RECTOR MAGNIFICUS

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DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP

WOENSDAG 24 NOVEMBER 1993 OM 13.45 UUR

DOOR

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Het onderzoek dat beschreven wordt in dit proefschrift werd gefinancierd door de stichting Psychon van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek.

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GENERAL INTRODUCTION

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The experiments presented in this thesis investigated the influence of several prenatal factors relevant to hormonal environment on the sexual differentiation of behavior, morphology and central nervous system in rats. The effects of such factors as prenatal sex composition of the litter and position in utero on the sexual differentiation of normally developed (i.e. untreated) male and female rats was examined. In addition, the effects of experimentally induced changes in the perinatal hormonal milieu on the central nervous system and behavior of male rats were assessed.

This general introduction provides an overview of the effects of hormones on reproductive morphology and behavior, and function and morphology of the central nervous system of mammals, with emphasis on rats. Current questions and hypotheses that led to the experiments presented in this thesis will be outlined.

I. BACKGROUND

A. Description of sex differences

In many mammalian species, a wide variety of both sexual and non-sexual behaviors differs markedly between males and females. Moreover, structures in the central nervous system hypothesized to underlie these behaviors, as well as genital morphology and the neural control of reproductive endocrine functioning, show clear sex differences as well.

1. Sex differences in genital morphology and reproductive behavior The most obvious area where the sexes differ is genital morphology. In males, internal genitals consist of epididymida, vasa deferens, prostate and seminal vesicles, and in females of oviducts, uterus and upper vagina. External genitals of males include scrotum, penile shaft and glans penis, and of females the vagina, clitoris and labia minora and majora.

In addition to the well-known sex differences in genital morphology, reproductive behavior of numerous mammalian species is sex dimorphic. Of the many species studied over the past decades, sex differences in reproductive behavior have probably been examined most extensively in rodents. Rodents show sex typic reproductive behavior, i.e., the behaviors involved in copulation are different for males than for females. In rats, the species investigated in the experiments presented in this study, the male approaches the female, investigates her genitals, then mounts her, often resulting in an intromission of the penis into the vagina. After a number of repeated intromissions, ejaculation occurs, followed by a refractory period during which the male does not display any interest in sexual activity. Mounting, intromitting and ejaculating are generally referred to as 'masculine' sexual behavior.

The female on the other hand, approaches the male as well, displaying a

variety of so called 'proceptive' behaviors, such as 'earwiggling', 'hopping', 'darting' and 'presenting', all of which serve to sollicit sexual behavior from the male. When mounted by the male, the female assumes the 'lordosis' position, in which she arches her back and stretches her hindlegs, a posture which allows intromission of the penis into the vagina. This component of the female's behavior is referred to as 'receptive' behavior (Beach, 1976).

The sex difference in these behaviors is a relative rather than an absolute one. That is, under certain conditions female rats may show high levels of mounting behavior (e.g. Södersten, 1972), and males may show lordosis (e.g. van de Poll and van Dis, 1977). Thus, both sexes are capable of displaying both the masculine and the feminine pattern of reproductive behavior, and differ mainly with regard to the likelihood with which they display sex typic patterns of behavior.

2. Sex differences in function and morphology of the central nervous system.
Reproductive behavior is dependent on endocrine events, the specifics of which will be outlined later. Neuroendocrine function, like sexual behavior, shows a clear sex difference in rats as well as in many other species. Typically, the anterior pituitary of females shows a cyclic release of luteinizing hormone (LH), which is necessary for ovulation, whereas a tonic pattern is present in males (Goy and McEwen, 1980).

Since LH secretion by the pituitary is under the control of the hypothalamus, the difference between males and females in pattern of LH secretion reflects a difference in the functioning of the central nervous sytem (CNS). The discovery earlier this century of this sex difference has been extended with the discovery of sex dimorphism in other CNS structures.

The search for morphological sex differences in the CNS has primarily focussed on structures which have been implicated in the expression of sexually dimorphic behavior. The preoptic area of the anterior hypothalamus (POA-AH) is one of many areas in the CNS which contains both estrogen and androgen receptors (Sar and Stumpf, 1975). Estrogen and androgen are gonadal steroid hormones important for the expression of sexual behaviors. Along with these other structures, the POA-AH has been implicated in the regulation of reproductive functioning through lesion and implantation studies (e.g. Malsbury, 1971, Christensen and Clemens, 1979, van de Poll and van Dis, 1979). This structure has been found to be morphologically differentiated by sex. Specifically, in 1978, a sexually dimorphic nucleus located in the POA (SDN-POA) was first descibed in rats (Gorski et al, 1978, Gorski et al., 1980). A similar sex dimorphic structure has since been identified in several other species (e.g. Commins and Yahr, 1984, Hines et al., 1985, Cherry et al., 1990), including man (Swaab and Fliers, 1985). In rats, the SDN-POA is severalfold (3-8 times) larger in males than in females. The involvement of the SDN-POA in the control of masculine sexual behavior in rats has been suggested both by studies in which lesions of this nucleus reduce masculine sexual behavior in males (de Jonge et al., 1989) and in females (Turkenburg et al., 1988), and by a report of a positive correlation between SDN-POA volume and sexual activity of males (Anderson et al., 1986).

Outside the brain, another sexually differentiated neural structure has been

described in the rat. The spinal nucleus of the bulbocavernosus (SNB) is located in the fifth and sixth lumbar segments of the rat spinal cord. The motoneurons that this nucleus consists of innervate the perineal muscles bulbocavernosus (BC) and levator ani (LA) which are connected to the penis and are critical for successful copulation. The SNB is sexually dimorphic both in terms of number of neurons (about 200 in males, about 60 in females) and size of neuron somas, which is larger in males than in females (Arnold and Gorski, 1984, Breedlove et al., 1982).

B. Sexual differentiation

The differences between the sexes described in the preceding section are perhaps most apparent in the adult organism. However, the full development of genital structures, sexual behavior, neuroendocrine function, and several sex dimorphic brain structures -a process referred to as 'sexual differentiation'-, depends primarily upon perinatal endocrine events. A brief overview of the important aspects of sexual differentiation is presented next.

1. Genital structures The chromosomal sex of an animal is established at the time of conception, when the sperm provides either an X or a Y chromosome to complement the X chromosome provided by the egg. Chromosomal sex determines whether testes or ovaries develop: if a Y chromosome is present, the Sry gene (sex determining region of the Y chromosome) causes testes to develop, whereas in the absence of the Y chromosome ovaries develop. The internal genital structures subsequently evolve prenatally from the wolffian ducts in the male, and from the mullerian ducts in the female. Both duct systems exist in males as well as females before differentiation of the gonads, but the type of gonads determines which ducts eventually develop. If testes are present, testosterone secreted by these gonads stimulate the development of the wolffian ducts into epididymides, vasa deferens and seminal vesicles, while Mullerian Inhibiting Substance (MIS), also produced by the testes, causes regression of the mullerian ducts. If, on the other hand, ovaries develop, the absence of MIS and androgens results in the development of the mullerian ducts into oviducts, uterus and the upper vagina, and regression of the wolffian structures. Testosterone secretion by the testes starts around day 14 of gestation in rats, and differentiation of internal genitals occurs entirely prenatally.

Like the male internal genital structures described above, the prostate and external genital structures are dependent on the presence of androgens. The specific androgen responsible for masculine development of genital structures derived from the urogenital sinus (prostate, scrotum and penis) is 5α-dihydrotestosterone (DHT). 5α-DHT is converted by the enzyme 5α-reductase from testosterone, which is secreted by the testes. In the absence of DHT, labia majora and minora and a clitoris develop (for an overview see Jost, 1970, Baum, 1979, Josso et al., 1993).

2. Neuroendocrine function. Like the differentiation of genital structures, the differentiation of reproductive neuroendocrine function is controlled by gonadal hormones during a restricted period around birth. In 1936, it was shown by Pfeiffer

(Pfeiffer, 1936) that the male pattern of gonadotropin release by the pituitary depends upon the presence of testes during early postnatal life. This tonic pattern characteristic of males could be induced in females by transplantation of a testis subcutaneously in the neck on the day of birth. Along the same line, the female pattern could be induced in males by castration at birth. More specifically, the cyclic pattern of LH release by the pituitary, characteristic of the adult female, only occurs when testosterone is absent during the neonatal period. The presence of testosterone during this critical period results in a tonic pattern of LH release in the adult male rat.

3. Sexual behavior Ever since the factors controlling differentiation of the genital tract and endocrine function were first outlined by Jost (1953), it has become clear that the organizing effects of hormones around birth are by no means limited to physiological and morphological characteristics of the organism. The suggestion that a similar hormonal process around the time of birth might underlie sex differences in reproductive behavior was put forward by Phoenix and colleagues (1959), who reported that female guinea pigs exposed to testosterone prenatally not only developed masculinized genitals, but in adulthood also displayed sexual behavior typical of males. This report marked the beginning of a new area of research in which this process was investigated in many mammalian species. Specifically, the relevant hormones, the critical period during which sexual differentiation takes place and the reproductive and non-reproductive behaviors affected were examined for many species. In this section, only the sexual differentiation of the rat will be reviewed.

The importance of the perinatal hormonal milieu for the differentiation of sexual behavior has now been firmly established in rats as well as many other species. The sex differences in sexual behavior observed in normally developed adult males and females can be reversed by manipulation of perinatal testosterone levels. Because the testes produce testosterone, higher levels of this androgen circulate in males than in females perinatally (Slob et al., 1980, Pang et al., 1979). Females perinatally treated with testosterone show high frequencies of mounts and intromissions in adulthood under the appropriate hormonal stimulation, and may even show ejaculatory behavior (Sachs and Thomas, 1985), such that their sexual behavior is indistinguishable from males. Males castrated at birth, and thus not exposed to testosterone during the neonatal period, do not show the full scale of masculine sexual behavior when they reach adulthood (see Baum, 1979). The development of the behavioral patterns characteristic for the male is referred to as 'masculinization'. The critical period for masculinization of behavior starts prenatally and extends untill approximately day 10 of postnatal life. Prenatally, the testes start secreting testosterone around day 14, and show a testosterone surge on days 18 and 19, which has been suggested to be critical for complete behavioral masculinization of male rats (Weisz and Ward, 1984). Postnatally, testosterone remains high in males untill about day 10 (Pang et al., 1979, Resko et al., 1968).

Apart from its masculinizing effect, testosterone exerts a 'defeminizing' effect, a process referring to the suppression of feminine behavioral characteristics. That

is, females treated with testosterone perinatally show low levels of receptivity and proceptivity in adulthood, whereas adult males, castrated at birth, under the appropriate hormonal conditions display receptive and proceptive behavior typical of females. Defeminization appears to occur primarily during the neonatal period, although evidence for some defeminization prenatally has been reported (Gladue and Clemens, 1978).

The observation that some hormone manipulations during the critical period increase masculinization without affecting defeminization or vice versa, showed that these are two distinct processes, rather than two extremes on the same dimension (Goy and Goldfoot, 1975).

In conclusion, the presence or absence of testosterone during a 'critical' period around birth determines the likelihood with which the animal will display a variety of behaviors in adulthood.

Aromatization In addition to the conversion via 5α-reductase to 5α-DHT, testosterone is converted to estrogen. In perinatal rats, this conversion occurs through aromatase by specific aromatase enzymes, and takes place in the CNS. Exposure of the CNS to estrogen around birth affects sexual differentiation of neuroendocrine function and sexual behavior, much as exposure to testosterone does around this time (e.g., Paup et al., 1972). Several lines of research have provided evidence that the aromatization of testosterone to estrogen contributes to defeminization and masculinization of the brain and sexual behavior in rats. First, the neonatal rat brain of both sexes possesses the enzymes needed for aromatization of androgen to estrogen (Reddy et al., 1974). Second, 5α-reduced androgens such as DHT, which cannot be aromatized to estrogen, are far less effective than testosterone in inducing defeminization in rats (Paup et al., 1972, Coniglio et al., 1973). Third, treatment with an estrogen antagonist blocks the effects of testosterone in neonatal females (McDonald and Doughty, 1972). Fourth, neonatal treatment with an aromatization inhibitor has inhibiting effects on defeminization of male rats similar to neonatal castration (Vreeburg et al., 1972, McEwen et al., 1977, Brand et al., 1991). Regarding masculinization, the perinatal aromatization of testosterone to estrogen is particularly important for the control of ejaculation (see Baum, 1979). With regard to the role of aromatization in sexual differentiation the androgen-insensitive (tfm) rat is interesting, since tfm males have reduced levels of androgen receptors, but normal levels of both estrogen receptors and aromatase. Although these genetic males are female in genital appearance, both gonadotropin release and sexual behavior of these males are masculinized, indicating that estrogen and not testosterone is primarily responsible for sexual differentiation of the brain and behavior (see Arnold and Gorski, 1984).

The question then arises as to why female fetuses, who are exposed to high levels of estrogen prenatally from the placenta and maternal body, do not show masculinization of the CNS like males do. It is currently believed that female fetuses are protected from the masculinizing effects of estrogen by alpha-fetoprotein, a serum protein which binds to estrogen and thus prevents it from crossing the blood-brain barrier and reaching estrogen receptors in the brain (Raynaud et al., 1971). Testosterone in contrast, enters the brain and is

subsequently aromatized to estrogen.

It seems inappropriate to conclude that sexual differentiation of the brain and behavior occurs entirely through estrogen. For example, treatment with an anti-androgen may also inhibit masculine differentiation (e.g., Gladue and Clemens, 1978).

4. Central nervous system In their first report on behavioral sexual differentiation, Phoenix et al. (1959) speculated that the behavioral changes observed in female guinea pigs after perinatal testosterone treatment might result from changes brought about in structures of the central nervous system. This suggestion originally met with great skepticism (Beach, 1971). Because both genital structures and behavior are affected by perinatal gonadal hormones, and because the motor components of sexual behavior are complementary to and dependent on genital structures (ejaculation for example is simply not possible without the appropriate genitals), it was argued that effects of perinatal hormones on behavior are mediated by changes in genital structures. That is, the reduction in masculine sexual behavior after neonatal castration of male rats was attributed to impaired penile development, rather than changes in the central nervous system. However, ample evidence has now emerged showing that some CNS structures involved in the control of sexual behavior show sex differences, and that these sex differences are dependent on the perinatal hormonal environment. Furthermore, the use of such techniques as neonatal hormone implantation directly into relevant CNS structures has provided evidence for effects of hormones directly on the CNS, as such procedures do not have any effect on peripheral morphology, but do affect both CNS morphology and sexual behavior (e.g. Christensen and Gorski, 1973, Swanson and Brayshaw, 1973, Swanson et al., 1991). Although sex differences in the CNS include many structures, only some related to sexual behavior will be dicussed here.

SDN-POA The volume of the SDN-POA is 3 to 8 times larger in males than in females (Gorski et al., 1978), and in addition, the SDN-POA is sex dimorphic in terms of neurochemicals in the cell bodies of neurons and in the fibers innervating the nucleus (Simerly et al., 1986). In the rat these sex differences have been shown to be dependent on the presence of testosterone around birth and can be prevented by manipulation of circulating levels of testosterone at this time (Jacobson, 1981, Simerly et al., 1985). The period during which testosterone permanently affects SDN-POA volume starts approximately at day 18 prenatally and extends till day 5 neonatally (Rhees et al., 1990a, Rhees et al., 1990b). The cellular processes through which differentiation of this nucleus occurs are not conclusive yet. Possible mechanisms through which steroids permanently affect the SDN-POA volume include prevention of cell death, stimulation of neurogenesis and stimulation of neuronal migration, mechanisms which may apply to the sex differences in neurochemicals and fibers of this nucleus as well (Gorski, 1987, de Jonge et al., 1990, Jarzab et al., 1990).

Accumulating evidence suggests that, like the differentiation of sexual

behavior, masculinization of SDN-POA volume is controlled by estrogen rather than testosterone. Treatment with an anti-estrogen inhibits the increase in SDN-POA volume in males, whereas anti-androgens do not have such an effect (Dohler et al., 1986). In females, perinatal estrogen treatment is as effective as testosterone in inducing normal masculine development of SDN-POA volume (Dohler et al., 1984).

Spinal nucleus of the bulbocavernosus (SNB) Early in development, both males and females have substantial numbers of motoneurons in the spinal nucleus of the bulbocavernosus (SNB), but consistent with other patterns of differentiation, in the absence of testosterone these neurons, as well as the perineal muscles to which they are connected, degenerate, resulting in the sex dimorphism observed in adulthood (Breedlove and Arnold, 1980, Breedlove et al., 1982, see for an overview Arnold and Gorski, 1984). Aromatization does not appear to play an important role for the differentiation of this nucleus, as in Tfm males, who are sensitive to estrogens but insensitive to androgens, the SNB is absent. Also, testosterone but not estrogen injected on neonatal day 2 will masculinize the SNB in females. The critical period during which this nucleus is masculinized starts prenatally and extends to day 6 postnatally with regard to the number of neurons, but continues much longer for the cell size, even after day 11 postnatally. It should be noted that dendritic growth of the SNB appears to be dependent not only on androgens level around birth, but also on androgens circulating during puberty.

In summary, regarding the sexual differentiation of genitals, neuroendocrine function, sexual behavior and brain structures important for the expression of these behaviors, the female is the default: in the absence of testosterone, the phenotype is female, whereas in the presence of testosterone differentiation is towards the male phenotype. These permanent, irreversible effects of testosterone during a restricted period around birth are generally referred to as the 'organizing' effect of testosterone.

Thus, brain, genitals, neuroendocrine function and behavior of each sex are perinatally 'primed' in a way that will enable adult reproductive physiology and behavior characteristic for that sex. These primed systems subsequently interact with gonadal hormones, which circulate in high levels again during puberty and then throughout adulthood. When threshold levels of gonadal hormones are attained in the adult animal, sexual behavior will become activated.

C. Activational effects of gonadal hormones

The role of gonadal hormones in adulthood must be distinguished from their organizational role in early development. In adulthood, hormones activate certain behaviors, and the extent of the activation depends upon the way the CNS has been organized by perinatal hormones. Testosterone secreted by the testes activates the 'masculine' behaviors in the male, whereas estrogen and progesterone, produced by the ovaries, activate 'feminine' behaviors in females. These 'activating' effects differ from organizing effects in that they are temporary and can be reversed by the withdrawal of hormones.

Female rats typically have a 4 or 5 day cycle, during which different levels of

hormones, predominantly estrogen and progesterone, are secreted by the ovaries. The display of feminine sexual behavior is controlled by the levels of and interaction between these hormones, resulting in a cyclic pattern of sexual behavior (see Morali and Beyer, 1979). On the day of estrus, high levels of both receptivity and proceptivity can be observed, whereas on other days of the cycle these behaviors are virtually absent. The day of estrus can be hormonally characterized by a prolonged period (>24 hours) of exposure to estrogen and short term exposure to progesterone (appr. 4-8 hours). Males show no consistent cyclic variation in sexual behavior, the result of the tonic release of LH, and therefore of testosterone.

Under normal conditions, testosterone activates masculine sexual behavior in rats. Testosterone may also activate masculine sexual behavior in normally developed females (Sodersten, 1972). Furthermore, some males, when primed with high doses of estrogen may show high levels of lordosis (van de Poll and van Dis, 1977). Yet the dose of estrogen needed to activate receptive behavior in males is much higher than that in females. To some extent, the sex difference in the display of sexual behavior then reflects differences in sensitivity to hormones, That is, males are less sensitive to the activating effects of estrogen and progesterone than females, and more sensitive to activating effects of androgens, a pattern which is perinatally organized.

Analogous to the organizational effect of testosterone, aromatization of testosterone to estrogen may play an important role in the activational effect of testosterone. Estrogen induces masculine sexual behavior (Sodersten, 1972), and the simultaneous administration of testosterone and an aromatization inhibitor reduces the effect of testosterone on masculine sexual responses (Kaplan and McGinnis, 1989). Non-aromatizable androgens, such as DHT, which cannot be converted to estrogen, are not as effective as testosterone in activating mounting behavior in females (van de Poll et al., 1986). However, the non-aromatizable synthetic androgen R1881 (Sodersten and Gustafsson, 1980), as well as DHT (Baum and Vreeburg, 1973), activated mounting behavior in castrated males. Nonetheless, DHT was less effective in this respect than DHT combined with estradiol benzoate (EB), suggesting aromatization plays a role in the activating effect of testosterone on masculine sexual behavior.

It should be noted that the traditional dichotomy of organizational and activational effects of hormones seems overly simplified. Several findings, for example, suggest that hormones may still have an organizational effect as late as during puberty (see Arnold and Breedlove, 1985).

II. VARIATION WITHIN EACH SEX

As was mentioned earlier, the sex differences in genital morphology and reproductive behavior are relative rather than absolute. Normally developed rats (i.e., that have not undergone manipulation of the hormonal environment around birth) of both sexes show variation in degree of masculinization and feminization, both in genital morphology at birth and sexual behavior in adulthood.

In rats, in addition to the internal genitals, the external genital morphology

differs between the sexes at birth. Although a penis or vagina has not yet fully developed at this time, the sexes can be distinguished by the distance from the anus to the genital orifice (anogenital distance, AGD), which at this time is much larger in males than in females. This sex difference is, like the difference in internal genitals, dependent on prenatal exposure to androgens. Females prenatally treated with androgen have an AGD indistinguishable from males (Gerall and Ward, 1966) and males prenatally exposed to anti-androgens have an AGD the size of those of females (Brand et al., 1990).

1. Variation among females

In addition to this sex difference in genital morphology at birth, there is also considerable variation in AGD within each sex. Because of its sensitivity to prenatal androgens, the size of a female's AGD relative to the AGD of other females is generally regarded a measure of morphological masculinization. A larger AGD for example places a female more towards the masculine end of the female/male continuum, although this AGD still falls well within the normal range of the female AGD.

This variation among females is also observed in the display of masculine sexual behavior. Mounting behavior may frequently be observed in females, yet there is great variability among females regarding the frequency with which they display this behavior. This variation cannot be ascribed to differences in adult androgen levels or experience with mounting behavior (de Jonge et al., 1986), and has therefore been hypothesized to reflect differences in amount of perinatal masculinization.

2. Hypothesized role for androgens in the natural variation among females in AGD at birth and mounting behavior in adulthood

Because prenatal androgens are important for masculinization of both AGD at birth and mounting behavior in adulthood, the variation in AGD as well as mounting behavior observed in normally developed females has been attributed to variation in exposure to androgens prenatally (Clemens et al., 1978). Detectable levels of androgens have been found in fetal females (Pang et al., 1979, Slob et al., 1980), and these levels, like AGD and mounting behavior, are subject to high inter-individual variability. Also, prenatal treatment with an anti-androgen reduced levels of mounting behavior in adult females (Stewart et al., 1971). Thus, females have been hypothesized to undergo some masculinization by prenatal exposure to endogenous androgens. The amount of masculinization then depends on the amount of androgen they are exposed to prenatally. Since the ovaries of females are relatively quiescent prenatally, the question arises as to the source of androgens in female fetuses.

3. Sources of androgens in fetuses

Several sources of androgens in female fetuses have been suggested. First, the placenta has been shown to produce androgens both in vitro (Chan and Leathem, 1975) and in vivo (Gibori and Sridaran, 1981), and has been suggested to be the major source of androgens in fetal females (Vreeburg et al., 1983).

Second, the ovaries of the mother produce androgens throughout pregnancy, although this production decreases during the second half of pregnancy (Gibori and Sridaran, 1981), the time when fetuses are affected most by their presence. Third, in 1971, Clemens and Coniglio hypothesized that female rats are exposed in utero to androgens from males with whom they share the uterine horn (Clemens and Coniglio, 1971). Rats are typically born in large litters (e.g. Wistar rats: appr. 12 pups per litter), which are prenatally divided over two uterine horns. Females from a uterine horn with a large number of males were reportedly more masculinized than females from predominantly female litters (Clemens and Coniglio, 1971). In male fetuses, the testes start secreting testosterone around day 14 of prenatal life and continue to do so throughout gestation. This testosterone was hypothesized to reach females sharing the uterine horn. This initial report marked the beginning of a series of experiments investigating whether female fetuses are masculinized by males in the same uterine horn, a phenomenon referred to as the 'intrauterine position phenomenon'.

4. Mechanism of androgen transport

Clemens and colleagues (1978) found that females showed morphological as well as behavioral masculinization which increased with the distance from the nearest male in the uterine horn. This observation led to the hypothesis that androgens secreted by males reach females in the same uterine horn by diffusion through the amniotic sac.

Since this original report, numerous studies, primarily using mice and gerbils, have provided support for the hypothesis that females are masculinized in utero by (presumably) androgens from males located next to them ('contiguity hypothesis'). Although negative findings have been reported as well (Gandelman and Kozak, 1988, Jubilan and Nyby, 1992, Simon and Colloger-Clifford, 1991), the majority of studies have found that female mice that develop between two males in utero (2M females) have a larger AGD at birth and show a variety of masculine behavioral patterns in adulthood which are less profound in females that resided between two females (0M females) (reviewed in vom Saal, 1989). Some of these findings have been replicated in gerbils (e.g. Clark and Galeff, 1988).

In rats, evidence for an effect of males on the sexual differentiation of adjacent females appears to be less conclusive however. Initially, no evidence was found that females from predominantly male litters were more masculinized behaviorally than females from predominantly female litters (Slob and van der Schoot, 1982, van de Poll et al., 1982). Another study, taking into account the position that females resided in relative to males in utero, found that 2M females had a larger AGD at birth than 0M females. and in addition became anovulatory at an earlier age than 0M females when given TP on day 3 (Tobet, Dunlap and Gerall, 1982). This treatment has been shown to result in premature sterility, the latency of which depends in part on the dosage of androgen. It was concluded that the earlier onset of sterility in the 2M group reflected exposure to a higher dose of androgen prenatally, presumably from adjacent males. In 1981, Meisel and Ward (Meisel and Ward, 1981) reported no difference in AGD or sexual behavior between 2M and 2F female rats, but proposed that androgens from males are transported

by the vasculature to females located rostrally. This alternative hypothesis was based on the observation that females that developed in utero with males located caudally had a larger AGD at birth and displayed more masculine sexual behavior in adulthood than females that developed in the absence of such males.

The idea that androgens could be transported in a rostral direction via the uterine bloodflow was derived from studies on luteolysis. In short, the research on luteolysis has suggested that substances may pass from the uterine vein to the uterine artery, which are in close approximation in the rat, and that the bloodflow in these vessels is primarily in a rostral direction in several species including rats, hamsters and guinea pigs (Del Campo and Ginther, 1972). It was hypothesized by Meisel and Ward (1981) that androgens secreted by males may follow the same route, thereby affecting females located rostrally, but not those located caudally. Thus, testosterone secreted by a male fetus supposedly enters the uterine vein, passes from the vein to the uterine artery and is transported in a rostral direction to the maternal part of the placenta of siblings located rostrally, where it enters the fetal placenta and affects the sexual differentiation of the fetus ('caudal male hypothesis').

These two hypotheses, one suggesting transport via diffusion through the amniotic sac, the other suggesting transport via the vasculature, have subsequently been tested in females from several species. In rats, when the two hypotheses were tested simultaneously, Richmond and Sachs (1984) found support for the 'caudal male' hypothesis but not the 'contiguity' hypothesis. Females with caudal males had a longer AGD at birth than females without such males, whereas AGD of females with two adjacent males did not differ from females with two adjacent females. In another study, female rats with caudal males were more sensitive to the effects of testosterone on extinction of conditioned taste aversion, than females without caudal males (Babine and Smotherman, 1984). In this study however, females were not compared on the basis of the presence or absence of adjacent males. In another species, the guinea pig, the presence of a caudal adjacent male is reportedly needed for behavioral masculinization, whereas the presence of merely a caudal or adjacent male is not sufficient (Gandelman, 1986). In mice and gerbils, although much evidence has been reported for a contiguous male effect, few attempts have been made to test the caudal male hypothesis in these species.

In conclusion, although there is much evidence that the sexual differentiation of females is affected by the presence of males in the uterine horn, the mechanism by which androgens from these males might be transported remains unclear.

5. Evidence for differences in prenatal androgen levels in females from different uterine positions

Morphological and behavioral differences between females from different intrauterine positions are supposedly the consequence of differences in prenatal androgen levels, resulting from the specific location a female resides in relative to males in utero. Studies in which actual androgen levels are measured in female fetuses from different intrauterine positions are therefore crucial to the question regarding the mechanism through which androgens might be transported from male to female fetuses. In both mice (vom Saal and Bronson, 1980) and gerbils

(Clark et al., 1991), female fetuses located in between two males reportedly have higher testosterone levels prenatally than females that are located between two females, thereby supporting the contiguity hypothesis. It must be noted however that in these species, androgen levels of females with and without caudal males were not compared, and possible effects of caudal males have thus not been assessed in either mice or gerbils. On the other hand, the caudal male hypothesis has received support as well from studies on prenatal androgen levels. In ferrets, female fetuses with more than one caudal male had higher androgen levels than females with either 0 or 1 caudal male, whereas there were no differences between females with 2 or with 0 adjacent males (Krohmer and Baum, 1989). In hamsters, levels of androgen were suppressed and levels of estrogen enhanced in males with females located caudally, but no effects of contiguity to males or females on hormone (androgen and estradiol) levels were found. In females from the same litters however, no effects of either adjacent or caudal male siblings were found (Vomachka and Lisk, 1986). Finally, in rats, no evidence has been found for effects of either caudal or adjacent males on androgen levels in female fetuses (Baum et al., 1991).

6. Variation among males in morphological and behavioral masculinization

Although the discussion has primarily focussed on variation in masculinization of females, the variation in masculinization observed in females is not restricted to this sex. Males of several species as well show considerable variation in AGD at birth and the frequency with which they display masculine sexual behavior in adulthood. Several studies have begun to investigate the role of intrauterine position in this variation in male mice (vom Saal et al., 1983) and gerbils (Clark et al., 1992). In male rats, however, the intra-uterine position phenomenon has not been investigated as of yet.

7. Tentative conclusions and contradictions

In conclusion, the intrauterine position phenomenon is as of yet not well established. Some of the inconsistency surounding this phenomenon may be related to the fact that different species were used across studies. Thus, in mice and gerbils, most studies report effects of male fetuses on adjacent females, although for mice negative findings have been reported as well. In several other species no effects of adjacent males have been found (Vomachka and Lisk, 1986, Krohmer and Baum, 1989, Baum et al., 1991), whereas in some of these same species effects of caudal males on female sexual differentiation have been reported (Vomachka and Lisk, 1986, Krohmer and Baum, 1989). It should be noted however that in mice and gerbils, the effects of caudal males on female sexual differentiation have not systematically been investigated. The inconsistencies among studies may reflect the fact that the mechanism by which intrauterine transport of androgens may occur, as well as the extent to which female fetuses are affected by the presence of males in the uterine horn, could differ across species. In addition, differences between strains within a single species may contribute to the lack of clear-cut effects. For example, strain differences in the sensitivity to hormones have been reported in rats (Brand and Slob, 1991). Various

strains of rats have been used to test the intrauterine position phenomenon, and these strain differences may have contributed to the inconsistent findings.

Contradictory findings may also be related to the fact that different methodologies have been used across studies investigating the intrauterine position phenomenon. For example, in the original study by Clemens et al. (1978), in which an effect of male fetuses on females was reported for the first time, some experimental females were born to mothers in whom one uterine horn had been taken out during pregnancy. Since stress during pregnancy has been shown to affect the prenatal hormonal environment and sexual differentiation of the offspring (Ward and Weisz, 1984), such a procedure might well confound the results.

Moreover, a problem inherent to the issue of prenatal environment and female masculinization is that studies examining the influence of male littermates on female sexual differentiation are not true experiments, as variables such as caudal males or adjacent males cannot be experimentally controlled in the laboratory. Thus, along with the independent variable, other prenatal factors which might affect sexual differentiation of the offspring may systematically vary, thereby obscuring the real effects of some variables, or ascribing effects to related, but uninvolved variables.

