SEXUAL DIFFERENTIATION OF THE BRAIN AND PARTNER PREFERENCE IN THE MALE RAT

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SEKSUELE DIFFERENTIATIE VAN DE HERSENEN EN PARTNER PREFERENTIE IN DE MANNELIJKE RAT

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CONTENTS

LIST OF ABBREVIATIONS

ABC	avidin-biotin-complex
AH	anterior hypothalamus
AMH	anti-müllerian hormone
ANOVA	analysis of variance
AOB	accessory olfactory bulb
AR	androgen receptor
Arc	arcuate nucleus
ATD	1,4,6-androstatriene-3,17-dione
BNST	bed nucleus of the stria terminalis
Chol	cholesterol
CNS	central nervous system
CTF	central tegmental field
DAB	3,3'-diaminobenzidine
DHT	dihydrotestosterone
DHTP	dihydrotestosterone propionate
E_2	estradiol
EB	estradiol benzoate
ER	estrogen receptor
FSH	follicle-stimulating hormone
GnRH	gonadotropin releasing hormone
INAH	interstitial nucleus of the anterior hypothalamus
IR	immunoreactivity
LH	luteinizing hormone
LSD	least significant difference
Me	medial amygdaloid nucleus
MeA	medial amygdaloid nucleus anterior
MeAD	medial amygdaloid nucleus anterodorsal
MeAV	medial amygdaloid nucleus anteroventral
MePD	medial amygdaloid nucleus posterodorsal
MePV	medial amygdaloid nucleus posteroventral
MN-POA/AH	male nucleus of the preoptic area/anterior hypothalamus
mPOA	medial preoptic area
mRNA	messenger ribonucleic acid
OVLT	organum vasculosum of the lamina terminalis
P	progesterone
PBS	phosphate-buffer saline
PVN	periventricular nucleus
PVP	periventricular preoptic area

LIST OF ABBREVIATIONS (continued)

sc	subcutaneous
SCN	suprachiasmatic nucleus
SDA	sexually dimorphic area
SDN-POA	sexually dimorphic nucleus of the preoptic area
SEM	standard error of the mean
SHy	septohypothalamic nucleus
SRY	sex determining region of the Y-chromosome
Ss	subjects
T	testosterone
TDF	testis-determining factor
TP	testosterone propionate
VIP	vasoactive intestinal peptide
VMH	ventromedial nucleus of the hypothalamus
VNO	vomeronasal organ
VP	vasopressin

Chapter I

GENERAL INTRODUCTION

The studies reported in this thesis have been conducted to investigate the sexual differentiation of partner preference in the male rat. Initially, the effects of neonatal inhibition of brain estrogen formation on later coital behavior and partner preference of the male rat were studied. Later studies were directed to the central nervous system and investigated the neural mechanisms regulating male coital behavior and partner preference. This first chapter provides an overview of the effects of gonadal hormones on the sexual differentiation of the brain, with emphasis on functional sex differences in behavior and neuroendocrine function in possible relation to morphological sex differences in the brain.

Sexual differentiation

In many mammalian species, males and females differ markedly in a wide variety of reproduction-related and in non-reproduction related behaviors. For instance, one easily observed sexually dimorphic behavior is the difference in urinary posture between adult male and female dogs. The male dog raises one of his hind legs to urinate, whereas the female dog usually adopts a squatting posture during urination. Furthermore, males and females of many species show sexually dimorphic mating postures. Female rodents stand immobile with arched backs and allow males to mount them. This mating posture is called lordosis. Males mount females and will normally not assume the lordosis posture when mounted by other males. In other vertebrate classes, for instance male canaries and zebra finches sing or vocalize in stereotypic manners normally not expressed in females (11,158,162). In these bird species, it has been found that the volume of the forebrain nuclei which control singing is significantly larger in males than in females (221). This sex difference correlates very nicely with the dimorphic control of song production in these species. Thus, structures in the central nervous system hypothesized to underlie sexually dimorphic behaviors, show clear sex differences as well.

The sex differences in genital morphology, in sexual and non-sexual behaviors and in structures and functions of the central nervous system associated with these behaviors are the most apparent in the adult organism. However, these sex differences are the outcome of a process called sexual differentiation which occurs perinatally. Sexual differentiation is a sequential process beginning at the establishment of the genetic sex at the time of conception, which results in the development of the gonadal sex and, under the influence of gonadal hormones, the formation of the phenotypic or body sex and the brain sex (see 223).

GONADAL DIFFERENTIATION

Genetic sex

The primary step in the process of sexual differentiation occurs at fertilization. An ovum can be fertilized by a sperm bearing either an X or a Y chromosome. In mammalian species, each individual possesses a set of paired autosomes and two sex chromosomes. Females are the homogametic sex (XX), males the heterogametic sex (XY). The chromosomal sex determines whether during embryonic development, the undifferentiated gonads will develop into testes or ovaries.

Gonadal sex

In mammals, the SRY gene (sex determining Y-chromosome gene) on the Y-chromosome encodes the testis-determining factor (TDF; 36,144,163,237). If the TDF gene is expressed by the undifferentiated gonads, then the TDF protein is produced and testes will develop. If the TDF gene is not expressed, ovaries will develop. It is possible for the TDF gene to be expressed in one gonad but not in the other, which leads to unilateral differentiation: a testis will develop on one side while an ovary develops on the other, suggesting that TDF functions only locally. Partial expression of the TDF gene can lead to incomplete gonadal differentiation, yielding an ovotestis.

Phenotypic or body sex

The development of the phenotypic sex consists of two events: the differentiation of the internal ducts and the differentiation of the external genitalia.

Differentiation of the internal ducts

Both male and female embryos have two sets of internal ducts, the Müllerian and Wolffian ducts. Once the undifferentiated gonad has differentiated into a testis, testosterone synthetized by and secreted from the Leydig cells of the fetal testes stimulate the development of the Wolffian ducts into the vasa deferentia, epididymides and the seminal vesicles, while the Müllerian ducts will regress under the influence of Anti-Müllerian Hormone (AMH), produced by the Sertoli cells of the fetal testes (37,150). In the absence of a Y-chromosome, ovaries develop. The ovaries in females do not secrete AMH and testosterone, thereby promoting the development of the Müllerian ducts into the uterus,

oviducts and the upper part of the vagina and the regression of the Wolffian ducts. In rats, testosterone secretion by the testes starts at around day 14 of gestation (rats have a gestation of 22 days). The differentiation of the internal genitalia occurs entirely prenatally.

Differentiation of external genitalia

The embryonic anlagen of the external genitalia are identical for both sexes. In humans and many other mammalian species, the primordial structures consist of 1) the genital tubercle, which becomes the penis in males and the clitoris in females; 2) the urethral genital folds, which become the shaft of the penis in males and the labia minora in females; and 3) the labioscrotal swellings, which become the scrotum in males and the labia majora in females. Masculinization of the external genitalia requires the presence of 5αdihydrotestosterone (DHT) converted from testosterone by the enzyme 5αreductase. In the presence of testes, DHT induces the differentiation of the primordial structures into the male direction. In the presence of ovaries, a condition associated with a lack of DHT, female external genitalia develop from the primordial structures (for an overview see 15,152,223).

SEXUAL DIFFERENTIATION OF THE BRAIN

Sexual differentiation of the brain in relation to coital behavior

Sexual differentiation of the brain in the rat

The sexual differentiation of the rat brain is orchestrated by gonadal steroids during a restricted developmental period and results in permanent changes in the hormonally sensitive neural substrates in the adult (191). In rats, this critical period, in which the animal is maximally susceptible to the organizing actions of steroids on neural tissue, extends approximately from Day 14 of prenatal life to Day 10 of postnatal life (e.g. 15). Prenatally, there is a testosterone surge on days 18 and 19 (21,318). Postnatally, there is an rise in serum testosterone in male pups directly after birth followed by a sharp decline approximately 6 hours after birth (23,63,275). Testosterone levels remain higher in males than in females until Day 10 (226,244), when they drop and remain low until the age of puberty. The large quantities of testosterone released into the circulation act on the rat brain to masculinize and defeminize the neural substrates that control gonadotropin secretion and sexual behavior in adulthood (e.g. 15,119,181,235). Masculinization of the brain results in the display of male-typical sexual behaviors (mounts, intromissions and

ejaculations) in adulthood. Defeminization results in a loss of the cyclic release of gonadotropins necessary for ovulation and of the display of female-typical sexual behaviors (lordosis, presenting, ear wiggle, hop-dart) in adulthood (e.g. 15,181). Thus, gonadally intact adult male rats display a pattern of copulatory behavior comprised of a series of mounts with pelvic thrusting and intromissions culminating in ejaculation. The development of the female rat brain proceeds in the absence of testosterone. In other words, the sexual differentiation of the brain is thought to be inherently female unless male differentiation is superimposed by testosterone during the critical period. This assumption is based upon the early observations that gonadectomy of female rabbit fetusses (151 cited in 78) or gonadectomy of newborn female rats (234) did not interfere with female differentiation, that is, the genital ducts. Gonadally intact female rats display periods of behavioral proceptivity, i.e. approaching the male partner, and receptivity, i.e. the display of lordosis in response to mounts, around the time of ovulation (27).

Numerous studies on mammalian species have shown that local conversion of androgens to estrogens is a crucial step in the sexual differentiation of the brain. Thus, estrogens mediates many of the effects of testosterone on brain function (e.g. 15,181,182,194). This represents the '*aromatization hypothesis*'.

· Aromatization hypothesis

The aromatization hypothesis proposes that testosterone secreted by the testes in male fetusses and newborns acts to masculinize and defeminize the developing brain after the intracellular metabolism of testosterone to estradiol by the enzyme aromatase. There are several lines of evidence that the aromatization of testosterone to estradiol is essential for masculinization and defeminization of the brain: 1) aromatizable androgens (e.g.Δ^4-androstenedione) mimic the masculinizing effect in the central nervous system of neonatally treated female rats (88,180,193). Aromatizable androgens (e.g. testosterone propionate (TP)), but not the nonaromatizable androgen dihydrotestosterone propionate (DHTP), are also able to prevent the demasculinizing effects of neonatal castration in the male rat (308); 2) treatment with an estrogen antagonist blocks the effects of testosterone in neonatal females (192); 3) 19-hydroxytestosterone, an intermediate in the aromatization process, is a more potent masculinizing agent than testosterone when administered to neonatal female rats (193); 4) neonatal administration of an aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD), via silastic capsules to male rats, strikingly increases males' ability to display female sexual behaviors not only after castration and treatment with ovarian hormones

in adulthood (66,194,314), but also if they remain gonadally intact (45). Male rats treated neonatally with ATD also show a cyclic secretion of gonadotropins, as evidenced by the occurrence of ovulation in ovaries grafted under the kidney capsule (314). Furthermore, ejaculatory behavior is impaired in these neonatally ATD treated males (45). Similar results are obtained with neonatal administration of the antiestrogen MER-25 to male rats (41,279): lordosis behavior is enhanced after administration of estrogen and progesterone in adulthood and ejaculatory behavior is reduced following testosterone treatment in adulthood; 6) neonatally castrated and DHTP-treated males show a significantly lower preference for an estrous female (vs a sexually active male) than control males under testosterone substitution in adulthood (44). Additionally, intact adult male rats, in which the conversion of testosterone to estradiol is inhibited pre- and/or neonatally by administration of the aromatase inhibitor ATD, show a significantly lower preference for the estrous female compared to control males (45).

It is assumed that circulating estradiol-17ß does not play a role in the sexual differentiation of the brain, because its passage into the brain is prevented by the presence of high concentrations of a plasma glycoprotein, alpha-fetoprotein, which binds estradiol with high affinity in rats (156). Presumably, alpha-fetoprotein reduces the free, biologically active fraction of estradiol to a concentration which is too low to enter the brain (e.g. 208,245). In both male and female rats, serum levels of estradiol decrease between the 21-day fetal stage and 24 h postpartum. In male rats, hypothalamic estradiol levels increase dramatically between 0 h *in utero* and 1 h after delivery and decrease between 2 and 24 h after birth(245). This hypothalamic estradiol surge is absent in female rats. The increase in hypothalamic estradiol in male rats is most likely due to the intracellularly conversion of testosterone to estradiol and not derived peripherally since testicular levels of estradiol are very low (245). Moreover, if the hypothalamic estradiol came from the testes, then an increase in serum estradiol would have been observed. In female rats, serum levels of estradiol rise dramatically beginning after postnatal Day 5 and reach a peak level around postnatal Day 15, then estradiol levels drop by postnatal Day 25 (77,196). Presumably, these increased serum estradiol levels will probably not reach the brain due to the presence of alpha-fetoproteins in the plasma thereby protecting the female rat brain from the masculinizing and defeminizing actions of estradiol. Indirect evidence that alpha-fetoproteins normally protect the female rat brain from estradiol was provided by the demonstration that neonatal administration of RU2858, which does not bind to alpha-fetoprotein, was nearly 100 times more potent than estradiol benzoate (EB) in defeminizing female rats (82). However, it was recently found that when 2-day old

female pups were treated with [^{3}H]estradiol via silastic capsules implanted subcutaneously in the neck, [^{3}H]estradiol was detected specifically bound to brain cell nuclei (208). This means that alpha-fetoproteins cannot entirely protect the female rat brain from estradiol. At present, it is unclear why the female rat brain is not masculinized and defeminized by these circulating concentrations of free estradiol. On the other hand, female rats, ovariectomized and chronically treated with high doses of testosterone or EB, can readily display all elements of masculine sexual behavior, i.e. mounts, intromission-like and ejaculation-like behaviors (20,26,93,222). These findings imply that considerable masculinization of the brain normally occurs in the female. It has been proposed that this masculinization may occur, because female rats are normally exposed to rather high levels of testosterone presumably of placental origin during fetal development (21,112,315). This masculinization of the female rat brain may also be due to the physiological levels of circulating estradiol. Döhler and co-workers (78) reported that neonatal treatment of female rats with an estrogen antagonist (tamoxifen) significantly inhibited the differentiation of a positive feedback mechanism for estrogen/progesterone stimulated release of luteinizing hormone (LH) and reduced the capacity to show lordosis behavior, but did not however, stimulate the display of male-typical sexual behavior. These findings suggest that not only male but also female sexual differentiation of the brain may be under the influence of estradiol (78). However, tamoxifen itself appears to have some estrogenic effects. Mathews and co-workers (186) reported that treatment with tamoxifen failed to block masculinization of the song control regions in male and femal zebra finches: tamoxifen even masculinized female zebra finches and hypermasculinized male zebra finches (186). Further evidence that estradiol is not required for the differentiation of cyclic gonadotropin release and the capacity to display female typical sexual behavior is that male rats neonatally castrated (309) or neonatally treated with ATD (314) can ovulate when implanted with ovarian grafts. Moreover, such male rats are highly receptive when tested in adulthood following castration and administration of exogenous estradiol and progesterone (29,66,314).

In support of the role of testosterone and estradiol in the organization of the brain in a male direction, it has been shown that the administration of exogenous testosterone to female rats during perinatal development, causes behavioral masculinization, i.e. higher frequencies of adult mounting behavior in response to testosterone, and behavioral defeminization, i.e. impaired lordosis behavior in adulthood in response to ovarian hormones (14,15,68,119,132,204). The absence of testosterone during the perinatal critical developmental period has the reverse effect. Males

castrated at birth exhibit in adulthood limited male copulatory behavior following treatment with TP and display lordosis after treatment with estradiol and progesterone (e.g. 122,320).

Sexual differentiation of the brain in hamsters, ferrets, and rhesus monkeys

In contrast to rats, perinatal exposure to testosterone and/or estradiol does not defeminize sexual behavior in some other species, such as hamsters, ferrets, and rhesus monkeys (reviewed in 15). In hamsters, the male is inherently capable of displaying high levels of both masculine and feminine sexual behaviors, depending on the exogenous hormones administered in adulthood, whereas the female displays feminine sexual behavior but relatively little mounting behavior after ovariectomy and testosterone treatment (15). Thus, male hamsters readily display lordosis when mounted by another male in response to the same doses of exogenous ovarian hormones necessary to elicit lordosis in ovariectomized females. Similarly, male ferrets can display male-typical sexual behaviors, i.e. neck-gripping, mounting, and pelvic thrusting behaviors which lead to the intromission of the penis into the female's vagina, as well as female-typical behaviors, i.e. a limp, unresisting posture when neck-gripped and mounted by a male (15,16). Female ferrets typically show little male-typical behavior, even after ovariectomy and treatment with testosterone (16). Thus, male ferrets are not defeminized with respect to receptive behavior, they are, however, defeminized with respect to proceptive behavior as a consequence of the perinatal action of testosterone and estradiol (16). In various nonhuman primate species, i.e. rhesus macaques (95,118) and stumptail macaques (218), defeminization does not seem to occur in response to perinatal androgens. Males will accept repeated mounting by other males, which may on rare occassions be accompanied by anal penetration and ejaculation by the mounting partner. Social relationships strongly influence the extent to which male macaques express the posture normally associated with feminine receptivity. For instance, males frequently signal subordinance to a more dominant animal by displaying the receptive posture (15,127). Administration of TP to pregnant rhesus monkeys between Days 40 and 100 of gestation does not defeminize the behavior of the female offspring: these androgenized females present to other animals as frequently as control females (and males) (120). Thus, perinatal exposure to testosterone does not completely defeminize sexual behavior in some mammalian species, i.e. hamsters, ferrets, rhesus monkeys (e.g. 15). This suggests that the display of female-typical coital behavior is not restricted to the female gender in hamsters, ferrets, and rhesus monkeys. It should be noted that lordosis behavior can be induced in castrated adult male rats by admin-

istering estrogen and progesterone, although the behavior displayed is usually less intense than that of female rats, and considerably more estradiol is required for its excitation (65,224,321). As previously described, the display of male-typical coital behavior is not restricted to the male gender in the rat; only the likelihood with which female-typical behavior or male-typical behavior are displayed, appears to be different between males and females. Thus, gonadally intact females are more likely to display female-typical coital behavior than males, and males are more likely to exhibit male-typical coital behavior than females (118).