Finally, the caudal male hypothesis assumes transport of androgens via the uterine vasculature. This transport, however, has never been directly established. Accordingly, the presumed mechanism of androgen transport underlying both the caudal male and the contiguity hypothesis await empirical demonstration.

8. CNS morphology and sexual behavior

Effects of intrauterine position on sexual differentiation presumably result from differences between animals from different intrauterine positions in prenatal exposure to androgens. These differences in androgen levels in turn supposedly permanently affect the CNS, which then results in changes in sexual behavior in adulthood. However, such processes are by no means well established.
In fact, it was not until fairly recently that sex differences in the CNS have been discovered and become subject of investigation (Raisman and Field, 1971). Although evidence has emerged implicating one sex dimorphic CNS structure, the SDN-POA, in the expression of sexual behavior, the exact relationship between perinatal hormonal environment, and SDN-POA and the expression of sexual behavior in adulthood is not clear.

III. OUTLINE OF THE EXPERIMENTS PRESENTED

This thesis presents experiments examining the influence of the prenatal hormonal environment on sexual differentiation of female and male rats. The effects of intrauterine position (i.e. the position of a fetus relative to male fetuses in the same uterine horn), and prenatal hormonal environment on morphological and behavioral masculinization of female rats were investigated. In addition, a parallel investigation on the intrauterine position effect in male rats was carried out. Finally, effects of experimental changes in the perinatal hormonal environment on the

sexual differentiation of CNS and sexual behavior of male rats were studied.

In the experiments described in this dissertation, 'contiguous' or 'adjacent' males are defined as those males who are positioned in utero next to an experimental animal, as established either through visual inspection during a ceasarean section of the mother, or through birth order in pups from mothers with only one uterine horn.

Studies on the intrauterine position phenomenon have typically used mice and gerbils. Few studies have focussed on the rat, and those that have, have yielded inconclusive results (Tobet et al., 1982, Clemens et al., 1978, Meisel and Ward, 1981, Richmond and Sachs, 1984). Therefore, in the experiments presented in this thesis, the intrauterine position phenomenon was studied specifically in the rat.

In those studies investigating the intrauterine position phenomenon in rats, different methodologies were used. In some studies, experimental animals were born to mothers who had undergone surgery during pregnancy (Clemens et al., 1978). In addition, in some studies pups were delivered naturally (Tobet et al., 1982), whereas in others, pups were delivered through caesarean section (Clemens et al., 1978). Since stress during pregnancy has been reported to have profound effects on sexual differentiation of the offspring, presumably through alterations in hormonal secretions by the fetuses (Ward and Weisz, 1984), surgical procedures carried out during pregnancy and around birth may have confounded results and contributed to the ambiguity surrounding the issue of the effects of intrauterine position on female sexual differentiation. The first experiment, described in Chapter 2, investigated the effects of caudal, as well as adjacent males in utero on behavioral sexual differentiation of females, while experimentally controlling for possible confounding effects of several methodological procedures used in previous studies.

Male rats, like females, show inter-individual variability with regard to genital and behavioral masculinization. In mice and gerbils, these differences have been associated with the uterine position of males (vom Saal, 1983, Clark et al., 1992). Thus far, the effect of the position in utero has not been investigated in male rats. In Experiment 2, presented in Chapter 3, the effects of caudal and adjacent males on sexual behavior of male rats were investigated.

Studies investigating the effect of male fetuses on sexual differentiation of siblings in the same uterine horn have typically categorized and compared subjects according to the presence or absence of either adjacent or caudal males. The inherent problem with these studies however is that they are not true experiments, in that variables cannot be controlled, and along with the independent variable (e.g., presence of caudal males), other variables (e.g. overall number of males, number of adjacent males) may systematically covary and perhaps contribute to, or obscure, the effect of the independent variable. In Experiment 3, (Chapter 4) the effects of several prenatal variables on female genital morphology were assessed simultaneously, using a statistical procedure that, while assessing the effect of a particular variable (e.g. number of adjacent males), controls for the effect of other

variables (e.g. number of males in uterine horn). In this way, the contribution of each factor independent of the others could be ascertained.

Support for the caudal male hypothesis in rats stems exclusively from studies measuring behavioral patterns, and inferences regarding intrauterine transport of androgen are based on behavioral differences observed between females from different intrauterine positions. However, no evidence for differences in prenatal androgen levels, presumably underlying behavioral differences in adulthood has been provided thus far in rats. In Experiment 4, described in Chapter 5, testosterone levels of female fetuses with and without caudal males were compared and in addition, these levels were related to several other prenatal factors of theoretical interest, such as sex ratio of the litter and androgen level in maternal blood.

The transport of androgens from male to female fetuses located rostrally is hypothesized to occur via the uterine vasculature. In Chapter 6, a description of the uterine vasculature of the rat is given, and a review is presented of the research on which the assumptions underlying the caudal male hypothesis are based.

Effects of intrauterine position on sexual behavior in adulthood are presumably mediated through differences in prenatal hormonal environment. This prenatal environment in turn, supposedly affects sexual behavior through changes in the CNS. However, the exact relationship between specific CNS structures supposedly involved in this process, such as the SDN-POA, and the expression of sexual behavior in adulthood is by no means clear. Therefore, two studies were performed to clarify the relationship between the perinatal hormonal environment, the CNS and sexual behavior in adulthood. In Studies 5 and 6 (Chapter 7) effects of manipulation of the perinatal hormonal environment on the SDN-POA and sexual behavior of male rats were examined. Changes in the SDN-POA, brought about by changes in the hormonal status around birth, were related to changes in sexual behavior in adulthood.

CHAPTER 2

MASCULINIZATION AND DEFEMINIZATION OF FEMALE RATS BY MALES LOCATED CAUDALLY IN THE UTERUS

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Physiology and Behavior 48:555-560; 1990

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CHAPTER 2

ABSTRACT

It is assumed that female rats are masculinized by the presence of males in the same uterine horn. Two hypotheses regarding the mechanism have been proposed: I. interamniotic diffusion of androgens (contiguity hypothesis) II. transport of androgens via the bloodflow (caudal male hypothesis). This study was designed to test these hypotheses while taking into account two previously uncontrolled factors: hemihysterectomy of the mother during pregnancy and birth by caesarean section. Pregnant females were hemihysterectomized during pregnancy or left intact, pups were born naturally or through caesarean section. Position in utero was determined. In adulthood all females were ovariectomized and tested for mounting behavior before and during testosterone treatment and lordosis behavior during estradiol treatment. It was found that females with males located caudally in the same uterine horn were more masculinized and defeminized than females without such males. Adjacent males had no influence on the behavioral sexual differentiation of females. These results confirm the 'caudal male hypothesis' rather than the 'contiguity hypothesis'. Hemihysterectomy during pregnancy prevented the 'caudal male effect'. Birth through caesarean section did not interfere with the caudal male effect.

INTRODUCTION

Normally developed female rats may frequently show high levels of mounting behavior. The frequency of this behavior in standardized tests, however, appears to be subject to large individual variability (e.g. Slob and van der Schoot, 1982, van de Poll et al., 1982) which cannot be reduced by adult hormone treatment and/or previous mounting experience (de Jonge et al., 1986).

Since perinatal androgens are important for the organization of sexual behavior in rats, it has been hypothesized that endogenous, prenatally circulating androgens may be responsible for this variability in female mounting behavior (Clemens et al., 1978). In support of this hypothesis detectable testosterone levels have been found in female fetuses (e.g. Baum et al., 1988, Slob and Vreeburg, 1985), and suppression of androgen action by prenatal administration of anti-androgens, such as cyproterone acetate (Stewart et al., 1971, Ward and Renz, 1972), or flutamide (Clemens et al., 1978) reduced adult mounting behavior in females.

Yet the source of these prenatal androgens in females remains controversial. Male siblings in utero have been suggested as one possible source of androgen. These androgens could reach the female fetus by diffusion through the amniotic membrane, such that females located between two males would be more masculinized (i.e. show more mounting behavior) than others ('contiguity hypothesis') (Clemens et al., 1978).

A different mechanism has been proposed by Meisel and Ward (1981), whose findings did not support the contiguity hypothesis. They suggested that vascular bloodflow, supposedly flowing from the cervix to the ovary, transports androgens to prenatal females such that females would be masculinized by

androgens secreted by males located caudally, i.e. 'upstream' from the female ('caudal male hypothesis').

While some studies on the effects of male siblings on mounting behavior favor the 'contiguity hypothesis' (Tobet et al., 1982) others support the 'caudal hypothesis' (Richmond and Sachs, 1984) (rats), (Vomachka and Lisk, 1986) (hamsters). Therefore definitive conclusions on the precise influence of prenatal male siblings on mounting behavior of adult female rats cannot yet be drawn.

We hypothesized that some of the controversies in the hitherto results might originate from differences in methodological procedures used. In some studies, pups were born through caesarean section, while in others, pups were delivered naturally (watched delivery). Furthermore, some mothers were hemihysterectomized during pregnancy and others were left intact. These factors might well be relevant, since stress during pregnancy has been shown to influence sexual differentiation of the offspring (Ward and Weisz, 1980, 1984, Rhees and Fleming, 1981, Ward, 1972, Ward, 1977, Herrenkohl, 1979).

We undertook this study to investigate whether male siblings influence mounting behavior according to the 'contiguity hypothesis' or according to the 'caudal male hypothesis', while taking into account two previously uncontrolled factors: 1. hemihysterectomy of the mother during pregnancy and 2. birth by caesarean section.

METHOD

Procedure

Twenty-two albino Wistar female rats (obtained from Harlan Sprague-Dawley, Zeist, The Netherlands) were housed three or four to a cage with food and water ad lib and kept on a reversed 14-10 hr light-dark cycle.

All females were time-mated in our laboratory. A two by two design was used with variables 'hemihysterectomy during pregnancy' and 'caesarean section'. Two weeks before impregnation the animals were divided into four groups in order to create a two by two design with variables 'hemihysterectomy during pregnancy' and 'birth by caesarean section': group A was hemihysterectomized 14 days before impregnation and delivered naturally (n=6), group B was hemihysterectomized between days 10 and 13 of pregnancy and delivered naturally (n=6), group C was hemihysterectomized during pregnancy (days 10-13) and pups were delivered by caesarean section (n=6) and group D was left intact during pregnancy and pups were delivered by caesarean section (n=3). Hemihysterectomy before pregnancy (group A) was performed in order to create a group that was left undisturbed during pregnancy and gave birth naturally.

Female pups that were born by caesarean section were fostered to a mother that had given birth 1 or 2 days before. All litters were culled or increased to 8-11 with 2 or 3 males. In our lab, we had no reliable equipment to measure anogenital distances, so no data were obtained for this measure. Animals were handled once a week throughout the experiment. At 21 days of age females were weaned and housed three or four to a cage under standard laboratory conditions. Bilateral ovariectomy took place between days 49 and 55 and two weeks later behavioral

testing started.

The animals were tested for mounting behavior on three consecutive days. Thereafter the animals received testosterone propionate (TP, 250 μg/day) for three weeks and were tested for mounting behavior once a week.

Three weeks after the last test for mounting behavior, the animals received daily injections of 1 μg estradiol benzoate (EB) for seven days. Tests for lordosis behavior were conducted on days 4, 6 and 8 of this treatment.

Hormones were dissolved in 0.1 ml olive oil and injected sc.

Behavioral tests

Ovariectomized females and sexually active males were used as stimulus animals in the tests for sexual behavior. Stimulus females were brought into heat by 40 μg EB 48 hours and 2.5 mg Progesterone (P) 4 hours before the test.

Tests for sexual behavior were carried out in semicircular cages (r=35 cm) under dim red light illumination during the first quarter of the dark period.

In the tests for mounting behavior, the experimental female was allowed to adapt to the environment for 5 minutes, before a stimulus female was introduced. For 15 minutes mounts with pelvic thrusts and intromission-like behaviors were scored. These two behaviors were taken together in the analysis of the data.

In the tests for lordosis behavior, a sexually active male was allowed to adapt to the environment for 5 minutes. After introduction of the experimental female, the male was allowed to mount the female 10 times. Lordosis was operationally defined as an arching of the back, lifting of the head and stretching of the hindlegs. To obtain 10 mounts, some females were exposed to more than one male. A lordosis quotient (LQ) was calculated for each female by dividing the number of times a lordosis was displayed by the number of mounts (10), multiplied by 100%.

Surgical procedures

All surgical procedures were performed under light ether anesthesia. Hemihysterectomy was performed by making one midline incision in the abdomen, ligating one uterine horn at both ends and removing it. Caesarean section was performed approximately 6 hours before expected parturition. The female was killed by cervical dislocation. Uterine horns were quickly removed through a midline incision in the abdomen. Pups were removed individually, laid out according to uterine position, cleaned, sexed and individually marked by toe-clips. They were fostered to a mother who had delivered approximately one or two days before.

With watched delivery, at the time of the expected parturition, an observer watched every 10 minutes to determine the onset of birth. Each pup was removed from the mother immediately after birth, sexed, toe-clipped and given back to the mother before the next pup was born.

Analysis of data

Added onto the two by two design (with variables 'hemihysterectomy of the mother during pregnancy' and 'birth by caesarean section') was a third variable: position in utero. With regard to this variable, two different classifications were

used.

Caudal male classification

To test the caudal male hypothesis females were grouped according to the mere presence (n=39) or absence (n=22) of caudal males in the same uterine horn regardless of the number of caudal males. This strategy is based upon Meisel and Ward (1981) who concluded that the presence of one male located caudally is as effective in inducing masculinization as is the presence of two or more. Consistent with their finding the present experiment found no significant behavioral differences between females with one and females with two or more males located caudally over the three tests ($F \leq 1.2$, $p \geq .28$ in all cases).

Contiguity classification

To test the 'contiguity hypothesis', females without adjacent males (fFf, n=24) were compared to females with two adjacent males (mFm, n=9).

Before TP treatment the females were tested on 3 consecutive days. In the data analysis these three sessions were collapsed to provide a single pre-test mean for each subject.

The data were subjected to four-way ANOVA involving three between-group factors: hemihysterectomy of the mother during pregnancy (HE), caesarean section (CS), **either** presence of caudal males (CAU) **or** adjacent males (ADJ) in utero, and one repeated measures factor tests (TEST) (four in the analysis of masculine sexual behavior and three in the analysis of feminine sexual behavior).

RESULTS

Masculine sexual behavior

Figure 1 shows mean mount frequencies of females grouped according to the 'contiguity classification'. Females of all groups displayed high levels of mounting behavior during testosterone treatment. Mount frequencies increased over the tests (TEST, $F(3,75)=30.93$, $p<.001$). No significant effects of the hemihysterectomy, caesarean section or contiguity of males ($F(1,25) < 3.9$, $p>.05$ in all cases) were found.

When data were analyzed using the 'caudal male classification' a significant effect of caudal males was revealed. Females with males caudally showed more mounting behavior than females without males caudally (CAU, $F(1,53)=4.46$, $p < .04$). Mounting increased over the tests (TEST, $F(3,159)=94$, $p < .001$), but no effects of the hemihysterectomy or caesarean section ($F(1,53)<.06$, $p>.8$) in all cases) were detected.

There was a significant interaction between the factors 'caudal males' and 'hemihysterectomy' ($F(1,53)=4.68$, $p=.04$). Further analysis (two-way ANOVA, factors CAU and TEST) showed that in the group of females from mothers that were not hemihysterectomized during pregnancy the effect of caudal males was highly significant (CAU, $F(1,26)=11.66$ $p=.002$), whereas in females born to mothers that were hemihysterectomized during pregnancy no such effect was present at all (CAU, $F()=.07$, $p>.7$) (Fig.2).

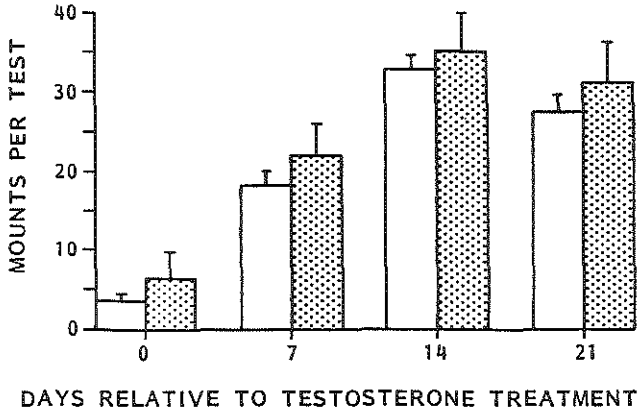

Fig.1 Frequency (mean ± SEM) of mounting behavior before and during testosterone treatment (TP, 250 µg/day) of adult female rats during 15-minute pair-test with estrous female. Solid bars: females without adjacent males prenatally (n=24); dotted bars: females with two adjacent males prenatally (n=9). Groups did not differ significantly.

In order to test whether **adjacent** caudal males rather than caudal males in general are important in inducing masculinization of females, females with caudal males from the 'no-hemihysterectomy-group' were subdivided into a group that had an adjacent caudal male (n=9) and a group that did not (n=9). No significant difference in mounting behavior between these groups was detected (ANOVA, factors: adjacent caudal male and tests, $F(1,16)=.33$, $p>.57$).

No significant correlation was found between number of caudal males and mean number of mounts on all tests for the 'no-hemihysterectomy-group with caudal males' (Spearman correlation coefficient=.005, $p>.49$).

As the statistics used in the analysis require independent observations and scores obtained from females from the same uterine horn do not meet this criterion, a post-hoc analysis was performed in which the 'caudal male' hypothesis was tested using only data from one randomly chosen female with or without caudal males per litter. Again this analysis (two-way ANOVA, factors: CAU and TEST) revealed that females with males located caudally (n=9) displayed significantly more mounting behavior than control females (n=9) ($F(1,16)=4.37$, $p=.05$).

There were five females that came from all-female litters. Mean mount frequencies (±SEM) for this subgroup for the four tests were 4 (±2.6), 16 (±4.9), 34 (±3.7) and 21 (±4.2), respectively.

Feminine sexual behavior

When data were analyzed according to the 'contiguity classification' no effect of contiguity to males (ADJ, $F(1,25)<.34$, $p=.57$) on lordosis quotient was found (see Fig. 3).

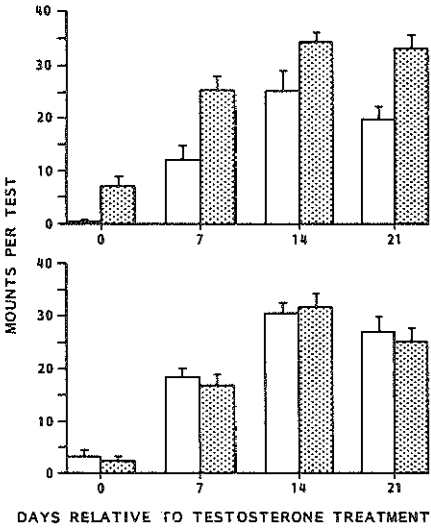

Fig.2 Frequency (mean ± SEM) of mounting behavior before and during testosterone treatment (TP, 250 µg/day) of adult female rats during 15-minute pair-test with estrous female. Solid bars: females without caudal males prenatally (n=10 for top figure, n=12 for bottom figure); dotted bars: females with caudal male(s) prenatally (n=18 for top figure, n=21 for bottom figure). Top: females born to mothers which were left undisturbed during pregnancy. Females with caudal males displayed significantly more mounting behavior, p<.003. Bottom: females born to mothers which underwent hemihysterectomy around day 11 of pregnancy. Groups did not differ significantly.

Hemihysterectomy during pregnancy of the mother had a significant effect on lordosis quotients: females from mothers that were hemihysterectomized during pregnancy had higher lordosis quotients than females from mothers that were not hemihysterectomized (HEMI, $F(1,25)=8.97$, $p=.006$). Caesarean section had a significant effect: females that were delivered by caesarean section displayed less feminine sexual behavior than females that were born naturally (CS, $F(1,25)=5.37$, $p=.03$). Lordosis quotients increased over the tests (TEST, $F(2,50)=4.09$, $p=.02$).

When data were analyzed using the caudal male classification females without caudal males prenatally tended to have higher lordosis quotients than females with caudal males, albeit this difference did not reach statistical significance (CAU, $F(1,53)=2.54$, $p=.12$). No other significant main or interaction effects were found either ($F(1,53)<2.6$, $p>.11$ in all cases).

Because hemihysterectomy during pregnancy had been shown to disrupt the effect of caudal males on mounting behavior, separate analyses (ANOVA, factors CAU and TEST) were run for the 'no-hemihysterectomy-group' and the 'hemihysterectomy-group' on lordosis behavior (see Fig. 4).

Again, as was the case for mounting behavior, in the 'hemihysterectomy-group' no significant difference between the groups in lordosis behavior could be

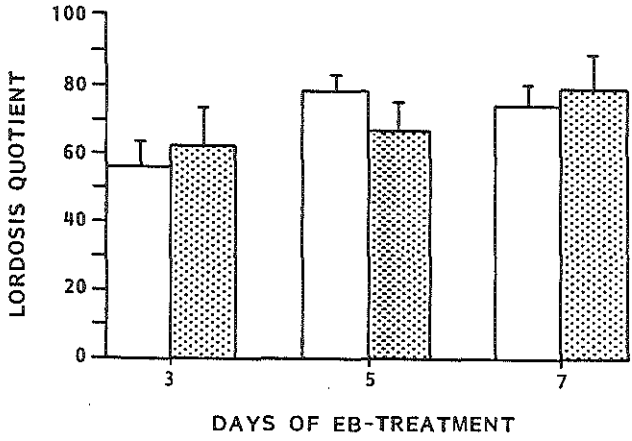

Fig.3 Frequency (mean ± SEM) of lordosis behavior following 3, 5 and 7 days of estradiol treatment (EB, 1 µg/day) of adult female rats during pair-test with active male (10 mounts). Solid bars: females without adjacent males prenatally (n=24); dotted bars: females with two adjacent males prenatally (n=9). Groups did not differ significantly.

detected (CAU, $F(1,31)=.01$, $p>.9$), whereas in the 'no-hemihysterectomy-group' females without males caudally displayed significantly more lordosis behavior than females with males caudally (CAU, $F(1,26)=6.02$, $p=.02$).

Mean lordosis quotients (±SEM) during the three tests for the five females from 'all-female' litters were 60 (±13.6), 80 (±8.9) and 64 (±11.7), respectively.

No significant differences were found when 'no-hemihysterectomy' females with an adjacent caudal male were compared with 'no-hemihysterectomy' females with non-adjacent caudal males (ANOVA, factors: adjacent caudal male and tests, $F(1,16)=.00$, $p=1.0$).

There was a significant negative correlation between mean lordosis quotient and number of caudal males in the 'no-hemihysterectomy-group with caudal males' ($n=18$), (Spearman correlation coefficient=-.4, $p<.05$).

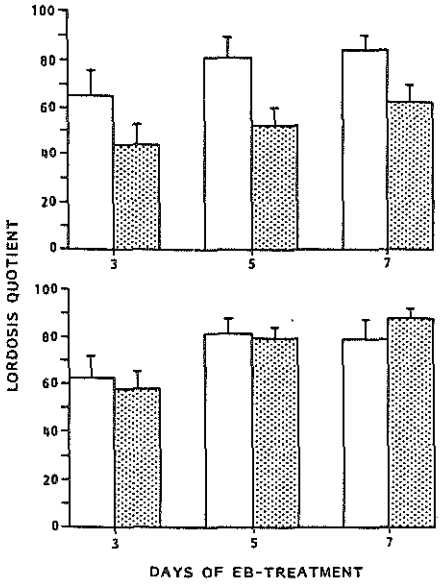

Fig. 4 Frequency (mean ± SEM) of lordosis behavior following 3, 5 and 7 days of estradiol treatment (EB, 1 µg/day) of adult female rats during pair-test with active male (10 mounts). Solid bars: females without caudal males prenatally (n=22); dotted bars: females with caudal male(s) prenatally (n=39). Top: females born to mothers which were left undisturbed during pregnancy. Females with caudal males had significantly lower lordosis quotients, p<.03. Bottom: females born to mothers which underwent hemihysterectomy around day 11 of pregnancy. Groups did not differ significantly.

DISCUSSION

The results obtained in this study confirm the idea that female fetuses are exposed to some masculinizing agent, presumably testosterone, from caudal males with whom they share the same uterine horn. The presence of adjacent male fetuses per se did not have such an effect on adult masculine and feminine sexual behavior of females. These results confirm the 'caudal male hypothesis' rather than the 'contiguity hypothesis'.

This 'caudal male effect' was disrupted by hemihysterectomy of the mother during pregnancy. Birth by caesarean section did not interfere with the 'caudal male effect'.

Females with males positioned caudally in the same uterine horn showed more mounting behavior before and during testosterone treatment and less lordosis behavior in adulthood than females with no male fetuses caudally, whereas adjacent males had no effect on behavioral sexual differentiation of the females.

These results support the work from other investigators who found higher frequencies of testosterone-induced mounting behavior (Richmond and Sachs,

1984, Meisel and Ward, 1981) in females that developed with males positioned caudally in the same uterine horn than in others. Although some studies have failed to find support for the caudal male hypothesis (Brand et al., 1990) hemihysterectomy of the mother during pregnancy may account for this discrepancy.

A new finding in the present study is the effect of male siblings located caudally in the same uterine horn on lordosis behavior of females. A decrease in feminine sexual behavior in females after prenatal administration of TP has been reported in many studies (see Ward and Ward, 1985), indicating that the critical period for defeminization starts prenatally.

Meisel and Ward (1981) reported that lordosis behavior was not affected by the presence of caudal males in utero. In their experiment females were injected with 10 μg EB, followed by an injection with 1 mg Progesterone and mean lordosis quotients were high in all groups. In the present experiment a lower dose of EB was used, wich appears to have allowed a more subtle distinction to be made between the various experimental groups than was possible in the work of Meisel and Ward (1981).

In the present study, the masculinizing and defeminizing effects of caudal males were disrupted by a hemihysterectomy of the mother during pregnancy. Stress during pregnancy has been reported to change the testosterone secretion of male fetuses (Ward and Weisz, 1980), such that the peak in testosterone levels in males which normally occurs on days 18 and 19 is advanced to day 17. Other evidence suggests that male offspring of stressed mothers are less defeminized and masculinized than controls (Ward, 1972, 1970). Thus, it is possible that in the present study the hemihysterectomy induced similar 'stress effects' in male fetuses, thus changing their testosterone secretion. One could hypothesize that females in the 'hemihysterectomy-group' were then less affected by testosterone from caudal males. Also, overall levels of lordosis behavior were somewhat higher in 'hemihysterectomy-females' than in the 'no-hemihysterectomy' group, but this difference was not statistically significant. Further research is necessary to examine whether the absence of an effect of caudal males in the 'hemihysterectomy-group' is due to the stress of the operation or to the specific operation itself.

Although in the 'adjacent males analysis' all females between two males were also females with at least one caudal male, this did not lead to significant differences between these females and females between two females. More than half (54%) of the animals in the latter group constituted of females with caudal males too and this may account for this absence of significant differences.

Unfortunately, there were only nine females that developed in utero between two males. In the analysis of main effects of uterine position, hemihysterectomy and caesarean section, no comparisons were made between individual cell means and so the small numbers were not likely to affect the validity of the conclusion. But because of these small cell numbers, specific conclusions about (the lack of) interaction effects are limited and must be drawn cautiously.

It is not clear how androgens may be transported from males to females located rostrally in utero. It has recently been argued (vom Saal et al., 1989) that the uterine blood flow in rats is not unidirectional, as was earlier suggested (Del

Campo and Ginther, 1972), but some other as yet unknown mechanism may be responsible.

Clemens et al. (1978) suggested that transportation of androgens from males to females is mediated by diffusion through the amniotic sac. This 'contiguity' hypothesis was derived from the observation that females that developed in utero between two males were more masculinised (i.e. displayed more mounting behavior and had greater anogenital distances at birth) than those in other uterine positions. A similar finding was reported by other authors (Tobet et al., 1982), who found that females that developed between two males became anovulatory at an earlier age when given a low dose of TP neonatally than females between two females. Meisel and Ward (1981) have argued that the results of Clemens et al. (1978) need not be contradictory to the caudal male hypothesis since females with two adjacent males have at least one male caudally. In light of the results of the present study, however, it seems unlikely that caudal males are responsible for the differences between females with two adjacent males and others as described by Clemens (1978), since in that study a hemihysterectomy was performed in all pregnant females, an operation that in the present experiment disrupted the 'caudal male effect'. No alternative explanation for their results can be given at this point.

Both hemihysterectomy of the mother during pregnancy and birth by caesarean section decreased lordosis quotients of female offspring, but these effects were only found when the 'contiguity classification' was used, i.e. when data of females between one male and one female were excluded. When data of all females were analyzed, no such effects were found. Caesarean section was performed approximately six hours before expected time of delivery, and the presence of caudal males had apparently already been effective in 'organizing' the downstream females.

In the present study we found that number of caudal males might have some influence on the degree of defeminization: there was a negative correlation between number of caudal males and lordosis behavior. No effects of number of caudal males on mounting behavior were found. This issue needs further investigation.

It should be noted that females without males caudally in the same uterine horn and even females from all-female litters exhibited high levels of mounting behavior. Apparently testosterone from male littermates can not be the only prenatal masculinizing agent, rather it seems to have an additive effect. Other sources of prenatal androgens have been suggested, including the placenta (e.g. Slob and Vreeburg, 1985) and the maternal ovaries (Sridaran et al., 1981, Witcher and Clemens, 1987).

CONCLUSIONS

Female rats are prenatally masculinized (i.e. show increased mounting behavior) and defeminized (i.e. show decreased lordosis behavior) by caudal males but not by adjacent males in the same uterine horn. These results consolidate the 'caudal male hypothesis' rather than the 'contiguity hypothesis'. Although the mechanism underlying this phenomenon is not yet clear, vascular flow between fetuses may be responsible.

This 'caudal male effect' is disrupted by hemihysterectomy of the mother during pregnancy. Whether this is due to the stress of the surgery or to the specific operation itself, remains to be investigated.

Birth by caesarean section does not interfere with the 'caudal male effect'.

It should be noted that the effect of male littermates is clearly additive: females without male fetuses caudally and females from all-female litters also display high levels of testosterone-induced mounting behavior.

ACKNOWLEDGEMENTS

The authors would like to express their appreciation to Jan van Ophemert for help with behavioral testing and maintenance of the animals and to Drs. J.J. van der Werff ten Bosch, D.L. Rowland, N.E. van de Poll and F.H. de Jonge for their helpful suggestions and critical reading of the manuscript.

CHAPTER 3

MALES LOCATED CAUDALLY IN THE UTERUS AFFECT SEXUAL BEHAVIOR OF MALE RATS IN ADULTHOOD

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CHAPTER 3

ABSTRACT

It has been suggested that the sexual differentiation of female rats is affected by androgens from male fetuses in the uterine horn (intrauterine position phenomenon). Effects of contiguous males, as well as of males located caudally in the uterus, have been reported. The present study investigated whether male rats, like females, are affected by the presence of either caudal or contiguous male littermates.

When tested in adulthood for sexual behavior, males that had male fetuses located caudally in the uterine horn showed shorter latencies to the first mount or intromission and shorter latencies to ejaculation, and exhibited more mounts and intromissions per minute than males that lacked caudal male siblings in the uterus. The presence of contiguous males did not significantly affect the parameters studied in this experiment.

INTRODUCTION

As a result of exposure to higher levels of testosterone perinatally, male rats show more masculine sexual behavior (mounts, intromissions and ejaculations) in adulthood than females (Baum, 1979). Testosterone secretion in male rat fetuses starts around day 14 of gestation, and peaks on days 18 and 19 (Baum et al., 1991, Slob et al., 1980, Ward and Weisz, 1980). This testosterone not only affects males' own sexual differentiation, but supposedly affects female fetuses in the same uterine horn as well (Clemens et al., 1978, Meisel and Ward, 1981, vom Saal and Bronnson, 1978).

The manner in which androgens from males reach female fetuses remains uncertain. It has been reported that females that develop in utero between two males are more masculinized behaviorally (i.e., show more mounting behavior) and somatically (i.e., have larger anogenital distances at birth) than females located between two females. This finding prompted the hypothesis that androgens from males reach females by diffusion through the amniotic membrane (contiguity hypothesis) (Clemens et al., 1978, vom Saal and Bronson, 1978). Support for this hypothesis has most often been provided in studies using mice and gerbils (e.g. (Clark et al., 1990, vom Saal, 1981, Zielinski et al., 1991, Zielinski et al., 1992), although non-supportive findings in mice and rats have been reported as well (Gandelman and Kozak, 1988, Houtsmuller and Slob, 1990, Meisel and Ward, 1981). In view of their non-supportive findings, Meisel and Ward (1981) proposed the alternative hypothesis that androgens from males reach females through the blood supply, which supposedly flows from the cervix in a rostral direction (Del Campo and Ginther, 1972). This hypothesis was derived from the observation that female rats that have males located caudally (i.e., females that are located 'upstream' from males with regard to bloodflow) in the uterus are somatically and behaviorally more masculinized than females without such males. Several, though not all (e.g. Baum et al., 1991, Brand et al., 1990), studies have provided subsequent support for this hypothesis in rats (Houtsmuller and Slob, 1990, Richmond and Sachs, 1984), hamsters (Vomachka and Lisk, 1986, and ferrets

(Krohmer and Baum, 1989).

In studies addressing this 'intrauterine position phenomenon', effects of male fetuses on females have been described extensively in a variety of species. Only recently it was reported that males, like females, may also be affected by hormones secreted by littermates in the same uterine horn. Specifically, male mice located between two females had higher levels of estradiol prenatally and showed, surprisingly, more mounting behavior and less aggression in adulthood than other males (vom Saal et al., 1983). Male gerbils that developed in utero between two males were found to mount females with shorter latencies, ejaculate after fewer intromissions, and sire more young than males that developed between two females (Clark et al., 1990, Clark et al., 1992). Therefore, sexual differentiation of male mice and gerbils may be affected by their position in utero relative to male and/or female littermates.

The present study was designed to investigate the effects of male littermates in the uterus on sexual differentiation of male rats. Males of known intrauterine position were repeatedly tested for masculine sexual behavior, and the effects of the presence in utero of adjacent, and of caudal males on several parameters of masculine sexual behavior were investigated.

MATERIALS AND METHODS

Twenty-three female Holtzman rats (Madison, WI) whose offspring were to become subjects for this study, were housed individually and kept on a 14-10 hr light dark cycle. Females were hemihysterectomized so relative pup position in utero could be determined by watching birth order (Tobet et al., 1982, Houtsmuller and Slob, 1990). Hemihysterectomy was performed under light ether anesthesia by making a midline incision in the abdomen, ligating the left uterine horn at both ends and removing it. The left ovary was left in situ. Two weeks after hemihysterectomy, females were mated at approximately the same time of the day. At the expected time of birth, an observer inspected the females every 10 min to determine the onset of parturition, so that order of birth could be established.

At birth each pup was toe-clipped and weighed, and position in utero was established. Animals were handled weekly throughout the experiment. At 21 days of age pups were weaned, and males were housed individually under standard laboratory conditions. Behavioral tests began when the male offspring were 17-18 weeks old. Three weekly tests for masculine sexual behavior were run.

Behavioral tests

Ovariectomized females were used as stimulus animals in the tests for sexual behavior. Stimulus females were brought into heat by 50 ug estradiol benzoate (EB) 48 hours (subcutaneous) and 2.5 mg progesterone (P) 17 hours (intramuscular) before the test. These hormones were dissolved in 0.1 ml of peanut oil.

Tests for masculine sexual behavior were carried out in rectangular cages (48 cm by 38 cm) under dim red light illumination during the first quarter of the dark period. The experimental animal was allowed to adapt to the testing environment

for 5 minutes before a stimulus female was introduced. Mounts, intromissions and ejaculation, as well as the latency to these behaviors, were scored. Testing ended after the animal ejaculated or, if no ejaculation occurred, after 45 minutes.

Analysis of data

Caudal male classification: To test for effects of caudal males, animals were grouped according to the presence (n=12) or absence (n=29) of caudal males in the uterine horn, regardless of the number of caudal males. This strategy was based on that of Meisel and Ward (1981), who concluded that the presence of one caudal male is as effective in inducing masculinization as is the presence of two or more. Our previous work confirmed that suggestion (Houtsmuller and Slob, 1990), and the present experiment found no statistically significant differences in measures of masculine sexual behavior between males with one and males with more than one caudal male ($F<1.1$, $p>.38$).

Contiguity classification: To test for effects of contiguous males, animals without contiguous males (n=15) were compared to animals with one (n=18) or two contiguous males (n=8).

Data on latencies and frequencies were analyzed using a 2 x 3 factorial ANOVA, with one between group factor being either 'caudal males' (CAUD) or 'contiguous males' (CONT), and one repeated measures factor (TEST) representing three successive tests for masculine sexual behavior. When data did not meet the requirement of homogeneity of variance, a logarithmic transformation was carried out before performing ANOVA. Numbers of animals responding (showing mounts and intromissions, or ejaculation) within the different groups were compared using Fisher's Exact test or the z-test. All probabilities represent two-tailed significance levels. Post hoc analysis was accomplished using the same procedure as for the overall analysis, with alpha adjusted downward to .01. Interaction effects are reported only when significant.

RESULTS

Latency to first sexual action (mount or intromission)

Figure 1 shows mean latency to the first mount or intromission, whichever came first, of males grouped according to the caudal male classification (top) and the contiguity classification (bottom). Animals that did not show any mounting behavior during the entire test were assigned a value of 2700 seconds (maximum length of the test in seconds, e.g. de Jonge et al., 1989). Males with caudal males showed significantly shorter latencies than males without caudal males (CAUD:$F(1,39)=7.53$, $p<.01$). Latencies decreased over tests for both groups (TEST:$F(2,78)=33.25$, $p<.001$). When data were analyzed according to the contiguity classification, groups did not differ (CONT:$F(2,38)=1.59$, $p=.216$). Latencies for all three groups decreased over tests (TEST:$F(2,76)=42.74$, $p<.001$).

Ejaculation latency measured from onset of testing

Fig. 2 shows mean ejaculation latencies for males, grouped according to both the caudal male classification (top) and the contiguity classification (bottom). Animals that did not ejaculate during the test were assigned a value of 2700 sec.

Mean ejaculation latencies were longer for males without caudal males than for males with caudal males (CAUD: $F(1,39)=4.21$, $p=.047$). Ejaculation latencies became shorter over tests (TEST: $F(2,78)=8.47$, $p<.001$). There was a marginally significant interaction effect between the factors CAUD and TEST ($F(2,78)=2.68$; $p=.063$). Further analysis revealed that ejaculation latencies became shorter over tests in the group with caudal males (TEST: $F(2,56)=23.71$; $p<.001$), but not in the group without caudal males (TEST: $F(2,22)=0.42$; $p=.665$). The difference between the groups was greatest, and significant, on test 3 ($F(1,39)=10.47$, $p=.002$.

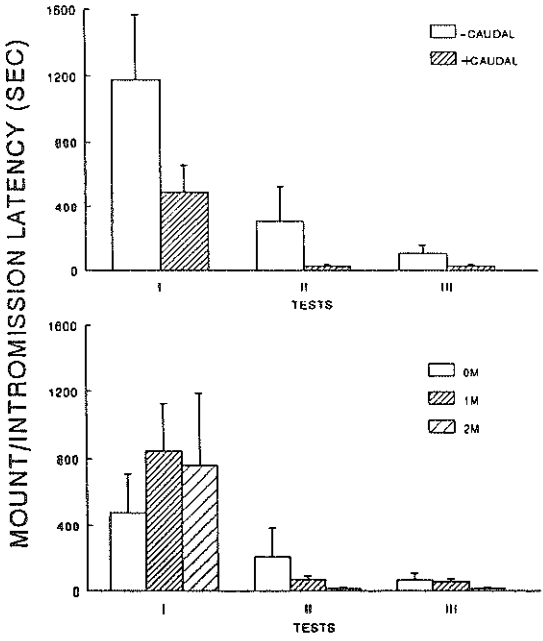

Fig.1 Mean (±sem) latency to the first mount or intromission, whichever came first, for males on three 45 minute tests for masculine sexual behavior. Males are grouped according to the presence of caudal males in utero (top, -caudal: no caudal males, +caudal: caudal males), and the presence of contiguous males in utero (bottom, 0M: no contiguous males, 1M: 1 contiguous male, 2M: 2 contiguous males).

When data were analyzed according to the contiguity classification, (Fig. 2, bottom) no significant differences among all three, or between 0M and 2M groups were found (CONT: $F<.59$, $p>.45$). Ejaculation latencies for all groups decreased over tests (TEST: $F(4,76)=16.56$, $p<.001$).

Means for ejaculation latency for responders only (i.e., ejaculators) are shown in table 1. Although these means generally show the same pattern as the means for the entire group, there were no statistically significant differences between the groups when data were analyzed according to either the caudal male

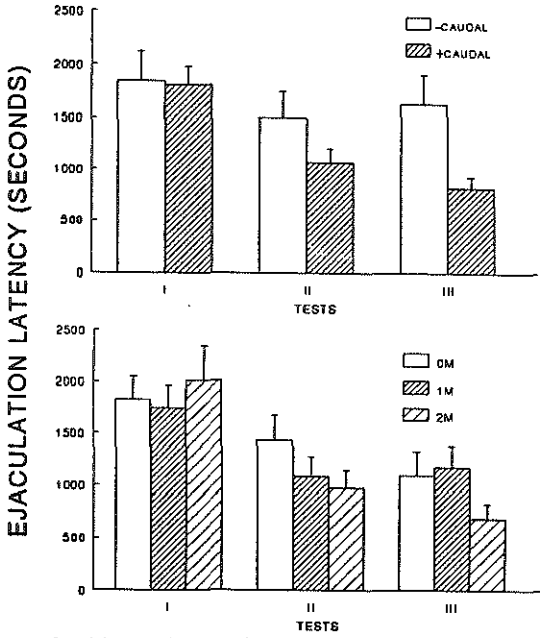

Fig.2 Mean (±sem) latency to ejaculation for males on three 45 minute tests for masculine sexual behavior. Subjects are grouped according to the presence of caudal males in utero (top, -caudal: no caudal males, +caudal: caudal males), and the presence of contiguous males in utero (bottom, 0M: no contiguous males, 1M: 1 contiguous male, 2M: 2 contiguous males).

Table I. Mean (±SEM) ejaculation latencies for males that ejaculated are shown for each of three tests for masculine sexual behavior. Males are grouped according to their position in utero relative to males.

	Caudal males				Contiguous males					
	Absent		Present		Zero		One		Two	
Tests	n	(n=12)	n	(n=29)	n	(n=15)	n	(n=18)	n	(n=8)
I	6	999	18	1264	9	1233	11	1122	4	1324
		(213)		(178)		(222)		(217)		(446)
II	10	1257	27	948	13	1241	16	886	8	982
		(242)		(113)		(222)		(141)		(166)
III	8	1096	28	745	13	849	15	873	8	686
		(231)		(100)		(189)		(145)		(145)

classification or the contiguous male classification (CAUD:$F<2.46$, $p>.12$,

CONT:$F<1.13$, $p>.33$).

Ejaculation latency from the first sexual action

Latency to ejaculation measured from the beginning of the testing session partly reflects latency to the first sexual action, as these measures correlated significantly ($r=.40$, $p<.01$). To obtain an estimation of ejaculation latency measured from the first sexual action, further analysis was performed using the variable 'ejaculation latency minus latency to the first sexual action'. For this analysis, males that did not ejaculate were assigned a latency of 2700 sec (maximum length of test session) minus latency to the first sexual action. Analysis was limited to test 3. For tests 1 and 2, this procedure would cause problems since animals that started mounting late and did not ejaculate would be assigned unjustly low values for ejaculation latency measured from the first mount or intromission (i.e., 2700 - latency to first mount or intromission). On test 3, all animals had short latencies to the first mount or intromission. This enabled us to use all animals in the analysis, including the ones that did not ejaculate during the test, who constitute an important group when investigating ejaculation latency. Means for ejaculation latency from first mount or intromission on test 3 for the two groups were: males with caudal males 783 ± 117; males without caudal males 1522 ± 253. ANOVA between the two groups indicated a significant effect ($F(1,39)=9.22$; $p=.004$).

Percentage of animals displaying mounts and intromissions, and ejaculation

Table II shows percentage of animals displaying masculine sexual behavior, and percentage of animals ejaculating for each test. Animals were grouped according to the caudal male classification, and according to the contiguity classification.

An overall analysis was carried out combining all three tests. However, because proportions compared only 2 groups at a time, for the contiguity classification, comparisons were made between the group without contiguous males and the group with two contiguous males, that is, the groups expected to show the greates difference. No significant differences between groups were found in proportions of animals showing mounting behavior using either classification ($z<0.4$, $p>.75$).

In contrast, the proportion of animals ejaculating was higher in the group with caudal males than in the group without caudal males ($z=2.13$, $p=.033$). Post hoc analysis showed that this difference was greatest on the third test ($p=.02$). When data were analyzed using the contiguous male classification, no significant differences between groups in proportions ejaculating were found ($z=0.19$, $p=0.85$).

Rate of mounts and intromissions

Males were tested for mounting behavior until they ejaculated, or, if no ejaculation occurred, for the duration of the 45 minute session. Therefore, test length varied between animals and between tests. To control for this variability, for each animal the number of mounts and intromissions was divided by the number of minutes that the animal was sexually active, that is, from the first mount or

intromission to ejaculation or the end of the test. This new variable, mounts and intromissions per minute, was used in the data analysis. Males with caudal males displayed somewhat more mounts and intromissions per minute (CAUD: $F(1,37)=3.74$; $p=.061$) (Fig. 3, bottom) than males without caudal males.

Table II. Percentage of males showing mounts and intromissions, or an ejaculation on three 45 minute tests for masculine sexual behavior. Males are grouped according to their position relative to males in utero.

	Caudal males		Contiguous males		
	Absent (n=12)	Present (n=29)	Zero (n=15)	One (n=18)	Two (n=8)
% mounting/ intromitting					
Test I	67	86	87	78	75
II	92	100	93	100	100
III	100	100	100	100	100
% ejaculating*					
Test I	50	62	60	61	50
II	83	93	87	89	100
III	67	97	87	83	100

**Overall percentage of males ejaculating was higher for males with caudal males than for males without caudal males in utero ($p=.033$). Post hoc tests indicated that this percentage was significantly higher on test 3 ($p=.020$).*

Frequency of mounts and intromissions increased over tests for both groups (TEST: $F(2,74)=15.83$, $p<.001$). When data were analyzed using the contiguity classification, no significant differences between groups were found (CONT: $F(2,36)=$, $p=.607$) (Fig. 3, bottom). Mean number of mounts and intromissions increased over tests (TEST: $F(2,72)=22.23$, $p<.001$).

Analysis of subgroup without contiguous males

In the group of males with caudal males, 76% had at least one contiguous male. Therefore, it could be argued that differences between this group and the group without caudal males may reflect differences between males with contiguous males and males without contiguous males. To rule out the possibility that some of the caudal male effect was contributed by a contiguous male effect, those animals that had no contiguous males were grouped according to the presence (n=8) or absence (n=7) of caudal males and compared on the dependent variables latency

to ejaculation, latency to first mount or intromission, and number of mounts and intromissions. Means are shown in table III.

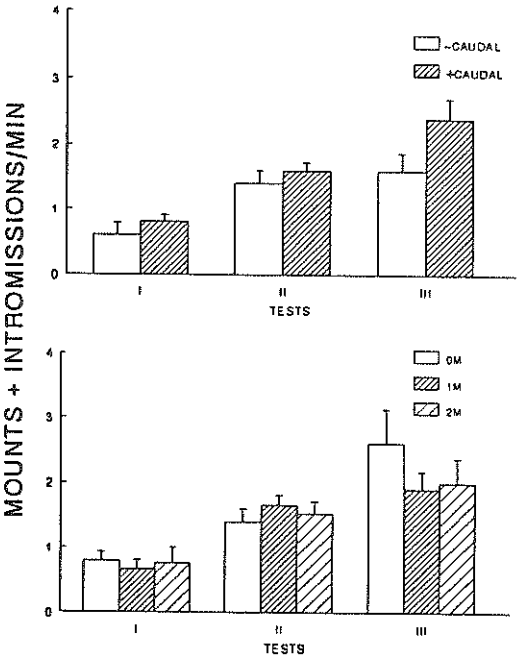

Fig. 3 Mean (±sem) number of mounts and intromissions per minute for males on three 45 minute tests for masculine sexual behavior. Subjects are grouped according to the presence of caudal males in utero (top, -caudal: no caudal males, +caudal: caudal males), and the presence of contiguous males in utero (bottom, 0M: no contiguous males, 1M: 1 contiguous male, 2M: 2 contiguous males).

Despite the low number of animals, males with caudal males showed marginally significantly shorter ejaculation latencies (CAUD: $F(1,13)=4.20$; $p=.061$). Ejaculation latencies decreased over tests (TEST: $F(2,26)=5.56$; $p=.01$).

Males with caudal males also displayed more mounts and intromissions per minute (CAUD: $F(1,13)=7.52$; $p=.017$). Mounts and intromissions increased over tests (TEST: $F(2,26)=20.84$; $p<.001$). There was a significant interaction between the factors CAUD and TEST ($F(2,26)=4.19$; $p=.026$). Further analysis showed that the difference between the groups was significant on test 3 ($F(1,13)=11.53$; $p=.005$).

Latency to first mount or intromission did not differ between groups (CAUD: $F(1,13)=2.45$, $p=.14$). These latencies decreased over tests in both groups (TEST: $F(2,26)=19.51$, $p<.001$).

Table III. Means (±SEM) for ejaculation latency, latency to first mount or intromission, and mounts and intromissions per minute on three tests for masculine sexual behavior, for males without contiguous males in utero. Subjects are grouped according to the presence or absence of caudal males in utero.

		Caudal males	
		Absent (n=8)	Present (n=7)
Ejaculation latency	Test I	1960 (300)	1660 (376)
	II	1786 (318)	1035 (294)
	III	1601 (313)	519 (200)
Latency first mount/intromission	Test I	746 (428)	166 (50)
	II	381 (332)	17 (7)
	III	114 (75)	14 (11)
Mounts+intromissions/min	Test I	0.69 (0.23)	0.91 (0.15)
	II	1.14 (0.24)	1.78 (0.29)
	III	1.42 (0.31)	4.03 (0.81)

DISCUSSION

The results of the present experiment lend support to the idea that androgens secreted by male rat fetuses affect the sexual differentiation of males located rostrally in the same uterine horn. In the present study, the number of males ejaculating was higher in the group with caudal males prenatally than in the group lacking caudal males. Males with caudal males also showed shorter latencies to the first mount or intromission, and shorter ejaculation latencies, than males without caudal males. Overall rate of mounting behavior was higher for males with caudal males as well. The presence of either one or two contiguous males in utero did not affect masculine sexual behavior of males, and in this respect, the present data provide support for the caudal male hypothesis rather than the contiguous male hypothesis.

Earlier reports have already shown that female rats with caudal males in utero are behaviorally and morphologically more masculinized than females that lack caudal male siblings (Houtsmuller and Slob, 1990, Meisel and Ward, 1981, Richmond and Sachs, 1984). Effects of male littermates on behavioral and somatic sexual differentiation of male fetuses have recently been reported for mice (vom Saal et al., 1983) and gerbils (Clark et al., 1990, Clark et al., 1992). The present results indicate that, consistent with other species, behavioral masculinization of male rats can be influenced by male fetuses in the same uterine horn. In contrast

with other species, however, the presence of caudal males rather than contiguous males appears to mediate this effect.

In the present experiment, there were no behavioral differences between males that had one, and males that had two or more caudal males in utero. This absence of increasing masculinization with an increasing number of caudal males is consistent with earlier reports examining female masculinization by males in the same uterine horn (Houtsmuller and Slob, 1990, Meisel and Ward, 1981). A satisfactory explanation for this finding may have to await a better understanding of the dynamics of uterine blood flow in the rat since contradictory findings regarding the direction of the blood flow in rodents have been reported (Del Campo and Ginther, 1972, Egund and Carter, 1974, Even and Dahr, 1992).

Our results show that in rats, males, like females, may be affected by the 'intrauterine position phenomenon', and this finding may partly explain the spontaneous variability in masculine sexual responses observed in rats in laboratory tests. In the present experiment, the effects of the presence of caudal males in utero on ejaculation latency of males became more profound over tests, that is, with increasing sexual experience. Specifically, in the group with caudal males the proportion of males that ejaculated during a 45 min test was higher after sexual experience than in the group without caudal males. Therefore, male rats that developed with males located caudally in utero might be at a reproductive advantage in that, given the same opportunities for sexual interaction, they are faster at developing 'efficient' sexual behavior. The question as to what reproductive consequences prenatal position relative to males has for male rats in a natural environment needs to be addressed. Several reports have begun to investigate such consequences in mice (Zielinski et al., 1991 Zielinski et al., 1992, Zielinski and Vandenbergh, 1991).

CHAPTER 4

A MULTIVARIATE APPROACH TO DETERMINING THE INFLUENCE OF MALE FETUSES ON NEONATAL GENITAL MORPHOLOGY OF FEMALE RATS

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CHAPTER 4

ABSTRACT

Among newborn females considerable variability is found in genital morphology (e.g., anogenital distance, AGD). Presumably, such differences are related to prenatal androgen exposure, with greater exposure resulting in larger AGD's and thus in a trend toward masculinization. The source of prenatal androgen in female fetuses is unclear, but a role for male uterine mates has been implicated. The present study investigated the effect of several prenatal factors related to number and position of males in utero on female AGD in two strains of rats. Because such prenatal factors often show systematic covariance, a methodology that enabled statistical control over variables that could not be experimentally controlled was used. Results confirmed the importance of caudal males to female AGD, and identified two additional variables salient to female genital masculinization, namely the distance of the female fetus from the nearest caudal male, and the overall number of males sharing the same uterine horn. Males positioned next to female fetuses exerted no significant impact on female morphological masculinization. There were no differences in the impact of any of these variables on AGD across strains, suggesting that type and strength of variables affecting female AGD are essentially the same for both strains.

INTRODUCTION

Sex differences in genital morphology in rats result from different exposure to prenatal androgens. Specifically, male neonates, exposed to androgens from their own testes, have a much greater distance between the anus and the genital orifice (anogenital distance [AGD]) than do females, who naturally lack prenatal exposure to high levels of androgens. Anti-androgens administered to male fetuses abolish anatomical (e.g., Gerall, 1973) differences with females, whereas androgen administration to female fetuses enlarges AGD such that these females are indistinguishable from males at birth. Thus, AGD has been used as an index at birth for prenatal androgen exposure, in both untreated and androgen-manipulated fetuses (e.g., Clemens, Gladue, & Coniglio, 1978).

Even among untreated, normally-developing females, there is substantial individual variability in AGD at birth. The fact that highly variable levels of testosterone have been found in such fetuses (e.g., Baum, Woutersen, & Slob, 1991) suggests that natural variation in prenatal levels of androgens may account for AGD differences among females. Since female fetuses presumably secrete no androgens of their own, the question arises as to the source (and therefore, source of variation) of androgens in the female fetus. The placenta has been identified as one likely source of androgen for the female fetus (Vreeburg, Groeneveld, Post, & Ooms, 1983; see also Slob & Vreeburg, 1985). Male fetuses sharing the same uterine horn may also contribute to the androgenization of females, an idea that has been supported in several species. Thus, the number of males within the uterine horn, as well as the position of the female relative to those males, reportedly influences androgen-related sexual development of the female fetus (e.g., mice: Vom Saal & Bronson, 1978; Zielinski, Vandenbergh, & Montano, 1991; rats:

Clemens et al., 1978; Meisel & Ward, 1981; gerbils: Clark & Galef, 1988, see also Brown-Grant & Sherwood, 1971). The robustness of the effect of male uterine mates on female masculinization remains somewhat unclear (Houtsmuller & Slob, 1990; Brand, Houtsmuller, & Slob, 1990), as does the physiological mechanism through which it might occur. Thus far two specific mechanisms of androgen transport from male to female uterine mates have been suggested: diffusion through the amniotic sac from adjacent males (known as the contiguity hypothesis, Clemens et al., 1978, Richmond & Sachs, 1984) and transport via the vasculature from males to females located rostrally (caudal male hypothesis, Meisel & Ward, 1981). However, evidence has been offered both supporting and refuting specific hypotheses regarding the importance of uterine position relative to males (Richmond & Sachs, 1984; Houtsmuller & Slob, 1990; Tobet, Dunlap, & Gerall, 1982, Baum et al., 1991) or the overall number of males in the uterus to female masculinization (Clark, Crews, & Galef, 1991; van de Poll, van der Zwan, van Ooijen, & Pater, 1982; Slob & van der Schoot, 1982). As a result, the issue remains to be clarified.

Some of the uncertainty surrounding the question of female masculinization by males in utero may arise from the use of different methodologies among studies (Houtsmuller & Slob, 1990) as well as inadequate strategies employed for analyzing such data. Specifically, experiments dealing with the influence of a prenatal variable on female masculinization may have been compromised by other prenatal factors that systematically vary with the variable of interest. For example, when determining an effect of caudal males, the number of adjacent males and overall number of males within the uterine horn may show systematic variation with the number of caudal males. Since analysis of variance, the most commonly employed technique for determining the impact of an independent variable in these studies, assumes that variation included in the error term is random (rather than systematic) (Cliff, 1987; Collyer & Enns, 1986), such analysis is not well-suited for this type of design. Other statistical procedures such as multiple regression analysis are more appropriate because they can provide statistical control for extraneous or other explanatory variables which cannot be controlled experimentally. With such a method, the influence of each selected factor, independent of the others (i.e., ceteris paribus), can be ascertained (Cliff, 1987; Johnston, 1984).

The present study attempted to identify various prenatal factors possibly affecting morphological masculinization, assessed through AGD at birth, of female rat fetuses. Specifically, the influence of four factors--the number of males in the uterine horn, the number of males located caudally to the female, the distance of the female to the nearest caudal male, and the number of males adjacent to the female--were examined. The effect of birth weight on AGD was taken into account while assessing these prenatal effects since the size of the neonate may affect its AGD (i.e., smaller animals may have smaller AGD's), independent of prenatal androgen exposure (Graham and Gandelman, 1986). Although such prenatal factors have been investigated individually in previous studies, no study has yet investigated them simultaneously to determine the relative contribution of each to the genital masculinization of female offspring in rats. With the use of multiple regression analysis, a model describing the influence of these prenatal variables on

morphological masculinization is presented.

METHOD

Subjects Sixty-five Holtzman and 29 Wistar female neonatal rats served as subjects for this study. These females were born to mothers who were provided with food and water ad lib, were kept on a 14-10 hr light dark cycle, and were housed individually beginning at least 48 hr before expected parturition. Pups were either delivered naturally, having been born to mothers hemihysterectomized before pregnancy so relative pup position in utero could be determined by watching birth order (Tobet et al., 1982; Houtsmuller and Slob, 1990; Brand et al., 1990), or were born through caesarean section under ether anesthesia approximately 6 hours before expected parturition. Because this difference in delivery has no effect on behavioral masculinization (Houtsmuller and Slob, 1990) and because any difference in birth weight that might have occurred was statistically controlled, no distinction was made between pups born through either procedure.

Procedure For natural deliveries, at the expected time of birth an observer inspected the females every 10 minutes to determine the time of the onset of parturition, so that order of birth could be established. At birth the position of each pup position in utero was recorded. Anogenital distance (AGD, distance from the center of the genital orifice to the center of the anus) was measured on this same day to the nearest 0.1 mm. AGD measurements were made blind to the uterine position of the pup by the same observer throughout the experiment. To assess the reliability of this measurement, for most pups a second observer (the same one throughout the experiment) independently measured AGD. The correlation between these independent measures was high ($r=0.77$, $p<.01$), indicating a reasonably reliable system of assessment. Furthermore, the means were highly similar (2.12 ± 0.06 and 2.08 ± 0.06) between observers. Therefore, in those cases where two measurements were taken, the mean for the two observations was used in data analysis.

Theoretical Model and Data Analysis Multiple regression analysis (Cliff, 1987) was used to construct a model for variables contributing to female AGD's at birth. Four variables were considered for entry into the regression equation, three having theoretical significance and one as a control. Those of theoretical interest included: the number of caudal males (#CAUD♂)(Meisel and Ward, 1981), the number of adjacent males (#ADJ♂)(vom Saal, 1981), and the overall number of males in the uterine horn (TOT#♂)(Clark et al., 1991). Increases in each of these variables would be expected to produce increases in AGD. Birth weight (BirthWt), although not a prenatal factor, was included as a control variable. This factor is known to correlate with AGD (e.g., in mice: Graham and Gandelman, 1986), and therefore its exclusion could lead to bias in the estimated impact of the other factors (i.e., omitted variable bias, Johnston, 1984).

Two additional variables were considered for analysis. One of these (BStrain) was a binary (dummy) variable representing differences in the intercept term for AGD between the two different strains of rats. Pooling data from both strains into a single analysis would enable more precise estimates of the contribution of each factor; yet, differences in AGD between the two strains could still be captured with this variable.

A second variable represented the interaction between the number of caudal males and the distance (DIST) (i.e., adjacent, one, two, or > two away) from the nearest caudal male. This interaction variable (DIST*CAUD♂) was based on the assumption that if #CAUD♂ did have an effect on AGD, the magnitude of this effect might depend on the proximity of the nearest caudal male (Gandelman, 1986). That is, the effect of an increase in caudal males would be greater the smaller the distance from the nearest caudal male.

The regression model was limited to the six variables presented above, though other variables were initially considered for inclusion (e.g., male to female ratio, distance of the female from the ovary). However, they were excluded because either they lacked theoretical justification, or they were statistically redundant and therefore may have resulted in multicollinearity problems (Johnston, 1984).

With all variables considered and assuming a linear relationship, the statistical model appears as:

(1) $AGD = \alpha + \beta_1 *\#ADJ♂ + \beta_2 *BirthWt + \beta_3 *TOT\#♂ + \beta_4 *\#CAUD♂ + \mu$

where

(2) $\alpha = \alpha_0 + \alpha_1 *BStrain$ (representing strain differences)

(3) $\beta_4 = \beta_5 + \beta_6 *DIST$ (representing the interaction)

and where μ denotes the error term and satisfies the assumptions of the classical linear regression model (Cliff, 1987). By substituting expressions (2) and (3) into (1), the estimating equation (4) is yielded. This equation was estimated using the ordinary least squares (OLS) procedure.

(4) $AGD = \alpha_0 + \alpha_1 *BStrain + \beta_1 *\#ADJ♂ + \beta_2 *BirthWt + \beta_3 *TOT\#♂ + \beta_5 *\#CAUD♂ + \beta_6 *DIST*\#CAUD♂ + \mu$

In equation (4) above, β_6*DIST*#CAUD♂ represents the interaction term, with DIST*#CAUD♂ representing the actual interaction variable, created by the product of DIST and #CAUD♂.

Before running the analysis, z-score transformations were performed on all continuous variables in order to standardize across strains. Analyses were carried out using the SPSS software program (SPSS, 1983), with all tests of significance being two-tailed.

RESULTS

Estimation of parameters and tests of hypotheses. Table 1 provides the results of the regression analysis. The overall F value ($F[6,87] = 4.22$; $p = .0009$) indicated that the joint effect of the six independent variables (see expression (4)) on AGD was significant. Tests of significance regarding individual variables, assessed through their respective t-values, indicated effects for number of males in the uterine horn (TOT#♂), the number of caudal males (#CAUD♂), and distance to the nearest caudal male (DIST*CAUD♂) at the .05 significance level, and for

Table 1. Results of regression model for AGD showing tests on individual variables.