Sexual differentiation of the brain in relation to sexual partner preference

Not only coital behavior, but also motivational aspects of sexual behavior are masculinized and defeminized by the presence of testosterone and/or estradiol during early development (1,45,66,91,313). Motivational aspects of sexual behavior can be studied by assessment of an animal's partner preference. In the rat, adult gonadally intact males prefer estrous females over males, and adult gonadally intact females prefer males over females (136,203). Male rats, castrated within 24 hours after birth, prefer an estrous female over a sexually active male, although this preference is lower compared to control males (44,91). Female rats treated with TP within 24 hours after birth prefer females over males (68). It has been shown that experimentally-induced variations in the availability of testosterone or estradiol during perinatal life also influences partner preference in other mammalian species, i.e. ferrets (22), hamsters (148), dogs (30), pigs (105), and zebra-finches (2). In humans, there are some examples of possible effects of prenatal steroidal changes on adult sexual orientation. Women exposed to elevated testosterone levels during gestation due to congenital adrenal hyperplasia reported increased levels of homosexual experience compared to control women (76,207). Furthermore, women with a history of prenatal exposure to diethylstilbestrol (DES), a nonsteroidal synthetic estrogen, show a higher incidence of bisexuality or homosexuality than control women (90,201).

Distribution and ontogeny of the aromatase enzyme in the brain

The recognition that aromatization of testosterone to estradiol is of fundamental importance for sexual differentiation has prompted a number of studies in which the distribution and ontogeny of aromatase activity

in the central nervous system were investigated. Aromatase, an enzyme of the cytochrome *P*-450 family, irreversibly converts testosterone into estradiol-17ß (e.g. 212). Early studies using crude brain dissections, have found that aromatase activity is concentrated in the diencephalon and amygdala of the rat (213,251,266). Later studies using punch microdissections have localized the major sites of activity within specific limbic nuclei and subregions, including the periventricular preoptic area (PVP), the medial preoptic area (mPOA), the bed nucleus of the stria terminalis (BNST), the ventromedial nucleus of the hypothalamus (VMH), and corticomedial amygdala (252). In the rat brain, the highest aromatase activity has been reported in the medial preoptic-anterior hypothalamic regions, BNST and medial amygdala (145,302). In the fetal rat brain, the first occurrence of aromatase immunoreactive cells have been detected in the POA on the 13th day of embryonic life, and additionally in the BNST on the 15th day of embryonic life. Aromatase immunoreactive cells have also been found in the medial amygdaloid nucleus and the VMH on the 16th day of embryonic life, and some have been detected in the arcuate nucleus (Arc) 3 days before birth. As gestation progresses, the number and the immunoreactivity of these cells gradually increase and peak within definite periods of perinatal life and thereafter decline or disappear (302). The distribution of aromatase-immunoreactive neurons is similar between the sexes, but males show a higher level of aromatase immunoreactivity than females throughout the pre- and postnatal stages (301,302). In other mammalian species, it has been found that the fetal guinea pig brain contains high levels of aromatase activity in POA, mediobasal hypothalamus, amygdala and septum during the critical period of sexual differentiation. However, no sex differences in aromatase activity have been found (61). Fetal ferrets have high levels of aromatase activity in the POA plus the mediobasal hypothalamus and in the temporal lobe. Furthermore, aromatase activity in POA and mediobasal hypothalamus is significantly higher in males than in females. In the adult ferret brain, aromatase activity is low in both sexes (300). Furthermore, aromatase activity was similar in the BNST, medial and lateral POA, medial and lateral amygdala, ventromedial hypothalamus, and parietal cortex of adult gonadectomized male and female ferrets given no concurrent steroid treatment. Daily injections with DHTP significantly stimulated aromatase activity in mPOA, medial amygdala and ventromedial hypothalamus of both male and female ferrets in adulthood(317).

In the adult rat brain, aromatase activity is higher in the male than in the female in the preoptic/anterior hypothalamic area (e.g. 285). This sex difference is abolished after castration of the male and restored by androgen treatment. Gonadectomy has no effect on aromatase activity in

the POA in the female. Thus, it seems that in the adult, the sex difference in aromatase activity is essentially due to the presence or absence of circulating testosterone (285). Exogenous testosterone administration induces a higher aromatase activity in male than in female rats in the BNST, PVP and VMH (250). By contrast, Jakab and co-workers (145) have failed to find any sex differences in the distribution and intensity of aromatase immunoreactivity in the adult rat. This absence of a sexual dimorphism in aromatase activity might be explained by a great variation in staining intensity between individual animals (145).

Distribution and ontogeny of the 5αreductase enzyme in the brain

As stated earlier, the differentiation of the external genitalia in the male depends on the conversion of testosterone to its 5αreduced metabolite, DHT, in the undifferentiated genital tissues. Metabolism of testosterone to DHT also occurs in the brains of fetal and adult individuals in a wide variety of vertebrate species.

Numerous *in vitro* and *in vivo* studies indicate that in many mammalian species an active 5αreductase system is present in all phases of development (fetal, young, adult and old) and in practically all brain structures (for overview see 53). In rats, no major quantitative differences have been found between the two sexes (184,199). In ferrets, relatively high levels of 5αreductase activity have been found in all brain regions accross all perinatal ages, as well as in gonadectomized adult animals (300). No sex differences in 5αreductase activity have been found at any of the postnatal ages studied. Prenatally, however, males have higher levels of 5αreductase activity than females only on day 8 before expected parturition in the POA and the mediobasal hypothalamus (300). Thus, DHT can be synthesized in the rat and ferret brain of both sexes during the critical period of sexual differentiation. However, a functional role of DHT in the sexual differentiation remains to be demonstrated. The formation of DHT in the hypothalamus might be of importance for the regulation of gonadotropin secretion. It has been found that DHT was more potent than testosterone in suppressing LH release (328). In contrast to aromatase, the 5αreductase enzyme is not affected by castration, steroid administration or afferent inputs to the hypothalamus (reviewed in 53). Furthermore, aromatase is found exclusively in neurons, whereas 5αreductase is present in both neurons and glia cells, although it is more active in neurons.

Steroid receptors

Many aspects of sexual behavior and neuroendocrine function are influenced by gonadal steroid hormones and the central nervous system is a major target for these gonadal steroids. Binding of the steroid hormone to specific receptors in the central nervous system is required for steroid hormone action. Receptors are large proteins found in the cytoplasm and/or nucleus of target cells. The steroid receptors have been divided into two groups on the basis of their amino acid structure: the glucocorticoid, mineralocorticoid, androgen and progesterone receptors as one group, and the estrogen, thyroid hormone, retinoic acid, and vitamin D3 as the other group (31). Steroid receptor proteins can be divided into four functional domains: the C-terminal domain which constitutes the hormone binding domain, a hinge region, a DNA-binding domain, and a N-terminal domain, which is highly variable in size and amino acid composition (e.g. 31).

The binding of the hormone to its receptor is the initial step in steroid action. This hormone-receptor complex is then 'activated' and binds to acceptor sites on the DNA (although binding to the nuclear matrix and nuclear membranes can also occur). This interaction of the hormone-receptor complex with the chromatin material can lead to initiation of mRNA transcription that ultimately leads to translation of specific proteins. Thus, steroid hormones exert their effects through an alteration of gene expression (see 59).

Androgen and estrogen receptor containing neurons can be detected in the perinatal brain of a number of species including hamsters, ferrets, mice, rats, and monkeys (for literature overview see 223), emphasizing the pivotal role of testosterone and estradiol in the sexual differentiation of the brain.

Androgen receptor

One specific characteristic of the androgen receptor is that both testosterone and DHT can bind to the receptor. The effects of androgens on sexual behavior and neuroendocrine function are mediated by androgen receptors in the central nervous system. Androgen receptors are widely distributed in the rat brain, with largest numbers of androgen receptor containing neurons in the mPOA and VMH, each of which is thought to play an important role in mediating the hormonal control of sexual behavior, as well as in the lateral septal nucleus, the medial and cortical nuclei of the amygdala, the amygdalohippocampal area, and the BNST (for overview see 272). Moreover, androgen receptor containing neurons are found in olfactory regions of the cortex and in both the main and accessory olfactory bulbs (272). In general, nuclear androgen

receptor levels are higher in neonatal male rats than in neonatal female rats; this difference is most clearly seen in the amygdala (195). In adulthood, male rats, castrated and treated with doses of testosterone that produce physiological circulating androgen levels, exhibit significantly higher levels of nuclear androgen receptor binding than like-wise treated females in the BNST, PVP, and VMH (250).

Estrogen receptor

Estrogen receptor containing neurons are found in brain areas known to be involved in gonadotropin release, such as the anteroventral, periventricular and arcuate nuclei. Furthermore, estrogen receptor containing neurons are found in the POA, BNST, Arc and the medial amygdala (272). In the rat brain, sex differences in estrogen receptor levels have been reported: the estrogen-binding capacity of the adult female PVP, mPOA, and VMH is greater than that of the adult male. No sex differences are found in the BNST and corticomedial amygdala (48). The sex differences found in the PVP and the medial preoptic nucleus are already present as early as 24 hours after birth (166). A sex difference in the VMH emerges later, between 5 and 10 days of age (166). It has been shown that estradiol treatment of ovariectomized females dose-dependently down-regulates estrogen receptor mRNA levels in cells of the ventrolateral portion of the VMH and the Arc (173,174). Moreover, sex differences have been found in basal estrogen mRNA levels in the ventrolateral portion of the VMH and in the Arc with males showing lower levels than females. In contrast to females, estradiol fails to down-regulate estrogen receptor mRNA levels significantly in males (173).

Sexual dimorphism in sexual and nonsexual behaviors

As desribed above, the initial step in sex determination, the development of a testis or an ovary, triggers a cascade of events that eventually results in sexually dimorphic behaviors in adulthood. Sexually dimorphic patterns in reproductive and nonreproductive behaviors are common in humans and nonhumans. The expression of many of the sexually dimorphic behaviors is dependent not only on the organizational, but also on the activational influences of gonadal hormones. During early development, the presence or absence of androgens determines the capacity to display masculine or feminine sexual behavior patterns. In the adult, testosterone secreted by the testes and the neural aromatization of testosterone to estradiol are necessary to stimulate male sexual behavior, whereas estrogen and progesterone produced by the ovaries are essential for female sexual behavior.

The studies reported in this thesis present a male rat, which has been

neonatally deprived of estrogenic stimulation and can readily display male-typical as well as female-typical sexual behavior in adulthood. Thus, such male rats are clearly intermediate between the extremes of normal male and female rats. Therefore, it was thought to be useful to describe not only the hormonal and neuronal regulation of male sexual behavior, but also the hormonal and neuronal regulation of female sexual behavior.

SEX DIFFERENCES IN NEUROENDOCRINE FUNCTION

Female laboratory rats display cyclic gonadal function, whereas male rats have a tonic gonadal function. Ovulation, i.e. the expulsion of eggs from the ovary occurs cyclically, whereas sperm production is constant. The reproductive behavior of the sexes follows these fundamentally different patterns of gamete production, with females displaying cyclic patterns of reproductive behavior and males displaying a continuous readiness to mate.

These sex differences in gonadal function are driven by sex differences in gonadotropin secretion from the anterior pituitary, i.e. LH and follicle stimulating hormone (FSH). Gonadotropin-releasing hormone (GnRH, also called LHRH for luteinizing hormone-releasing hormone) is secreted in pulsatile fashion from the median eminence of the hypothalamus and drives the pituitary to a pulsatile release of LH and FSH, which subsequently drives gonadal function. In the intact female rat, the cyclic release of FSH and LH is the result of positive (stimulatory) and negative (inhibitory) feedback control by ovarian hormones acting at both the hypothalamus and the pituitary gland. Female rats typically have a 4 or 5 day cycle. The estrous cycle consists of the following stages: 1) proestrus (lasts 12-14 hours), estrus (lasts 25-27 hours), metestrus (lasts 6-8 hours), and diestrus (lasts 55-57 hours). The secretion of LH is low from late estrus to early proestrus as a result of the negative feedback provided by estrogen and progesterone secreted by the ovaries (107). Most likely, the steroids exert their negative feedback on LH secretion by modifying the amplitude and frequency of the LH pulses (107). The most potent ovarian steroid for inhibiting LH secretion is estrogen (261). On the afternoon of proestrus, the circulating levels of LH begin to increase rapidly and ultimately reach peak levels a couple of hours later. This rapid surge of LH induces follicular rupture and ovulation and stimulates the secretion of progesterone from the ovaries. Numerous studies have shown that the rising level of estradiol is the major positive feedback stimulus for the ovulation-inducing surge of LH on proestrus

(for overview see 107). Ovarian estradiol, secreted from metestrus through early proestrus, and ovarian progesterone, secreted during the afternoon of proestrus, sensitize the pituitary gland to respond to become more responsive to hypothalamic GnRH. A further increment in LH release from the pituitary gland is the result of an enhanced secretion of GnRH in the portal blood. This enhanced release of GnRH occurs just prior to the surge of LH on proestrus. There is little doubt that the increased output of GnRH by the medial basal hypothalamus is caused by an enhanced secretion of estradiol on diestrus exciting GnRH neurons in the preoptic-anterior hypothalamic area, either directly or indirectly, to release a surge quantity of GnRH into the portal blood. The physiological relevance of progesterone in the preovulatory LH surge on proestrus is not that clear. Presumably, progesterone does not enhance GnRH secretion in the portal blood (259). However, there is a large body of evidence that circulating progesterone concentrations during the estrous cycle participate in a positive manner in the preovulatory surge (reviewed in 153). In rats, there is no luteal phase, because the corpora lutea are not maintained after ovulation in unmated rats.

Normal male rats, when implanted with an ovary, do not ovulate. This sexual dimorphism in the ability to support ovulation is not caused at the level of the pituitary, but reflects changes induced by early testosterone and/or estradiol exposure in the hypothalamus. Normal females are capable of showing a preovulatory LH surge after adult gonadectomy and treatment with a large dosage of estradiol, whereas males are not (217; for overview see 87). However, neonatal treatment of female rats with testosterone eliminates their later capacity to exhibit estrogen-induced LH surges, whereas neonatal castration makes it possible for males to show a preovulatory LH surge in response to estrogen. Neonatally castrated males with an ovarian graft and a vaginal graft can ovulate, although after a limited number of cycles, ovulation ceases to occur and constant vaginal cornification follows (309). A similar cessation of ovarian cyclicity has also been reported in female rats, which were treated neonatally with a low dose of androgen (e.g. 295). Presumably, the sexual differentiation of the central nervous control of gonadotropin release starts prenatally (309).

SEX DIFFERENCES IN SEXUAL BEHAVIOR

Females and males of many species show sexually dimorphic mating postures (Figure 1). Female rodents stand immobile with arched backs and allow males to mount them. This mating posture is called lordosis (Fig 1C). Males mount females and will normally not assume the lordosis

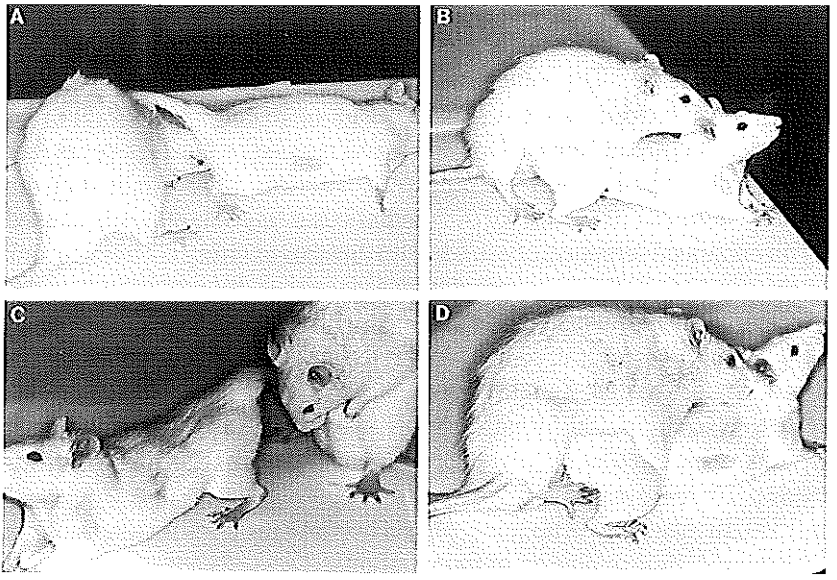

Figure 1. SEXUAL BEHAVIOR IN RATS. A male first investigates the perineal region of the female (A). If the female is in estrus, the male mounts her with his forepaws clasped against her hindquarters. This tactile stimulation causes her to display the lordosis posture, that is, arching her back and deflecting her tail (B). After a few seconds, the male dismounts the female and grooms himself (C). After several mounts and intermissions, the male ejaculates (D).

posture when mounted by other males.