Variable	t-value	p
BStrain	.15	.880
#ADJ♂	-1.05	.299
BirthWt	1.66	.101
#CAUD♂	1.97	.051
DIST*CAUD♂	-1.96	.051
TOT#♂	2.53	.013

birth weight (BirthWt) at the .10 significance level. Neither the number of adjacent males (#ADJ♂) nor differences between strains (BStrain) had sufficiently high t-values to reject the null hypothesis.

Estimation of parameters and tests of hypothesis of the revised model. As neither #ADJ♂ nor BStrain significantly impacted AGD, these variables were eliminated from the model. Because BirthWt was marginally significant, this variable was retained to prevent omitted variable bias (Johnston, 1984). The revised model yielded a significant F value ($F[4,89]=5.98$; $p=.0003$) indicating a joint effect for all four variables. As shown in Table 2, t-tests on individual variables indicated significant effects for #CAUD♂, TOT#♂, and, at marginal levels, DIST*CAUD♂ and BirthWt.

Table 2. Results of the revised model for AGD showing tests on individual variables.

Variable	Standardized Beta	t-value	p
BirthWt	.189	1.83	.071
#CAUD♂	.627	2.66	.009
DIST*CAUD♂	-.434	-1.90	.061
TOT#♂	.262	2.42	.018

Impact magnitude of individual factors. Inspection of the standardized beta coefficients generated by the revised model (Table 2) enables assessment of the direction and magnitude of the effect on AGD for each of the four variables included in the analysis. The direction of impact (indicated by sign) for each variable was consistent with prior theoretical expectations. In addition, magnitude of beta coefficients indicated that #CAUD♂ exerted the strongest effect, followed in descending order by DIST*CAUD♂, TOT#♂, and BirthWt. These coefficients, expressed in standardized units, represent the change (measured in standard deviation units) in AGD that results from a change of one standard deviation in the selected independent variable. For example, regarding the variable #CAUD♂, a one

standard deviation increase in the number of caudal males produces a 0.628 standard deviation increase in AGD.

Goodness of Fit. For the revised model, where all variables are measured in standardized units, $R = .47$, and $R^2 = .23$.

Test for strain differences in the contribution of factors. Various strains of rats have been shown to be differentially sensitive to androgens (Whalen, Gladue, and Olsen, 1986; Brand and Slob, 1991), and therefore female fetuses from different strains may be differentially influenced by prenatal factors. Accordingly, additional analysis was carried out to test the hypothesis that the impact on AGD for each variable in the revised regression model differed between the two strains of rat. That is, that the slope (beta) coefficients for the two strains were different. To test the null hypothesis that the slopes did not differ, a binary variable representing the difference between strains was used to construct interaction terms with each independent variable which appeared in the revised regression equation. Thus, each newly constructed variable, designated with a prefix "B" (B-#CAUD♂, B-TOT#♂, B-DIST*CM, B-BirthWt), represented the interaction between the specified independent variable and the difference on that variable between strains.

Table 3. Results of regression model testing for strain differences.

Variable	t-value	p
B-BirthWt	- .10	.921
B-#CAUD♂	.07	.944
B-DIST*CAUD♂	- .29	.770
B-TOT#♂	-1.27	.209

The results of this analysis, presented in Table 3, indicated no difference between strains for the impact of any individual variable in the revised model. That is, none of the t-values obtained from the interaction variables representing strain differences were significant at either the .05 or .10 level. To determine whether the joint effect of these variables on the slope of the AGD regression line was significant, the Wald F-test (Johnston, 1984) was used. For this analysis, $F[3,89] = 1.04$; $p > .10$, indicating again that the overall slopes did not differ for the two strains.

DISCUSSION

Using a procedure that enabled statistical control over variables that could not be experimentally controlled, the present study has confirmed the importance of caudal males in female morphological masculinization, and has identified two additional prenatal factors relevant to the masculinization of female rat fetuses. Thus, the number of caudal males, the distance to the nearest caudal male, and the overall number of males in the uterine horn were found to exert significant and unique effects on female AGD. In contrast, the number of males adjacent to a female fetus did not

significantly affect AGD. Nor were there differences between Holtzman and Wistar rats in the impact of any of these variables on AGD, suggesting that both the type and strength of factors influencing AGD were essentially the same across strains.

This analysis has also enabled, for the first time, information on the relative contribution of each of the three factors impacting AGD. As indicated by beta coefficients, the number of caudal males and distance to the nearest caudal male clearly exerted the strongest effects, while the overall number of males in the uterine horn exerted a significant, but lesser effect. Furthermore, this methodology has provided a means for controlling birth weight across subjects, a variable that correlates with AGD and therefore may have confounded earlier attempts to relate AGD to prenatal hormonal influences (Graham and Gandelman, 1986).

Previous research on the impact of ipsi-uterine horn males on female masculinization has suggested that androgens are transported from male siblings to females either through the amniotic sac (Clemens et al., 1978) or via rostral flow of the uterine vasculature (Meisel and Ward, 1981). In general, such studies indicate the possible role that the female's position relative to male fetuses plays in the masculinization of females, and in this respect, the present findings are consistent with previous theorizing. Discrepancies among studies regarding the hypothesized mechanism of female androgenization (e.g., caudal vs. contiguous effect) may be a reflection of the complex and perhaps, multiple mechanisms through which male androgens are transported to female fetuses, at least in the rat. However, some of the discrepancy among studies may also be explained by the fact that previous experiments have not adequately controlled for variables which may systematically vary with the one being investigated. Thus, in such experiments the number of caudal males and the number of adjacent males are likely to be correlated, and together these factors may covary with the overall number of males within the uterine horn or with the females's distance from the nearest caudal male. Such covariation is likely to contribute to inconsistent results, and may partly account for the rather elusive caudal and/or adjacent male effects. However, our analysis, which controls for such problems, indicates a robust impact for caudal males, such that an increasing number of caudal males results in larger AGD's. In contrast, no impact for adjacent males was apparent. Thus our findings provide further support for the idea that androgens are transported to females from caudal males. The actual mechanism through which these androgens might be transported has yet to be understood, as a recent report failed to find evidence for androgen transport via the uterine vasculature in the rat (Even, Dahr, and vom Saal, 1992).

The present findings expand previous reports by identifying two prenatal factors, previously unreported, that impact AGD in the female rat. The first is the interaction between the number of caudal males and distance of the female from the nearest caudal male. Thus, the closer the nearest caudal male, the greater the impact for the number of caudal males on female AGD. This interaction further defines the relationship between caudal males and AGD size, and is tenable assuming transport of androgens in a rostral direction through the uterus. This relationship may also explain previous research on male and female rats which, while showing an effect for the presence of caudal males on masculinization, showed no effect for increasing the number of caudal males beyond one (Meisel and Ward, 1981; Houtsmuller and Slob,

1990; Houtsmuller, Juranek, Gebauer, Slob, and Rowland, 1993). If, as is suggested by the present study, the effect of caudal males depends on their distance from a particular 'target' fetus, the relationship between the number of caudal males and masculinization of a fetus might be obscured when the distance from those caudal males is not taken into account.

Above and beyond the contribution of caudal males, the present study indicates a role for the overall number of males in the uterine horn on female AGD, independent of where those males are positioned relative to the female. A parallel relationship between the number of males in the uterine horn and the testosterone levels of female fetuses, independent of their uterine position, has recently been reported in gerbils (Clark et al., 1991). While several reports have failed to find an effect for the number of males in a litter on behavioral sexual differentiation in females (Slob and van der Schoot, 1982; van de Poll et al., 1982), in these studies litters were divided over both uterine horns. In the present study only litters within individual uterine horns were investigated. Assuming that a local mechanism within a single uterine horn accounts for the transport of androgens from males to females, females would be masculinized only by males within the same uterine horn. That is, no relationship between the number of males in both uterine horns and masculinization of females would be expected. Further research is necessary to ascertain whether the effect of number of males in the uterus is local, being confined to individual uterine horns, or more general, encompassing both uterine horns and perhaps even involving overall maternal circulation (see Clark, Crews, and Galef, 1993). Whatever the case, the fact that the overall number of males affects female AGD argues strongly for a mechanism beyond merely rostral transport in the androgenization of female fetuses.

In most studies on prenatal masculinization reported so far, the effect of one or two relevant variables has been investigated, with groups formed and compared on the basis of the presence or absence of those variables (e.g., caudal males, adjacent males). In contrast, the present experiment used procedures that enabled the simultaneous assessment of the unique contribution of a number of theoretically-salient prenatal variables to the morphological masculinization of females. In this way, a more precise estimate of factors influencing prenatal masculinization of females has been determined. Such a methodology may provide an appropriate strategy for future investigations of prenatal environment and behavioral masculinization of female rats, a process which presumably relies on the same physiological mechanisms as morphological masculinization. A further challenge will be that of identifying the sources of the remaining 75% unexplained variance in neonatal female AGD.

CHAPTER 5

PLASMA TESTOSTERONE IN FETAL RATS AND THEIR MOTHERS ON DAY 19 OF GESTATION

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CHAPTER 5

ABSTRACT

Plasma testosterone levels were higher in pooled samples from male fetuses than from female fetuses on day 19 of pregnancy. Plasma testosterone from female fetuses with males located caudally in the uterus was higher than from females that lacked such males. Testosterone level of both male and female fetuses was correlated with maternal testosterone. No correlation was found between maternal testosterone and number of males in the litter, male-to-female ratio, or litter size. These results corroborate earlier findings of a sex difference in plasma testosterone levels on fetal day 19 in rats, and provide support for the hypothesis that female rats receive androgens from males located caudally in the uterus. No evidence was found that testosterone of pregnant females is affected by the sex ratio or size of her litter.

INTRODUCTION

There is considerable variation among normally developed female rats in the frequency with which they display masculine sexual behavior (mounting) in adulthood. Since perinatal androgens are important for the organization of this behavior (see Baum,1979), it has been suggested that this variation among females stems from variation in the amount of prenatal androgen exposure. Indeed, detectable testosterone levels have been found in female fetuses, and these levels differ substantially among individuals (Baum et al., 1991, Slob et al., 1980, Weisz and Ward, 1980). Since there is no evidence to suggest that female fetuses themselves produce androgen, the question arises as to the source of this testosterone. The placenta (Gerall and Ward, 1966, Vreeburg et al., 1983), the ovaries (Sridaran et al., 1981) of the mother, and male fetuses sharing the same uterine horn (Clemens et al., 1978) have been suggested as possible sources.

While evidence purporting an influence of androgens from male uterine mates has been offered in a number of studies (e.g., rats: Clemens et al., 1978, Meisel and Ward, 1981, gerbils: Clark and Galef, 1988, mice: vom Saal, 1981, Zielinski and Vandenbergh, 1991), there is uncertainty about the mechanism through which such androgens might actually reach female fetuses in utero. Diffusion through the amniotic sac to adjacent females (Clemens et al., 1978), and transport of androgens via the vasculature to females from males located caudally (i.e., caudal male effect, Meisel and Ward, 1981) have both been proposed as possible mechanisms. Previous experiments in rats based upon sexual differentiation have preferentially supported a caudal male effect in that females as well as males having caudal males undergo greater morphological and behavioral masculinization in utero than same-sex siblings without caudal males (Babine and Smotherman, 1984, Richmond and Sachs, 1984, Houtsmuller and Slob, 1990, Houtsmuller et al., 1993a, Houtsmuller et al., 1993b).

Actual evidence that prenatal testosterone levels differ between female fetuses occupying different uterine positions is scant and inconclusive. In mice (Vreeburg et al., 1983) and gerbils (Clark et al., 1991), prenatal testosterone level is reportedly higher in females with two adjacent males than in females without adjacent males. In ferrets, higher testosterone has been found in females with two caudal males than in females with one or none (Krohmer and Baum, 1989). In hamsters, androgen levels

were lower and estradiol levels elevated in males with females located caudally (Vomachka and Lisk, 1986). In contrast, in rats there is no evidence from hormone studies for transport of hormones, particularly androgen, from males to either adjacent or rostral females. Baum et al. (1991) analyzed androgen levels of female fetuses, but failed to find support for androgen transport between fetuses. However, in that study the number of animals included for testosterone analysis specifically on days 18 and 19 of gestation was small (i.e., 3, 2 and 15 with 0, 1 and >1 caudal male, respectively). Since the sex difference in prenatal androgen level of rats is most profound on days 18/19 of gestation, resulting from an androgen surge in males that is critical to behavioral masculinization of the fetus (Baum et al., 1991, Slob et al., 1980, Weisz and Ward, 1980), the lack of effect seen in the study by Baum et al. (1991) may have resulted from the limited group sizes on these critical gestational days. Therefore, the first purpose of the present study was to reexamine whether female fetuses with caudal males have higher testosterone levels, specifically on day 19 of pregnancy, than female fetuses without caudal males.

While much research has focussed on the influence of androgens from male uterine mates, other androgen sources may also contribute to prenatal masculinization of female rats. For example, some females born from all-female litters display high levels of masculine sexual behavior in comparison with females born from other all-female litters. Yet all such females lack prenatal exposure to androgens from males fetuses. Since pregnant rats show considerable differences in blood levels of androgens (Gibori and Sridaran, 1981), and since testosterone administration to pregnant females results in increased masculinization of female offspring (e.g., Even et al., 1992, Slob et al., 1983, Ward and Renz, 1972 but see also Ito et al., 1986), differences in endogenous androgen among pregnant females may account for some variation in masculinization of female offspring born to different mothers. Therefore, the second purpose of the present study was to investigate the relationship between testosterone level of female fetuses and that of maternal blood.

Finally, male fetuses themselves may be an important source of androgen in pregnant females, as is known to be the case in the rhesus monkey (Resko, 1970). A recent report indicates significant correlations in gerbils between number and proportion of males in a litter, and testosterone in maternal blood (Clark et al., 1993). Specifically, the higher the number of males in the litter, the higher the maternal androgen level, suggesting that male fetuses contribute significantly to androgen levels in maternal circulation. Since androgens in maternal blood may in turn influence masculinization of fetuses, male fetuses could affect female fetuses via the maternal circulation. Therefore, the third purpose of the present study was to investigate the relationship between testosterone level in maternal blood as well as that in female fetuses on the one hand, and several litter parameters, such as number of males, number of females, male-to-female ratio, and litter size on the other.

METHOD

Twenty-one Wistar females (TNO, Zeist), housed 2-3 to cage and kept on a 12-12 hr light schedule, were mated in our laboratory. On day 19 of pregnancy (day of impregnation = day 0), blood from the mother was collected from the orbital plexus

under light ether anaesthesia. Immediately thereafter, caesarean section was performed by making a midline incision in the abdomen. The two uterine horns were spread out, and pups were removed from the horns and laid out according to their uterine position. Blood from the fetuses was collected through an incision in the carotid artery and vein. Since blood from individual fetuses was insufficient for measurement of hormones, samples were pooled. Blood from individual females was pooled with that of females from similar positions according to the caudal male hypothesis (i.e., with or without caudal males). Blood from male fetuses was pooled irrespective of their position in utero.

Hormone measurements

Testosterone levels were estimated in plasma by a radioimmunological method (Verjans et al., 1973) in which 5α-dihydrotestosterone cross-reacts for 43.2%. Inter- and intraassay coefficients of variation were 13.4% and 5.6%, respectively.

Data analysis

To test the caudal male hypothesis, differences between pooled samples from female fetuses with caudal males ($n=28$) and without caudal males ($n=12$) were tested using Student's t-test.

Correlations were performed using the Spearman test as not all variables were normally distributed. Number of animals was not equal for all correlations, since blood samples of females were pooled using the presence or absence of caudal males in utero as a criterion. Female fetuses represented in one pool sometimes differed on other variables (e.g., number of rostral males) and therefore had to be excluded from analyses involving that particular variable.

Correlations were also calculated between testosterone from pooled samples of females, and mean testosterone from pooled samples of female siblings. In this way, the relationship between testosterone in females and that of the rest of the females in the litter could be examined, and thus, the influence of factors affecting the litter as a whole, rather than specific individuals, could be determined.

Consistent with theoretical expectations, one-tailed tests were used to determine caudal male effects. For all other analyses, two-tailed p values are reported.

RESULTS

Blood was collected from 106 female fetuses, 114 male fetuses and 21 mothers. One female appeared not to be pregnant and was excluded from the analysis. Blood samples of female fetuses were pooled based on the presence or absence of caudal males. Number of fetuses per pooled sample ranged from 1-4 (mean=2.3) for females and 1-5 (mean=2.8) for males.

Testosterone levels were significantly higher in male than in female fetuses ($p<.01$) (Table 1).

Testosterone of female fetuses and the presence of caudal males

The plasma testosterone level of females with caudal males in the uterine horn was significantly higher than that of females without such males ($p=.05$) (Table 1).

Table 1. Mean (± SE) plasma testosterone of fetal rats and their mothers

	n	Plasma testosterone level (nmol/l)
Mothers	20	2.5 ± 0.11
Male fetuses	40	2.8 ± 0.18
Female fetuses	40	1.0 ± 0.15*
with caudal males	28	1.1 ± 0.20
without caudal males	12	0.7 ± 0.15§

* $p < .01$, compared to male fetuses
§ $p = .05$, compared to female fetuses with caudal males

In addition, testosterone of females correlated significantly with the number of caudal males in the same uterine horn (0 to 4) (Table 2).

Testosterone level of female fetuses correlated positively with the number of males in the same uterine horn. In addition, for female samples, the number of caudal males correlated with the overall number of males in that uterine horn ($r = .44$; $p = .01$). These two relationships suggest that the correlation between testosterone and the number of caudal males may have occurred because of an effect from the overall number of males in the same uterine horn, rather than specifically from caudal males. To eliminate this possibility, the correlation between female fetal testosterone and the number of rostral males was also calculated: testosterone of females was not signficantly correlated with the number of rostral males.

Fetal testosterone, maternal testosterone and sex composition of the litter (Table 2)

Testosterone levels of both female and male fetuses were positively correlated with maternal testosterone. No relationships between sex composition of the litter and testosterone levels of fetuses or mother were found, that is, between number of males in the entire litter, male-to-female ratio, or littersize on the one hand, and plasma testosterone in female fetuses, in male fetuses, or in maternal blood on the other. Testosterone of female fetuses was significantly correlated with testosterone of female siblings, and testosterone of males was marginally correlated with testosterone of male siblings.

Table 2. Correlations among maternal and fetal testosterone and variables related to the uterine environment.

	Plasma testosterone levels of:					
	female fetuses	n	male fetuses	n	mothers	n
number of caudal males	.35*	30				
number of rostral males	-.02	24				
number of males in uterine horn	.24	30				
maternal testosterone	.26*	40	.39**	40		
testosterone same-sex siblings	.39**	33	.29#	33		
number of males in both horns	.13	40	-.11	40	-.18	20
male/female ratio	.19	40	-.21	40	.08	20
litter size	-.15	40	.10	40	-.33	20

p=.06
* p<.05
** p<.01

DISCUSSION

The present study confirms earlier reports which have shown that in fetal rats, testosterone content is significantly higher in males than in females on day 19 of gestation (Baum et al., 1991, Slob et al., 1980, Weisz and Ward, 1980). This study also provides evidence for the first time that female rat fetuses that develop in utero with male siblings located caudally have higher testosterone levels on day 19 of gestation than females that lack such males. Furthermore, in female fetuses, a higher number of caudal males is associated with a higher testosterone level. Previous experiments have provided evidence that female rats with caudal males in utero are more masculinized than females without caudal males, both in genital morphology (i.e., anogenital distance, an androgen-sensitive measure) and in masculine sexual behavior in adulthood (Meisel and Ward, 1981, Richmond and Sachs, 1984, Houtsmuller and

Slob, 1990, Houtsmuller et al., 1993b). Although it has long been assumed that this increased masculinization in females having caudal males is due to exposure to higher levels of androgens prenatally (Meisel and Ward, 1981), the present study provides positive evidence for such differences in prenatal testosterone levels between female fetal rats with and without caudal males in utero. This finding is in accordance with an earlier study reporting higher testosterone levels in female ferrets with two caudal males than in females with one or no caudal male (Krohmer and Baum, 1989), but contradicts another study (Baum et al., 1991) in which no difference was found in androgen levels of female rat fetuses with and without caudal males. In this latter study (Baum et al., 1991), Baum and his coworkers measured androgens on days 17-22 of gestation, whereas in the present study testosterone was measured specifically on day 19. Since several reports have suggested that the sex difference in testosterone prenatally is restricted to days 18 and 19, due to a testosterone surge in male fetuses on these days (Hoepfner and Ward, 1988, Baum et al., 1991, Weisz and Ward, 1980) although sex differences on other days of gestation have been reported as well, Slob et al., 1980), the difference between females with and those without caudal males may be apparent only on these two days. Although androgen measured in fetuses on days 18/19 was analyzed separately from other gestational days by Baum et al. (Baum et al., 1991), the subgroup for days 18/19 was small. Our ability to detect a difference in androgen on day 19 between females with and without caudal males may have resulted from our larger sample size. Nonetheless, while the present study supports an effect for caudal males on female androgenization, the physiological mechanism underlying the transport of these androgens to females remains unclear (e.g., see Even et al., 1992).

Although the correlation between testosterone levels of female fetuses and number of caudal males was statistically significant, only about 12% (r^2) of the variance in testosterone level of female fetuses was explained by the number of caudal males. A partial explanation for this low covariance is that the group lacking caudal males also had remarkably high testosterone levels. This general idea is consistent with behavioral observations, which show that some females without caudal males may show high levels of masculine sexual behavior, suggesting substantial prenatal masculinization (Meisel and Ward, 1981, Houtsmuller and Slob, 1990) independent of caudal males. Thus, while caudal males may increase masculinization of females, their presence accounts for only part of the masculinization of females.

Testosterone level of female fetuses was significantly correlated with that of female siblings, suggesting less variation of testosterone within litters than across litters. Strong variation across litters in morphological masculinization of females (i.e., anogenital distance) has also been reported in mice (Zielinski et al., 1991). Together, these findings suggest that in addition to factors which androgenize specific individuals in the uterus (e.g., caudal males, placenta), there may also be significant factors affecting the litter as a whole (e.g., androgens in maternal blood). The present study attempted to specify the relevance of two such factors which might impact the litter as a whole: testosterone level in maternal circulation, and the number of males in the litter.

Regarding the former, small but significant positive correlations between testosterone level in maternal blood and that of female as well as male fetuses were

found, suggesting that maternal blood is a possible source of androgens affecting the whole litter. While there remains some question regarding the extent to which maternal testosterone can impact behavioral masculinization of female fetuses (Ito et al., 1986, Slob et al., 1983) it is commonly accepted that systemic testosterone administration to pregnant females results in behavioral masculinization of female offspring (e.g., Gerall and Ward, 1966, Ward and Renz, 1972), presumably through the transfer of androgens from maternal blood to fetal circulation.

Alternatively, it may be that fetal testosterone production affects the androgen level of the mother, rather than vice versa. The placentas become an important source of androgen in maternal circulation between days 12-18 of pregnancy (Gibori and Sridaran, 1981). In addition, a recent report has suggested that testosterone level of pregnant gerbils increases with the number of males in the litter (Clark et al., 1993). Consistent with this finding, testosterone level in one non-pregnant female from the present experiment (excluded from the analysis) was considerably lower (0.2 nmol/l) than that in pregnant females (mean:2.5 ± .11). However, we found no significant correlations between maternal testosterone and overall number of males in the litter, male/female ratio, or litter size in these females. In this respect, our finding corroborates an earlier study (Ward and Weisz, 1984) which also reported no significant correlation between number of males or litter size and testosterone in maternal blood in rats. Therefore, although the present study has established a relationship between maternal and fetal testosterone levels in the rat, and thus reiterates the importance of factors that might impact the litter as a whole, no definite conclusion can be drawn at this point regarding the direction of that relationship.

The present study found no evidence to support the idea that male fetuses influence plasma testosterone levels of all female fetuses equally within the litter, regardless of uterine position or horn, via the general maternal circulation. Were female fetuses affected by male fetuses mainly general through maternal circulation, a relationship between female fetal or maternal testosterone and the number or ratio of males might be expected. However, none of these variables were found to covary significantly.

In conclusion, the present study suggests that variation in testosterone level in female fetuses on day 19 of pregnancy can partly be explained by the presence or absence of caudal males. Furthermore, fetal testosterone level of both males and females correlated positively with maternal testosterone and mean testosterone of same-sex siblings, but no relationship was found between maternal testosterone and sex ratio of the litter or litter size. Further research needs to investigate the extent to which fetuses are affected by endogenous androgens in the maternal circulation, and to identify other factors that might affect the testosterone level of the litter as a whole. In addition, as caudal males accounted for only a small portion of the variance in testosterone, other potential sources of androgens which might affect individual fetuses within the uterus need to be investigated. For example, testosterone production by the placenta during the second half of pregnancy has been demonstrated (Gibori and Sridaran, 1981), and several reports have identified the placenta as a major source of androgen in the female fetus (Slob and Vreeburg, 1985, Vreeburg et al., 1983).

CHAPTER 6

THE CAUDAL MALE HYPOTHESIS AND THE UTERINE VASCULATURE OF THE RAT

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CHAPTER 6

ABSTRACT

Female rats have been suggested to be exposed in utero to androgens from male fetuses with whom they share the uterine horn. Two hypothesis regarding transport of androgens from males to females in utero have been proposed. The 'contiguity' hypothesis suggests transport through the amniotic sac, such that females that are located between two males are exposed to higher levels of androgens, and consequently are more masculinized, than females that develop between two females. The 'caudal male' hypothesis assumes transport via the uterine blood vessels, such that females are exposed to androgens from males located rostrally from them. Results from several experiments from our lab have preferentially supported the 'caudal male' hypothesis, while providing no evidence for the 'contiguity' hypothesis. The 'caudal male' hypothesis is based on two assumptions. First, testosterone is assumed to pass directly from the uterine vein to the uterine artery, and secondly, blood flow in uterine vein and artery is assumed to follow a rostral direction. Since these assumptions are critical to the hypothesis, the research on which each is based is reviewed here.

Female rats may display high levels of mounting behavior in adulthood, and there is considerable variability among females in the frequency with which they display this 'masculine' sexual behavior (de Jonge et al., 1986). Since the sexual differentiation of this behavior presumably is to a large extent dependent on the presence of androgens around birth, it has been suggested that some females are prenatally exposed to higher levels of endogenous testosterone than others, and as a result are more masculinized. Indeed, detectable levels of testosterone have been found in female fetuses (e.g., Pang et al., 1979, Slob et al., 1980, Weisz and Ward, 1980), and females that are prenatally exposed to anti-androgens show lower levels of mounting behavior than controls (Stewart et al., 1971).

The question then arises as to where this endogenous testosterone in female fetuses comes from. As possible sources the placenta (Vreeburg et al., 1983, Slob and Vreeburg, 1985), the ovaries (Whitcher and Clemens, 1981) and adrenals of the mother, and male fetuses in the same uterine horn (Clemens et al., 1978) have been suggested. Regarding the manner in which testosterone secreted by males might reach the female, two hypotheses have been proposed. 1. Testosterone diffuses through the amniotic sac, such that females are masculinized by androgens from contiguous males (contiguity hypothesis, Clemens et al., 1978). 2. Testosterone secreted by males passes from the uterine vein directly to the uterine artery, and is transported by the arterial blood, which supposedly flows in a rostral direction, masculinizing females that are located rostrally (caudal male hypothesis, Meisel and Ward, 1981). Evidence supporting (Gandelman, 1986, Houtsmuller and Slob, 1990, Houtsmuller et al., 1993, Richmond and Sachs, 1984, Vom Saal, 1989, Zielinski et al., 1991) and refuting (Simon and Cologer-Clifford, 1991, Brand et al., 1990, Baum et al, 1991) each hypothesis has been provided, leaving the issue unresolved.

The caudal male hypothesis: transport of steroids via the uterine vasculature

The hypothesis that rat fetuses are masculinized by androgens from males located caudally (caudal male hypothesis), for which support was found in previous experiments in our laboratory (Houtsmuller and Slob, 1990, Houtsmuller et al., 1993a, Houtsmuller et al., 1993b, Houtsmuller et al., 1993c), is based on two assumptions concerning the vasculature of the rat. The first assumption is that androgens, secreted by male fetuses and transported by the uterine vein, pass directly from the vein to the uterine artery (Meisel and Ward, 1981). The second assumption is that uterine arterial and venal blood flows in a rostral direction, such that androgens from males are transported to fetuses located rostrally and not to those located caudally (Meisel and Ward, 1981). These two assumptions then lead to the hypothesis that androgens secreted by males pass from the uterine vein to the uterine artery and are subsequently transported to fetuses located rostrally (Meisel and Ward, 1981). These assumptions stem from an extensive body of research on luteal regression in several species, carried out two decades ago (e.g., McCracken et al., 1971, Del Campo and Ginther, 1972). Since the assumptions about the uterine vasculature of the rat are of utmost importance for the caudal male hypothesis, the research on which each is based will be reviewed here. First, a short description of the uterine vasculature of the rat, based upon work of other authors as well as our own observations, will be given. Then the research on luteal regression upon which the two abovementioned assumptions are based will be reviewed shortly. Possible consequences of several aspects of the vasculature and the bloodflow for the caudal male hypothesis will be discussed.

Uterine vasculature of the rat

The following description of the uterine vasculature of the rat is based upon work of others as well as observations from our lab. The vasculature of 5 pregnant females was observed on day 19 or 20 of pregnancy. Females were anaesthesized with ether, a midline incision in the abdomen was made and uterine horns were carefully exposed. Excess fat was removed untill anatomy of the vasculature could be readily observed.

Anatomy

Arteries. The uterine artery arises as a branch of the internal iliac artery and runs parallel to the uterus throughout its course (Fig.1). Del Campo and Ginther (1972) report that on the right side, the uterine artery terminates in the ovary, with an anastomosis between the ovarian and uterine artery near the ovary, whereas on the left side the uterine and ovarian artery form a junction. The ovarian artery arises from the abdominal aorta close to the origin of the renal arteries and passes ventrolateral to the ovary (Greene, 1963, Del Campo and Ginther, 1972). According to Wells (1964), the left ovarian artery arises from the left renal artery, whereas the right ovarian artery arises from the aorta. Our observations of the anatomy of these arteries were in accordance with the descriptions and pictures provided by the authors mentioned above.

Veins. The uterine vein joins the iliac vein at the caudal end and is in close apposition

with the uterine artery throughout its course (Fig.1). At the rostral end, the uterine vein joins the ovarian vein to form the uteroovarian vein, which runs parallel to the ovarian artery and joins the vena cava (right) or the renal vein (left). The ovarian vein follows the ovarian artery before forming a junction with the uterine vein.

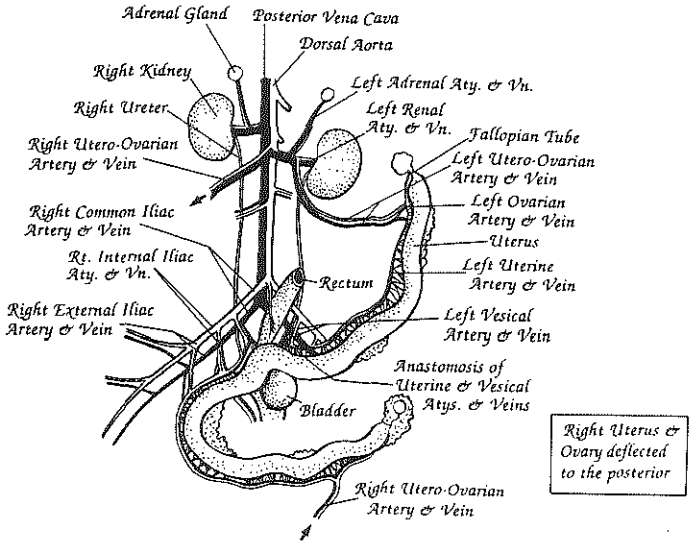

Fig. 1 Uterine vasculature of the rat. After: Wells, 1964.

The luteolytic effect: passage of substances from vein to artery.