Sexual behavior in rats

Male rat sexual behavior

It is necessary to distinguish coital behavior from the behavior that precedes it. This so-called precopulatory behavior can involve olfactory and gustatory investigation of the female, especially her perineal region and can last as little as a few seconds or be completely absent in sexually experienced rats (Fig 1A). Typical male coital behavior consists of three components: 1) mounting, 2) intromission, and 3) ejaculation (Figure 1). Mounting is operationally defined as the male assuming a copulatory position, i.e. dorsally and from the rear, but failing to insert his penis into the female's vagina (Fig 1B). Mounting is mostly associated with thrusting motions of the hindquarters (pelvic thrusts). Intromission can be defined as the penis entering the vagina for a very short period of time (1-2 sec) during a mount. Intromission is commonly followed by a short period of genital autogrooming (Fig 1C). After a number of repeated

mounts and intromissions, ejaculation occurs (Fig 1D). Ejaculation is the forceful expulsion of semen from the male's body. Ejaculation is behaviorally defined as the culmination of vigourous intravaginal thrusting accompanied by the arching of the male's spine and often the lifting of the forepaws off the female prior to withdrawal (for overview see 197). Ejaculation is commonly followed by a period of genital autogrooming, after which the male enters a period of sexual inactivity, i.e. the postejaculatory period, which lasts several minutes. The postejaculatory period consists of two phases: an absolute refractory period and a relative refractory period (28). Male rats normally vocalize at ultrasonic frequencies (22 kHz) after an ejaculation. This vocalization continues during 50% to 75% of the postejaculatory period (197).

Precopulatory behavior is taken as one index of sexual motivation. Motivational aspects of sexual behavior can be studied by assessment of an animal's partner preference. Partner preference is usually tested by giving a choice between a sexually active male and an estrous female. Another choice stimulus may be that between an anestrous female and an estrous female. In most studies, the time spent in the proximity of each stimulus animal is used as a measure of preference. Gonadally intact males prefer estrous females over males. This preference occurs even without prior sexual experience (136). Subjecting a male rat to various degrees of sexual exhaustion by repeated ejaculations results in clear alterations in partner preference. Increased sexual exhaustion results in a progressive increase in time spent away from both stimulus animals and a shortening of the time spent near the female. By contrast, males that are sexually aroused by a few intromissions without ejaculation spend more time with the female and less time away from the stimulus animals (311). In addition, partner preference of males at various ages is differentially affected by sexual experience. A few contacts with a female are sufficient to induce a preference for a female in young males (between 70 and 90 days of age), whereas sexually unexperienced older males (150 days of age) require larger amounts of stimulation to show a preference for the female (312).

Hormonal regulation of male sexual behavior

Endogenous testosterone activates male sexual behaviors. Castration leads to a dramatic reduction in sexual behavior which is restored by treatment with TP (e.g. 28). Not only coital behavior is diminished, also motivational aspects of sexual behavior are affected. When given free access to a sexually active male and an estrous female, intact male rats spend significantly more time with an estrous female. After castration, this preference declines rapidly and is restored by testosterone treatment (136). As described previously, testosterone can be converted to DHT

and estradiol. Postcastration treatment with DHT is not sufficient to activate coital behavior (99) and induces only a low preference for the female (311). However, estradiol is very effective in activating male mating behavior in castrated rats (65,278), whereas estradiol treatment together with DHT treatment will restore mating behavior to the level found in gonadally intact males (100) or testosterone treated males (18). Treatment with estradiol alone, fails to induce a female-oriented preference in males (311).

In general, it is thought that estradiol affects the central nervous system to promote sexual behavior, whereas DHT is important to restore penile tactile sensitivity to precastration levels (197). However, DHT also acts in the central nervous system. Lodder and Baum (178) reported that DHT stimulated mounting behavior in long-term castrated EB-treated male rats after bilateral pudendal nerve transsection. This finding suggests that DHT and estradiol act synergistically in the rat brain to activate mounting behavior.

Neuronal regulation of male sexual behavior

Historically, brain lesioning techniques have been used to find out where in the brain sexual behavior is regulated. The assumption behind these brain lesions, is that removal of a critical component of the neural mechanism regulating sexual behavior should result in disruptions of this behavior. The initial attempts were based on the removal of large areas of the brain. Removal of the neocortex in male rats disrupts copulation, with the greatest disruption when the frontal cortex is removed (169,172). More recently, researchers have tried to limit the lesions more precisely to discrete brain areas (197). In fact, lesion techniques have become more sophisticated, i.e. instead of large destructive electrolytical lesions, axon-sparing excitotoxic lesions are made by infusion of the NMDA receptor agonist, quinolinic acid. In addition to lesion studies, researchers have also performed electrical stimulation and recording studies to locate the neural circuitry of male sexual behavior. Brain implant studies have been used to unravel the sites of hormone action in the central nervous system.

· The olfactory bulbs

In many rodent species, olfaction is not only crucial for successful copulation, but is also crucial in mediating many other social interactions. Experimental blocking of the smell usually results in social and sexual behavior deficits (197). In vertebrate species, the olfactory system consists of two morphologically and functionally different systems: 1) the main olfactory system, and 2) the vomeronasal or accessory olfactory system (123). The olfactory bulbs are located at the front of the brain and

consist of two anatomically distinct regions, the primary olfactory bulbs (part of main olfactory system) and the accessory olfactory bulbs (part of vomeronasal system). At the rear of the nasal cavity lie bipolar cells in the olfactory neuroepithelium. Their axons terminate in the primary olfactory bulbs. A portion of the neuroepithelium is grouped in the vomeronasal organ (VNO), a structure that lies on each side of the nasal septum located near the floor of the nasal cavity (211). The VNO is highly developed in rodent species, but is regressed in humans. The receptors of the VNO connect mainly to the accessory olfactory bulb (AOB) via the vomeronasal nerve, also called the nervus terminalis (3,211,326). The primary effect of olfactory bulb removal in the male rat is a reduction in the percentage of males that achieve ejaculation (170,171,198,316,323). This failure to ejaculate stems from both an inability of some males to initiate copulation (170,171,316,323) as well as an inability to sustain copulation once initiated (198). The male rats with olfactory bulbectomy that do ejaculate, show longer intromission and ejaculation latencies than controls (170). These findings suggest that both sexual motivation and performance are impaired after bulbectomy. This is confirmed by Edwards and co-workers (89), who found that males in which the olfactory bulbs were removed, failed to show a preference for an estrous female over an anestrous female. Thus, in the rat, inputs from the primary olfactory bulbs and the VNO have an important, but not critical, role in copulation. However, damage to the olfactory system of male hamsters produces severe copulatory deficits. Bilateral removal of the olfactory bulbs in male Syrian hamsters completely eliminates copulation (reviewed in 197). As does olfactory bulbectomy, cutting the efferent pathways of the olfactory bulb by transecting the lateral olfactory tract eliminates copulation in male hamsters (72). For nonrodent species, the olfactory system may not be as important for copulation as in rats and hamsters. Olfactory bulbectomy or damage to the nasal epithelium has no effect on copulation in dogs, cats, rhesus monkeys, or sheep (197).

· Amygdala

Efferents from the primary and accessory olfactory bulbs travel to the amygdala, an almond-shaped structure located in each temporal lobe of the brain. The role of the amygdala in the control of copulation in rodents has been assessed by lesions of two regions of the amygdala: the basal and lateral nuclei (basolateral amygdala) and the corticol and medial nuclei (corticomedial amygdala). Lesions of the basolateral nuclei of the amygdala do not disrupt sexual behavior in either male rats (111,133) or hamsters (176). By contrast, lesions of the corticomedial nuclei of the amygdala can disrupt mating in male rats (111) and male hamsters (176). Male rats with such lesions have longer ejaculation latencies and fewer

ejaculations to sexual exhaustion than do control males (111,133).

· Amygdaloid efferents

Efferents from the corticomedial amygdala travel via the stria terminalis, innervating the BNST as well as the mPOA (165). Lesions of the stria terminalis or the BNST have induced copulatory deficits in male rats similar to those seen following lesions of the corticomedial amygdala. Giantonio and co-workers (111) reported that lesions of the stria terminalis significantly increase ejaculation latencies of male rats. In other studies of male rats, lesions of the stria terminalis (228), or BNST (94,305), significantly increase the number of intromissions preceding ejaculation, the interintromission interval, and consequently, the ejaculation latency. The similarity of the effects of lesions of the BNST and the corticomedial amygdala suggests that little or no processing of copulation information occurs in the neurons of the BNST and that this nucleus serves primarily to relay information from the amygdala to other areas, such as the mPOA (197).

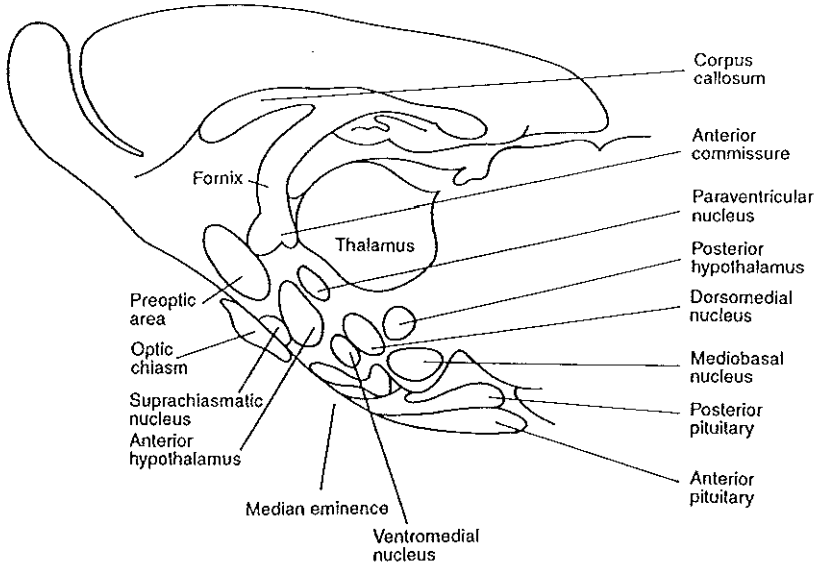

Figure 2. SAGITTAL SECTION OF THE PREOPTIC AREA AND THE HYPOTHALAMUS OF THE RAT. The preoptic area appears to play an important role in the neural regulation of male copulatory behavior. Lesions of the preoptic area elimate male sexual behavior in virtually every vertebrate species examined. The preoptic area regulets endocrine function by interacting with hypothalamic nuclei. After RJ Nelson (1995). An Introduction to Behavioral Endocrinology, Sinauer Associates, Inc. Publishers, Sunderland MA.

· Medial preoptic area

The mPOA lies along both sides of the midline of the brain anterior to the hypothalamus (Figure 2). The mPOA appears to play an important role in the neural regulation of male copulatory behavior. Bilateral lesions of the mPOA severely disrupt masculine coital function in many vertebrate species (reviewed in 197). Heimer and Larsson (134) have published the first study on the effects of medial preoptic lesions on copulation in male rats, in which they showed that lesions disrupted male coital behavior. In contrast to lesions, electrical stimulation of the mPOA accelerates ejaculation in male rats (183,306). Additionally, gonadal hormones act in the mPOA mediating sexual behavior. Implantation of crystalline testosterone into the mPOA of castrated male rats facilitates mating in all animals; in contrast, castrated rats with crystalline cholesterol implanted into the mPOA do not copulate (64). Christensen and Clemens (55) have compared the efficacy of pellets of testosterone and estradiol implanted into the mPOA of long-term castrated male rats to activate copulation. Only 30% of the males implanted with testosterone copulate to ejaculation, whereas 70% of the males implanted with estradiol ejaculate. In a follow-up study, Christensen and Clemens (56) have infused solutions containing controlled doses of testosterone (10 g/day) or estradiol (5 g/day) into the rostral anterior hypothalamus. These treatments only stimulate mounting in castrated male rats. Concurrent treatment with the aromatization inhibitor ATD blocks the increase in mounting induced by testosteron infusion without affecting mounting activated by estradiol treatment. These results suggest that testosterone exerts its effect on mating behavior via aromatization to estrogen. This is confirmed by a recent study (58), in which it was found that mounting and ejaculation significantly decreased in gonadally intact male rats after bilateral implantation of the nonsteroidal aromatase inhibitor Fadrozole in the POA. As described in the section on SEXUAL DIFFERENTIATION OF THE BRAIN, neurons in the mPOA contain large quantities of androgen and estrogen receptors, which are necessary for steroid hormone action. The inability of males with mPOA lesions to mate is probably not caused by deficits in sexual arousal. Male rats with medial preoptic lesions fail to mount females even when tested 8 months after surgery, although they will press bars or run mazes to gain access to females (113,134). Thus, the mPOA seems to be involved in consummatory, rather than appetitive, aspects of sexual behavior (see 98). However, Paredes and Baum (227) have shown that in ferrets appetitive aspects of sexual behavior also are regulated in the mPOA. When tested in a T-maze after gonadectomy and treatment with EB, male ferrets with bilateral lesions of the mPOA approach a stimulus male significantly more often than control males do. The latter prefer to approach the stimulus female (227).

· Midbrain

The primary efferents of the mPOA project to the midbrain by the medial forebrain bundle (294). However, it should be kept in mind that most interactions of the mPOA with other brain regions involve reciprocal connections (269). Thus, there is a dense projection from the central tegmental field (CTF) to the mPOA. The elimination of mating by mPOA lesions is thought to be the result of an interruption of efferents to the midbrain (reviewed in 197). Several midbrain regions which are innervated by the medial forebrain bundle, have been studied for their possible role in the regulation of mating behavior. Lesions of the lateral tegmental field, dorsal and medial to the substantia nigra, disrupt mating in male rats (42,43,129). The anatomical connections of the peripeduncular nucleus indicate that this nucleus relays sensory information from the pudendal nerve (51) which is connected to the penis, to the mPOA, at least via the amygdala (185). Consistent with these effects of lesions is electrophysiological evidence that lateral tegmental neurons are active during copulation in male rats (267). Thus, the neurons in the lateral tegmental area form an important component of the neural pathway for mating.

· Nucleus Accumbens

The nucleus accumbens is a brain region, which has been implicated in sexual reward (98). Indeed, a chronoamperometric study by Mitchell and Gratton (205) has shown that male rats exposed to soiled bedding from estrous females have an increase in the release of the neurotransmitter dopamine in the nucleus accumbens. This response becomes larger and peaks earlier in time with repeated presentations of the male to soiled bedding from estrous females. When male rats are exposed to other males' bedding or anestrous females' bedding, however, dopamine does not increase (205). Similar results have been obtained by Louilot and coworkers (179). A neural pathway for activation of dopamine release by terminals in the nucleus accumbens, may be formed by afferents from the medial amygdala, which in turn receives afferents from the AOB. Such a projection from the medial amygdala to the nucleus accumbens has been found in male hamsters (114). Another neural pathway that might activate the nucleus accumbens, runs from the mPOA, which has an output to the ventral tegmental area, where information is relayed to the ventral striatum and in particular to the nucleus accumbens, which in turn projects to the basal ganglia. Thus, the nucleus accumbens forms the neural interface between regions assessing the animal's motivational state and motor systems capable of organizing movements that permit the animal to execute behaviors.

Female rat sexual behavior

Female rat sexual behavior is characterized by a cyclic pattern of behavioral proceptivity, i.e. approaching the male partner (thereby hopping and darting, presenting and earwiggling), and receptivity, i.e. the display of lordosis in response to mounts (27). Around the time of ovulation, high levels of female sexual behavior can be observed, whereas on other days of the estrous cycle these behaviors are virtually absent.

Hormonal regulation of female sexual behavior

The copulatory behavior of female rats usually coincides with ovulation. Because females ovulate periodically, copulatory behavior is observed in cycles called estrous cycles. Mating behavior coincides with the presence of preovalutory follicles and often stops with the onset of corpus luteum activity. As described previously, gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus in a pulsatile pattern, stimulates the anterior pituitary to release FSH and LH. FSH causes the follicles of the ovaries to grow, and as the follicles develop, estradiol and progesterone are produced in increasing concentrations. On the afternoon of proestrus, the circulating levels of LH begin to increase rapidly and ultimately reach peak levels a couple of hours later. This rapid surge of LH induces follicular rupture and ovulation and stimulates the secretion of progesterone from the ovaries (for overview see 107). The elevated estrogen and progesterone blood levels on proestrus stimulate female sexual behavior. The elevated progesterone levels will eventually terminate estrous behavior (121,333,334). In ovariectomized females, behavioral estrus can be induced by appropiately timed injections of estradiol and progesterone.

With respect to appetitive aspects of sexual behavior, gonadally intact females prefer males over females around the time of ovulation (203). This preference of females for stimulus males disappears after ovariectomy and is restored by estrogen treatment (67,203,274).

The adult hormonal environment appears to interact with sexual experience to determine partner preference of female rats. Female rats, ovariectomized in adulthood and treated with TP show a preference for the stimulus male, but after being allowed to sexually interact with a male, switch to a preference for the stimulus female (274). De Jonge and co-workers (69) have compared the partner preferences of sexually experienced versus sexually naive females, ovariectomized as adults and given oil or TP. Naive females receiving oil show no preferences; when treated with TP they prefer males. But females with mount experience (experience with females) prefer females, independent of adult hormone treatment. A similar effect of sexual experience on partner preference of

females has been found by Vega Matuszczyk and Larsson (310). Sexually naive females, ovariectomized in adulthood and treated with androgens (TP or DHT or the synthetic nonaromatizable androgen R 1881) show a preference for males. However, sexual experience abolishes this preference for males. Sexually naive females, ovariectomized in adulthood and treated with estradiol, show no preferences. However, sexual experience induces a preference for males.

Neuronal regulation of female sexual behavior

Almost all knowledge concerning neural regulation of female sexual behavior has come from studies on the neural control of lordosis behavior in rats (230). Several brain areas have been discovered to be essential for lordosis.

· *Ventromedial nucleus of the hypothalamus*

In the ventrolateral portion of the VMH, roughly 30% of the neurons have significant estrogen concentrations. Some of these estrogen-binding neurons send their axons to the midbrain central gray (230). Local implants of estrogen (255) and progesterone (256) in the VMH can facilitate lordosis behavior, and implants of antiestrogens can reduce it (230). Lesions of the VMH reduce the frequency of lordosis (92).

· Medial preoptic area

The net effect of activated neurons in the POA is an inhibition of female repoductive behavior. In female rats, bilateral lesions of the mPOA facilitate lordosis (141,236,322). Electrical stimulation of preoptic neurons has the opposite effect: it suppresses the lordosis reflex in female rats (229).

· Lateral septum

Electrolytic lesions in the lateral septum clearly increase lordosis behavior in the female rat (214,215,216). Thus, the net effect of the septál efferents seems to be inhibitory with respect to lordosis.

· Olfactory bulbs

Olfactory inputs have shown to be inhibitory with respect to female reproductive behavior. Moss (210) surgically removed the olfactory bulbs from estrogen-progesterone-primed female rats and found increases in lordosis behavior. Furthermore, it has been found that removal of the olfactory bulbs enhances sexual receptivity in normal, cycling female rats (8). Therefore, the overall net effect of olfactory input must be inhibitory with respect to lordosis behavior. The effect of olfactory bulbectomy cannot be due to a reduction of vomeronasal input, since

olfactory signals processed by the AOB appear to stimulate several aspects of reproductive function in the female rat: a facilitation of ovulation, the release of LH, and sexual receptivity by exposure of the female to male rats and/or odors of male rats (9,33,147,157). These facilitory effects as well as the tendency of the female to seek the proximity of a sexually active male are blunted or blocked by removal of the VNO (147,157,243,248,258). The VNO projects solely to the AOB, which in turn has efferents to the medial amygdala. Additionally, the VMH has been demonstrated to be directly linked to the vomeronasal projection circuit via afferents from the medial amygdala, which strengthen the anatomical link between the vomeronasal system and lordosis behavior (159,165). This is confirmed by the findings of Masco and Carrer (185), who reported that lesions of the medial amygdala strongly suppress lordotic responsiveness, whereas stimulation of this region can enhance lordosis behavior in ovariectomized female rats, treated with estradiol and progesterone. Additionally, lesions of the posterior cortical amygdala have been reported to suppress preference of female rats for an intact male, an effect that is also observed by resection of the VNO (248).

SEX DIFFERENCES IN NEURONAL MORPHOLOGY AND FUNCTION

The functional sex differences in behavior and neuroendocrine function are presumably related to morphological differences between the brains of males and females. Such evidence for the existence of sexually dimorphic brain structures became available in 1971, when Raisman and Field (239) using electron microscopy, discovered that the dendrites of the POA receive a higher proportion of afferent inputs into dendritic spines in females than in males. Furthermore, neonatal castration of male rats created the female phenotype of dendritic organization, whereas neonatal administration of TP to females created the male phenotype (239,240,241). In 1976, Nottebohm and Arnold (221) working with zebra finches and canaries reported that the volume of the forebrain nuclei which control singing is significantly greater in males than in females. This sex difference correlates very nicely with the dimorphic control of song production in these species, i.e. only males sing. To date, almost all brain regions involved in the regulation of sexual behavior, have been found to be sexually dimorphic. Within the scope of this thesis, it is interesting to note that most sex dimorphisms in brain structures and functions are under the influence of perinatal estrogens derived from the neural aromatization of testosterone. Thus, neonatal inhibition of brain

estrogen formation in male rats might create the female phenotype or an intermediate between the male and female phenotypes of many of the sex dimorphisms described in the next paragraphs.

Sexual dimorphism in the medial preoptic area/anterior hypothalamus

Following the work of Nottebohm and Arnold (221), Gorski and coworkers found a nucleus in the rat POA, which is significantly larger in males than in females. This nucleus has been labeled the sexually dimorphic nucleus of the preoptic area (SDN-POA) (115,116). Homologous structures have been identified in other species, such as the gerbil (the sexually dimorphic area (SDA) and its pars compacta (SDApc); 60), guinea pig (sexually dimorphic preoptic area (SD-POA; 49,138), and the ferret (the male nucleus of the POA/anterior hypothalamus (MN-POA/AH; 299). A sexually dimorphic nucleus has also been discovered in the POA of humans (288). Because this nucleus resembles the SDN-POA of rats and is larger in males than in females, it was also named the SDN-POA. The presence of galanin (109), galanin mRNA (40), thyrotropin-releasing hormone (104) supports the possible homology with the SDN-POA in the rat (39,271). In studies by Allen and co-workers (7) and LeVay (177), another subdivision of the human POA has been used, i.e. into 4 smaller subregions called the Interstitial Nuclei of the Anterior Hypothalamus (INAH), abbreviated INAH-1, INAH-2, INAH-3 and INAH-4. Using this anatomical classification scheme, INAH-1 is considered to be equivalent to the SDN-POA and in contrast with the findings of Swaab and Fliers (288), no sex difference has been found in this nucleus (7). The controversy about the sexually dimorphism of the human SDN can be explained by the strong age-dependence of the sex difference (291). In males, a major reduction in SDN cell number has been observed between the age of 50 and 60 years, so that the sex difference becomes small. In females over 70 years of age, cell death has been found to be more prominent than in males, increasing the sex difference again (139). This sex difference in the pattern of ageing, and the fact that sexual differentiation of the human SDN only occurs after the fourth year of age (289) might explain why Allen and co-workers (7), who had a sample of human adults biased for age, did not find a significant sex difference in the size of the SDN or INAH-1 (292). The volumes of INAH-2 and INAH-3 are reported to be larger in men than in women (7), which is in contrast with later findings by Levay (177), who has reported a sex difference in INAH-3, but not in INAH-2. The latter

controversy can also be explained by age-dependent sexual dimorphism. INAH-2 shows only a sex difference after the child bearing age and in a 44 year old woman who had a hysterectomy with ovarial removal 3 years prior to death (7), while LeVay (177) studied only young gonadally intact women. In addition, LeVay (177) has reported that the volume of INAH-3 is significantly smaller in homosexual men than in heterosexual men, and resembles the volume of INAH-3 of heterosexual women. The homosexual men used in the study by LeVay (177) all died from AIDS-related illness. Their brains have been compared with those of men whose sexual orientation is unknown, but assumed to be heterosexual, and who have died primarily from other causes. Another sexually dimorphic structure in the human hypothalamus is the anterior commissure: it was found to be 12% larger in females (5). In addition, the anterior commissure was larger in homosexual men than in heterosexual men and women (6).

The volumes of the rat SDN-POA and the ferret MN-POA/AH are not affected by adult hormonal status (115,299), whereas the volume of the gerbil SDA is significantly increased in the presence of testosterone (60). Although the volume of the MN-POA/AH is not measurably affected in the ferret, the cells in the MN-POA/AH are 20% larger in the presence of gonadal steroids (299).

Sex differences in the POA morphology depend on the action of gonadal steroids during critical periods early in development. Testosterone administered neonatally to female rats increases the volume of the SDN-POA in adulthood, whereas neonatal castration of male rats reduces the volume of the nucleus in adulthood (115,128,143). However, these neonatal manipulations do not result in complete sex reversal of the size of the SDN-POA. Neonatally androgenized female rats have SDN volumes that are larger than the volume in normal females, but smaller than the volume in normal males. Apparently, the sexual differentiation of the SDN begins prenatally. This possibility is emphasized by the findings of Döhler and co-workers (79,80), who have administered testosterone pre- and neonatally to females. The volume of SDN in such females in adulthood becomes indistinguishable from that in normal males. It should be noted that multiple steps in the differentiation process may act cumulatively or sequentially, inducing new stages which are more sensitive to the next hormone-dependent stimulus (106,297). This means that testosterone given to neonatally gonadectomized male and female rats during the critical period elicit a larger SDN-POA in males than in females in adulthood (143).

Furthermore, it has been found that the sexual differentiation of the SDN-POA depends on the estrogenic metabolite of testosterone

(79,81,143). Male rats treated prenatally or pre- and neonatally with the aromatase inhibitor ATD, have smaller SDN volumes in adulthood compared to control males (142). The largest reduction in SDN volume is found in the pre- and neonatally ATD treated males (142).

The actual function of the SDN-POA in controlling the expression of sexual behaviors is still not clear. Lesions restricted to the SDN show either no effect or a very small effect on masculine sexual behaviors in rats (10,70) and ferrets (54), though in male gerbils SDA lesions disrupt mating (327). A study by Hennessey and co-workers (135) suggests that the SDN-POA in male rats may be involved in the inhibition of feminine sexual behavior. Bilateral lesions of the medial POA/AH augment the display of lordosis in male rats treated with estrogen and progesterone. The most effective feminizing brain lesions are those which bilaterally destroyed a substantial portion of the medial POA encompassing the SDN. If the SDN is involved in inhibition of female sexual behavior then bilateral lesions of the SDN in female rats would enhance their proceptive and receptive behaviors. However, it was found that bilateral lesions of the SDN in ovariectomized female rats which were treated with testosterone in adulthood, did not affect their female sexual behavior, but did affect their masculine sexual behavior (304).

Sexual dimorphism in the suprachiasmatic nucleus and the arcuate and ventromedial nuclei of the hypothalamus

Sex differences at the ultrastructural level have been found in the suprachiasmatic nucleus (SCN). More synapses (5-30%) are observed in male than in female rats (124,125,175). In addition, the volume of the SCN is larger in male than in female rats (247). In humans, no sex difference has been found in the total number of vasopressin-expressing neurons of the SCN, but the shape of the vasopressin-containing subnucleus of the SCN was sexually dimorphic, i.e. it was elongated in women and more round in men (288). Furthermore, there is an age-dependent sex difference in the vasoactive intestinal polypeptide (VIP) cell population of the SCN: young men had twice as many VIP expressing SCN neurons as young women, whereas in the middle-aged group, the women had twice as many VIP SCN neurons as the men (330).

Ultrastructural sex differences have also been found in the Arc (187,188) and the VMH (189,190) of the rat. In the Arc, female rats have approximately twice as many synapses on dendritic spines than males, but half as many synapses on perikarya. In the ventrolateral portion of the VMH,

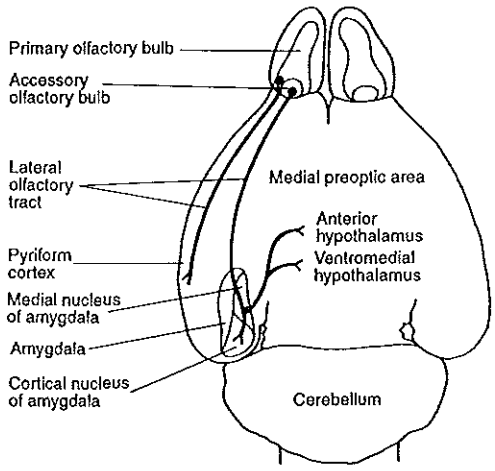

Figure 3. SCHEMATIC NEURAL CIRCUIT OF THE RAT OLFACTORY SYSTEM. Information from the primary olfactory bulbs is relayed to the piriform cortex and eventually travels to the amygdala. Information from the accessory olfactory bulbs travels directly to the cortical nucleus of the amygdala. From the amygdala, pheromonal/chemosensory information travels to the medial preoptic area via the stria terminalis and the bed nucleus of the stria terminalis. The medial preoptic area connects to the anterior and ventromedial hypothalamus. After RJ Nelson (1995), An introduction to Behavioral Endocrinology. Sinauer Associates, Inc. Publishers, Sunderland MA.

males have approximately 33% more axodendritic synapses (spines and shafts) than females. These synaptic sex differences in the Arc (188) and the VMH (189) have been attributed to the action of neonatal testosterone and/or estradiol.

Sexual dimorphism in the vomeronasal system

It has been found that the vomeronasal systeem (Figure 3) is a sexually dimorphic network with male rats having more vomeronasal receptors and a larger number of neurons throughout this chemosensitive pathway (reviewed in 123,263) than female rats. A brief synopsis of sexual dimorphisms at various levels of the vomeronasal system is provided below.

Vomeronasal organ

In the rat, the VNO is a sexually dimorphic organ organized by gonadal steroids during the critical period of sexual differentiation (262). Males have greater values than females with respect to VNO volume, neuroepithelium volume and number of olfactory receptors. Male rats castrated on the day of birth show a female-like appearance of the VNO in adulthood. Females treated with testosterone on the day of birth show

a male-like VNO in adulthood. Furthermore, it has been found that the structure of the VNO also depends on the level of gonadal steroids during adulthood. Gonadectomy of the adult rat produces a decrease in the neuroepithelial height in both males and females.

Accessory olfactory bulb

The AOB, an olfactory structure embedded in the dorsocaudal portion of the olfactory bulb(Figure 3), shows sexual dimorphism in several structural aspects (e.g. 249,265). The AOB volume is larger and there are more mitral cells in male than in female rats (249,264). Furthermore, males have larger mitral somata and more dendritic branches growing out from the main dendritic stalks (50). These examples of sexual dimorphism depend on the action of gonadal steroids during early development. Male rats, neonatally castrated, show a female-like organization of the AOB, whereas females, neonatally treated with androgens, show a male-like organization of the AOB (263). The number of AOB granule cells is also sexually dimorphic with males having more light and dark granule cells than females. Castration of males and androgenization of females on the day of birth reverse the number of light granule cells with the castrated males showing female numbers and the androgenized females showing male numbers. Castration of males also reduces the number of dark granule cells to female numbers, but androgenization of females does not cause any increment in the number of dark granule cells (123). The lack of an effect of postnatal testosterone treatment on the dark granule cells in females, can probably be explained by the fact that AOB granule cells have two neurogenetic periods. The first takes place prenatally (Days 17-19 of gestation), while the second occurs postnatally between Days 1-20 (25,287). Thus, the AOB dark granule cells apparently have a period of maximal susceptibility to the organizational effects of androgens that is both different from and occurs later than in the AOB light granule cells (123).

The bed nucleus of the stria terminalis

Although the overall volume of the BNST does not appear to be sexually dimorphic, volumetric sex differences have been found in smaller divisions of the BNST. The BNST is not a homogeneous structure in terms of sex differences as two different patterns of sexual dimorphism take place. In some regions, males show greater morphometric values than females, while in others females show greater morphometric values than males. For instance, the volume of the medial posterior division of the BNST is larger in the male rat than in the female rat (71). However, the volume of the medial anterior region of the BNST is larger in females than in males (71). In humans, a clear sex difference in the so-

called 'darkly staining posteromedial component of the BNST' has been found by Allen and Gorski (4). The volume of this area is 2.5 times larger in males than in females. Recently, Zhou and co-workers (332) have found sex differences in a more rostral part of the BNST, the central part of the BNST (BNSTc), in humans. This is the part of the BNST that is innervated by VIP fibers from the amygdala. The BNSTc volume in heterosexual men was 44% larger than in heterosexual women. The BNSTc volume of heterosexual men and homosexual men did not differ in any statistically significant way. Interestingly, a small volume of the BNSTc has been found in male-to-female transsexuals. Its size is only 52% of that found in reference males (332).

The bed nucleus of the accessory olfactory tract

The bed nucleus of the accessory olfactory tract shows sexual dimorphism in that males have a larger volume and more neurons in this nucleus than females (123). Castration of male rats and testosterone treatment of female rats performed on the day of birth, reverse these measures (123).