In short, results from research on luteolysis has suggested that the nongravid uterus secretes a luteolytic agent (presumably the prostaglandin $PGF_{2\alpha}$), which passes from the uterine vein to the uterine artery, and is consequently transported to the adjacent (ipsilateral) ovary, where it causes luteal regression (see Niswender and Nett, 1988).

This idea is based on results from a myriad of studies carried out by several research groups. These studies have shown that in several bicornaute species (i.e. having two separate uterine horns), the luteolytic effect of the uterus primarily affects the corpora lutea in the ipsilateral, and not the contralateral ovary, suggesting a local rather than a systemic pathway. Unilateral hysterectomy results in maintenance of the corpora lutea in the ovary on the ipsilateral side, while regression of the corpora lutea occurs in the contralateral ovary (e.g. pseudopregnant rats and guinea pigs: Butcher et al., 1969, pseudopregnant hamsters: Duby et al., 1969, cattle: Ginther, et al., 1967).

Regarding the way transport of the luteolytic factor from the uterus to the ipsilateral ovary takes place, the vascular system was strongly implied. Ligation of artery and vein prevented luteolysis on the side of the ligation in rats, guinea pigs and sheep (rats and guinea pigs: Butcher et al., 1969, sheep: McCracken et al., 1971). Further evidence for transport of the luteolytic factor by the local vasculature was provided by a series of anastomoses studies, carried out by Ginther and coworkers

(for a review, see Ginther, 1974). In unilaterally hysterectomized sheep and cattle, the corpus luteum was maintained on the intact side, but regressed when the uterine vein draining the intact side was surgically anastomosed to the hysterectomized side, such that the uterine vein on the hysterectomized side carried venous blood from the intact uterine horn. Similarly, luteolysis occurred when the ovarian artery was anastomosed from the intact to the hysterectomized side (sheep: Ginther et al., 1973, Mapletoft and Ginther, 1975, cattle: Mapletoft et al., 1976). Hence, these experiments provided strong indications that a luteolytic factor secreted by the uterus reaches the ovary by transport via the uterine vein and ovarian artery.

Interestingly, anatomical studies of the uterine vasculature showed that in species in which a local pathway for luteolysis is involved, uterine artery and vein are in close apposition and frequently in contact with each other, thereby presumably providing opportunity for substances to pass from the vein to the artery (rat, guinea pig, hamster: Del Campo and Ginther, 1972, cattle: Mapletoft et al., 1976, sheep: McCracken et al., 1971, Del Campo and Ginther, 1974, Ginther et al., 1973, Ginther and Del Campo, 1973, Mapletoft and Ginther, 1975). (It should be noted however, that other authors reported negative results for guinea pigs (Egund and Carter, 1974)). In species in which a systemic pathway is involved on the other hand (horse, rabbit) there is very little contact between artery and vein (Del Campo and Ginther, 1973, Ginther et al., 1972). Also, in sheep, the walls of artery and vein are relatively thin at the point of apposition (Ginther and Del Campo, 1974). Furthermore, surgical separation of uterine artery and vein, such that they were no longer in contact, prevented luteolysis in sheep (McCracken et al., 1971). Thus anatomical arrangements of the vasculature were suggested to allow for passage of substances from vein to artery.

Evidence for the actual passage of substances from vein to artery was further provided by an experiment in which after injection of tritium-labeled prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) into the uterine vein of a ewe, more radioactivity associated with $PGF_{2\alpha}$ was detected in the uterine artery than in the iliac artery (for details see McCracken et al., 1971, McCracken et al., 1972), suggesting direct passage of $PGF_{2\alpha}$ between artery and vein. After injection of labeled $PGF_{2\alpha}$, water or testosterone in the testicular vein of rats (Free et al., 1973), wallabies and rams (Jacks and Setchell, 1973, Ginther et al., 1974) these labeled substances were detected in larger amounts in the testicular artery than in the iliac or femoral artery. Furthermore, the testosterone concentration in the testicular artery was reportedly higher than in the intestinal artery or abdominal aorta in rams (Ginther et al., 1974) and rats (Free et al., 1973). It should be noted, however, that negative results were reported as well (Coudert et al., 1974).

How passage of substances between artery and vein might occur is not clear. No vascular connections have been reported so far. Del Campo and Ginther (1974) suggest transport might occur through the intercellular spaces of the intervening tissues. As no evidence favoring any mechanism has been provided yet, the issue awaits further investigation.

In conclusion, several lines of research have provided evidence that substances may pass from the uterine artery to the uterine vein in several species. Evidence that testosterone specifically can pass from vein to artery has been provided as well. However, not all findings have been demonstrated in rats specifically. Research is

necessary to validate further the idea that such passage of androgens may occur in the uterine vasculature in rats. For example, anastomoses studies in rats, as well as measurements of testosterone levels in blood collected simultaneously from the iliac artery and the rostral part of the uterine artery of rats pregnant with male fetuses could provide valuable information.

Direction of the uterine bloodflow.
Figure 1 shows that ovarian and uterine artery are connected and form a loop. With regard to the caudal male hypothesis, the question then arises as to what the direction of the bloodflow is. More specifically, does arterial blood coming from the caudal end of the uterus (and presumably carrying testosterone from males located caudally) reach fetuses located at the rostral end, as is assumed by the caudal male hypothesis (Meisel and Ward, 1981). And second, is venal blood from the uterus drained in a cranial direction, such that passage from vein to artery of testosterone from a male will only occur rostrally from that male, affecting only fetuses located rostrally.

Uterine artery. The caudal male hypothesis, which assumes blood in the uterine artery and vein to flow in a cranial direction was based on the study by Del Campo and Ginther (1972). These authors concluded on the basis of changes in the diameter of the uterine artery that blood flow in this vessel is primarily in a cranial direction in guinea pigs, rats and hamsters. In the guinea pig, the diameter of the uterine artery decreased in the cranial direction, being 1.0 mm at the caudal end and 0.5 mm at the cranial end. No actual data were reported for the rat or the hamster in this report. The conclusion that blood flow in the uterine artery is in a cranial direction was confirmed by Egund and Carter (1974), who on the basis of an angiographic study concluded that in the pregnant guinea pig, the uterine artery predominates (i.e. supplies most of the blood) over the ovarian artery. The diameter of the ovarian artery at the end of gestation was at its origin about 0.7 mm, whereas that of the uterine artery was about 1.2 mm. Thus, most of the blood supplying the pregnant uterus of the guinea pig (the authors suggest 75%) comes from a caudal direction, whereas relatively little (25% according to the authors) is provided by the ovarian artery and comes from the cranial direction. Also, contrast medium injected into the uterine artery in vivo, did not always reach the most rostral placenta, and never reached the ovary. Contrast medium injected into the ovarian artery entered the most cranial placenta, and usually only that one. These findings support the hypothesis that blood flow in the uterine artery of the guinea pig is in a cranial direction, providing support for the caudal male hypothesis. However, some of the blood, to the most rostral fetus(es) may be provided by the ovarian artery and thus come from a cranial direction. Research investigating this phenomenon in rats specifically is needed to generalize this finding to rats.
Uterine vein. Del Campo and Ginther (1972) concluded on the basis of an increase in the diameter of the uterine vein in the cranial direction that uterine blood was drained in a cranial direction in the rat, guinea pig and hamster. The same conclusion was reached by Egund and Carter (1974), who angiographically studied the uterine vasculature of the guinea pig. These authors report that the ovarian vein is widely dilated in the pregnant guinea pig, whereas the uterine vein is relatively small. This

observation led them to conclude that the ovarian vein is the main path of drainage in the guinea pig. However, Del Campo and Ginther (1972) suggest that a small part of the blood, at the caudal end of the uterus probably drained in a caudal rather than a cranial direction, emptying into the iliac vein.

The caudal male hypothesis and the uterine vasculature of the rat.

The 'caudal male' hypothesis as postulated by Meisel and Ward (1981) proposed that blood flow in the uterus is in a cranial direction. The figure they provide in their paper however ignores the connection between ovarian artery and uterine artery, and therefore the arterial loop that supplies the uterus (Fig. 2).

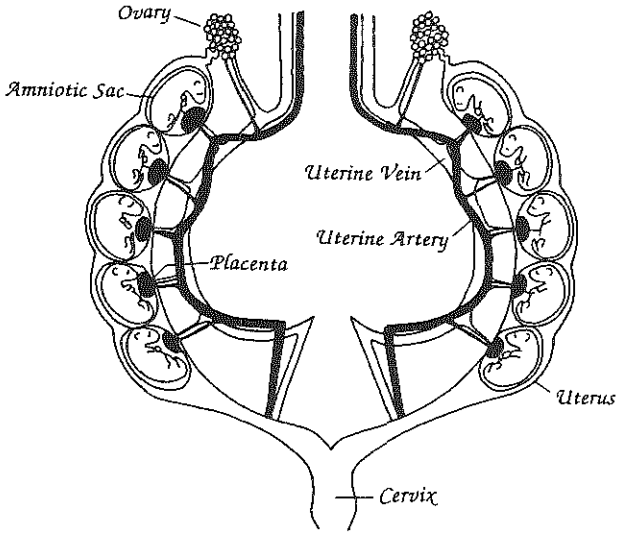

Fig. 2. Uterine vasculature. After: Meisel and Ward, 1981.

Vom Saal and colleagues have recently argued that arterial and venal blood flow in the *mouse* uterus is bidirectional (1991) (Fig. 3). Although the studies discussed in the present paper clearly report that also in the rat both uterine artery and uterine vein form a loop with the ovarian artery and vein, respectively, they also suggest that in rats, guinea pigs and hamsters most of the arterial and venal blood flows in a cranial direction. Thus, results from Del Campo and Ginther (1972), Egund and Carter (1974) and Vom Saal and Dahr (1991) are in accordance in that they all contend that the uterus is supplied by an arterial loop and drained by a venal loop. They differ however in the relative amount of blood that is supplied and drained by the ovarian artery and vein, respectively. Since different species were examined in these studies, the difference in conclusions may reflect a difference between species in hemodynamics of the uterus. It is in this respect interesting to note that evidence for the contiguous male hypothesis has been provided primarily in mice (reviewed in Vom Saal, 1989) and gerbils (e.g. Clark and Galef, 1988), whereas evidence for the caudal male hypothesis comes primarily from studies using rats (e.g. Tobet et al., 1983, Babine and

Smotherman, 1984, Houtsmuller and Slob, 1990, Houtsmuller et al., 1993a, 1993b, 1993c).

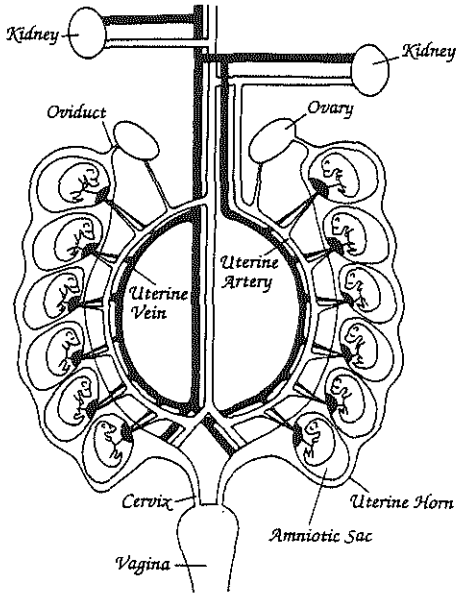

Fig. 3. Uterine vasculature. After: vom Saal and Dahr, 1991.

A recent study suggested that in rats, testosterone implanted into the amniotic sac of a fetus was transported to adjacent fetuses, and more so to the one on the caudal, than on the rostral side (Even et al., 1992). However, in our experiments measuring morphological and behavioral masculinization, no indications of such transport of androgens from males to adjacent fetuses were ever found, i.e., females that developed in utero between two males were not more masculinized morphologically or behaviorally than females without adjacent male siblings (Houtsmuller et al., 1990, Houtsmuller et al., 1993b, see also Houtsmuller et al., 1993a). The findings of Even et al. (1992) therefore await further investigation. It should be kept in mind that processes observed under highly unusual circumstances, such as surgery, in which the animal is anaesthesized, put on its back and cut open, do not necessarily reflect what happens under natural circumstances, i.e. in a moving, unaneasthesized animal.

To determine the direction of the uterine blood flow in rats, two experiments were carried out.

Experiments determining direction of uterine blood flow.

Pregnant female Wistar rats were housed 2-3 to a cage under standard laboratory conditions.

Procedure I. On day 20 of pregnancy, (day of impregnation = day 0, day of parturition is usually day 22), six females were anaesthesized with ether. A midline incision was made which exposed the uterine horns and chest. The heart was exposed by cutting open the chest, and a canula was inserted in the aorta. India ink was injected into the canual and the movement of the dye into the uterine horns was recorded with a Grundig VHS camera.

Procedure II. On day 20 of pregnancy, 3 females were anaesthesized with ether. The uterine blood flow was assessed by means of a ^{99m} Tc-diethylenetriamine pentaacetic acid (^{99m}DTPA) scan, after an intravenous injection in the tongue of ^{99m}Tc-Methylacetylglycineglycol (^{99m}TcMagg^3, Mallinckrodt, Petten, The Netherlands). Radioactivity was viewed with a nuclear Chicago gamma camera coupled to a PDP 11/34 computer, using GAMMA II software (Digital Equipment Company). Data were collected for 1 minute at 5 second intervals, and stored on a magnetic disc.

Our examinations of the direction of the bloodflow in pregnant rats did not yield consistent results. That is, in procedure I bloodflow in the uterine vessels of aneasthesized pregnant female rats appeared to be highly sensitive to manipulation of the uterus, vessels and fetuses. In procedure II radioactivity was distributed rapidly throughout the body. It was impossible to determine subtle differences between the two ends of the uterine horn. Therefore, no conclusions regarding the direction of the bloodflow in rat uterine artery and vein could be drawn.

ACKNOWLEDGEMENTS
The authors wish to express their appreciation to Dr. P van der Schoot, J. v. Ophemert and T. van Aaken for their help with the experiments.

CHAPTER 7

PERINATAL HORMONAL ENVIRONMENT, SDN-POA VOLUME AND SEXUAL BEHAVIOR OF MALE RATS

1. NEONATAL PROGRAMMING OF ADULT PARTNER PREFERENCE IN MALE RATS

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In: M. Haug et al. (eds) The development of sex differences and similarities in behavior, 1993, 33-49. Kluwer Academic Publishers, The Nederlands.

2. SDN-POA VOLUME, SEXUAL BEHAVIOR AND PARTNER PREFERENCE OF MALE RATS AFFECTED BY PERINATAL TREATMENT WITH ATD

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CHAPTER 7

Neonatal Programming of Adult Partner Preference in Male Rats

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Estradiol, derived from aromatization fo testosterone (T), is essential during a critical period around the time of birth for masculinization and defeminization of adult sexual behavior in male rats (e.g. Baum, 1979). The role of estradiol in organizing adult sexual orientation, as indicated by partner preference behavior, has received relatively little attention (Adkins-Regan, 1988). Recently we have published that neonatal treatment with the aromatase inhibitor ATD (1,4,6-androstatriene-3,17-dione) significantly affected adult partner preference behavior of male rats (Brand et al., 1991). Compared to control males these ATD males showed a significantly lower preference for the estrous female partner. These findings prompted us to assume that T, through its metabolite estradiol, plays a role in the organization of adult partner preference in male rats.

The effects of neonatal Atamestan (1-methyl-1,4-androstadiene-3,17-dione, another aromatase inhibitor) on partner preference and sexual behavior in adult male rats was investigated in Experiment 1.

In Experiment 2, the ontogeny of partner preference behavior of males rats was investigated in neonatal ATD and cholesterol treated males before, during and after puberty.

It has been suggested that the volume of the sexual dimorphic nucleus of the preoptic area (SDN-POA) is organized by the estradiol metabolite of testosterone (Döhler, 1984). Perinatal treatment with ATD could therefore affect the SDN-POA volume in male rats. This was investigated in Experiment 3.

General method

Animals

Experimental animals were Wistar albino rats ; stimulus animals were F1 hybrids of two inbred Wistar strains (R x U). They were housed two to four to a cage with food and water available *ad lib* and kept in a 14hr light 10hr dark cycle (lights on : 5:30 PM to 7:30 AM). Temperature in the animal room ranged from 20 to 22°C.

M. Haug et al. (eds.), The Development of Sex Differences and Similarities in Behavior, 33–49.

Treatments

Three experiments are reported (see also Table I for a comprehensive overview of the various experimental designs).

Table I Overview of the various experimental designs to study of perinatal endocrine manipulations on sexual orientation of adult male rats.

Number exept	Treatment		Number of litters	Number of male young used	Number of adult behavioral tests (age at time of testing : weeks (w) or days (d))			
	Prenatal injection	Neonatal Silastic implant			Partner preference tests (F vs M) ; with sexual interaction : prevented	allowed	Sexual pair tests vith : estrous F	active M
1*	-	empty	5	12				
	-	ATD	8	11	1(12 w)	3(14,16,17 w)	4(18,20,22,29w)	1(19 w)
	-	Atamesta n	2	14				
2*	-	cholestero l	4	9	9(32-60 d)	2(88,90 d)	-	-
				9	7(63-84 d)	2(89,91 d)	-	-
	-		8	19	9(32-60 d)	2(88,90 d)	-	-
		ATD		20	7(63-84 d)	2(89,91 d)	-	-
3*	solvent	empty	4	14(55)#				
	ATD	empty	4	12(5)#	7	6	1	1
	ATD	ATD	6	18(5)#	(11-19 w)	(20-25 w)	(27 w)	(26 w)

* all animals were left intact ; # 5 males out of each group were used for SDN-POA study ; Atamestan = 1-methyl-1,4-androstadiene-3,17-dione ; AZTD=1,4,6-androstatriene-3,17-dione ; M (male) ; F (female)

Female rats were timed mated (Day of mating = day 0 of pregnancy) and parturition occurred 22 days later.

Prenatal treatment (Experiment 3) consisted of daily injections to the mothers with ATD (1,4,6-androstatriene-3,17-dione ; 5 mg/day) or solvent (propylene glycol ; O.1 ml/day) from days 10-22 of pregnancy.

Neonatal treatment consisted of implantation (between 3 and 9 hours after birth s.c. in the back, under ice anaesthesia) of a Silastic capsule filled with ATD, Atamestan (1-methyl-1,4-androstadiene-3,17-dione), cholesterol or with nothing (Experiment 1-3). The implants were removed when the pups were 21 days of age. Pups were weaned at 21 days of age and housed two to four to a cage of same sex and treatment. The animals were left undisturbed until the onset of behavioral testing.

Stimulus animals were sexually active male and ovariectomized female rats. The latter were brought into estrus with 30 µg estradiol benzoate (EB) 24-48h prior to testing followed by 2.5 mg progesterone (P) 3-4h before testing.

CHAPTER 7

Behavioral testing

Three-compartment partner preference test

A test box (Figure 1) made of gray perspex with a transparent front was used (Slob et al., 1987) which had three compartments (60 x 30 x 40 cm each) with a small opening (13 x 12 cm) in both partitions near the front window.

These openings could be closed by a sliding door. Stimulus animals could be put in the left and right compartments. The incentives, an estrous female and a sexually active male, wore either a leather harness which was attached with a stainless-steel string to the rear of the compartment or no harness when placed behind a wire mesh separation halfway down the lateral compartment. Tethered animals had a limited action radius. They were adapted to the tethering device during 1hr in the week before testing. When physical interaction was prevented by wire mesh, the experimental animal could only see, smell, and hear the incentives.

Behavioral tests lasted 15 minutes. Before testing all three animals (one experimental, two incentives) were put in the box, one in each compartment, with the sliding doors closed, for 15-20 min of adaptation. At the beginning of the test the sliding doors were removed and the experimental animal could freely move around and interact with the stimulus animals or sit before the wire mesh separation. Time spent in each compartment was recorded. To quantify partner preference, a preference score was calculated for each test by subtracting time spent in the compartment containing the sexually active male from time spent near the estrous female (the prevailing method in our laboratory, adopted from Edwards and Pfeifle, 1983). Thus, a positive score indicates preference for the estrous female ; a negative score indicates preference for the sexually active male. In the tests in which interaction was possible, the sexual behaviors with the incentives were also scored. Only ejaculation frequencies are presented.

Pair test with estrous female

In these tests, lasting 15 minutes, semicircular cages measuring 62 x 40 x 36 cm were used. Before the test the experimental animal was put in the cage for a 5-minutes adaptation period. At the beginning of the test an estrous female was put in the cage. Various behaviors were scored. For this report the number of ejaculations were analysed.

Pair test with sexually active male

For these tests, carried out also in semicircular cages, sexually active males were used. After a 5-minutes adaptation period of the stud males, the experimental males were put in the boxes. The lordosis responses of the experimental male to the mounting of the stud male were recorded. The test lasted until the experimental male had received 10 mounts or, at the longest, 10 minutes. Since many males were not very attractive and did not receive 10

mounts, we have included in the analyses the lordosis quotients of those experimental males that received three or more mounts.

Hormone assay

Blood was collected for testosterone assays in Experiments 1 and 2. Testosterone concentrations were estimated in serum by radioimmunoassay, without chromatography using the prevailing method in our laboratory (e.g. Baum et al., 1988). The interassay and intraassay coefficents of variation were 13.4 % and 5.6 %, respectively. In Experiment 2 also the estradiol concentrations were measured. Intra- and interassay coefficients of variation were less than 15 % and less than 19 % for the estradiol assay (e.g. Kwekkeboom et al., 1990).

Statistics

Most data were analysed using One- or Two-way ANOVA (Perlman, 1986). For further analysis the least significant difference (LSD) procedure (Kirk, 1968) was used.
The numbers of animals displaying some type of behavior were analysed using Fisher exact two-tailed probability analysis or the Binomial test when appropriate. The data of Experiment 3 were analysed using Kruskall-Wallis or Mann-Whitney analysis.

Experiment 1

Test procedure

The males were subjected to four consecutive partner preference test (see Table I). In test 1, sexual interaction was prevented by wire mesh ; in tests 2-4 sexual interaction with the tethered stimulus animals was possible. The experimental males received a s. c. injection with progesterone (0.5 mg) which was given 4-7h prior to preference test 4. Also, four pair tests with an estrous female were carried out (S1-S4) ; tests S1 and S4 without any pretreatment, test S2 after 3 s. c. daily injections with 20 µg EB/day, test S3, 25-30 minutes after one i. p. injections with 2 mg/kg Yohimbine, an α^2-adrenoreceptor blocker with sexual behavior stimulating properties (e.g. Clark et al., 1984). Lordosis behavior was studied in a pair test with a sexually active male (test L), without any treatment. Blood was collected from the orbital plexus under light ether anaesthesia following the last pair test (30 weeks of age).

Partner preference behavior

Partner preference scores are depicted in Figure 2.

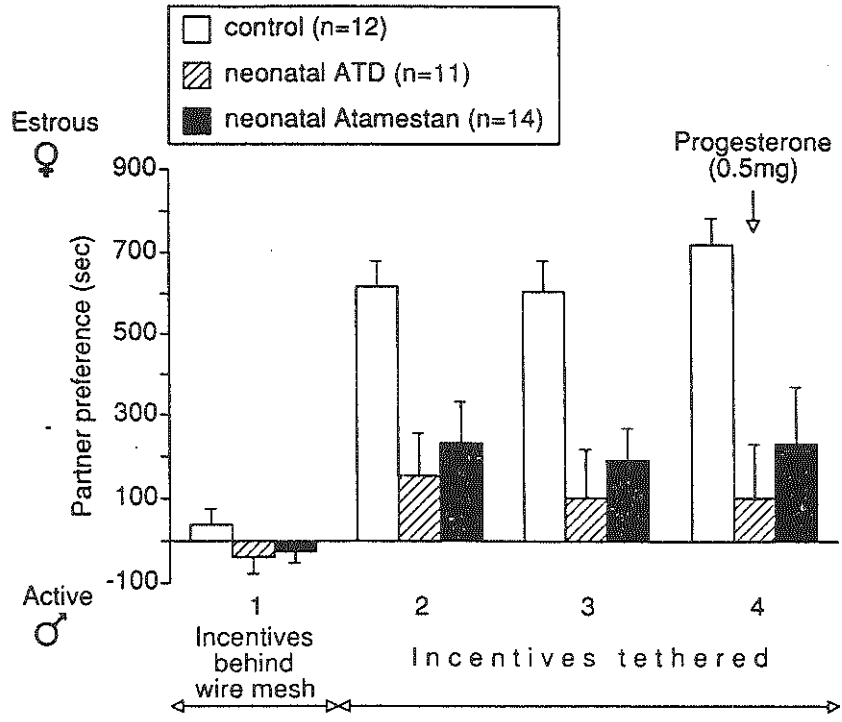

Figure 2 Mean (± SEM) preference (seconds) for an estrous female over a sexually active male of intact adult male rats after neonatal ATD, Atamestan or control treatment. See also text and Table I for experimental details.

One-way ANOVA of test 1 did not show a significant group difference ($F(2/34) = 1.44$, n.s.). Two-way ANOVA of tests 2-4 revealed a significant group difference ($F(2/34) = 11.32$, $P < 0.0005$), no significant effect of tests ($F(2/68) = 0.41$, n.s.), and no significant groups x tests interaction ($F(4/68) = 0.28$, n.s.). Further analysis of the group difference (LSD(5 %) = 233.2 sec.) showed that control males had significantly higher preference scores for the estrous female than neo-ATD and neo-ATA males ; the latter two groups did not differ.

Sexual behavior with estrous female

Ejaculation behavior during partner preference and pair testing is shown in Figure 3.

Fisher's two-tailed probability analysis, performed on the total number of males ejaculating at least once during partner preference tests 2, 3 and 4 (combined), showed that a lower number of neo-ATD and neo-ATA males ejaculated than controls (control vs neo-ATD : $P < 0.001$; control vs neo-ATA : $p < 0.02$). Neo-ATD and neo-ATA males did not differ.

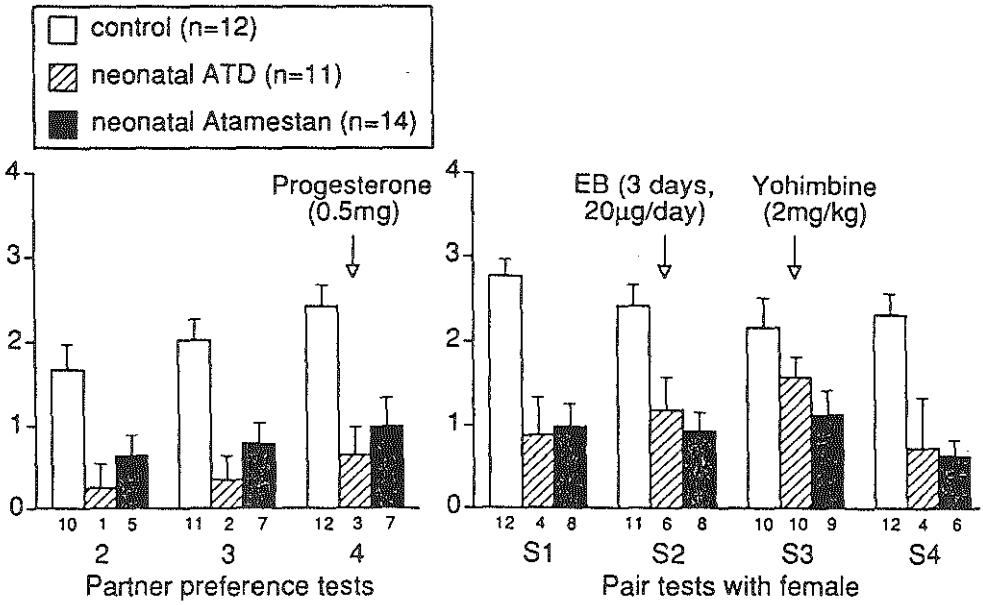

Figure 3 Mean (± SEM) ejaculation frequency during partner preference tests (2-4) and pair tests with an estrous female (S1-S4) of intact adult male rats after neonatal ATD, Atamestan or control treatment. The digits below the bars indicate the number of males per group ejaculating during the test. For details see text and Table I.

Two-way ANOVA of the ejaculation frequencies displayed during pair testing, showed a significant group difference ($F(2/34) = 17.66$, $P < 0.0005$), no significant effect of tests ($F(3/102) = 1.70$, n.s.), and no significant interaction ($F(6/102) = 1.27$, n.s.). Further analysis of this group difference (LSD(5 %) = 0.65 ejac.) showed that neo-ATD males and neo-ATA males had lower ejaculation frequencies than controls in all 4 pair tests.
Yohimbine only increased the number of neo-ATD males that ejaculated : after a single injection (test S3) almost all ATD-males ejaculated (10 of 11), whereas in tests before (S1) and after Yohimbine (S4) only 4 of 11 males ejaculated (10 of 11 vs 4 of 11, $p < 0.001$, binomial test). Such stimulatory effect of Yohimbine was not found in neo-ATA and control males. Adult treatment with EB had no effect in any group.

Lordosis behavior with active male

Lordosis data are shown in Figure 4.
In this experiment many males were not very attractive, i.e. they were not readily mounted by the stimulus male.

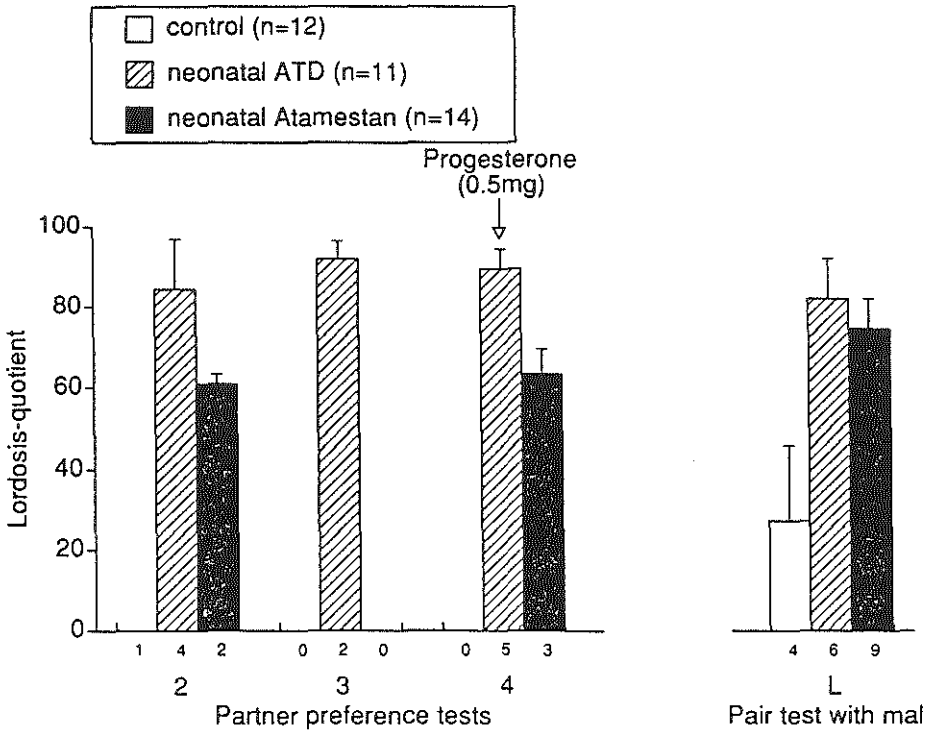

Figure 4 Mean (± SEM) lordosis quotients of intact male rats during partner preference tests 2-4 and pair test with a sexually active male (test L) after neonatal ATD, Atamestan or control treatment. The digits below the bars indicate the number of males per group that were mounted by the sexually active male and that contributed to the calculated lordosis quotients. Test 4 was carried out 4-7h after a s.c. injection with 0.5 mg progesterone.

The number of males that had received 3 or more mounts by the stimulus male in at least one of the partner preference tests 2-4 were : control : 1 of 12, ATD 6 of 11, and Atamestan : 3 of 14. Fisher's exact two-tailed probability analysis did not show differences between groups (control vs neo-ATD, $P = 0.07$; control vs neo-ATA, $P = 1.00$; neo-ATD vs neo-ATA, $P = 0.11$). It is remarkable that neo-ATD and neo-ATA males that were receptive, showed fairly high lordosis quotients ; the one control male allowing mounts from the stimulus male during partner preference testing did not show lordosis.