Amygdala

The amygdala receives not only afferents from the AOB and is part of the vomeronasal projection pathway, but receives also afferents from the midbrain CTF and is part of a somatosensory neural pathway. Furthermore, neurons in the medial amygdala contain androgen- and estrogenreceptors and the aromatase enzyme, necessary for the conversion of testosterone to estradiol. Significant sex differences in various morphological characteristics of the medial amygdaloid nucleus are apparent in various species. In rats, the volume of the medial amygdaloid nucleus is approximately 20% larger in males than in females, whereas the volume of the lateral amygdaloid nucleus does not differ between the sexes (206). At the ultrastructural level, males have approximately 25% more synapses ending on dendritic shafts in the medial amygdaloid nucleus than females (219,220).

The medial amygdaloid nucleus may be part of a sexually dimorphic neural circuit (269,270) that also includes the SDN-POA and the BNST.

Sex differences in synapse number may depend on the early action of gonadal steroids. In the medial amygdaloid nucleus of rats, neonatal castration of males decreases the number of shaft synapses in adulthood to female levels, and neonatal administration of testosterone (Day 5) to females increases the number of shaft synapses to male levels (219).

Sex differences in the amygdala are manifested after the period considered to be critical for hormone action. For instance, sex differences in volume of the medial amygdaloid nucleus are not observed until postnatal Day 21 during normal development (206). Similarly to the

differentiation of the SDN-POA, it has been postulated that the estrogenic metabolite of testosterone mediates the sexual dimorphism in the medial amygdaloid nucleus of the rat (206,219). Treatment of female rats with estradiol for the first 30 days of life results in a male-like organization of the medial amygdaloid nucleus both with respect to shaft synapses (219) and nuclear volume (206).

Sexual dimorphism in neurotransmitter and neuropeptide systems

Another type of morphological sex difference has become obvious with the application of immunocytochemical techniques for localizing neurotransmitter-associated or neuropeptide systems (for literature overview see 298). In the next sections, sex dimorphisms in the distribution of vasopressin and GnRH are described.

Sex dimorphism in vasopressin projections

Vasopressin-immunoreactive projections in the brain have now been extensively traced (73). In rats, all vasopressin-immunoreactive projections of the BNST and the medial amygdala appear to be denser in males than in females. Consistent with these differences, males have about two or three times more vasopressin-immunoreactive cells in the BNST. Using *in situ* hybridization, nearly twice as many medial amygdaloid cells labelled for vasopressin mRNA are present in males than in females (reviewed in 73). Moreover, male rats have a denser vasopressinergic innervation of the lateral septum than females (74). The vasopressin-immunoreactive projections of the BNST, medial amygdala and lateral septum are extremely sensitive to gonadal hormones. After gonadectomy, BNST and medial amygdaloid cells lose their vasopressin immunoreactivity and can no longer be labelled for vasopressin mRNA; treatment with gonadal hormones prevents these changes (reviewed in 73). Male rats castrated on the day of birth or at postnatal day 7 had fewer vasopressin-immunoreactive cells in the BNST and the medial amygdala and a lower density of vasopressin-immunoreactive fibers in the lateral septum than male rats castrated on day 21 or control males (73). These findings suggest that androgens influence the differentiation of vasopressin-immunoreactive neurons around day 7 after birth.

There is some evidence that vasopressin is involved in sexual behavior (282,283,284). For instance, it has been found that an intracerebroventricular injection of vasopressin inhibits (283) and of a vasopressin antagonist stimulates (282) lordosis behavior in ovariectomized female rats. These findings suggest that vasopressin has

an inhibitory effect on female sexual behavior. Although it is not known via what mechanism vasopressin exerts this action, the target area might for example be the sexually dimorphic innervation of the lateral septum, since various studies have demonstrated that the septal nuclei are involved in the (predominantly) inhibitory control of feminine sexual behavior (117,214,329). However, a direct link between the vasopressinergic innervation of the lateral septum and female sexual behavior has never been tested.

Sex dimorphism in the GnRH system

Harris and Jacobsohn (131) established that the hypothalamus, and not the pituitary gland itself, is primarily reponsible for the sexual dimorphism in the preovulatory release of gonadotropins. Within the central nervous system, GnRH containing neurons are diffusely distributed across the basal forebrain of all mammals. In rats, the basal forebrain population of GnRH neurons numbers approximately 1000-1600. In rostral regions of the diagonal band of Broca and the POA surrounding the organum vasculosum of the lamina terminalis (OVLT), the vascular structure at the tip of the third ventricle, GnRH neurons are positioned medially and dorsally. In more caudal regions, i.e. in the caudal POA and anterior hypothalamic area, GnRH neurons are positioned more ventrolaterally. Finally, a small number of neurons are found ventral or lateral to the Arc in the basal hypothalamus (reviewed in 160). In both males and females, quantitative estimations show that the number of GnRH neurons is greatest in the POA. The classical projection of GnRH-containing axons is to the median eminence from where GnRH is released into the portal vessels and is transported to the pituitary gland, where it modulates the synthesis and secretion of gonadotropins. Electrical stimulation of the preoptic GnRH neurons, in either male or female rats, causes GnRH secretion leading to LH release. The increase in the concentration of GnRH in the portal blood is linearly related to the strength of the stimulating current (230). In ovariectomized females, estrogen treatment increases the number of GnRH cell bodies in the POA, presumbably caused by an increase in GnRH synthesis. However, the effects of estradiol on GnRH synthesis will not be exerted on GnRH neurons themselves, but must be mediated by another class of neurons, since none of the estrogen-concentrating neurons has immunoreactive GnRH in the cytoplasm (230).

Perhaps most important for female reproductive behavior are the GnRH axonal terminals found in the midbrain central gray, a site important for lordosis behavior, suggesting a link between the GnRH system and lordosis behavior. The behavioral importance of GnRH-

containing terminals, of which the GnRH content can be influenced by estrogen, is confirmed by the findings that GnRH administered to the midbrain central gray potentiate lordosis behavior, whereas an GnRH antiserum can block lordosis behavior (230). In addition, bilateral lesions of the medial amygdala reduce mating-induced lordosis behavior, presumably by a reduced activation of the GnRH neuronal system (242). Double labeling immunocytochemistry of the brain of the repeatedly mated females for Fos protein (the product of the immediate-early gene *c-Fos*, which can be used as a marker for neuronal activity, see later in the section **Activation of neural structures by mating associated stimuli**) and GnRH revealed that a significantly lower percentage of the GnRH neurons in females with bilateral lesions of the medial amygdala exhibited Fos protein-immunoreactivity than the controls.

Sex differences have been found in the population of GnRH neurons acutely following gonadectomy. In male rats, the population of GnRH neurons of males, one day after castration is considerably smaller than that of intact males. This is predicted given the rise in LH in male rats at this time, which presumably results from increased release of GnRH. The population of GnRH neurons of females, one day after ovariectomy is not as small as that of males, one day after castration. In female rats, there is a delayed rise in serum LH compared to that in male rats following gonadectomy. Therefore, a reduction in the population of GnRH neurons occurs in females on day 6 following ovariectomy, as LH levels at this time are elevated (reviewed in 160).

Immunocytochemical visualization of immediate early genes, such as *c-Fos*, has been used to investigate the relationship between the LH surge and increased GnRH neuronal activity. Since Fos is a nuclear protein, its colocalization by immunocytochemistry with the predominantly cytoplasmic GnRH is easily accomplished. In the rat, Fos protein can be detected in GnRH neurons on the afternoon of proestrus and during a steroid-induced LH surge (see 268). The activated neurons are concentrated around the OVLT and represent only 40% of the total. Mating (mounts, intromissions and/or ejaculations) also increases *c-Fos* expression in GnRH neurons in female mice (325) and in female rats, but not in male rats (62). In ferrets, the pulsatile secretion of LH rises and lasts for more than 12 hr in females after receipt of an intromission, whereas in males that achieved an intromission, both LH and testosterone secretion are either reduced or unchanged (52,167). The female ferret ovulates reflexively in response to somatosensory stimulation received during mating in contrast with the female rat, which is a spontaneous ovulator (137). Lambert and co-workers (168) have reported that intromissive stimulation activates forebrain GnRH neurons, as determined by colabelling with Fos protein, in female ferrets, but not in males.

Apparently, the sexually dimorphic pattern of LH secretion that occurs in ferrets after mating reflects a selective activation of GnRH neurons in the female forebrain.

SEX DIFFERENCES IN NONSEXUAL BEHAVIORS

Experimental investigations of the effects of gonadal hormones on sex differences in nonsexual behaviors have been guided by concepts and methods applied to the study of sexual behavior (32). A brief synopsis of sex differences in various nonsexual behaviors is provided below.

Activity

Sex differences in activity in rats have been usually tested in running wheels or in the open-field test. On both measures, females are more active than males. Wheel running is strongly dependent on activational effects of gonadal hormones in both sexes. Gonadectomy depresses wheel run activity in both sexes, abolishing the sex difference (see 32). Hormone replacement stimulates wheel running in both males and females. The activity is positively correlated with estrogen dosage in both sexes. The activational effect of testosterone requires aromatization to estradiol (254).

Organizational effects of gonadal steroids on wheel running have also been reported. Neonatal treatment of female rats with high doses of TP reduces the response to activating effects of estrogen in adulthood (for overview, see 32). This organizational effect of testosterone on wheel-running activity does not require aromatization to estradiol, since neonatal treatment of females with EB does not reduce the response to the activating doses of estrogen in adulthood (110).

Another measure of activity is the open-field test. There are no clear activational effects of gonadal steroids on open-field activity (273). However, gonadal steroids exert prominent organizational effects on open-field behavior. Neonatal treatment of females with TP depresses open-field activity (e.g. 38,231). Male rats castrated neonatally are generally more active than control males (149).

Social behaviors

Aggression has not been studied as extensively in the laboratory rat as in the mouse. Although sex differences have been demonstrated in rats, the magnitude of the differences is rather small. Independently of what

method is used to elicit aggressive behavior, activational influences of testosterone can be demonstrated unequivocally. Thus, castration reduces shock-elicited fighting (35), as well as aggression directed toward an intruder placed in the resident's home cage (12) or intermale aggression stimulated by the presence of an estrous female (296). The mechanism by which testosterone activates aggression in male rats has received little attention. Christie and Barfield (57) have found that testosterone and estradiol are equally potent in restoring aggression towards an intruder by castrated resident males. Nonaromatizable androgens, i.e. DHT, are less potent but more effective than control injections.

An unresolved question is whether or not there are organizational effects of gonadal steroids on aggressive behavior in rats. Barr and co-workers (13) have observed that males are more likely to initiate attacks than females and that neonatal castration greatly reduces attack even when testosterone replacement is given in adulthood.

By contrast, Van de Poll and co-workers (307) have found no sex difference in the overall amount of fighting induced by adult testosterone treatment. However, males and females do differ in their response to estradiol, which stimulates fighting in males but not in females.

Activation of neural structures by mating associated stimuli

As described in the section ***sexual behavior in rats***, virtually all the early knowledge about the neural basis of sexual behavior has been derived from experiments in which mating was studied in rats after lesions, electrical stimulation of certain brain regions, or the implantation of small quantities of steroid hormones in discrete brain areas. Over the last 10 years, immunocytochemical methods have been used for investigating brain structures involved in the regulation of sexual behavior. Especially, the immunocytochemical visualization of the protein products of immediate-early genes, such as *c-Fos* and *c-Jun*, has been used to provide information about the neural pathways which are activated in response to a variety of peripheral, sensory, as well as internal, homeostatic stimuli (e.g. 17,46,83,84,103,161,232,324). For example, light pulses administered during the dark phase of the light/dark cycle activate *c-Fos* in those neurons of the hamster and rat SCN which receive retinal inputs (164,257). However, the possible physiological significance of the increased neuronal expression of *c-Fos* is not known. It has been found that mice, in which the *c-Fos* gene has been experimentally disabled, exhibit a stunted growth of the long bones, severe osteopetrosis, and lymphopenia associated with ossification of the bone marrow spaces (24). It has been postulated that *c-Fos* expression might be required for

the programmed cell death that normally occurs in the nervous system, bone, and other somatic tissues during development (276,277). Sexual behavior of these male mice with a null mutation of the *c-Fos* gene was to some extent affected: the onset of mounting was slower and the subsequent mounting rate was significantly lower in homozygous mutants than in control mice; intromissive and ejaculatory behavior were equivalent between mutant and control mice (24).

ACTIVATION OF NEURAL STRUCTURES IN MALES BY GENITAL/ SOMATOSENSORY INPUTS

Numerous studies have shown that mating with an estrous female significantly increases *c-Fos* expression in neurons located in four specific brain areas of male rats: the mPOA, BNST, the medial amygdala, and midbrain CTF (17,19,245,319). Previous experiments have shown that bilateral lesions of each of these regions in the male rat disrupt coital behavior to varying degrees. Baum and Everitt (17) reported that unilateral lesions of the olfactory peduncle significantly reduce *c-Fos* expression in the ipsilateral medial amygdala, but not in other structures, in male rats of which coital interaction with estrous females is restricted to mount-thrust and occasional intromission due to repeated application of a lidocaine anaesthetic to the penis. These results suggest that olfactory inputs, possibly of vomeronasal origin, contribute to the induction of *c-Fos* in the medial amygdala. Unilateral lesions of the mPOA fail to reduce *c-Fos* expression in the ipsilateral or contralateral medial amygdala or CTF following ejaculation. By contrast, combined, unilateral lesions of the medial amygdala and CTF, significantly reduce *c-Fos* expression in the ipsilateral BNST the mPOA. These findings suggest that afferent inputs from the CTF, probably of genital-somatosensory origin, and from the medial amygdala, probably of olfactory-vomeronasal origin, interact to promote neuronal activity, and the resultant induction of *c-Fos* in the ipsilateral BNST and mPOA (17).

ACTIVATION OF NEURAL STRUCTURES IN FEMALES BY GENITAL/SOMATOSENSORY INPUTS

It has been found that copulation with intromission and ejaculation (received) produces a dramatic induction of Fos protein-immunoreactivity in estrogen-concentrating brain regions, such as the lateral septum,

mPOA, BNST, VMH, lateral habenula and medial amygdala, in ovariectomized female rats, which were treated with estradiol and progesterone (233). Similar increments in *c-Fos* expression have been obtained by stimulation of the vaginal cervix with a glass rod in both hormone-treated and untreated female rats. Mechanical stimulation of the flanks produces a smaller induction of *c-Fos* in these rats, whereas hormone treatment alone has no effect (233). Apparently, genital stimulation carried by the pelvic nerves induces the forebrain *c-Fos* response. This is confirmed by the findings of Rowe and Erskine (253), who reported that bilateral transsection of the pelvic nerve eliminated the increases in *c-Fos* expression in the mPOA and the medial amygdala after receiving intromissions from males. Transsection of the pelvic nerve also prevents the occurrence of pseudopregnancy, which is defined as the occurrence of any functional luteal phase in a non-pregnant cycle (97). In rats, the cycle begins anew after ovulation occurred, unless the female mates. If she receives sufficient cervical stimulation and the mating is sterile, i.e. the female does not become pregnant, the corpora lutea remain functional for approximately 14 days before regressing. Because this causes a number of physiological changes which resemble pregnancy, this state of sustained corpus luteum function is called pseudopregnancy. One of the hormones involved in pseudopregnancy and pregnancy in the rat is prolactin, which is released from the pituitary and stimulates the formation and maintenance of the corpora lutea. In a later stage of the pregnancy, prolactin is important in the development of the mammary glands and promotes lactation in female mammals. Several brain areas, such as the mPOA, SCN, and dorsomedial/ventromedial nuclei of the hypothalamus have been shown to influence the duration or circadian aspects of the prolactin surges (108,126,146).

Presumably, the mating-induced activation of the mPOA and the medial amygdala is responsible for initiating the prolactin surge; the mating-induced activation of the periventricular nucleus (PVN), VMH and CTF may be caused by non-specific stressful stimuli (PVN) or by afferent input important for the display of lordosis behavior (VMH, CTF) (253).

ACTIVATION OF NEURAL STRUCTURES IN MALES BY VOMERONASAL/ CHEMOSENSORY INPUTS

In rodent species, reproductively relevant pheromonal cues are detected by receptors in the VNO, which in turn transmit this information centrally via the AOB, the medial amygdala, BNST and the mPOA (see

Figure 3). Monitoring *c-Fos* expression in the vomeronasal projection pathway has provided effective means of studying the role of vomeronasal/chemosensory factors in determining the activity of neural structures which control the expression of sexual behavior. Exposure to chemosensory stimuli derived from vaginal secretions or urine-soaked bedding, has been shown to activate the vomeronasal projection pathway. For instance, in male hamsters exposure to females' vaginal secretions augmented *c-Fos* expression in the AOB, posterior portions of the medial amygdala and the posteromedial portion of the BNST (101,102). Similar results have been obtained by Bressler and Baum (47): male rats exposed to soiled bedding from estrous females show an induction in *c-Fos* expression at each level of the vomeronasal projection circuit.

In several studies (17,86,101), surgical disruption of the vomeronasal inputs to the brain are found to cause partial attenuation of mating-induced increments in Fos protein in different parts of the vomeronasal projection circuit.