Fisher's exact two-tailed probability analysis of the number of males being mounted 3 times or more by the sexually active male (during test L) did not reveal statistically significant differences. One-way ANOVA of the lordosis quotients (of the responders) in test L showed a significant group difference ($F(2/16) = 5.95$, $P < 0.02$). Further analysis (LSD(5%) = 35.8) showed that controls had lower lordosis quotients than neo-ATD and neo-ATA males ; the latter two groups did not differ.

Hormone data

Testosterone levels at 30 weeks of age can be seen in Table II.
One-way ANOVA of the serum T levels showed no significant group difference ($F(2/30 = 1.75$, n.s.). From these data it appears that neonatal ATD or Atamestan treatment had no effect on adult endogenous testosterone levels in these gonadally intact males.

Table II Blood serum levels (mean ± SEM) of testosterone and estradiol in male rats during 2 experiments.

Number expt	Neonatal treatment (Silastic implant)	Age in weeks (w) or days (d)	Number of animal samples	T-levels (nmol/l)	E_2-levels (pmol/l)
1*	empty		11	7.3 + 1.0	-
	ATD	30 w	9	5.2 + 0,3	-
	Atamestan		13	5.2 + 0.7	-
2*	cholesterol	60 d	6	19.6 + 3.2	58.3 + 4.3
	ATD	60 d	13	18.4 + 2.6	56.4 + 5.5
	cholesterol	90 d	17	14.4 + 1.5	58.2 + 5.3
	ATD	90 d	36	26.1 + 2.7	54.1 + 1.3

* All animals were left intact ; Atamestan = 1-methyl-1-1,4-androstadienen-3,17-dione ; ATD = 1,4,6-androstatiene-3,17-dione

Experiment 2

In Experiment 1, clear effects were found of neonatal treatment with aromatase inhibitors on adult 'sexual orientation' and sexual behavior. In Experiment 2, the ontogeny of these effects was investigated.

Test procedure

Firstly, the animals were tested for partner preference behavior with sexual interaction prevented by wire mesh. Half of the males was tested 9 times between days 32-60 (group 1), the other half, 7 times between days 63-90 (group 2). Both groups then received two tests in which sexual interaction with the tethered stimulus animals was possible (see also Table I).

Partner preference behavior

The preference data are shown in Figure 5.
Tests without sexual interaction were analysed separately from tests in which sexual interaction was possible. Tests 1-9 from group 1 were analysed

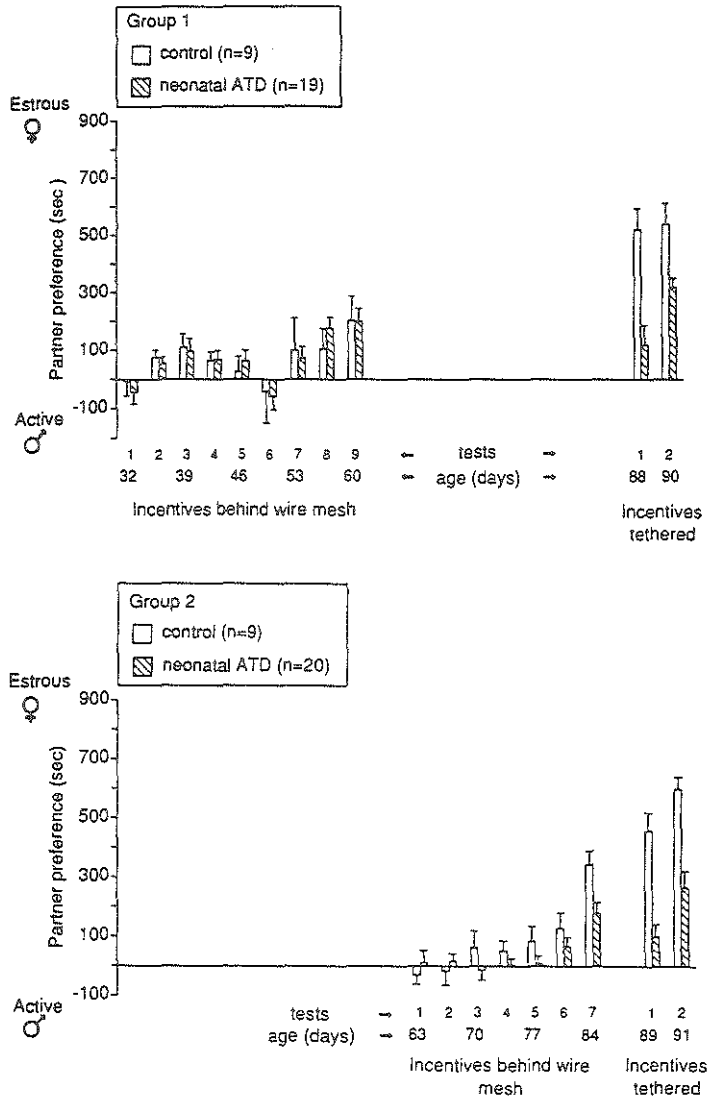

Figure 5 Mean (± SEM) preference for an estrous female over a sexually active male of male rats neonatally treated with ATD or cholesterol. The animals were tested with incentives behind wire mesh or with tethered incentives with sexual interaction possible (see also Table I for details).

separately from tests 1-7 from group 2. Two-way ANOVA of tests 1-9 of group 1 revealed no statistically significant group difference ($F(1/26) = 0.001$, n.s.), a significant effect of tests ($F(8/208) = 6.16$, $P < 0.0005$), and no significant interaction ($F(8/208) = 0.26$, n.s.). Further analysis of the difference between tests (LSD(5 %) =89.6 sec.) showed a significant increase in preference for the estrous female between tests 1 and 2, as well as between tests 7 and 9. Between tests 2 and 7 the preference scores were similar. Surprisingly, however, the preference scores in test 6 were quite different from the other tests. In test 9 (60 days of age) neo-ATD and control males both showed a similar preference for the etrous female partner.

The preference scores of group 2 (test 1-7) are depicted in Figure 5 (bottom). Overall, there were gradually increasing preference scores for the estrous female. Two-way ANOVA showed no significant group difference ($F(1/28) = 2.34$, n.s.), a significant effect of tests ($F(6/168) = 13.24$, $P < 0.0005$) and a borderline significant interaction ($F(6/168) = 2.08$, $P < 0.06$). Further analysis of this interaction (LSD(5 %) = 96.05 sec.) indicated that only during test 7 (84 days of age) ATD males had significantly lower preference scores than controls.

The preference scores of groups 1 and 2 of the tests in which sexual interaction was possible are also shouwn in Figure 5 (right and side). Two-way ANOVA revealed a significant difference between groups ($F(3/53) = 15.92$, $P < 0.0005$), a significant effect of tests ($F(1/53) = 16.72$, $P < 0.0005$), and no significant interaction ($F(3/53) = 0.90$, n.s.). The preference scores of test 2 are significantly higher than those of test 1. Further analysis of the difference between groups (LSD (5 %) = 136.3 sec.) showed that both ATD-groups (not different from each other) had lower preference scores than both control groups. The control groups did not differ.

Sexual behavior with estrous female

Sexual behavioral data during the two partner preference tests in which sexual interaction was possible are shown in Figure 6.

Since only a few ATD males ejaculated, the number of the males that ejaculated at least once during tests 1 and 2 (combined) was used. Fisher's exact two-tailed probability test showed that less ATD males ejaculated than controls in groups 1 and 2 ($P < 0.002$).

Hormone levels

Mean testosterone and estradiol levels are shown in Table II. Samples of Experiment 1 and 2 were analysed separately in assays approximately 6 months apart.

One-way ANOVA of the T-levels at 90-91 days of age showed a significant group difference ($F(1/51) = 8.11$, $P = 0.006$), i.e. ATD males had higher T-levels than controls. Two-way ANOVA of the T-levels of those males that were bled both at 60 and 90-91 days of age revealed a significant group difference

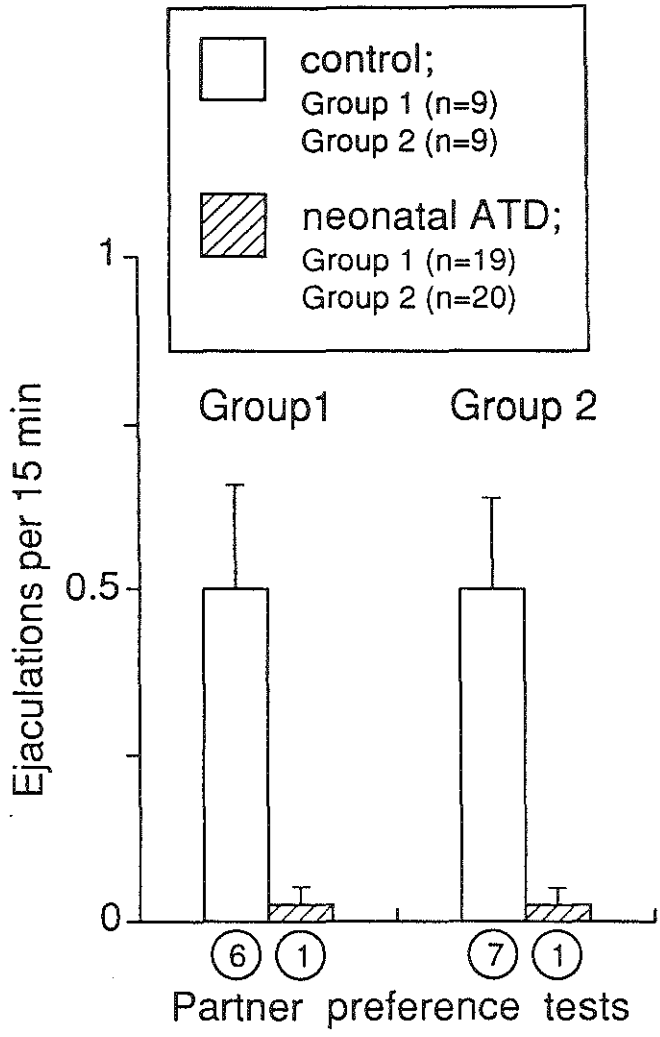

Figure 6 Mean (± SEM) ejaculation frequencies during partner preference tests in which sexual interaction was possible of the same males as in Figure 5. The digits below the bars indicate the number of males that ejaculated at least once during one of those tests.

($F(1/17) = 7.00$, $P < 0.02$), a significant effect of age ($F(1/17) = 6.91$, $P < 0.02$), and a significant interaction ($F(1/17) = 8.95$, $P = 0.008$). Further analysis of this interaction (LSD(5 %) = 13.79 nmol/l) showed that at 60 days of age the levels of T did not differ. At 90-91 days of age the T-level in the ATD males was significantly higher than the control males.

One-way ANOVA of the E_2-levels at 90-91 days of age did reveal no difference ($F(1/51) = 1.00$, n.s.). Two-way ANOVA of the E_2-levels of those males that were bled both at 60 and 90-91 days of age showed no significant difference between groups ($F(1/17) = 0.20$, n.s.) or age ($F(1/17) = 0.29$, n.s.) and no significant interaction ($F(1/17) = 0.005$, n.s.).

Experiment 3

We published recently about the effects of perinatal endocrine manipulations, using ATD, on adult partner preference and sexual behavior in male rats (Brand et al., 1991). The SDN-POA of a representative sample of these animals was studied in Experiment 3 (Houtsmuller et al., 1992).

Previous experimental procedure (Brand et al., 1991)

Three experimental groups were used : (1) control (n=14), (2) prenatal ATD (pre-ATD ; n=12), (3) pre- and neonatal ATD (preneo-ATD ; n=18).

From 11 weeks onward, the males were subjected to 13 weekly partner preference tests. In tests 1-7 sexual interaction was prevented. In tests 8-13 sexual interaction with the tethered incentives was possible. In tests 6, 10 and 12, 8-OH-DPAT (a serotonin agonist) was injected 30 minutes prior to testing. In tests 4, 5, 9, 11 and 13 saline (2 ml/kg) was injected 30 minutes before testing.

One and 2 weeks following the last partner preference test the males were pair tested with a sexually active male or with an estrous female.

Present experimental procedure

Detailed data will be presented elsewhere (Houtsmuller et al.,1992). At 28 weeks of age, 5 males of each group were randomly chosen for histological examination of the SDN-POA. They were injected with pentobarbital (Nembutal, 0.5 ml/rat i. p.). All males were then perfused intracardially with saline followed by 500 ml fixative (4 % paraformaldehyde, pH = 7.2). The brains were removed and stored in fixative at 4°C for one day. Subsequently the brain was dehydrated and embedded in paraffin. Serial 6 µm frontal sections were cut according to the coronal plane of the atlas of the rat brain by Paxinos and Watson (1986), mounted upon chrome-aluminium-coated slides and stained with thionin.

Morphometry

Area measurements of the SDN were performed bilaterally by means of a Calcomp 2000 digitizer connected to a VAX 1/780 computer, using a Zeiss microscope with x10 and x40 (plan) objectives respectively, and x12.5 plan oculars. The volume of the SDN was determined by integrating area measurements from the first to the last cell-containing sections. These measurements were taken twice. The mean of these two volumes was calculated and used in the statistical analysis (Kruskall-Wallis and Mann-Whitney U).

SDN-POA data

The SDN-data are shown in Figure 7.

SDN volume

The three groups differed significantly (Kruskall-Wallis, $P = 0.007$). The SDN of the control group was significantly (Mann-Whitney) larger than the SDN of the pre-ATD ($P = 0.036$) or preneo-ATD males ($P = 0.009$). The SDN of pre-ATD males was larger than the SDN of the preneo-ATD males (M-W, $P = 0.047$).

Summary of behavioral data

Detailed behavioral data of these animals will be presented elsewhere (Houtsmuller et al., 1992). In summary the results are as follows. Male rats perinatally treated with ATD had significantly lower preference scores for the estrous female than pre-ATD or control males. The latter 2 groups did not differ. In pair tests with an estrous female none of the 5 preneo-ATD males ejaculated, whereas 4 of 5 controls and pre-ATD males did. In a pair test with a sexually active male, all 5 preneo-ATD males were mounted (mean lordosis quotient 78 ± 9 %), whereas 2 of 5 pre-ATD and 0 of 5 control males were mounted.

General discussion

In the first experiment, carried out in gonadally intact male rats, the effects of neonatal ATD treatment on adult partner preference found earlier (Brand et al., 1991) were replicated : neonatally ATD treated males had significantly lower preference scores for an estrous female than controls. Neonatal treatment with Atamestan had similar effects on adult partner preference behavior of male rats. Thus, the present study confirms the hypothesis from our laboratory (Brand et al., 1991; Brand and Slob, 1991) that estradiol derived from testosterone plays a significant role in programming adult male rat partner preference behavior.

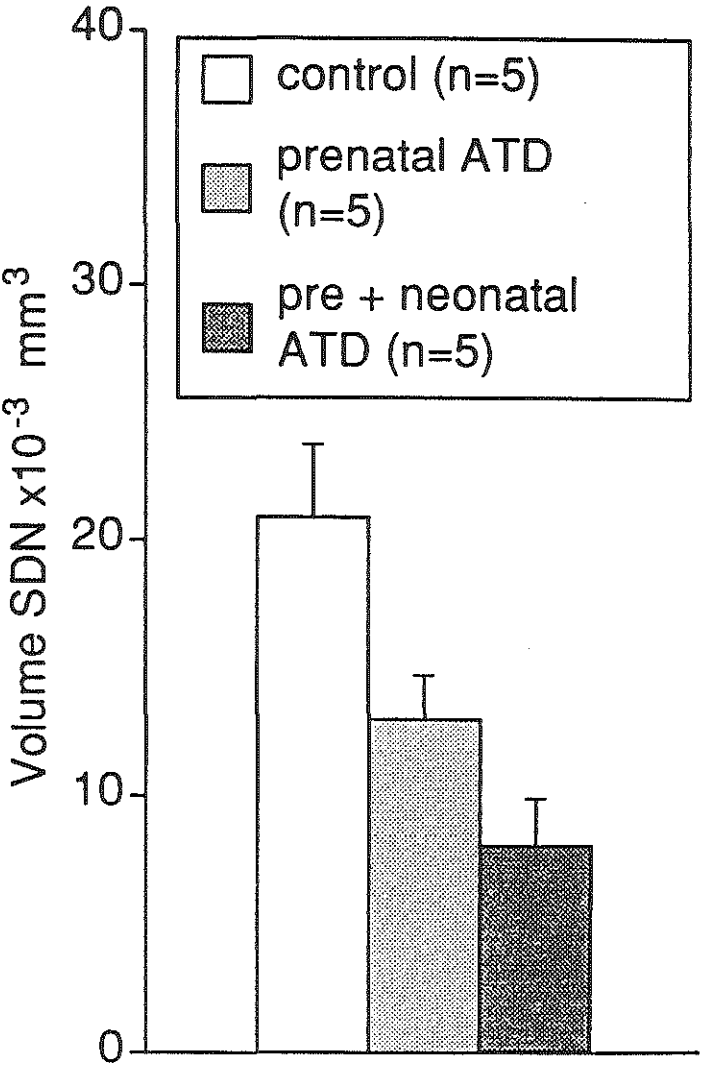

Figure 7 Mean (± SEM) volume of sexually dimorphic nucleus of the preoptic area (SDN-POA) of adult male rats after perinatal ATD or control treatment.

Similarly to the altered adult sexual partner preference, sexual behavior with an estrous female or with a sexually active male was affected by the neonatal ATD or Atamestan treatment. Neo-ATD and neo-ATA males displayed "bisexual" behavior, i.e. they responded complementary to the partner : displaying mounts and intromissions when paired with the estrous female and lordosis behavior when mounted by the stud male. Neo-ATD and neo-ATA males showed significantly lower ejaculation frequencies than controls, in partner preference tests and in pair tests. Thus, neo-ATD and neo-ATA males are less 'masculinized' and less 'defeminized' in adulthood.

In the second experiment the ontogeny of partner preference behavior was investigated. It appeared that the different partner preference behavior of neo-ATD males (a lower preference for the estrous female) first became apparent at the age of around 84 days, i.e. postpubertally. This suggests that testicular hormones play a significant role in the expression of this behavior. Future research into the effects of castration and subsequent subtitution with testicular hormones could support or reject this supposition.

In the third experiment it was found for the first time that the volume of the SDN-POA was larger in control males than in pre-ATD and preneo-ATD males. The SDN-POA volume of the pre-ATD males was also larger than the SDN-POA volume of preneo-ATD males. It thus seems that neonatal as well as prenatal E_2 has a programming effect on the volume of the SDN-POA. Whether there is a causal relationship between the altered volume of the SDN and the altered partner preference and sexual behavior is not clear. However, the smaller volume of the SDN following perinatal ATD treatment is in line with the hypothesis that E_2 programs the volume of this nucleus (Döhler et al., 1984).

In conclusion, neonatal estradiol programs adudlt partner preference and sexual behavior in male rats. The difference between ATD treated and control males becomes apparent after puberty (around 80 days of age) and is presumably dependent on the activating action of endogenous testicular hormones. The volume of the SDN-POA, a sex-dimorphic nucleus, is programmed by pre- as well as neonatal E_2. Whether there is a causal relationship between the volume of this nucleus and partner preference and sexual behavior remains to be investigated.

Acknowledgements

Thanks are due to J. van Ophemert, S. M. Haensel, M. Koolen and F. Jonker for support with animal testing and maintenance. Dr. F. H. de Jong is thanked for supervising the hormone assays and Professor J. J. van der Werff ten Bosch for reading and commenting on an earlier version of this manuscript. Mr. A. Houba, Schering Ned. Inc, is thanked for providing the aromatase inhibitor Atamestan.

2. SDN-POA VOLUME, SEXUAL BEHAVIOR AND PARTNER PREFERENCE OF MALE RATS AFFECTED BY PERINATAL TREATMENT WITH ATD

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CHAPTER 7

ABSTRACT

The present study investigated 1) the importance of the aromatization process during the perinatal period for the development of the sexually dimorphic nucleus in the preoptic area of the hypothalamus (SDN-POA) of male rats, and 2) the relationship between SDN-POA volume and parameters of masculinization in male rats that were treated perinatally with the aromatase-inhibitor ATD. Males were treated with ATD either prenatally or pre- and neonatally, or with the vehicle. Masculine sexual behavior as well as partner preference were investigated in adulthood. Thereafter, animals were sacrificed and SDN-POA volume was measured. SDN-POA volume was reduced in both the prenatally and the pre- and neonatally treated group, with a larger reduction in the latter than in the former group. Combined pre- and neonatal ATD treatment resulted in reduced frequency of mounts, intromissions and ejaculations, as well as a reduced preference for a female over a male. SDN-POA size was significantly and positively correlated with frequency of masculine sexual behavior, as well as preference for a female over a male.

INTRODUCTION

Recently it was found in our laboratory that perinatal treatment of male rats with the aromatase-inhibitor ATD (1,4,6-androstatriene-3,17-dione), which blocks the aromatization of testosterone to estrogen, impairs masculine sexual behavior, enhances feminine sexual behavior and decreases the preference for an estrus female over a sexually active male (Brand et al., 1991). The differentiation of masculine and feminine sexual behavior in various mammals has long been known to be directed by estrogens aromatized from androgens around birth (Baum, 1979, Goy and McEwen, 1980), and in addition, partner preference behavior has now been suggested to be dependent on the same process (female ferrets: Baum et al., 1990, male rats: Brand et al., 1991, female rats: Brand and Slob, 1991).

At the level of the central nervous system, the medial preoptic area (MPOA) of the hypothalamus has been strongly implicated as an important site for both gonadotropin release and the expression of masculine and feminine sexual behavior (e.g. Christensen and Clemens, 1979, van de Poll and van Dis, 1979, Malsbury, 1971, Hart, 1974, Slimp et al., 1978). Within the MPOA, a sexually dimorphic nucleus (SDN-POA), which is severalfold larger in males than in females, was first described in rats (Gorski et al., 1978, Gorski et al., 1980). A similar sex-dimorphic nucleus has subsequently been identified in several other species, (gerbil: Commins and Yahr, 1984, guinea pig: Hines et al., 1985, ferret: Tobet et al., 1986, Cherry et al., 1990) including man (Swaab and Fliers, 1985, de Jonge et al., 1990).

The sexual differentiation of the SDN-POA shows a close parallel with the sexual differentiation of behavior. First, the size of the SDN-POA of the adult rat is dependent on the presence of androgens during the perinatal period (Jacobson et al., 1981), as is behavioral sexual differentiation (see Baum et al., 1979, Goy and McEwen, 1980). Second, several findings suggest that the conversion of these androgens to estrogens perinatally is a prerequisite for the masculine development of the SDN-POA (Dohler et al., 1986, see also Dohler et al., 1984b). The importance of the aromatization process

during the perinatal period for behavioral sexual differentiation has also been well documented (Baum, 1979). The first purpose of the present study therefore was to investigate the importance of the aromatization process during the critical period for the development of the SDN-POA in male rats by determining the effect of perinatal treatment with the aromatase inhibitor ATD on SDN-POA volume in adulthood.

Because of its sex dimorphism, its location within the MPOA, and the parallels between its development and behavioral sexual differentiation, the specific involvement of the SDN-POA in the regulation of sexual behavior has been investigated in several studies. It has been found that the volume of the SDN-POA correlates significantly with sexual performance and testosterone levels in males (Anderson et al., 1986). Moreover, lesions of this nucleus reportedly decrease masculine sexual behavior in several species, indicating the involvement of this nucleus in the expression of masculine sexual behavior (male rats: de Jonge et al., 1989, female rats: Turkenburg et al., 1988, male gerbils: Commins and Yahr, 1984, male ferrets: Cherry and Baum, 1990). It should be noted however, that negative findings have been reported as well (Arendash and Gorski, 1983).

Therefore, the second purpose of this study was to study SDN-POA volume in relationship to differences in partner preference and masculine sexual behavior, induced through perinatal ATD treatment. Subjects randomly drawn from a larger group of ATD treated males described elsewhere (Brand et al., 1991) were re-examined following determination of SDN-POA volume.

METHOD

Animals and hormone treatment

Animals were housed 2-4 to a cage with food and water ad lib and kept on a reversed 14-10-hr light-dark cycle.

Pregnant Wistar females received daily subcutaneous injection of ATD (5 mg in 0.1 ml propylene glycol) (n=8) or propylene glycol (0.1 ml) (n=4) from days 10-22 of pregnancy (day of impregnation=day 0). Within 9 hr of birth male pups from ATD mothers received a subcutaneous silastic implant (inner diameter 1.5 mm, outer diameter 2.1 mm, length 5 mm) filled with ATD (n=18), which was removed again after 21 days, or no implant (n=12). Thus, three groups were formed: males that were treated pre- and neonatally with ATD (pn-ATD) (n=18), males that were treated only prenatally with ATD (pre-ATD) (n=12), and males, whose mothers received injections of propylene glycol during pregnancy, and who served as control subjects (CONTROL) (n=14). All animals were behaviorally tested in adulthood and the results have been described elsewhere (Brand et al., 1991). For the purpose of the present experiment, SDN-POA volume was measured in a subgroup of 15 animals, 5 drawn from each experimental group. This number proved sufficient to yield significant differences among groups. Selection of animals was random and did not take into account the results from the behavioral tests, since the primary goal of the present experiment was to determine the effect of perinatal ATD treatment on SDN-POA size. Had behavioral results been used as a selection criterion, it would not have been possible to distinguish between effects of experimental treatment on the one hand, and other, unknown variables which may have determined sexual behavior in these animals on

the other hand.

Stimulus animals used in the behavioral tests were sexually active males, and females brought into heat by 30 ug EB (24-48 hours before testing) and 2.5 mg P (3-4 hours before testing). These hormones were dissolved in olive oil and injected subcutaneously.

Procedure

At 21 days of age, pups were weaned and housed 2-4 to a cage of same sex and treatment. They were left undisturbed until behavioral testing started, when the animals were 11 weeks old.

Males were subjected to 13 weekly partner preference tests. In tests 1-7 no sexual interaction with stimulus animals was possible, as stimulus animals were separated from the experimental animals by wire mesh. In tests 8-13 sexual interaction was possible. (See Behavioral tests for details). Two weeks after the last preference test, the males were tested for masculine sexual behavior.

For purposes other than the present study, males were injected with 8-OH-DPAT prior to partner preference tests 6, 10 and 12, and the results of these tests are not included here. Prior to tests 4, 5, 9, 11 and 13, animals were injected with a saline solution (1 ml). One week after the last partner preference test, males were tested for feminine sexual behavior, the results of which were presented elsewhere (Brand et al., 1991).

After all behavioral testing was completed, the animals were sacrificed, brains were weighed and SDN-POA volumes were measured.

Behavioral tests

Partner preference.

Partner preference tests were carried out in a test box with three compartments (60x30x40 cm each) (Slob et al., 1987), in which a stimulus male was placed in one lateral compartment and a stimulus female in the other. Experimental animals could move freely from one compartment to the next through a small opening (13x12 cm) in the partitions separating the compartments.

In the tests without interaction (1-7), a wire mesh separated the experimental animals from the stimulus animals, allowing the animals to have non-physical sensory contact with each other. In the tests with interaction (8-13), each stimulus animal was tethered by a harness attached to the back wall of the compartment, limiting the action radius of the stimulus animals. In these tests physical interaction was possible between the experimental male and stimulus animals.

Prior to the test, stimulus and experimental animals were adapted to the test environment for 15 minutes. During this adaptation time, the openings in the partitions were closed by sliding doors, so that no interaction between animals was possible. These doors were then removed, and for 15 minutes behaviors were observed through the transparent front. Time spent in each compartment was recorded for the experimental male, and when interaction was possible, mounts, intromissions, ejaculations and lordosis were scored.

Masculine sexual behavior

The test for masculine sexual behavior was carried out in semicircular cages (radius=36 cm). Males were allowed a 5 min adaptation period before a stimulus female was introduced. For 15 min, mounts, intromissions and ejaculations, as well as the latencies to these behaviors were scored.

Autopsy procedures

Upon completion of the behavioral tests the animals were injected with pentobarbital (Nembutal, 0.5 ml/rat IP). All animals were then perfused intracardially with saline followed by 500 ml fixative (4% paraformaldehyde, pH-7.2). The brains were removed and stored in fixative at 4 C° for one day. Subsequently the brains were dehydrated and embedded in paraffin. Serial 6 μ frontal sections were cut according to the coronal plane of the atlas of the brain of Paxinos and Watson (Paxinos and Watson, 1986), mounted upon chrome-alum-coated slides and stained with thionin.

Morphometry

Area measurements of the cross-sectional SDN were performed bilaterally by means of a Calcomp 2000 digitizer connected to a VAX 11/780 computer, using a Zeiss microscope with x10 and x40 (plan) objectives respectively, and x12.5 plan oculars. The volume of the SDN was determined by integrating area measurements from the first to the last SDN cell-containing sections. To insure reliability, these measurements were taken twice under blind conditions, with a correlation of .70 ($p=.002$) between the first and second measurement. The resulting mean of these two volumes was used in the data analysis.

Data analysis

Data on SDN-POA volume and behavioral measures were subjected to a nonparametric analysis of variance (Kruskall-Wallis), comparing the subgroups pn-ATD ($n=5$), pre-ATD ($n=5$), and CONTROL ($n=5$). When justified, individual group comparisons were made using the nonparametric Mann-Whitney test. Since there was overlap in SDN-POA volume between the groups, and since SDN-POA volume has been suggested to be a strong predictor of sexual activity in males (Anderson et al., 1986), correlations between SDN-POA volume and several behavioral parameters were calculated in addition to the analyses of variance, which merely tested differences between groups. For these correlations the three groups were combined as SDN-POA data comprised a normal distribution (Kolmogorov-Smirnov Goodness of Fit Test, $p=.96$, see Fig.3). Correlations were calculated using the Pearson procedure (when data met the requirement of a normal distribution) or Spearman procedure (when this requirement was not met), and are reported only when significant. Differences in number of animals ejaculating were calculated using the Fisher Exact two-tailed-probability test.

RESULTS

All animals appeared healthy throughout the experiment. Genitalia of males treated

with ATD were normal in appearance at birth and continued to developed normally throughout the course of the experiment.

SDN-POA volumes

Mean volumes of the SDN-POA for the three groups are shown in Fig. 1. An overall significant difference between groups was found ($p = .007$). Subsequent analysis showed that SDN-POA volumes of the pn-ATD group were significantly smaller than those of both the pre-ATD and the CONTROL-group ($p = .047$ and .009, resp.), and that SDN-POA volumes of the pre-ATD group were significantly smaller than those of the CONTROL-group ($p = .036$). Mean brain weights (CONTROL: 1.9 g, pre-ATD: 2.0 g and pn-ATD: 1.9 g) did not differ among groups (ANOVA, $p > .10$).

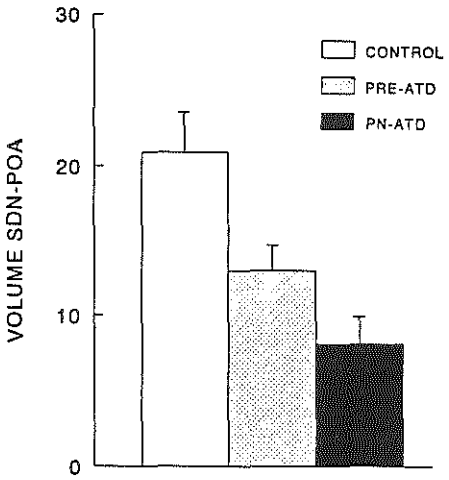

Fig. 1. Mean (±SEM) SDN-POA volume (x 10^{-3} mm^3) of males treated with ATD pre- and neonatally (pn-ATD), prenatally, (pre-ATD) and control males (control).

Behavioral tests

As there were statistically significant differences between the groups in SDN-POA volume, behavioral data of these subgroups were analyzed and correlated with SDN-POA volume.