ACTIVATION OF NEURAL STRUCTURES IN FEMALES BY VOMERONASAL/CHEMOSENSORY INPUTS

As described in the section on *neuronal regulation of female sexual behavior*, olfactory cues processed by the VNO and the AOB appear to stimulate several aspects of reproductive function in the female rat. Removal of the VNO impairs the ability of exposure to male rat odors to enhance ovulation (147), LH secretion (33) and the expression of lordosis (243,258). Dudley and co-workers (86) have investigated the differential effects of olfactory vs somatosensory stimulation on *c-Fos* expression in the vomeronasal projection circuit. They have found that both exposure to male-soiled bedding or repeated mating with males induce similar amounts of Fos protein-immunoreactivity in the mitral and granule cell layers of the AOB. The *c-Fos* expression in the AOB is entirely mediated by olfactory stimulation, since the AOB receives its afferents solely from the VNO. By contrast, differences between the two sensory stimuli have been noted in the medial amygdala, BNST and VMH. Exposure to male-soiled bedding for 60 min produces scattered staining in the medial amygdala, BNST, and VMH, whereas 60 min of repetitive mating results in a more dense distribution of Fos protein-immunoreactive neurons in these areas. Strikingly distinct patterns of *c-Fos* expression are observed in the medial amygdala, BNST and VMH following 120 or 180 min of repetitive mating. These patterns are not present in animals exposed to male odors (86). These data suggest that

genital/somatosensory stimulation rather than olfactory stimulation mediates *c-Fos* expression in the medial amygdala, BNST and VMH. This supposition is supported by the findings of Bressler and Baum (47), who have found that olfactory/chemosensory stimulation fails to induce *c-Fos* expression in the VMH and CTF, sites at which mating has previously been shown to activate *c-Fos* expression.

The VMH, a nucleus critical for the display of lordosis in female rats (230), is directly linked to the vomeronasal projection circuit via afferents from the medial amygdala (159,165). Dudley and Moss (85) reported that bilateral lesions of the AOB decrease lordotic responsiveness and reduce mating-induced *c-Fos* expression in the medial amygdala and the BNST (the mPOA was not taken into account). The decrease in lordotic behavior is probably due to a disruption of the input from the AOB to the medial amygdala.

Circadian rhythms

The studies presented in this thesis show that sexual behavior of male rats, in which brain estrogen formation has been inhibited neonatally by administration of the aromatase inhibitor ATD, fluctuate over the dark phase of the light/dark cycle. Therefore, it was thought to be useful to describe briefly the neural regulation of circadian rhythms by the SCN.

The vast majority of biochemical, physiological, and behavioral phenomena are expressed rhythmically. Circadian rhythms are endogenous oscillations with a period of approximately 24 hour. Circadian rhythmicity has been found in gonadotropin levels, in prolactin, in testosterone and in melatonin in a wide variety of species (for overview see 303). It has now been established that a bilaterally paired structure, the SCN, located in the anterior hypothalamus, contains a master circadian clock that regulates most, if not all, endogenously generated circadian rhythms (303). The SCN receives direct neural input from the retina via the retinohypothalamic tract, which is involved in relaying synchronized light-dark information to the SCN. Bilateral lesions of the SCN disrupt a number of reproductive rhythms in rats and hamsters, including those of circulating prolactin and gonadotropin levels, pineal melatonin, sexual behavior in males, sexual receptivity in females, and the timing of ovulation (96,155,225,238,281,286). Likewise, bilateral lesions of the SCN disrupt many metabolic rhythms, such as feeding, drinking, locomotor activity, body temperature and sleep-wake (for overview see 303).

The most convincing evidence that the SCN contains a circadian clock is the finding that *in vitro* a number of rhythms persists. In both hypothalamic slice and organ culture preparations, rhythms have been observed in neural firing, vasopressin release, and glucose metabolism (303).

In the rat, the SCN can be subdivided in a small rostral component and a large caudal area with dorsomedial and ventromedial components (209). Although the role of the SCN in the control of various circadian rhythms is well known, the actual mechanism by which the SCN controls these rhythms is not known with certainty.

The rat transfers its rhythmicity into the brain presumably by its vasopressinergic projections (154). It has been found that the vasopressinergic projections of the SCN closely follow the projections of the SCN as established by anterograde techniques (34,140).

Swaab and Hofman (290) reported that the size of the vasopressin-containing subnucleus of the SCN is correlated with sexual orientation, i.e. it is twice as large in homosexual men compared to heterosexual men. The relation of the number of peptide expressing neurons in the SCN and sexual orientation seems to be quite specific for vasopressin, since VIP neurons did not show a difference between homosexual and heterosexual men (331). In the rat, it has recently been found that the SCN of pre- and neonatally ATD-treated males contained more vasopressin-expressing neurons than those of prenatally ATD-treated and control males. The latter two groups did not differ (293). These findings suggest a possible role for perinatal estradiol in the differentiation of vasopressin-expressing neurons in the rat SCN.

Techniques for measuring sexual behavior

Obviously, studies conducted to investigate the hormonal and/or neuronal regulation of sexual behavior must use reliable and valid measures for sexual behavior. For instance, the measures of sexual behavior in a test may vary considerably as a function of many situational factors, including the stage of the female partner's cycle, time of test, test duration, size of test chamber, time of adaptation to the chamber, age, and sexual experience of the tested animals (197). Especially the time of day can play a role in the expression of sexual behavior. For example, male rats display more mounting and show shorter latencies to first ejaculation as the dark period of the light/dark cycle progresses (75,130,280).

Moreover, as described in the section *male rat sexual behavior*, it is necessary to distinguish appetitive from consummatory aspects of sexual

behavior. A short synopsis of techniques for measuring sexual behavior is provided below.

OBSERVATION AND RECORDING OF CONSUMMATORY RESPONSES

In most mating tests for coital behavior, one male and one female are introduced into a glass enclosure (usually an empty aquarium) or a semicircular cage. If a male is tested for coital behaviors, the male is first placed in the cage for an adaptation period. Then an ovariectomized female, which has been brought into behavioral estrus by injecting EB and progesterone, is introduced in the cage and the test begins. Various sexual behaviors can be scored, such as number of mounts, intromissions, and ejaculations and latencies to first mount, intromission, and ejaculation.

OBSERVATION AND RECORDING OF APPETITIVE RESPONSES

Historically, appetitive aspects of sexual behavior have been tested in a so-called 'obstruction apparatus' (e.g. 202,260). A male is placed on one side of an obstacle, traditionally an electrified grid, and a female is placed on the other. After each mount or intromission, the male is returned to the other side of the obstacle. The amount of electric current the male withstands to reach the female is taken as a measure for sexual motivation. Alternatively, a male must press a bar or do some other task in order to gain access to a female.

Another method for determining sexual motivation is the measurement of partner preference. Two or more choices are presented, and the one selected most of the time, i.e. significantly more than 50% of the test duration, is assumed to be preferred. A variety of methods and types of apparatus have been used for measuring partner preference: 1) double choice runways or T-mazes (e.g. 227), 2) arenas with stimulus animals in wire-mesh cages or stimulus animals tethered (e.g. 310), 3) a residential plus maze consisting of a central box and four arms extending to peripheral boxes which could contain food, water, or stimulus animals (e.g. 200), and 4) a three-compartment box with the stimulus animals either tethered or placed behind a wire mesh separation in the two lateral compartments (e.g. 45). In some experiments, the subjects are not allowed to mate with the stimulus animals, so that the measures would more accurately reflect appetitive/motivational aspects of mating prefer-

ence unconfounded by consummatory aspects (1). A disadvantage of preventing sexual contact between the subject and the stimulus animals, is that it remains unclear whether the choice was made on the basis of 1) sexual preference as opposed to social preference, 2) a tendency to attack the stimulus animal, and 3) curiosity evoked by novelty (197).

Scope of this thesis

This thesis presents studies investigating the effects of neonatal inhibition of brain estrogen formation on the sexual differentiation of partner preference in male rats. It had been shown before that estradiol is essential for the masculinization and defeminization of coital behavior. Relatively less attention has been paid to the organization of motivational aspects of sexual behavior. In all experiments presented in this thesis, brain estrogen formation has been inhibited by neonatal administration of the aromatase inhibitor ATD through silastic capsules. Within 2-4 h after birth, these silastic capsules were implanted sc in the back of the newborn male. Control males have been implanted with silastic capsules which are either empty or contain cholesterol (a hormonally-inert precursor of testosterone). The implants were removed either at the end of the critical period, that is, at the age of 10 days, or at weaning, that is, at the age of 21 days.

In chapter 2, a new semiautomated test apparatus for studying partner preference in the rat is described. It should be noted, however, that in chapters 3, 4, and 6, an earlier version of the three-compartment box was used for measuring partner preference (274). In chapter 3, the effects of castration in adulthood and subsequent hormone treatment with estradiol and DHT on partner preference of neonatally estrogen-deprived males, were investigated. In chapter 4, the possible interaction between neonatal hormone treatment and postweaning housing conditions on later partner preference and sexual behavior has been investigated. Since male rats, neonatally deprived of estrogenic stimulation, can readily display masculine sexual behaviors as well as feminine sexual behaviors, in contrast to control males, one can hypothesize that the sexual experiences of ATD males housed in groups might affect their partner preference differently from ATD males housed singly or from control males. In addition, we have changed the choice of stimulus animals: subjects were given free access to an ATD male (instead of a normal male) and an estrous female. This has been done to investigate whether there is a difference between singly housed and group-housed ATD males in the preference for a stimulus ATD male over a non-treated stimulus male. Chapter 5 was

aimed to investigate sex differences in odor preference and partner preference. We have investigated what stimulus characteristics of active males versus estrous females are responsible for the previously observed preference of males for females, of females for males, and of ATD males for males: are chemosensory or more distal cues like seeing and hearing the estrous female/active male or the reward of sexual activity with the stimulus animals responsible for the observed differences in partner preference? To that end, ATD males, normal males and females were first given free access to soiled bedding from estrous females and soiled bedding from sexually active males. Then, they were given free access to an estrous female and a sexually active male behind a wire mesh separation and then to tethered stimulus animals.

In chapter 6, the duration of the critical period for the organization of partner preference and coital behavior was determined and the existence of nocturnal fluctuations in sexual behavior of neonatally estrogen-deprived male rats was investigated. Neonatal male rats received ATD or cholesterol, beginning directly after birth (Day 0) or on Day 2 or Day 5 after birth. In adulthood, partner preference and coital behavior of such males have been studied early and late in the dark phase of the light/dark cycle. In chapter 7, we have questioned whether or not the nocturnal fluctuations in adult partner preference of neonatally estrogen-deprived males, parallels rhythmic changes in endogenous reproductive hormones. To that end, blood of adult ATD and control males has been collected early and late in the dark phase. Other groups of adult ATD and control males have been tested for partner preference before castration and after castration with subsequent testosterone treatment using silastic implants. The latter treatment ensures a constant blood level of testosterone, thereby eliminating any endogenous rhythm in testosterone and gonadotropins. In chapter 8, the possible contribution of the SCN to the nocturnal fluctuations in sexual behavior of ATD males was investigated through lesions of the SCN. We have investigated the question whether ATD males' partner preference would alter after SCN lesions.

Chapters 9 and 10 were completely dedicated to the central nervous system. In chapter 9, the effects of neonatal inhibition of brain estrogen formation on adult neural *c-Fos* expression to mating and pheromonal stimulation in the male rat, were examined. It has been found that the volume of the SDN-POA is dependent on the presence of perinatal estrogens. We have asked the question whether the neural *c-Fos* expression to intromissive stimulation will be lower in neonatally ATD-treated males than in neonatally cholesterol-treated males, in a manner corresponding to the reduced volume of the SDN-POA in such ATD males vs control males. We have also questioned whether the mating-induced Fos protein-immunoreactivity within the medial amygdala and CTF which

project to the mPOA may differ between ATD and control males. In rats, the vomeronasal projection circuit has been found to be sexually dimorphic, with males having more vomeronasal receptors and a larger number of neurons throughout this chemosensitive pathway. Presumably, the sexual differentiation of the vomeronasal system is under influence of perinatal estrogens. Therefore, in the second experiment of chapter 9, we have enquired whether chemosensory cues (derived from either the urine and feces of estrous females or of sexually active males) may reveal differences in neural *c-Fos* expression in the vomeronasal projection to the mPOA and/or the nucleus accumbens, a brain region which has been linked to reward and (sexual) motivation, between ATD and control males. Chapter 10 was aimed to investigate the distribution of androgen and estrogen receptors in the central nervous system of neonatally estrogen-deprived males. It has been found that there is a differential effect of estradiol on the activation of partner preference: ATD males, castrated in adulthood and treated with estradiol, show a clearcut preference for males, whereas like-wise treated control males prefer females. In addition, sex differences have been reported with the estrogen-binding capacity of the female hypothalamus and POA being greater than that of a male. Sex differences have also been reported in androgen-binding capacity in the brain with males showing a higher binding-capacity in the BNST, POA, and VMH than females. Therefore, we have investigated the possible contribution of postnatal estradiol in the sexual differentiation of brain estrogen and androgen receptors using immunocytochemical techniques to localize androgen and estrogen receptors in the rat brain.

In chapter 11, the main results and some future lines of research to further study the role of perinatal estradiol in the sexual differentiation of the male rat brain will be discussed.

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Chapter II

A SEMIAUTOMATED TEST APPARATUS FOR STUDYING PARTNER PREFERENCE BEHAVIOR IN THE RAT

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A semiautomated test apparatus for studying rat partner preference behavior.

PHYSIOL BEHAV **56**(3) 597-601, 1994. A semiautomated three compartment box (3CB) for studying partner preference behavior of rats is decribed. This apparatus automatically records the rat's time spent in each compartment, as well as the locomotor activity (i.e., the number of visits an animal pays to each compartment). Software was developed for calculating partner preference scores. Behavioral testing in the semiautomated 3CB, which is a modification of an earlier version, is less time consuming and less laborious. Three 3CBs can be observed simultaneously by two trained observers, and the behavioral interactions of three experimental animals with the stimulus animals can be observed and scored by hand. The use of the new apparatus was validated by studying adult partner preference behavior of neonatally ATD-treated male rats. The collected data fully corroborate previous results, obtained in the earlier version of the 3CB, again revealing the behavioral bisexual nature of these ATD males. A new finding was the much higher locomotor activity of the ATD-males compared to controls.

To study motivational aspects of sexual behavior in rats, a partner preference behavior test using a three-compartment box (3CB) was developed (11). Stimulus animals could be placed in the lateral compartments of the 3CB, either behind a wire mesh separation, which prevents sexual interaction, or tethered with a rope to the rear of one of the lateral compartments, which makes sexual interaction possible. The experimen-

tal animal could move around freely and interact with the stimulus animals or sit in the empty middle compartment. The time that the experimental animal spent in each compartment was recorded by hand, with one observer per 3CB (1-3, 11). Also, the various behaviors had to be scored manually. This manner of recording appeared to be time consuming and laborious.

Therefore, it was desirable to automate the 3CB. To this end, the apparatus was changed such that the time spent in each compartment was recorded automatically. This article contains a description of the semiautomated 3CB and its validation with sexually experienced male rats treated neonatally with the aromatization inhibitor ATD (4, 7, 8, 12).

DESCRIPTION OF THE SEMI-AUTOMATED THREE COMPARTMENT BOX

Figure 1 shows a artistic drawing of the semiautomated three compartment box (wall height 40 cm) and the tethering device of the stimulus animals. The box is made of gray perspex with a Plexiglass front; it has three compartments (60 x 30 x 40 cm each) with a small opening (13 x 12 cm) in both partitions near the back. These openings can be closed by a sliding door. Stimulus animals are tethered with a rope to the front of one of the lateral compartments, and the experimental animal freely moves around. Movement of the experimental animal from one compartment to an other is registered with position-sensitive tilt platforms placed

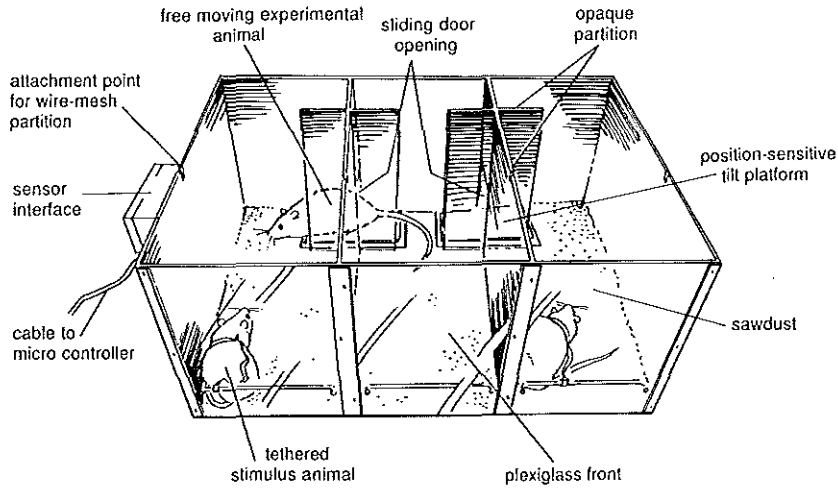

FIG. 1. Semiautomated three-compartment box (3CB) for partner preference testing. A enlargement of one stimulus animal with the tethering device is depicted in the lower left-hand corner.

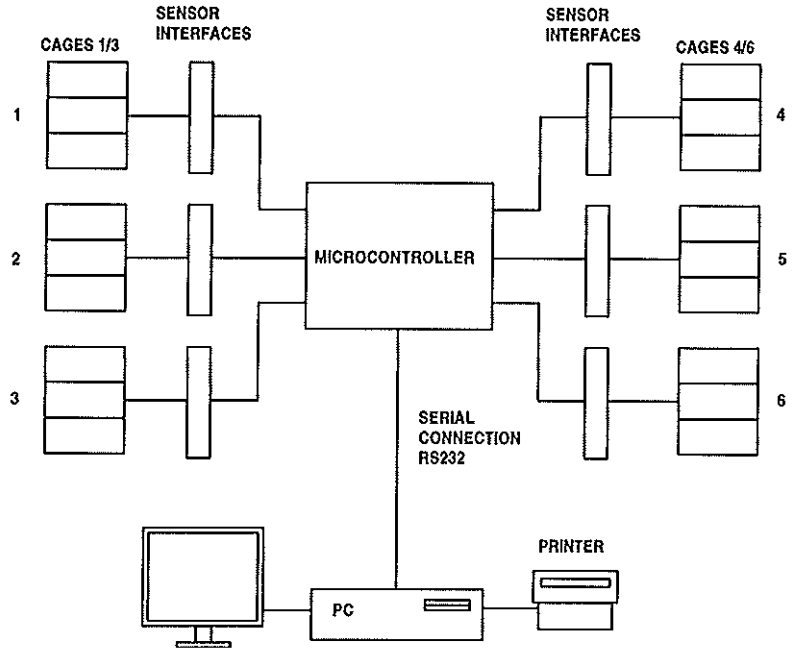

FIG. 2. System block diagram. The numbers (1, 2, 3; 4, 5, 6) refer to the semiautomated three-compartment boxes. See the text for details.

in each opening. The height of the openings can be lowered to prevent the experimental animal from jumping over the tilt platforms. The tilt platforms are made of gray perspex and can easily be removed for cleaning purposes. The position of the tilt platform is determined using a magnetic field. Two small samarium cobalt magnets are fixed in the opposite ends of each platform. To detect the magnetic fields, sensors are placed in the bottom part of the box. Any good lab could build such an apparatus.

Data collection

Figure 2 shows a diagram of the system. To make the data suitable for computer processing, the sensors in the bottom of the cage are connected with an interface that digitizes the sensor signals. The data of the sensor interfaces are collected by a microcontroller which can monitor three cages simultaneously (cages 1-3 or cages 4-6). The microcontroller transfers the data to a serial RS232 output, which is connected with the serial input port of a personal computer (PC). An Olivetti M24 computer attached to a Star LC-10 printer is used.

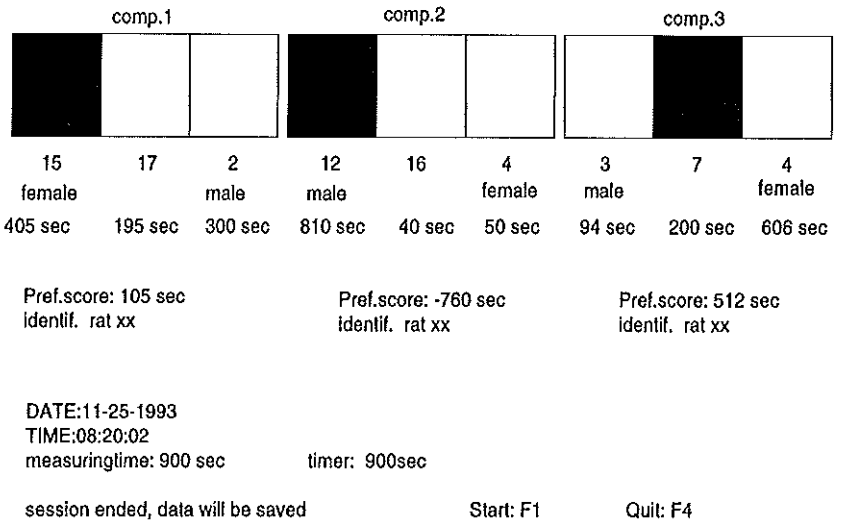

FIG. 3. Schematic drawing and other relevant information of the three semiautomated three-compartment boxes on the monitor of the personal computer. See the text for details.

Representation of the results

Software has been developed to draw schematically the three cages and their compartments on the monitor of the PC (Fig. 3). When an animal moves from one compartment to another, the corresponding compartments are highlighted on the monitor. Additionally, on-line calculations are made for a preference score, and the number of visits an animal pays to each compartment are counted. A preference score is calculated by subtracting the time spent in the compartment containing the active male from the time spent in the compartment containing the estrous female (11).

Testing procedure

Before testing begins, two stimulus animals and the experimental animal are put in the test apparatus, one in each compartment, with the sliding doors closed, for 15-min adaptation. The stimulus animals are either tethered with a rope to the front of one of the lateral compartments (sexual interaction possible) or placed behind a wire mesh separation (no sexual interaction possible). Additional data (i.e., the position of the stimulus animals) essential for calculating a preference score, the duration of testing and a file name are fed into the computer. At the beginning of the test, the sliding doors are removed and the experimental animal can freely move around and interact with the stimulus animals or sit before

the wire mesh separation. Three 3CB (three experimental animals) can be observed simultaneously by two trained observers, and the behavioral interaction of the experimental animals with the stimulus animals can be scored. The data of each test are stored on the hard disk under a chosen file name and can be printed at the end of testing for processing.

SUPPORTING EXPERIMENT

Adult partner preference behavior was studied in male rats neonatally treated with ATD (1,4,6-androstatriene-3,17-dione). ATD blocks the aromatization of testosterone to estradiol (4, 5, 7, 12). Within 2-4 hr after birth, male rats (Wistar, RP strain) received subcutanously (SC) a silastic capsule (i.d. 1.5 mm; o.d. 2.1 mm; length 5 mm) containing ATD or cholesterol. The capsules were removed at weaning (21 days of age). The stimulus animals were sexually active males obtained from a commercial breeder (Harlan Sprague-Dawley Company, Zeist, The Netherlands), because at that time no locally bred adult males were available, and estrous females (locally bred; Wistar RP strain). The latter were ovariectomized and brought into behavioral estrous by injecting 20 g estradiol benzoate, 24-48 h before testing followed by 1.0 mg progesterone 3-4 h before testing. These hormones were dissolved in olive oil and injected sc in the neck. Behavioral testing commenced at the age of approximately 4 months. To get sexual experience, the males were first subjected to two pair tests (duration 15 min) with an estrous female (all males displayed intromission behavior) and two pair tests (duration 15 min) with an active male [only ATD males (five of nine) showed lordosis behavior]. After these pair tests, the male rats were subjected to three partner preference behavior tests with sexual interaction possible (tethered stimulus animals). All tests were carried out in the early portion (between 0900-1100 h) of the dark phase of the light-dark cycle (lights off from 0745 to 1745 h). This is the time in which ATD males were found to be sexually active both with the stimulus female and the stimulus male (i.e., when they were behaviorally most bisexual) (1-3).

Figure 4 shows the partner preference data. Two-way analysis of variance (ANOVA) (6) on the preference scores [Fig. 4(A)] revealed a significant effect of groups, $F(1,16) = 35.23, p < 0.001$). The ATD male rats showed either a partner preference for the active male or no partner preference, whereas control males clearly preferred the estrous female partner. ANOVA on the time spent in the empty
middle compartment [Fig. 4(B)] showed no significant differences between ATD and control male rats and no effect of testing. The time

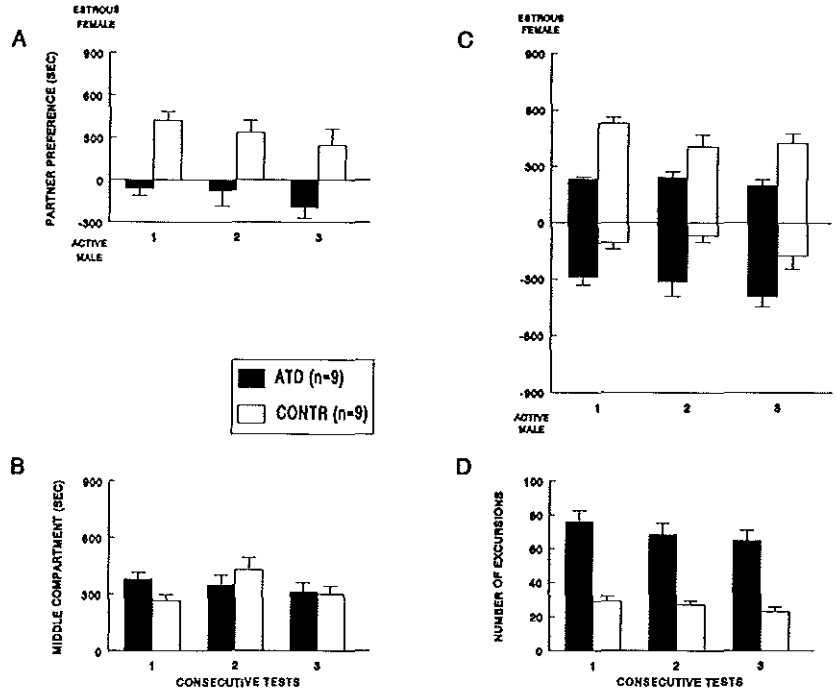

FIG. 4. Partner preference behavior of adult gonadally intact male rats, neonatally treated with the aromatization inhibitor ATD (black bars) or cholesterol (open bars). (A) Partner preference behavior, (B) time spent in the empty middle compartment, (C) time spent with the estrous female and the time spent with the active male, (D) total number of visits to the compartments of the experimental animals. Values are means and SEM.

spent with the estrous female and the time spent with the active male are shown in Fig. 4(C). From looking at this figure, it is clear that ATD-males spent more time with the active male and less time with the estrous female than control males.

The mean total number of visits are depicted in Fig. 4(D). ANOVA showed a significant group effect, $F(1,16) = 133.2$, $p < 0.001$. ATD-males paid significantly more visits to the stimulus animals and the empty middle compartment than controls.

The partner preference behavior data of the present study corroborate earlier results (1, 3): neonatal ATD treatment to male rats significantly affected their adult partner preference behavior. Compared to control males, such ATD males show no (or a very low) preference for the estrous female partner or a preference for the male partner. A new finding from the present study is the much higher locomotor activity of the ATD males. Although we had this impression already from earlier experiments (ATD males running from one side to the other more frequently than controls), thanks to the new semiautomated 3CB this supposition was confirmed

and substantiated.

We don't know whether or not ATD males are generally more active or whether it is linked to the method of testing in the 3CB with stimulus animals. Current experiments (Verzijden and Van der Helm, 1994, unpublished results) show a higher locomotor activity of ATD males independent of the stimuli offered [e.g., ovariectomized female vs. estrous female; castrated male vs castrated testosterone-treated male; bedding only (i.e., odors) of such stimulus animals]. Sex differences are reported in locomotor activity with females showing more activity than males (9,10). It is possible that the higher locomotor activity of ATD-males is a manifestation of the incomplete defeminization process. More research is needed to answer this question.

In conclusion: the new semiautomated 3CB is a very useful apparatus for studying partner preference behavior and associated locomotor activity of rats.

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Chapter III

HORMONAL REGULATION OF ADULT PARTNER PREFERENCE BEHAVIOR IN NEONATALLY ATD-TREATED MALE RATS

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SUMMARY

Male rats, neonatally treated with ATD (1,4,6-androstatriene-3,17-dione), which blocks the aromatization of testosterone into estradiol (E_2), were tested for adult partner preference behavior (PPB; estrous female vs active male). Castration caused a decrease in preference for the female partner in all males, with ATD males showing lower preference for the female partner than controls. Long-term castrated males did not show preference for either partner. Precastration levels of PPB in control males occurred after treatment with E_2 or dihydrotestosterone (DHT) plus E_2. DHT alone had no effect on PPB. With E_2 alone, the ATD males clearly preferred the male partner. When DHT was added, these ATD males showed no preference for either partner or a low preference for the female partner. In conclusion, adult PPB in male rats is activated by endogenous T or by both its metabolites (DHT and E_2) or by E_2 alone. ATD males showed a much lower preference for the female. There was a differential effect of DHT and E_2: DHT had no effect, but E_2 clearly caused ATD males to prefer the male partner and control males to prefer the female partner.

INTRODUCTION

The role of hormones in organizing and activating adult sexual orientation or partner preference behavior has received relatively little atten-

tion (Adkins-Regan, 1988). Partner preference could be defined as the preference of an animal to spend time or interact sexually more with one animal than with the other, when given the choice between two stimulus animals (e.g. an estrous female and a sexually active male). Only recently, Brand, Kroonen, Mos, and Slob (1991) reported that the estradiol (E_2) metabolite of endogenous testosterone plays an important role in the organizing of adult partner preference behavior as well as the organizing of sexual behavior. Contrary to controls, adult male rats treated neonatally with 1,4,6-androstatriene-3,17-dione (ATD), an aromatase inhibitor which blocks the conversion of testosterone into E_2 (e.g., Kaplan & McGinnis, 1989), exhibited very low or no preference for an estrous female. ATD males readily displayed masculine sexual behaviors (mounts and intromissions), but did not ejaculate when paired with an estrous female, and they displayed feminine sexual behavior (lordosis) when paired with a sexually active male (Brand et al., 1991). All these experiments were carried out with gonadally intact animals.

To investigate the relative contribution of testicular hormones to partner preference behavior, we undertook a comprehensive study. In this investigation, the ontogeny of partner preference behavior in ATD and control males was studied, as well as the effects of castration and subsequent hormone treatment. The ontogeny data have been published separately (Brand, Houtsmuller, & Slob, 1993). The present paper deals with the castration and hormone-treatment data.

From the ontogeny study (Brand et al., 1993) it appeared that the specific partner preference behavior of ATD males first became apparent at the age of about 84 days, that is, after puberty. This suggests that testicular hormones play an important role in the expression of partner preference behavior. Hence, the effects of castration in adulthood and subsequent hormone treatment with one or two of the testosterone metabolites on partner preference and sexual behavior were investigated. Castrated males received either dihydrotestosterone (DHT) followed by E_2 or E_2 followed by DHT.

METHOD

Animals

Experimental animals were Wistar albino rats (R strain). The male stimulus animals were F1 hybrids of two inbred Wistar strains (R x U). The female stimulus animals were Wistar albino rats (R strain). Two to four rats were housed in a cage with food and water available ad libitum and kept in a 14-hr/10-hr light/dark cycle (lights were on from 5:45 p.m. to 7:45 a.m.). The temperature in the animal room ranged from 20°C to 22°C.

Treatment

Female rats were time mated (day of mating = day 0 of pregnancy), and parturition occurred 22 days later. Within 3 to 9 hours after birth, small silastic implants (inner diameter 1.5 mm; outer diameter 2.1 mm; length 5 mm) containing ATD or cholesterol were placed subcutaneously in the back of the newborn males under ice anesthesia (one treatment per litter). The implants were removed when the pups were 21 days of age. In the present experiments, 37 ATD males (out of 7 litters) and 17 control males (out of 4 litters) were used.

The pups were weaned at 21 days of age and then housed 2-4 to a cage with other rats which had received the same treatment. They were left undisturbed until the onset of behavioral testing. The rats were randomly divided into two groups of approximately equal size and were tested for partner preference behavior around the time of puberty, that is, between 32 and 60 days of age (Group 1) or between 63 and 84 days of age (Group 2; for behavioral data see Brand et al., 1993).

At the age of 91 days (13 weeks), all rats were castrated under ether anesthesia through a midline abdominal incision. Stimulus animals were sexually active males and estrous females. The latter were ovariectomized and brought into behavioral estrus with an injection of 20 g of estradiol benzoate 24-48 hr prior to testing, followed by an injection of 2.5 mg progesterone 3-4 hr before testing. These hormones were dissolved in olive oil and injected sc in the neck.

Hormone treatments consisted of sc implantation of silastic capsules containing crystalline hormone. DHT implants were 3.0 cm long, with an inner diameter of 1.5 mm and an outer diameter 2.1 mm. E_2-implants were 2.5 cm long and had an inner diameter of 0.5 mm and an outer diameter of 1.0 mm.

Behavioral Testing

All behavioral testing was carried out during the first part of the animal's dark period, that is, between 9:00 a.m. and 12:00 a.m.

Three-compartment partner preference test. We used a test box made of gray perspex with a transparent front; it 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 (Slob, de Klerk, & Brand, 1987). These openings could be closed by a sliding door. Stimulus animals wore a harness that was attached with a stainless-steel wire to the rear of one of the lateral compartments. The tethered animals thus had a limited action radius.

Before testing, the two stimulus animals, and the experimental animal were put in the test apparatus, one in each compartment, with the sliding doors closed, for a 15-20-min adaptation period. At the beginning of the

test (which lasted 15 minutes), the sliding doors were removed, and the experimental animal could freely move around and interact with the stimulus animals. The time spent in each compartment was recorded. At the same time, various sexual behaviors with the stimulus animals were scored.