Partner preference

In order to obtain a score for the preference for an estrous female, the time (in seconds) spent in the compartment of the stimulus female was divided by the total time spent in the compartments of the stimulus female and male. Fig. 2 presents two mean preference scores, one for 6 tests in which physical interaction between experimental and stimulus animals was not possible, and one for 4 tests in which such interaction was allowed.

There was no overall significant difference between the groups on mean preference score over tests without interaction (K-W, $p = .174$). Groups did differ significantly with respect to mean preference for a female on the tests with interaction

(K-W, p=.009). Preference for a female on these tests was significantly lower for the pn-ATD group than both other groups (p=.009 in both cases). No significant difference was found between the pre-ATD and the CONTROL-group (p=.917). Combining over groups, SDN-POA volume correlated positively and significantly with preference for a female on both the tests without (r=.55, p=.017), and with interaction (r=.66, p=.003).

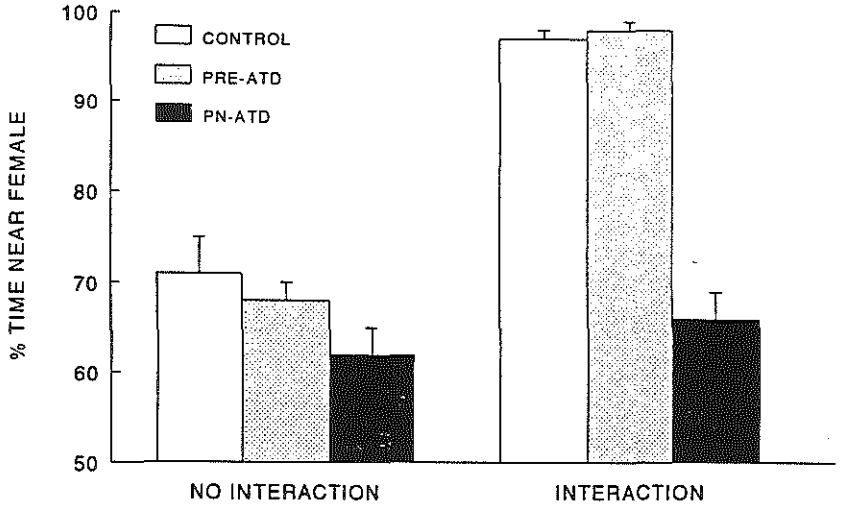

Fig. 2. Preference for an estrous female over a sexually active male, expressed as the mean (±SEM) amount of time spent near the female as a percentage of the total time spent near male and female. Bars represent a mean for 6 tests on which interaction was not possible as stimulus animals were separated from experimental animals by wire mesh (no interaction), and a mean for 4 tests in which stimulus animals were tethered and interaction between experimental animals and stimulus animals was possible (interaction). For explanation of treatments, see Fig.1

Sexual behavior during partner preference tests with interaction.

Fig. 3 shows the distribution of SDN-POA volumes and frequency of masculine sexual behavior during the partner preference tests across groups.
Frequencies of mounts plus intromissions during the preference tests did not differ between groups on any of the tests (p=.128, means ± sem: pn-ATD:15.9 ± 3.1, pre-ATD:21.6 ± 3.7, CONTROL:23.3 ± 1.2). There was however a significant correlation between mean frequency of mounts and intromissions, and SDN-POA volume (r=.67, p<.01). In addition, mean number of ejaculations during these tests differed significantly across groups (p=.01), with animals from the pn-ATD group ejaculating less than both other groups (p<.04 in both cases). The difference between the pre-ATD group and the CONTROL group did not reach statistical significance (p=.11). The correlation between SDN-POA volume and mean number of ejaculations was positive and significant (r=.79, p<.001).

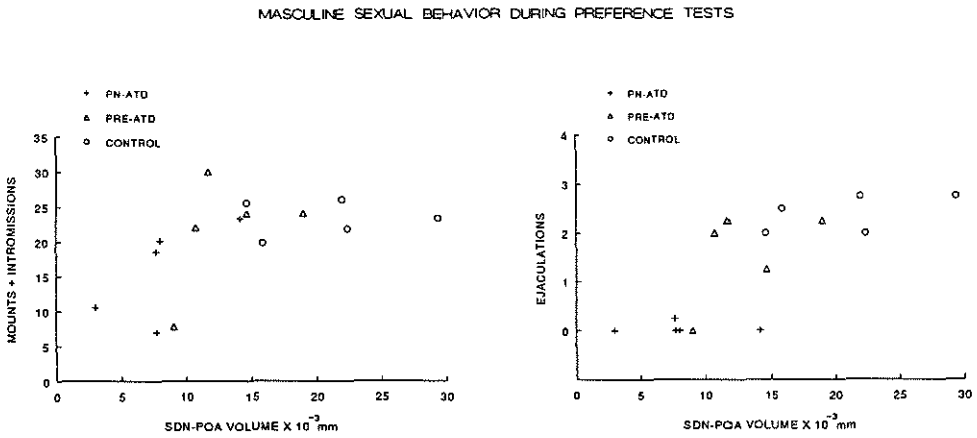

Fig. 3. Relationship between SDN-POA volume, and mean number of mounts plus intromissions, and ejaculations for individual animals during 4 partner preference tests in which interaction with stimulus animals was possible. For explanation of treatments, see Fig.1.

Masculine sexual behavior during pair test

Mean number of mounts and intromissions during a test which paired the male with an estrous female for 15 minutes differed significantly across groups ($p = .031$, Fig. 4).

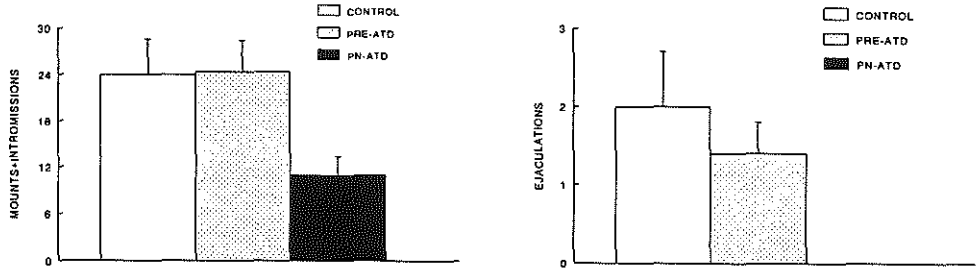

Fig. 4. Mean (±SEM) number of mounts, intromissions, and ejaculations during a test with a receptive female. For explanation of treatments, see Fig.1.

Mounts plus intromissions were significantly lower for the pn-ATD group than both the pre-ATD and the CONTROL group ($p < .04$ in both cases). Number of ejaculations was significantly different across groups as well ($p = .029$). Animals from the pn-ATD group did not ejaculate and differed significantly from both other groups ($p < .029$ in both cases). There was no difference between the pre-ATD group and the CONTROL group. SDN-POA volume and number of ejaculations during the pair test correlated

significantly ($r=.73$, $p=.001$).

DISCUSSION

The results of the present study show that in addition to behavioral sexual differentiation, SDN-POA volume of male Wistar rats is affected by perinatal treatment with the aromatase-inhibitor ATD. SDN-POA volume was significantly reduced by both pre- and perinatal (pre- and neonatal) treatment with ATD, with perinatal treatment being the most effective. The reduction in SDN-POA volume by combined pre- and neonatal ATD treatment was concomitant with reduced preference for an estrous female over a sexually active male and with reduced masculine sexual behavior. Furthermore, SDN-POA volume was positively and significantly associated with several parameters of masculine sexual behavior.

These results corroborate earlier studies, which suggest that the aromatization of androgens to estrogens perinatally is a prerequisite for the 'masculine' development of the SDN-POA. For example, when administered perinatally, the non-steroidal estrogen DES is as effective as testosterone in increasing SDN-POA size in female rats (Dohler et al., 1984a). Pre- and postnatal treatment with an estrogen antagonist reduced the size of the SDN-POA in males, whereas similar perinatal treatment with an anti-androgen did not have such an effect (Dohler et al., 1986). In male ferrets, prenatal ATD treatment blocked the formation of a similar sex dimorphic nucleus (Cherry et al., 1990). The conclusions from these reports are consistent with the results of the present study, which show that inhibition of aromatization of testosterone in males during the perinatal period significantly affects the development of the SDN-POA.

Combined pre- and neonatal ATD treatment was more effective in reducing SDN-POA volume than prenatal treatment alone. Several studies so far have indicated that the critical period during which the SDN-POA is sensitive to circulating gonadal hormones starts prenatally and extends into the neonatal period. That is, both prenatal and neonatal exposure to testosterone are effective in enlarging SDN-POA volume (e.g. prenatal: Ito et al., 1986, Rhees et al., 1990a, Anderson et al., 1985, neonatal: Jacobson et al., 1981, Rhees et al., 1990b). Moreover, neither neonatal androgen or estrogen treatment is as effective as combined pre- and neonatal treatment in increasing SDN-POA volume (Dohler et al., 1984a). Our results are therefore consistent with the view that both pre- and neonatal exposure to estrogen are required for normal development of the SDN-POA in males. However, it cannot yet be ruled out that the greater effect in the combined pre- and neonatal treatment group might have resulted from the greater cumulative dose of ATD, as there was a longer period of treatment in this group.

Differences in SDN-POA volume were accompanied by major differences in behavior. That is, males that were pre- and neonatally treated with ATD and thus had smaller SDN-POA volumes, showed a reduced preference for a female over a male on tests in which interaction with stimulus animals was possible, and reduced levels of masculine sexual behavior during partner preference tests (no ejaculations) as well as during a test in which males were paired with an estrous female (fewer mounts and intromissions, and no ejaculations). These results reiterate the importance of the

perinatal hormonal environment for partner preference, which has only recently been demonstrated in male (Brand et al., 1991) and female rats (Brand and Slob, 1991), and female ferrets (Baum et al., 1990), and further support the idea that perinatal exposure to estrogen aromatized from testosterone is a requirement for full behavioral masculinization of male rats (e.g., Baum, 1979). The lack of differences between groups in partner preference scores where no interaction was possible may have been due to the low n's in the present experiment, as previous results did indicate such a difference (Brand et al., 1991).

The concomitance of changes in SDN-POA volume and changes in behavior in the present study is significant since lesion and implantation studies have implicated the SDN-POA in the expression of masculine sexual behavior (rats: de Jonge et al., 1990, Turkenburg et al., 1988, gerbils: Commins and Yahr 1984, ferrets: Cherry and Baum, 1990). Although the effect of perinatal ATD treatment on behavior of male rats has been reported previously (Brand et al., 1991), a possible mechanism through which ATD might affect behavior, specifically through changes in SDN-POA volume, is suggested by our findings. The strong positive relationship between ATD-induced differences in SDN-POA volume and frequency of ejaculations corroborates an earlier report (Anderson et al., 1986) which also reported such a correlation. The present study expands this relationship to include mounts and intromissions. Taken together, these studies lend support to the idea that the SDN-POA may be involved in the expression of masculine sexual behavior. Interestingly, our results suggest that the subjects in fact comprise two groups, one which ejaculates, the other which does not (see Fig. 3). Although not central to the present hypothesis, if two groups are established based upon behavioral criteria (ejaculators versus non-ejaculators) a significant difference in SDN-POA volume is found ($p<0.02$, both for ejaculations during preference tests and during pair test). It appears that SDN-POA volume smaller than $10x10^{-3}$ mm^3 characterizes non-ejaculators, whereas volumes larger than $16x10^{-3}$ mm^3 are typical of ejaculators.

Our data also provide evidence for a positive relationship between SDN-POA volume and preference for a female in tests with and without interaction. Measures of partner preference when tested without the possibility of interaction with stimulus animals are generally considered parameters of sexual motivation (e.g., Meyerson and Lindstrom, 1973). The SDN-POA thus far has been associated with consummatory aspects of sexual behavior (mounts, intomissions and ejaculations), but not motivational aspects (Everitt, 1990). In fact, partner preference of female rats was previously shown to remain unaffected by a lesion of the SDN-POA (Turkenburg et al., 1988). However, our finding that in males, larger SDN-POA volume is associated with a higher preference score for a female, even when no sexual interaction with the female is possible and thus consummatory aspects of sexual behavior are irrelevant, might serve to stimulate further research delineating the role of the SDN-POA in partner preference of males.

The results from the present experiment thus show a clear effect of perinatal ATD on SDN-POA volume in adulthood, and suggest a positive relationship between SDN-POA volume and consummatory aspects of masculine sexual behavior (mount, intromission and ejaculation frequency) and to a lesser extent partner preference (time spent with a female when no physical interaction is possible). The correlations found

between SDN-POA volume and several parameters of behavioral masculinization indicate that in general, larger SDN-POA volumes were associated with increased behavioral masculinization. However, because of the limitations of a correlational design, further research is necessary to specify the relationship between SDN-POA volume, partner preference and masculine sexual behavior. In addition, the relationship between more subtle variations in SDN-POA volume, as occurred within individual treatment groups, and behavioral parameters was not examined due to the low n's in these groups. Future research needs to investigate possible behavioral differences associated with these more subtle differences in SDN-POA volume.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. D.L. Rowland for general comments on the manuscript, and Dr. D.F. Swaab for his consultation regarding measurements of the SDN.

CHAPTER 8

GENERAL DISCUSSION

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In this thesis experiments are presented that systematically investigated the effect of male fetal rats on the sexual differentiation of siblings with whom they share the uterine horn. Specifically, the effect of males in utero on genital morphology at birth and sexual behavior in adulthood of female siblings was examined; the effect of males in utero on *male* siblings was studied as well. In addition, since changes in prenatal androgen levels are assumed to underlie effects of males in utero on the sexual differentiation of females, and since effects of prenatal hormones on behavioral sexual differentiation are presumably mediated by changes in the central nervous system (CNS), the relationship between the perinatal hormonal environment, and sexual differentiation of reproductive behavior and CNS in rats was studied.

Specifically, it was investigated whether the sexual differentiation of rats is affected by the prenatal position they occupy relative to males in the uterus. Previous studies have investigated this 'intrauterine position phenomenon' in a number of species, and two hypotheses regarding this phenomenon have emerged. One assumes transport of androgens from males to females in utero via diffusion through the amniotic sac, such that females are masculinized by adjacent males ('contiguity hypothesis' Clemens et al., 1978). An alternative hypothesis suggests transport of androgens from males via the uterine vasculature to females located rostrally, such that females are masculinized by males located caudally, but not those located rostrally ('caudal male hypothesis', Meisel and Ward, 1981). Indirect evidence based upon the morphology and adult sexual behavior of females has been provided both supporting and refuting each hypothesis, leaving the issue unresolved. The experiments presented in Chapters 2-5 of this thesis systematically examined the intrauterine position phenomenon in rats, while attempting to identify several factors that may have contributed to the contradictory results reported so far.

Effects of caudal males

In Experiment 1 (Chapter 2) it was shown that when factors relating to prenatal stress from surgery are controlled, females that developed without caudal males are less masculinized (i.e., showed less mounting behavior) and more defeminized (i.e., showed less receptive behavior) than females with caudal males. In Experiment 2, (Chapter 3) it was shown that male rats, like females, are affected by the presence of males located caudally in utero. Males that developed in the presence of such males showed more masculine sexual behavior in adulthood and shorter latencies to sexual behavior and ejaculation, than males that developed in the absence of such males. Moreover, in Experiment 3 (Chapter 4), genital morphology at birth (anogenital distance (AGD), an androgen-sensitive parameter) of female rats from two strains, was shown to increase with the number of males located caudally in utero. In Experiment 4 (Chapter 5), it was found that prenatal androgen levels of female fetuses with caudal males are higher than of females without caudal males, on day 19 of gestation, a critical day for sexual differentiation of the CNS.

Thus, in general the present investigation shows that both female and male rats

that develop in the uterus with males located caudally are more masculinized than their same-sex siblings that develop without such males. These results provide support for the idea that androgens are transported from male fetuses to siblings located rostrally, but not those located caudally, relative to these males in the uterus (caudal male hypothesis). No support was found that androgens are transported from males to adjacent siblings (contiguity hypothesis) in any of the experiments described here. That is, females or males with two adjacent males in utero were not more masculinized than their same-sex siblings without such males.

Differences between species

Support for the 'contiguity' hypothesis stems primarily from studies using mice and gerbils. In these species, a myriad of experiments have suggested that female mice and gerbils that are located between two males (2M females) in utero, differ from females that do not have such males (0M females), on a number of morphological and behavioral traits (mice: reviewed in vom Saal, 1989, gerbils: e.g., Clark and Galef, 1988, Clark et al., 1992). Results from our experiments suggest strongly that such an effect does not occur in rats, but, alternatively, that in the rat natural variation within the sexes in morphological and behavioral masculinization can partly be attributed to the number of caudal males in utero. These results thereby provide robust confirmation of the caudal male hypothesis first put forward by Meisel and Ward (1981).

Since effects of adjacent males have been reported extensively in mice and gerbils, whereas the present investigation found no such effect in rats, differences between species in the mechanism of transfer of androgens from male to female fetuses may account for the lack of consistency across species. It is important to note however, that in mice and gerbils, virtually no attempts have been made to test for the caudal male effect. It is therefore premature to rule out the possibility of an effect of caudal males in these species. In order to eliminate this possibility and assume a species difference, future studies need to systematically investigate possible effects of caudal males in utero on sexual differentiation of mice and gerbils.

Although the contiguity hypothesis has been supported by many studies using mice and gerbils, inconsistencies are apparent. Some recent reports have failed to find evidence for the contiguity hypothesis in mice (Gandelman and Kozak, 1988, Simon and Colloger-Clifford, 1991, Jubilan and Nyby, 1992). Surprisingly, although 2M females have been shown to differ from 0M females on a number of traits, no studies have reported an effect on masculine sexual behavior of females, a behavior which has been shown to be sensitive to perinatal androgen levels in female mice (Gandelman and Kozak, 1988). In fact, a recent report failed to find an effect of adjacent males on masculine sexual behavior (Simon and Colloger-Clifford, 1991). Thus, some questions concerning this phenomenon remain unanswered.

Consistency across strains

Two strains of rats were used in the present investigation: Wistar and Holtzman. In both strains, effects of caudal, but not adjacent males on the sexual differentiation of females were found on genital masculinization of females. Of the two previous studies in rats that reported an effect of adjacent males, one used Holtzman (Clemens

et al., 1978) and the other Sprague-Dawley (Tobet et al., 1982) rats. In the study using Sprague-Dawley rats (Tobet et al., 1982), the authors were unable to differentiate between an effect of adjacent, and an effect of caudal males because most of the females with two adjacent males also had caudal males. In a later study, using this same strain of rat, when the two hypotheses were simultaneously tested, support for the caudal male hypothesis but not the contiguity hypothesis was found (Richmond and Sachs, 1984). In the study using Holtzman rats (Clemens et al., 1978), only the contiguity hypothesis was tested. The caudal male hypothesis was not tested and therefore the results could not be construed as evidence against a caudal male effect. Our study simultaneously assessed the effects of adjacent, and of caudal males on genital morphology of Holtzman females, and clearly found an effect of caudal rather than adjacent males. Thus, the effect of caudal males in rats seems to be fairly consistent across strains, at least those strains studied so far (Holtzman, Wistar, Sprague-Dawley, Long-Evans), and strain differences therefore do not seem responsible for the contradictory results, although it is possible that strains may differ in the extent to which females are affected by androgens from males prenatally. However, in our investigation, no such difference was found between Holtzman females and Wistar females (Experiment 3).

Effects of surgical procedures used in previous studies on the intrauterine position phenomenon

In addition to different strains, previous studies have used different methodologies. Because we hypothesized that this use of different methodologies may have contributed to the controversy surrounding the intrauterine position phenomenon, in exp 1 (Chapter 2), the effects of males on the sexual differentiation of female siblings were examined while controlling for possible confounding effects of two such surgical procedures previously used in studies on the intrauterine position phenomenon: hemihysterectomy of the mother during pregnancy, and birth through caesarean section. Hemihysterectomy was used in a previous study to increase the chance of all-female litters (Clemens et al., 1978). Rats have two uterine horns, and birth order therefore does not reflect position in utero. Caesarean section has therefore been used to allow investigators to establish position in utero (Clemens et al., 1978, Richmond and Sachs, 1984). Experiment 1 showed that hemihysterectomy of the mother midway during pregnancy disrupted the 'caudal male effect', whereas birth through caesarean section did not affect this phenomenon. Neither procedure affected sexual differentiation of the offspring. The finding that hemihysterectomy during pregnancy disrupted the caudal male effect is important since this procedure was used in the study by Clemens et al. (1978), in which an effect of adjacent males on female sexual differentiation was reported in Holtzman females. Although our results do not explain why an effect of adjacent males was observed in this study, they do imply that in future studies on the intrauterine position phenomenon this procedure (hemihysterectomy during pregnancy) should be avoided.

The mechanism through which hemihysterectomy might disrupt the effect of caudal males remains unclear. Stress during pregnancy has been shown to severely affect the prenatal hormonal environment and the sexual differentiation of the male offspring. Specifically, males from mothers who were subjected to stress during

pregnancy reportedly are less masculinized and more defeminized than controls (Ward, 1972), presumably resulting from changes in the hormone secretion in these males. Normal male fetuses show a testosterone surge on day 18 and 19 of gestation. In males from stressed mothers the surge in testosterone level occurs on day 17 with a sharp decline immediately thereafter, and this rise in testosterone is hypothesized to be too early and/or too short for full masculinization and defeminization of the fetus (Ward and Weisz, 1984). In our experiment, if the hemihysterectomy of the mother induced a similar phenomenon in male fetuses, testosterone secretion of the male fetuses may have been inadequate to masculinize females located rostrally.

Another possible explanation for the lack of a caudal male effect after hemihysterectomy of the mother during pregnancy is that the procedure of removing a uterine horn may have affected the direction of the bloodflow in the remaining uterine horn. During the removal of the one uterine horn, both the ipsilateral uterine and ovarian vessels are ligated, and perhaps this affected the hemodynamics of the contralateral horn, and thereby the transport of androgens via the vasculature in this remaining horn. However, this effect appears to be a transient one since an effect of caudal males on masculinization and defeminization was apparent in females from mothers that were hemihysterectomized before pregnancy.

Birth by caesarean section did not disrupt the effect of caudal males on female masculinization. Caesarean section is often used as a means of establishing position in utero relative to males (e.g. vom Saal, 1989, Gandelman, 1986), and it is therefore important that in our experiment, this procedure did not affect the sexual differentiation of the offspring, nor the effect of male siblings on the sexual differentiation of the offspring. The sex difference in prenatal androgen levels is most profound on days 18 and 19 of gestation, days thought to be critical for masculinization in rats (Weisz and Ward, 1980, Baum et al., 1991). Thus, androgens from caudal males presumably have already affected siblings at the time of birth, or shortly preceding birth when a caesarean section is typically carried out.

Effects of intrauterine position in males

In Experiment 2, (Chapter 3) an effect of male fetuses on the sexual differentiation of male siblings was reported for the first time in rats. The research on the intrauterine position phenomenon has thus far primarily focussed on females. Recently, effects of position in utero on male mice (vom Saal et al., 1983, Even and vom Saal, 1991, Nonneman et al., 1992) and gerbils (Clark et al., 1989, Clark et al., 1992) have been reported. In these species, effects of males on adjacent males in utero were found, whereas in our study no such effects were observed. In contrast, effects of caudal males on behavioral masculinization were shown. Males with prenatal caudal males showed more mounting behavior in adulthood, had shorter latencies untill they started sexual activity with an estrous female, and after sexual experience had shorter ejaculation latencies. Therefore, males with caudal males might be at a reproductive advantage since they develop 'efficient' sexual behavior faster than others. Thus, naturally occurring variation among males may partly stem from differences in position in utero relative to males. This finding is consistent with the idea that such variation reflects variation in sensitivity in neural tissue (see Sachs

and Meisel, 1988), rather than testosterone levels in adulthood. Adult plasma testosterone levels have been shown to be largely unrelated to individual differences as long as they are within normal limits (e.g., Damassa et al., 1977).

Statistical control of variables that cannot be controlled experimentally

Typically, studies investigating the intrauterine position phenomenon have used analysis of variance to test differences between groups (e.g., females with vs female without adjacent males, females with vs. females without caudal males). Random assignment of subjects to different groups is not possible in such studies, and therefore, effects of independent variables can, in fact, not be independently assessed. For example, 2M females by definition never develop in the absence of caudal males, since one of the two adjacent males is always located caudally. Thus, along with the independent variable (such as adjacent males), other, possibly confounding, variables (such as caudal males) may vary systematically. As a consequence, effects of one variable may be wrongly ascribed to another variable, or effects of one variable may be confounded by another variable. These consequences are particularly relevant to the literature on the intrauterine position phenomenon in mice, since in these studies the only comparison that has been typically made is between animals with and without adjacent males. Any difference between those groups has consequently been attributed to adjacent males. In the present investigation, this problem was partly obviated by the statistical testing of both hypotheses from data obtained from single experiments; i.e. by grouping subjects according to both the contiguity and the caudal male classification. Since in our experiments, no differences were ever observed when animals with adjacent males were compared to animals without such males, the possibility that the difference between animals with and those without caudal males was really due to an effect of adjacent males can be ruled out. However, several problems remained. First, assessment of the effect of one variable independent of another, covarying variable, which might well be relevant (such as total number of males), is not possible by forming and comparing groups with analysis of variance (ANOVA). Secondly, birth weight is another variable that cannot be controlled experimentally. When assessing the effect of prenatal variables on genital morphology (AGD) this variable needs to be taken into consideration, since birth weight and AGD are related (i.e., larger animals tend to have larger AGD's, e.g.,mice: Graham and Gandelman, 1986). Therefore, variables affecting birth weight may, in doing so, also indirectly affect AGD. Thus, variables identified to affect AGD may in reality affect AGD only *through* an effect on birth weight. To deal with this problem, some research groups therefore, when assessing effects of prenatal factors on AGD, have used the ratio of AGD/birth weight, rather than simply AGD as the dependent variable (e.g. Meisel and Ward, 1981). However, in doing so, the problem is not adequately resolved, as any variable that affects birth weight may also affect the AGD/birth weight ratio (e.g., nutrition, size of the litter, etc.). These considerations have therefore led us to use an alternative statistical approach (multiple regression, Experiment 3), which allowed for simultaneous assessment of the effect of several prenatal variables on AGD at birth of females.

Simultaneous assessment of effects of several prenatal variables

The effects of caudal males, adjacent males and total number of males in the uterine horn on genital morphology at birth were assessed while the effect of birth weight was statistically controlled. Using multiple regression, the effect of each of these variables on AGD at birth was assessed independent of the effects of the other variables. This analysis found that as the number of caudal males increases, AGD of female newborn rats increases. No effect was found for the number of adjacent males. A similar increase with the presence of caudal males was already earlier reported (Meisel and Ward, 1981, Richmond and Sachs, 1984). Our finding, again, provided support for the hypothesis that androgens from males are transported to females located rostrally in utero.

Experiment 3 also found that AGD increased with the *total* number of males in utero, independent of their position relative to the female. Thus, in addition to caudal males, the total number of males in utero affects morphological masculinization of female rats, independent of the location of these rats. Other authors have reported that the total number of males may affect female masculinization as well. Specifically, a recent report suggested a relationship between number of males and prenatal androgen levels in female gerbils (Clark et al., 1991). Prenatal testosterone level of female gerbils increased with both the presence of adjacent males, and the total number of males. An earlier report indicated that an effect of adjacent males was reduced when the total number of males was statistically controlled (Tobet et al., 1982). Our study for the fist time provides information on the effect of each of the variables (adjacent males, caudal males and total number of males) independent of each other, and gives an indication of the magnitude of the contribution of each.

The mechanism through which females may be affected by all males in a uterine horn, in addition to the ones located caudally, remains as of yet unclear. In our experiment, fetuses generally came from only one uterine horn, and it was therefore impossible to determine whether this effect is local, i.e. confined to the uterine horn, or general, i.e., males affecting females in both uterine horns. This latter possibility might involve transport of androgens via the maternal circulation not just to fetuses in the same uterine horn, but also to the contralateral horn. It was recently reported that in gerbils, androgen level of a pregnant female increases with the number of males in her litter, suggesting transport of androgens from male fetuses to the maternal circulation (Clark et al., 1993).

Analysis of prenatal testosterone levels of fetuses and their mothers in Experiment 4 however provided no indications for such transport in rats. Testosterone level of female fetuses did not increase with the total number of males in both uterine horns, or the male-to-female ratio. Testosterone level of the mother did not show any sytematic variation with these variables either, suggesting that in rats, androgens from male fetuses do not affect the androgen level of the mother, or of female fetuses without regard for the position they occupy relative to these males. An earlier study did not find a relationship between number of males in the litter and testosterone in maternal blood either (Ward and Weisz, 1984).

Thus, using a procedure in which birth weight was statistical controlled, and the effect of caudal, adjacent and total number of males in the uterine horn were simultaneously assessed, it was found that AGD of newborn female rats increases with

the number of caudal males as well as the total number of males, but not with the number of adjacent males.

The caudal male effect was further specified in Experiment 3. Specifically, the effect of caudal males on AGD decreased with the distance from the nearest caudal male. Thus, the farther away from a caudal male a female was located, the less the effect of the caudal male. This finding is interesting with regard to the assumed transport of androgens via the vasculature underlying the caudal male hypothesis. If androgens are transported via the uterine vasculature from males to fetuses located rostrally (Meisel and Ward, 1981), the concentration of androgens in the uterine artery transporting these androgens might be expected to decrease as the distance from the male increases. Our findings are consistent with this prediction, providing further support for the caudal male hypothesis. An earlier study reported a similar 'distance-dependent' caudal male effect: female guinea pigs were reportedly masculinized by the presence of an adjacent caudal male, whereas a caudal male that was not adjacent had no effect (Gandelman, 1986). However, further research is needed to corroborate these findings.

Lack of difference between females with one and those with more than one caudal male

In the experiments reported here, when females or males were classified according to the presence of 0, 1 or more than 1 caudal male, a difference between the group without caudal males and the groups with caudal males was typically observed, but no difference between the group with 1, and with more than 1 caudal male (Experiments 2 and 5). Previous studies have reported similar findings (e.g., Meisel and Ward, 1981). A possible explanation for this lack of difference stems from the finding in Experiment 3 that for genital masculinization, the effect of caudal males is dependent on the distance from those caudal males. From this finding it follows that the number of caudal males alone does not determine the effect, but that the distance from the nearest male needs to be taken into account as well. If such is the case, females with more than one caudal male would not necessarily be more masculinized than those with only one caudal male, i.e., two distant caudal males may exert an equivalent, or even less effect than 1 proximal caudal male. Further research is needed to validate this suggestion however.

Prenatal androgen levels

Results from Experiments 1 and 3 clearly showed that females with caudal males are more masculinized than females without such males. The morphological and behavioral differences between females with, and those without caudal males are assumed to be caused by differences in prenatal androgen level. However, to date no evidence has emerged that androgen levels in females with caudal males are indeed higher prenatally than in females without caudal males. In mice and gerbils, two species in which an effect of adjacent males has repeatedly been shown, higher androgen levels were reported in females with two adjacent males, than in females without adjacent males (mice: vom Saal and Bronson, 1980, gerbils: Clark et al., 1991). In rats, however, Baum et al. (1991) found no evidence for transport of androgens from males to either adjacent or rostral females. In that study, androgen

was measured on days 16-20 of gestation. The sex difference in prenatal androgen level is most striking, and according to some authors only apparent, on days 18 and 19 (Weisz and Ward, 1980, Slob et al., 1980). Therefore, effects of caudal males on androgen level of females may also be most apparent on these days. In the study by Baum et al. (1991), although the subgroup of fetuses in which androgen was measured on days 18 and 19 was analyzed separately, the number of animals in this subgroup may have been too low to detect a subtle difference in androgen level. Indeed, as shown for the first time in Experiment 4, when plasma testosterone level was analyzed of female fetuses of gestational day 19 only, plasma testosterone level on gestational day 19 is higher in females with caudal males than in females without such males. These increased testosterone levels are probably responsible for increased masculinzation and defeminization observed in females with caudal males.

Mechanism of transport.

The mechanism supposedly underlying the caudal male effect is transport of androgens via the uterine vasculature. Two assumptions underlie this hypothesis. The first assumes transport of androgens from the uterine vein to the uterine artery, such that androgens secreted by males may be transported into the circulation of siblings. The second assumes that the direction of the bloodflow is primarily in a rostral direction in the uterine vasculature, such that androgens from males are transported only to siblings located rostrally, and not those caudally.

Both assumptions stem from research on luteolysis, which has suggested that substances including androgens may be transported from the uterine vein to artery, and that in the uterine artery and vein, bloodflow is primarily in a rostral direction in rats, guinea pigs and hamsters (Del Campo and Ginther, 1972, Egund and Carter, 1974). Some controversy about the possibility of intrauterine androgen transport via the vasculature exists however. A recent report concluded that in the mouse, bloodflow is bidirectional in the uterine loop (vom Saal and Dhar, 1992). Another study suggested that in rats, labeled testosterone injected into a fetus is transported to adjacent fetuses only, and more so to the fetus on the caudal, than on the rostral side (Even et al., 1992). This finding is obviously in discordance with the results of previous studies (e.g., Richmond and Sachs, 1984, Gandelman, 1986, Babine and Smotherman, 1984) and the experiments presented here. However, it should be noted that in these studies, pregnant rats were anaesthesized, positioned on the back, and a midline incision was made through which the uterine horns were taken out of the body and spread out. Processes observed under such circumstances do not necessarily provide an accurate representation of what happens under natural circumstances, i.e., in the free-roaming, non-anaesthesized, intact animal. Our own observations of the uterine bloodflow of the rat (Chapter 6) yielded no consistent findings. Bloodflow appeared to be highly sensitive to manipulation of the uterus, fetuses and vessels. It is however difficult to assess directly intrauterine transport of androgen or the direction of the bloodflow without manipulation of the uterus. Thus, further study and perhaps new techniques are necessary to give conclusive evidence about transport of androgens in utero. Until such evidence emerges, transport of androgens via the vasculature remains hypothetical.

Finally, the caudal male hypothesis assumes that androgens from males cross

the placenta of siblings and thus enter the circulation of these siblings. Some questions remain concerning such placental transfer. For example, only a small part of androgens injected into the mother were found in the fetal circulation of guinea pigs (Vreeburg et al., 1981, Despres et al., 1984, see also Slob et al., 1983). However, supraphysiologial maternal levels of androgen have clearly been shown to masculinize and defeminize offspring in numerous species.

Magnitude of caudal male effect

It should be noted that although the effect of caudal males on the sexual differentiation of female and male siblings is significant in rats, the magnitude of the effect is limited. The number of caudal males explained about 12% of the variance in prenatal testosterone level of females (Experiment 4). The factors number of caudal males, distance from a caudal male, total number of males and birth weight together explained about 25% of the variance in AGD, leaving 75% unexplained. Moreover, since females as well as males without caudal males may display high levels of masculine sexual behavior, and females without caudal males also have high levels of testosterone prenatally, the effect of caudal males should be viewed as an additional effect.

Thus, sources of androgens other than merely males in utero must be responsible for some of the variation in masculinization among females. The ovaries of the fetus are relatively quiescent. The ovaries of the mother on the other hand produce androgens, but this androgen production decreases during the second half of pregnancy, the time when fetuses are sensitive to the organizing effects of hormones (Gibori and Sridaran, 1981). The placenta finally has been shown to produce androgens (Chan and Teathem, 1975, Gibori and Sridaran, 1981) and has been suggested to be a major source of androgen production in the female fetus (Vreeburg et al., 1983).

Possible consequences of the intrauterine position phenomenon

The obvious question that arises concerns the relevance of the intrauterine position phenomenon. Vom Saal (1989) has argued that this phenomenon may be adaptive in that offspring from one female will, as a result of differences in exposure to androgens, vary with regard to phenotype. Thus, individuals from one litter will differ on traits such as attractivity and aggression, which in mice have been related to intrauterine position. This way, i.e., with this variation within litters, chances are increased that under many sets of circumstances, each of which requires different traits to ensure survival and reproduction, *some* of the offspring will be adapted to survive. For example, under conditions of limited food supplies, aggression may be a trait that increases chances of survival, whereas on the other hand, when the percentage of males in a population is low, attractivity will increase chances of reproduction. In rats, no systematic studies have yet investigated such consequences as reproduction and survival. However, since results from the experiments presented here suggest which fetuses are likely to be exposed to more androgens (i.e. those with caudal males) in the rat, future studies should investigate the consequences of such subtle differences in exposure to androgens for situations in a more natural setting in this species. Our findings that females with caudal males are more

defeminized may indicate a reproductive disadvantage for these females, however, actual parameters of reproductive success, such as number of pregnancies and number of live offspring have not been measured to date. Also, in Experiment 2, males with caudal males were faster at developing 'efficient' sexual behavior (i.e. shorter latencies to ejaculation) than males without caudal males, when given the same opportunities for sexual interaction. Thus, these males might be at a reproductive advantage. However, actual impregnations, semen characteristics and offspring should be examined in order to draw consequences about the long-term significance of the intrauterine position phenomenon.

The significance of the increase in mounting behavior observed in females with caudal males is unclear. Mounting behavior in females has been interpreted both as a proceptive behavior (Beach, 1976) and as a measure of masculinization (Baum, 1979). Whatever its meaning, mounting behavior in adulthood is sensitive to perinatal androgen level, and as such may be used as an assay of such exposure.

Perinatal androgens, sexual behavior and SDN-POA volume in adulthood

The effects of position in utero relative to males has been assumed to result from differences in prenatal androgen levels. Indeed, the present investigation found that female fetuses with caudal males have higher testosterone levels than females without caudal males. Such effects of the perinatal hormonal environment on sexual behavior in adulthood are presumably mediated by changes brought about in the CNS. However, sex dimorphic structures involved in the expression of sexual behavior have only recently become the subject of investigation (e.g. Anderson et al., 1986), and the exact relationship between such sex dimorphic structures and sexual behavior is not well understood. The sexually dimorphic nucleus of the preoptic area (SDN-POA) is dependent on the presence of perinatal androgens and has been implicated in the expression of masculine sexual behavior in male rats. For the scope of this thesis, the importance of the perinatal hormonal environment for the masculine development of the SDN-POA as well as for behavioral masculinization were investigated. In Experiment 5, it was shown that perinatal inhibition of the aromatization process affected several parameters of sexual behavior in male rats. That is, males in whom the perinatal aromatization from testosterone to estrogen was inhibited, showed reduced levels of masculine sexual behavior, increased levels of feminine sexual behavior and a reduced preference for an estrous female over a sexually active male. In Experiment 6, the volume of the SDN-POA in a subgroup of these males was studied, in order to examine a) the effects of perinatal inhibition of aromatization on SDN-POA volume in adulthood, and b) the relationship between SDN-POA volume and parameters of behavioral masculinization measured in Experiment 5. Both pre- and perinatal treatment with an aromatase-inhibitor significantly reduced SDN-POA volume, with perinatal treatment being the most effective. These results corroborate previous experiments, which showed that perinatal estrogen treatment is as effective as testosterone in masculinizing SDN-POA volume (Dohler et al., 1984), and that treatment with an estrogen antagonist perinatally results in a reduction in adult SDN-POA volume, whereas treatment with an androgen antagonist does not (Dohler et al., 1986). These and our results descibed herein indicate that in male rats, the masculine development of the SDN-POA is dependent on the presence of estrogen,

aromatized from testosterone around birth. In the animals from our experiment, the reduction in SDN-POA volume resulting from the perinatal treatment with an aromatase inhibitor corresponded to the reduction in masculinization of sexual behavior. That is, frequency of masculine sexual behavior as well as preference for a female, measures which have been shown to be dependent on the perinatal masculinizing effect of androgens, corresponded to SDN-POA volume. Although previous studies have shown an involvement of the SDN-POA in the expression of masculine sexual behavior, they have not suggested the involvement of this nucleus in partner preference. Further studies therefore need to clarify the relationship between SDN-POA and partner preference.

General conclusions

In rodents, sex differences in genital morphology, CNS and reproductive behavior have convincingly been shown to depend to a large extent on the organizing effects of hormones during a restricted period around birth (see Baum, 1979, Feder, 1981). Results from previous and the present experiments suggest that variation within each sex can partly be attributed to differences in exposure to hormones prenatally as a result of different positions relative to males in utero. The specifics of this process may differ across species. In this thesis, evidence was provided that in rats, females located rostrally from males are exposed to higher androgen levels and are more masculinized and defeminized morphologically and behaviorally than females located rostrally. Sex differences in many non reproductive areas (e.g., aggression, learning and memory) have been reported in addition to sex differences in reproductive behavior. One study has reported an intrauterine position effect on the sensitivity to the effects of testosterone on extinction rate of acquired taste aversion in female rats (Babine and Smotherman, 1984), indicating that variation among females and among males, in areas other than those directly associated with reproduction may also be related to the position fetuses occupy relative to male fetuses in utero.

Effects of intrauterine position relative to males have usually been limited to morphological and behavioral traits. Since effects on behavior are supposedly mediated through effects on the CNS, future studies need to investigate intrauterine position effects on sex dimorphic structures in the CNS which may be involved in the expression of sex dimorphic behaviors. Such studies would help to clarify further the relationship between perinatal hormones, CNS and behavior in adulthood.

The intrauterine position phenomenon has been investigated primarily in rodents. However, data from other species are gradually accumulating as well. In swine, sows born in predominantly female litters in adulthood have larger litters than those coming from litters with a high proportion of males (Edgerton and Cromwell, 1986). However, when the effect of adjacent males in utero on parameters relevant to reproduction, such as attractivity, length of the estrous cycle or ovulation rate of females was investigated, no consistent pattern of differences was observed (Rohde Parfet et al., 1990b). In pigs, defeminization occurs after birth (Ford and D'Occhio, 1989) and effects of prenatal exposure to androgens may therefore not be expected to affect reproductive parameters. In male swine testes weight or semen characteristics were not affected by adjacent males (Rohde Parfet et al., 1990a), although males with two adjacent males gained more weight under restricted feeding

conditions than males without adjacent males.

In humans, although the importance of experience and environment are readily recognized, several studies have suggested that some sex differences may be related to biological factors. Cognitive functioning and sexual orientation for example are two parameters in which sex differences have repeatedly been observed (see Hines, 1993 for an overview). Regarding cognitive functioning, although men and women are similar in overall intelligence, specific intellectual functions seem to differ between the sexes. Females in general perform better on verbal tasks, whereas males possess better spatial abilities. Studies on women or men who were exposed to abnormal hormone levels around birth, either through a genetic defect or because their mothers were prescribed hormones during pregnancy, have indicated a possible role for prenatal hormones in the organization of these sex differences in spatial abilities. For example, women exposed to high levels of androgen around birth because of Congenital Adrenal Hyperplasia (CAH, in which the adrenals produce an excess of androgens) have enhanced visuospatial abilities. Men who had lowered levels of androgen around birth because of indiopathic hypogonadotropic hypogonadism (IHH, in which androgen and estrogen levels are low during early development) showed reduced visuospatial abilities. Although far from being conclusive, such data suggest a possible role for perinatal hormones on aspects of intellectual functioning in humans.

Studies on sexual orientation have yielded no clear results regarding the influence of the prenatal hormonal environment. A possible influence of prenatal hormones on sexual orientation has been suggested. For example, women exposed to high levels of estrogen prenatally (women whose mother was prescribed the synthetic estrogen DES during pregnancy) are more likely to have a bisexual or homosexual orientation than their unexposed sisters (35%). Thus, although factors other than hormonal environment during early development contribute significantly to sexual orientation, given for example that still 60-70% of the DES women has a heterosexual orientation, a role for perinatal hormones has been indicated in sexual orientation (Ehrhardt et al., 1985). It should be noted that in this respect that recently, evidence for a role for genetic factors in human male sexual orientation was provided (Hamer et al., 1993).

The findings described above then suggest that some of the sex differences regarding human characteristics may be related to the perinatal hormonal environment. Since humans are not a litterbearing species, the intrauterine position phenomenon may not seem directly relevant for the understanding of human development. However, there are likely to be other sources of variation in hormone levels in humans, such as adrenals, ovaries, or environmental factors or drugs subtly affecting hormone production. Studies investigating the effect of perinatal hormones on sex differences in humans typically examine groups that have been exposed to abnormal hormone levels around birth. Animal studies investigating the intrauterine position phenomenon may perhaps provide a model on how within each sex, subtle natural variations in the prenatal hormonal environment may account for subsequent natural variation in behavior and reproductive functioning.

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SAMENVATTING

Sekseverschillen in lichamelijke kenmerken zoals de uitwendige genitaliën, morfologie en funktie van het centraal zenuwstelsel (CZS) en ook de expressie van bepaalde gedragingen worden voor een belangrijk deel bepaald door de aanwezigheid van geslachtshormonen vroeg in de ontwikkeling. Mannelijke genitaliën en CZS structuren, en een tonische hormoonproduktie zijn aanwezig wanneer gedurende een 'kritische periode' rond de geboorte het mannelijk geslachtshormoon testosteron (dat gemaakt wordt door de testes) aanwezig is geweest, en ook de voor mannelijke dieren kenmerkende gedragingen treden op in volwassenheid wanneer testosteron perinataal aanwezig geweest is. Dit permanente effekt van testosteron op het zich ontwikkelende organisme wordt een 'organiserend' effekt genoemd. Wanneer geen of weinig testosteron circuleert in de perinatale periode, ontwikkelt het dier zich in een 'vrouwelijke' richting. Deze ontwikkeling kenmerkt zich vooral door vrouwelijke genitaliën, een cyclische hormoonproduktie en het vertonen van vrouwelijke gedragingen. Onder natuurlijke omstandigheden worden in mannelijke dieren hogere testosteronnivo's aangetroffen rond de geboorte dan in vrouwelijke dieren.
Sekseverschillen in gedrag komen wellicht het duidelijkst tot uiting in seksueel gedrag. Bij seksuele interactie tussen mannetjes- en vrouwtjesratten benadert enerzijds het mannetje het vrouwtje en beklimt haar een aantal malen, waarbij hij soms zijn penis in de vagina brengt (intromissie). Wanneer het mannetje het vrouwtje een aantal malen heeft beklommen volgt een ejaculatie. Het vrouwtje anderzijds benadert het mannetje en vertoont een aantal 'proceptieve' gedragingen (b.v. oortrillen, 'darting', waarbij ze hard loopt en plotselingtot stilstand komt) waarvan verondersteld wordt dat ze tot doel hebben het mannetje tot beklimgedrag aan te zetten. Wanneer zij beklommen wordt vertoont het vrouwtje 'lordosis' waarbij ze een holle rug maakt en kop en achterlichaam opheft (receptief gedrag). Deze houding maakt het mogelijk dat het mannetje zijn penis in de vagina brengt.

Een belangrijk aspekt van de sekse-verschillen in gedrag is dat ze relatief zijn. Zo vertonen mannelijke ratten onder bepaalde omstandigheden vrouwelijk seksueel gedrag, en worden bepaalde mannelijke gedragingen ook frekwent door vrouwtjes vertoond. Sommige vrouwtjes vertonen bijvoorbeeld veel beklimgedrag wanneer zij geconfronteerd worden met een receptief vrouwtje. Er zijn behoorlijke individuele verschillen wat betreft de frekwentie waarmee vrouwtjes dit gedrag vertonen. Omdat de belangrijke rol van perinataal testosteron voor dit mannelijk seksueel gedrag duidelijk is aangetoond, is gesuggereerd dat de variatie onder vrouwtjes wat betreft dit gedrag veroorzaakt wordt door verschillen in perinatale blootstelling aan testosteron. Inderdaad is in vrouwelijke foeten testosteron aangetoond, en verschillen vrouwelijke foeten onderling wat betreft de hoeveelheid testosteron die prenataal circuleert. Bovendien vertonen vrouwtjes die prenataal behandeld zijn met een anti-androgeen minder beklimgedrag.

Omdat verondersteld wordt dat vrouwelijke foeten zelf geen testosteron produceren, rijst de vraag naar de bron(nen) van dit testosteron. Behalve de ovaria van de moeder en de placenta, die beiden testosteron produceren, zijn ook mannelijke

foeten in dezelfde uterushoorn gesuggereerd als mogelijke bronnen. De testes van mannelijke foeten beginnen androgenen te produceren rond dag 14 na conceptie, en de zwangerschap van de rat duurt ongeveer 22 dagen, zodat mannetjes in de uterus gedurende ongeveer 8 dagen testosteron produceren. Wat betreft het transport van testosteron van mannetjes naar vrouwtjes in dezelfde uterushoorn zijn twee mechanismen gesuggereerd. De 'contiguiteit' hypothese is gebaseerd op de observatie dat vrouwtjes die in de uterushoorn tussen twee mannetjes liggen, bij de geboorte mannelijkere genitaliën hebben en meer mannelijke gedragingen vertonen in volwassenheid dan vrouwtjes die zich tussen twee vrouwtjes bevonden. Deze hypothese veronderstelt dat testosteron dat door mannetjes geproduceerd wordt door het amnionvlies getransporteerd wordt en zo de naastliggende foeten bereikt. Een alternatieve hypothese suggereert transport van androgen via de uterus vasculatuur, zodat testosteron van mannelijke foeten via het bloed in de uterus getransporteerd wordt naar foeten die rostraal (i.e. in de richting van het ovarium) ten opzichte van van hen liggen ('caudale mannen' hypothese). Deze hypothese voorspelt dat vrouwtjes die in de uterus mannetjes caudaal ten opzichte van hen hebben meer gemasculiniseerd zullen zijn wat betreft genitale morfologie en gedrag dan vrouwtjes zonder zulke mannetjes.

In onderzoeken waarbij muizen en woestijnratten worden gebruikt zijn voornamelijk aanwijzingen gevonden voor de 'contiguiteit' hypothese. Vrouwelijke muizen en woestijnratten die tussen mannetjes liggen in de uterus hebben hogere prenatale testosteronnivo's, mannelijkere genitaliën bij de geboorte en vertonen meer mannelijke gedragingen in volwassenheid. Onderzoek aan ratten heeft een minder duidelijk beeld opgeleverd: resultaten van sommige studies suggereren dat vrouwtjes die tussen mannetjes liggen meer gemasculiniseerd zijn dan vrouwtjes tussen vrouwtjes, terwijl andere onderzoeken dit niet konden bevestigen en daarentegen een masculiniserend effekt van caudale mannetjes aantoonden.

Vier experimenten die beschreven worden in dit proefschrift werden opgezet om de rol van mannelijke foeten in de uterus in de variatie in masculinisatie van genitaliën en gedrag van ratten te onderzoeken. In Experiment 1 (Hoofdstuk 2) werden de twee hypothesen wat betreft androgeen transport in de uterus, i.e. de 'contiguiteit' hypothese en de 'caudale mannen' hypothese, getoetst. Tevens werd in dit experiment de invloed onderzocht van twee procedures die voorheen gebruikt zijn in soortgelijke experimenten, omdat het gebruik van deze procedures wellicht heeft bijgedragen aan de tegenstrijdige resultaten. Zo werd in sommige experimenten tijdens de zwangerschap een uterushoorn van de moeder verwijderd. Bovendien werden proefdieren in sommige onderzoeken geboren via een keizersnede, in andere op natuurlijke wijze. Omdat stress tijdens de zwangerschap de seksuele differentiatie van de jongen belangrijk kan beinvloeden, kunnen procedures zoals een keizersnede of het verwijderen van een uterushoorn tijdens de zwangerschap de resultaten beinvloeden van experimenten waarbij de seksuele differentiatie van de jongen onderzocht word. In Experiment 1 bleken vrouwelijke ratten met caudale mannetjes in de uterus meer mannelijk seksueel gedrag te vertonen in volwassenheid dan vrouwtjes zonder zulke mannetjes. Er waren geen verschillen tussen vrouwtjes die tussen twee mannetjes en vrouwtjes die tussen twee vrouwtjes lagen. Bovendien bleek dat het 'caudale mannen effekt' niet optrad wanneer een uterushoorn van de moeder verwijderd werd tijdens de zwangerschap, wellicht als gevolg van de stress die deze procedure met zich meebrengt. Geboorte via een keizersnede had geen

effekt op de seksuele differentiatie van vrouwtjes, noch op het 'caudale mannen effekt'.

Effekten van positie ten opzichte van mannetjes in de uterus zijn vooral onderzocht bij vrouwelijke dieren. In muizen en woestijnratten zijn ook effekten van positie in de uterus ten opzichte van mannetjes op seksueel gedrag van mannetjes aangetoond. Omdat er ook bij mannelijke ratten sprake is van individuele verschillen in frekwentie van mannelijk seksueel gedrag, werd in Experiment 2 (Hoofdstuk 3) onderzocht of deze variatie verklaard kan worden door de positie die mannetjes hebben ten opzichte van andere mannetjes in de uterushoorn. Mannetjes met caudale mannen bleken meer mannelijk seksueel gedrag te vertonen, en na seksuele ervaring sneller te ejaculeren dan mannetjes zonder caudale mannetjes in de uterus. Er waren wederom geen verschillen tussen dieren die tussen twee mannetjes, en dieren die tussen twee vrouwtjes lagen.

Een inherent probleem in het onderzoek naar de invloed van positie in utero op seksuele differentiatie is dat de onafhankelijke variabelen (aanwezigheid van caudale mannen, aanwezigheid van naastliggende mannen) niet experimenteel gemanipuleerd kunnen worden. Daarom zijn de faktoren: 'caudale mannetjes' en 'naastliggende mannetjes' niet onafhankelijk van elkaar: dieren die tussen twee mannetjes liggen hebben per definitie een caudaal mannetje. Als gevolg daarvan kan het effekt van caudale of aangrenzende mannetjes niet goed onafhankelijk van elkaar worden onderzocht. Bovendien kunnen met de onafhankelijke variabele een aantal andere variabelen systematisch varieren die wellicht ook een invloed uitoefenen op seksuele differentiatie. Een dier dat bijvoorbeeld de uterushoorn deelt met een groot aantal mannetjes heeft meer kans om caudale mannetjes te hebben dan een dier in een uterushoorn met een laag percentage mannetjes. Verschillen tussen dieren met en zonder caudale mannen kunnen in dat geval in werkelijkheid verschillen zijn tussen dieren met een hoog en met een laag percentage mannetjes in de uterus. In Experiment 3 werd daarom simultaan de relatieve invloed van een aantal relevante variabelen op de genitale masculinisatie van vrouwelijke ratten van twee stammen onderzocht. De variatie in genitale masculinisatie van vrouwtjesratten bleek voor een deel verklaard te kunnen worden door het aantal caudale mannetjes, door de afstand tot die caudale mannetjes en door het totaal aantal mannetjes in de uterushoorn. Vrouwtjes die tussen twee mannetjes lagen in de uterus bleken somatisch (i.e. wat betreft genitaliën) niet meer gemasculiniseerd dan vrouwtjes zonder naastliggende mannetjes.

Aanwijzingen voor masculinisatie van vrouwelijke ratten met caudale mannetjes kwamen tot nu toe van somatische en gedragsobservaties, en deze masculinisatie wordt verondersteld het gevolg te zijn van verschillen in prenataal testosteronnivo. In Experiment 4 werd een dergelijk verschil inderdaad aangetoond: vrouwelijke foeten met caudale mannetjes hadden een hoger testosteronnivo dan vrouwtjes zonder caudale mannetjes.

De manier waarop testosteron getransporteerd wordt van mannetjes naar rostraal gelegen vrouwtjes is onderhevig aan discussie. De 'caudale' hypothese is gebaseerd op twee aannames: dat testosteron direkt van de uterusvene naar de uterusarterie getransporteerd kan worden, en dat het bloed in uterusvene en -arterie in een rostrale richting stroomt. Deze aannames zijn recentelijk echter ter discussie gesteld. In Hoofdstuk 6 wordt een overzicht gegeven van de literatuur waarop deze aannamen gebaseerd zijn.

Er zijn aanwijzingen dat het centraal zenuwstelsel een belangrijke medierende

rol speelt bij de organiserende invloed van perinataal testosteron op gedrag in volwassenheid. De precieze relatie tussen perinatale hormonen, centraal zenuwstelsel en seksueel gedrag in volwassenheid is echter niet duidelijk. Experiment 5 en 6 werd opgezet om deze relatie nader te onderzoeken. Testosteron wordt in het centraal zenuwstelsel omgezet naar oestradiol, en oestradiol is verantwoordelijk voor een belangrijk deel van de masculiniserende werking van testosteron. In Experiment 5 bleken mannelijke ratten die perinataal behandeld werden met een aromatase-remmer, d.w.z. een stof die de omzetting van testosteron naar oestradiol remt, in volwassenheid minder gemasculiniseerd te zijn in verschillende aspekten van seksueel gedrag. Om een duidelijker beeld te krijgen van de relatie tussen de perinatale aromatase van testosteron, het centraal zenuwstelsel en seksueel gedrag in volwassenheid werd in Experiment 6 de seks-dimorfe nucleus van het preoptisch gebied (SDN-POA) van deze dieren onderzocht. Het volume van de SDN-POA, dat in volwassenheid 3-8 keer zo groot is in mannetjes dan in vrouwtjes, is afhankelijk van de aanwezigheid van testosteron rond de geboorte. De SDN-POA van mannetjes die perinataal waren blootgesteld aan een aromatase-remmer bleek kleiner dan die van controle-mannetjes. Deze nucleus was eerder door een aantal experimenten in verband gebracht met de expressie van mannelijk seksueel gedrag. In Experiment 6 bleek de mate waarin de perinatale behandeling het volume van de SDN-POA had beinvloed te corresponderen met de mate waarin het seksueel gedrag van deze dieren was beinvloed. Dat wil zeggen, er was een correlatie tussen de frekwentie van mannelijk seksueel gedrag en de voorkeur voor een bronstig vrouwtje boven een seksueel aktief mannetje enerzijds, en het volume van de SDN-POA anderzijds.

Op grond van deze studies kan geconcludeerd worden dat een aantal faktoren van belang is voor de natuurlijke variatie in morfologische en gedragsmatige masculinisatie van vrouwelijke zowel als mannelijke ratten. Deze masculinisatie wordt beinvloed door de aanwezigheid van caudale mannetjes in de uterus, waarbij ook de afstand tot die caudale mannetjes van belang is. Tevens speelt het totaal aantal mannetjes in de uterushoorn, onafhankelijk van hun positie in de uterus, een rol in de natuurlijke variatie in masculinisatie van de genitaliën van vrouwtjes. Deze variatie in masculinisatie als funktie van de aan- of afwezigheid van caudale mannetjes wordt ook gezien in prenataal testosteronnivo van vrouwtjes. Er werden geen aanwijzingen gevonden voor een masculiniserend effekt van aangrenzende mannetjes op de seksuele differentiatie van rat. Caudale mannetjes en totaal aantal mannetjes verklaren samen ongeveer 25% van de natuurlijke variantie in somatische masculinisatie. Een deel van de overige variantie kan wellicht verklaard worden door de placenta, die testosteron produceert. Het effekt van caudale mannen echter kan wellicht als model dienen voor de manier waarop subtiele verschillen in prenatale testosteronnivo's een deel van de variantie binnen een sekse kunnen bepalen.

DANKWOORD/ACKNOWLEDGEMENTS

Hoewel dit proefschrift alleen op mijn naam staat, hebben vele mensen belangrijk bijgedragen aan het uiteindelijke resultaat. Een aantal van hen wil ik hier met name bedanken.

Allereerst wil ik de mensen van de vakgroep 'Endocrinologie en voortplanting' en van de vroegere vakgroep 'Endocrinologie, Groei en Voortplanting' bedanken voor de leuke tijd die ik daar gedurende de eerste twee jaren van mijn onderzoek en tijdens het schrijven van dit proefschrift heb gehad. Met name Jan van Ophemert, op wie een belangrijk deel van het werk van de 'Gedragsgroep" steunt, bij bedank ik voor zijn hulp bij het uitvoeren van de experimenten tijdens de eerste twee jaar van mijn onderzoek. Pieter van der Schoot bedank ik voor zijn vakkundige hulp bij de vasculatuur experimenten.

Francien de Jonge heeft mij en enkele van mijn artikelen vele malen voorzien van waardevolle professionele adviezen. De promotiecommissie bedank ik voor het lezen en beoordelen van dit proefschrift.

Mijn ouders bedank ik voor hun onvoorwaardelijke steun. Mijn vader heeft bovendien min of meer vanaf mijn geboorte getracht mijn interesse voor de wetenschap te wekken, en dit proefschrift is mede daarvan het resultaat.

Mijn broer Michiel heeft een cruciale rol gespeeld bij de laatste fase van mijn promotie, door een vrij weekend te besteden aan het editen van dit proefschrift, dat zonder hem een chaos geworden zou zijn.

Jenifer Juranek, Chris Gebauer and Chris Kotrba are gratefully acknowledged for their excellent work in the lab. Jenifer especially I thank because her competence and dedication to the experiments were of great value to me during my first year in Valparaiso. I am also indebted to her for teaching me everything I could ever possibly want to know about fast food in America. Marylyn Gebhardt provided me with unconditional moral support during the time that I worked in the lab of the Psychology Department in Valparaiso, and is much thanked for that. Jim Thornton I thank for spending days of his vacation in Europe trying to teach me statistics, and of course for convincing me that the only way to learn about multiple regression was to go to Paris.

Nanne van de Poll begeleidde mij bij mijn eerste schreden op het onderzoekspad, en werd daarna mijn 'tweede' promotor. Ik bewonder zijn kennis op het gebied van hormonen en gedrag, maar bovenal bewonder ik de manier waarop hij steeds mijn zelfstandigheid stimuleerde, terwijl hij mij af en toe door krachtdadig optreden van vreselijke fouten weerhield. De balans tussen zijn gebrek aan bemoeizucht en zijn hulp en steun wanneer ik daarom vroeg heb ik altijd zeer gewaardeerd.

Koos Slob was mijn promotor. Zijn doorzettingsvermogen en zijn onwil om zich neer te leggen bij het idee dat iets 'niet kan' hebben mij door menige impasse gesleept. Ik bewonder zijn enorme energie en nimmer aflatende enthousiasme, en ben hem dankbaar voor zijn steun bij mijn plan om mijn onderzoek in de Verenigde Staten voort te zetten. Maar bovenal bedank ik hem voor zijn bereidheid om altijd, ook

voort te zetten. Maar bovenal bedank ik hem voor zijn bereidheid om altijd, ook wanneer ik hem tot regelrechte wanhoop dreef, weer overnieuw te beginnen.

And of course I thank David, who experienced, all the zeniths and nadirs of my research, and was, and is, invaluable to me. He was involved in many of the studies, and read all versions of nearly all articles presented here. I admire the way he changes, with a few suggestions, a chaotic article into a readable one. I also thank him for always making the distinction between the details and the bigger picture, both in research and in life in general.

CURRICULUM VITAE

Els Houtsmuller werd geboren op 19 augustus 1963 in Utrecht. Zij deed in 1982 eindexamen aan het Stedelijk Gymnasium te Schiedam, waarna zij een aanvang maakte met de studie Psychologie aan de Universiteit van Amsterdam. Als afstudeerrichting koos zij Fysiologische Psychologie. In het kader van een afstudeerstage deed zij een half jaar onderzoek bij het Nederlands Instituut voor Hersenonderzoek te Amsterdam, onder leiding van Prof. dr. Nanne van de Poll. Aansluitend op het doctoraal examen in 1988 werd zij aangesteld als onderzoeker in opleiding (OIO), in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), bij de afdeling Endocrinologie, Groei en Voortplanting, thans Endocrinologie en Voortplanting, van de Erasmus Universiteit Rotterdam. Het onderzoek dat beschreven wordt in dit proefschrift werd uitgevoerd onder leiding van Prof. dr. Koos Slob.