To quantify partner preference, we calculated a preference score for each test by subtracting the time spent in the compartment containing the sexually active male from the time spent in the compartment containing the estrous female (the method used by Edwards & Pfeifle, 1983). Thus, a positive score indicates a preference for the estrous female; a negative score indicates a preference for the sexually active male.

Pair test with sexually active male. For these tests, which were carried out in semicircular cages, sexually active males were used. After a 5-min adaptation period for the stimulus animal, an experimental animal was put in the cage. The lordosis responses of the experimental male to the mounting of the stimulus male were recorded. The test lasted until the experimental male had received 10 mounts or 10 min had elapsed. We included in the data analyses the lordosis quotients for all males that received 4 or more mounts by the stimulus male.

Test Procedure

At the age of 12 weeks, the males were subjected to two consecutive partner preference tests 2 days apart (Tests 1 and 2). Then they were castrated. One week later, partner preference testing was resumed on a weekly basis during Weeks 14 through 18 (Tests 3 - 7). At the age of 27 weeks, the males were randomly divided into two groups.

Group A males started with DHT treatment alone for 5 weeks (Tests 11-15), followed by treatment with DHT and E_2 for 3 weeks (Tests 16-18). These animals were tested for partner preference behavior between 27 and 39 weeks of age. Group B males started with 3 weeks of E_2 treatment (Tests 11-13), followed by treatment with E_2 and DHT for 5 weeks (Tests 14-18). These animals were tested behaviorally between 50 and 62 weeks of age. Both groups were tested three times for partner preference behavior during the 3 weeks before hormone implants were given.

Hormone assay and blood colllection

DHT levels were estimated with the radioimmunological method for testosterone (described by Verjans, Cooke, de Jong, de Jong, & van der Molen, 1973), using ^{3}H-DHT as the label and DHT as the standard. Estradiol (E_2) was estimated with a commercially obtained kit (DPC, Los Angeles, Ca). Intra- and interassay coefficients of variation were less than 12-15% for DHT and less than 15-19% for E_2. Blood was collected

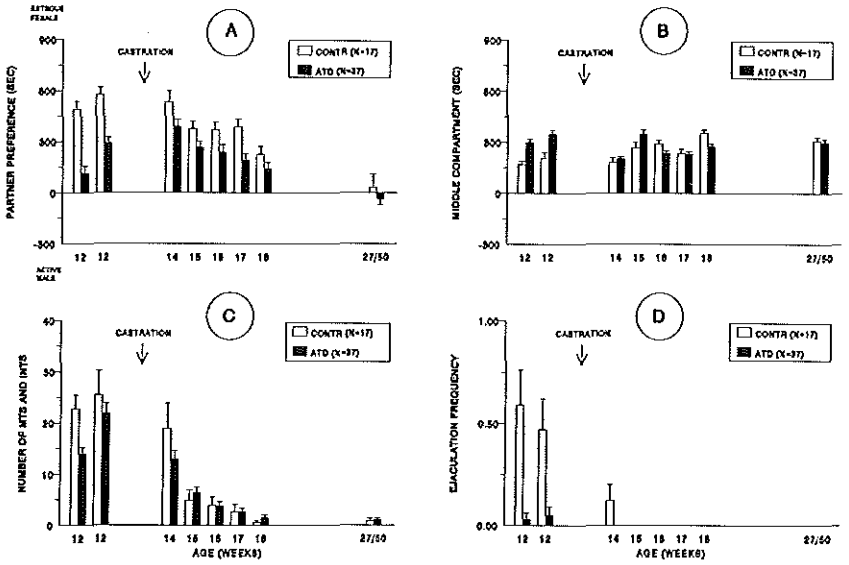

Figure 1. Effects of castration on partner preference and sexual behavior of adult male rats after neonatal ATD or cholesterol treatment. (Panel A shows the mean preference for an estrous female over a sexually active male. Panel B shows the mean time spent in the empty middle compartment of the three compartment box. Panel C shows the mean number of mounts and intromissions with the estrous female during partner preference testing. Panel D shows the mean ejaculation frequency. The bars for Weeks 27/50 show the mean results of the first tests in the hormone replacement experiment, which was conducted when Group A was 27 weeks of age and Group B was 50 weeks of age. Bars indicate standard error of the mean. CONTR = control rats; ATD = rats treated neonatally with 1,4,6-androstatriene-3,17-dione.)

from the orbital plexus under light ether anesthesia.

Statistical analysis

The behavioral data were subjected to two-way analysis of variance (ANOVA), followed by the least significant difference (LSD) procedure (Kirk, 1968). Only significant F values are presented. The lordosis and ejaculation data were analyzed with Fisher's exact one- or two-tailed probability test (Perlman, 1986). The $p < .05$ level was used as the upper limit for statistical significance.

Pre- and postcastration data were analyzed separately by means of two-way ANOVAs. Animals that had low E_2 (< 100 pmol/l) levels or low DHT (< 0.5 nmol/l) levels, or both, following hormone treatment were excluded from all data analyses. Thus, 5 ATD males and 3 control males were omitted from Group A, and 5 ATD males and 2 control males were omitted from Group B.

RESULTS

Effects of Castration

Partner preference behavior. Mean partner preference scores are presented in figure 1A. ANOVA of precastration data showed a significant effect of group, $F(1,52) = 47.24, p < .001$, and of testing, $F(1,52) = 15.31, p < .001$. Thus, ATD males showed a much lower preference for the female partner than did controls. After castration (Tests 3-7; Weeks 14-18), there remained an overall difference in partner preference, $F(1,52) = 8.81, p = .005$, and a significant decrease in preference scores in both ATD and control males, $F(4,208) = 12.92, p < .001$. After long-term castration (Test 8, Weeks 27 or 50), ATD and control males no longer differed, nor did they prefer one partner over the other.

Before castration, ATD males spent significantly more time in the empty middle compartment than did controls (see Figure 1B), $F(1,52) = 20.43, p < .001$. There also seemed to be an overall increase in time spent in the middle compartment, $F(1,52) = 3.96, p = .052$. ANOVA on the postcastration middle-compartment data revealed a significant effect of test, $F(4,208) = 12.99, p < .001$, and a significant Group x Test interaction, $F(4,208) = 4.21, p = .003$. LSD analysis (5% = 60 s) indicated that males in both groups showed an increase in the time spent in the middle compartment, with the lowest values recorded at 14 weeks. In addition, it also seems that, after castration, ATD and control males no longer differed in the time spent in the middle compartment. After long-term castration (Test 8, Weeks 27 or 50), ATD and control males spent an equal amount of time in the middle compartment.

Sexual behavior with tethered estrous female. The mean number of mounts plus intromissions during partner preference testing is shown in Figure 1C. Before castration, ATD males showed significantly fewer mounts and intromissions than did controls, as shown by a significant effect for group, $F(1,52) = 4.30, p = .043$. Animals in both groups showed an increase in activity, as demonstrated by a significant effeect for test, $F(1,52) = 8.40, p = .004$. After castration, there was a rapid decrease in the number of mounts and intromissions for both ATD and control males, $F(4,208) = 34.01, p < .001$.

Ejaculation data is presented in Figure 1D. During the two precastration tests, about 50% of the control males ejaculated (8 and 7 of 17), whereas only about 5% of the ATD males did so (1 and 2 of 37; Fisher's exact two-tailed probability test, $p < .001$). Castration immediately caused virtually all males to cease ejaculating.

Effects of Treatment With DHT Followed by E_2

Partner preference behavior. Partner preference scores for Group A

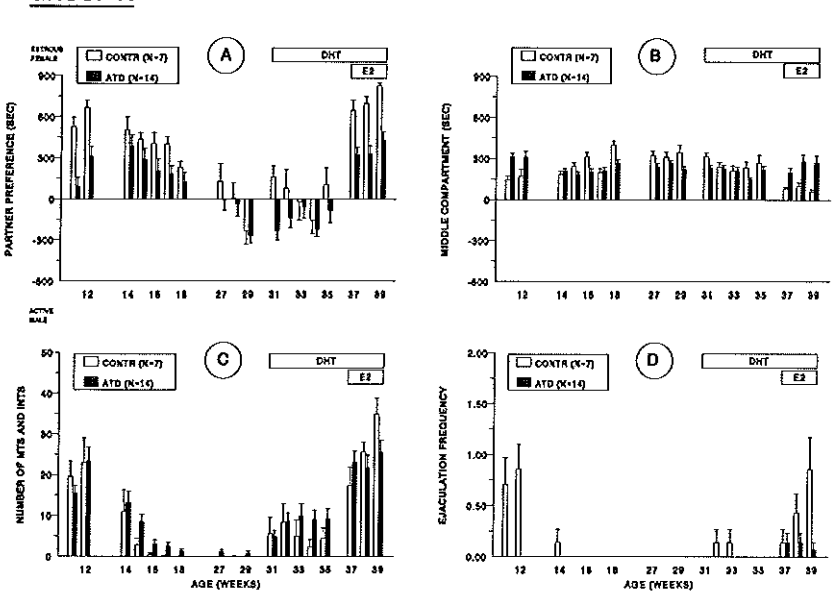

Figure 2. Effects of hormone treatment on ATD-treated and cholesterol-treated male rats in Group A (treated with DHT followed by E_2). (For comparison, the castration data for these males are also shown. Panel A shows the mean preference for an estrous female over a sexually active male. Panel B shows the mean time spent in the empty middle compartment of the three compartment box. Panel C shows the mean number of mounts and intromissions with the estrous female during partner preference testing. Panel D shows the mean ejaculation frequency. Bars indicate standard error of the mean. CONTR = control rats; ATD = rats treated with 1,4,6-androstatriene-3,17-dione; DHT = dihydrotestosterone; E_2 = estradiol.)

are presented in Figure 2A (for comparison, the pre- and postcastration data for this group are also shown; in the previous section, they were combined with the data for Group B). ANOVA on the data of Tests 8-18 (Weeks 27-39) revealed a significant group effect, $F(1,190) = 9.48, p = .006$, and a significant test effect, $F(10,190) = 24.87, p = .001$. Subsequent LSD analysis (5% = 232 s) revealed that the preference for the female significantly increased in all males after E_2 treatment, with ATD males showing significantly less preference for the female partner than did controls (5% = 423 s). DHT treatment alone did not affect preference behavior, with one exception: In the first week of DHT treatment, control males showed a significant increase in preference for the female partner.

ANOVA (Tests 8-18; Weeks 27-39) on the time spent in the empty middle compartment (see Figure 2B) revealed an overall effect of testing, $F(10,190) = 2.41, p = .01$), and a significant Group x Test interaction, $F(10,190) = 4.83, p < .001$. Further LSD analysis (5% = 102 s) showed that control males spent significantly less time in the middle compartment after combined DHT and E_2 treatment.

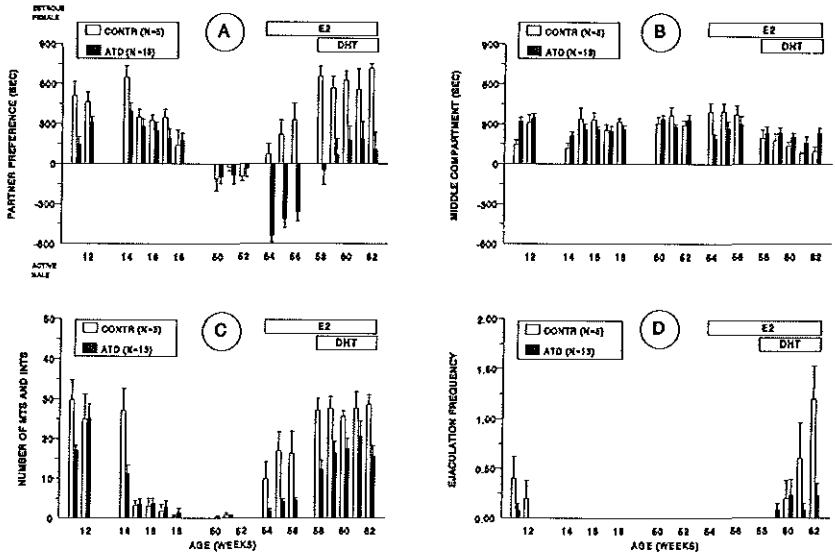

Figure 3. Effects of hormone treatment on ATD-treated and cholesterol-treated rats in Group B (treated with E_2 followed by DHT). (For comparison, the castration data for these males are also shown. Panel A shows the mean preference for an estrous female over a sexually active male. Panel B shows the mean time spent in the empty middle compartment of the three compartment box. Panel C shows the mean number of mounts and intromissions with the estrous female during partner preference testing. Panel D shows the mean ejaculation frequency. Bars indicate the standard error of the mean. CONTR = control rats; ATD = rats treated neonatally with 1,4,6-androstatriene-3,17-dione; E_2 = estradiol; DHT = dihydrotestosterone.)

Sexual behavior with tethered estrous female. The number of mounts and intromissions is shown in Figure 2C. ANOVA (Tests 8-18; Weeks 27-39) revealed a significant effect of test, $F(10,190) = 35.86, p < .001$, and a Group x Test interaction, $F(10,190) = 1.83, p = .058$. LSD analysis (5% = 7) showed a significant increase in the sexual activity of males in both groups after DHT treatment. A further significant increase occurred after E_2 treatment. There were no statistical significant differences between ATD and control males.

Ejaculation behavior is shown in Figure 2D. With DHT treatment alone, only one control male ejaculated. During the combined DHT and E_2 treatment, about 60% of the control males ejaculated (4 of 7), whereas only about 14% of the ATD males (1 or 2 of 14) did so (Fisher's exact two-probability test, $p = .12$).

Effects of Treatment With E_2 Followed by DHT

Partner preference behavior. Partner preference scores are presented in Figure 3A (for comparison, the pre- and postcastration data for this

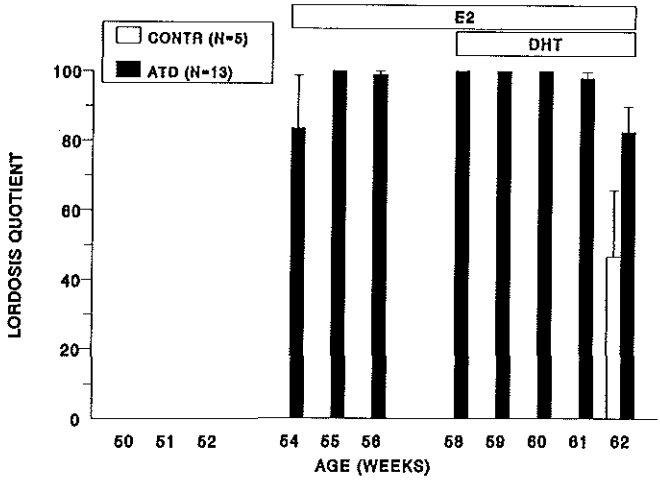

Figure 4. Mean lordosis quotient of ATD-treated and cholesterol-treated male rats during partner preference testing and in a pair test (Week 62) with a sexually active male. (Only males that received four or more mounts by the stud male are included. Bars indicate standard error of the mean. CONTR = control rats; ATD = rats treated neonatally with 1,4,6-androstatriene-3,17-dione; E_2 = estradiol; DHT = dihydrotestosterone.)

group are also shown). ANOVA (Tests 8-18; Weeks 50-62) revealed a significant effect of group, $F(1,16) = 13.94, p = .002$, a significant effect of test, $F(10,160) = 15.62, p < .001$, and a significant Group x Test interaction, $F(10,160) = 4.74, p < .001$. Further analysis with LSD (5% = 259 sec) indicated that during E_2 treatment ATD males showed a clear-cut preference for the sexually active male, whereas control males showed a significant increase in preference for the female partner. During the combined E_2 and DHT treatment, a significant increase in preference for the female partner was observed in all males, with ATD males showing significantly less preference for the female partner than did controls.

ANOVA (Tests 8-18; Weeks 50-62) on the time spent in the empty middle compartment (see Figure 3B) showed a significant effect of test, $F(10,160) = 5.22, p < .001$, and a significant Group x Test interaction, $F(10,160) = 2.61, p = .006$. Further LSD analysis (5% = 125 sec) indicated that during E_2 treatment (Weeks 54-56) ATD males spent significantly less time in the middle compartment. After additional DHT treatment (Weeks 58-62), control males spent significantly less time in the middle compartment than did ATD males.

Sexual behavior with tethered estrous female. The mean numbers of mounts plus intromissions are shown in Figure 3C. ANOVA (Tests 8-18; Weeks 50-62) indicated a significant group effect, $F(1,16) = 11.38, p = .004$, a significant test effect, $F(10,160) = 32.02, p < .001$, and a

significant Group x Test interaction, $F(10,160) = 2.51, p < .008$. Further analysis with LSD (5% = 7) showed that after E_2 treatment a significant increase in sexual activity occurred in control males but not in ATD males. During the combined E_2 and DHT treatment, all males displayed significantly more mounting and intromitting, with ATD males exhibiting significantly lower levels of sexual activity than controls.

Ejaculation behavior is shown in Figure 3D. E_2 treatment alone had no effect, but additional DHT treatment did increase ejaculation frequency: About 80% of control males ejaculated (4 of 5), whereas only about 20% of ATD males did so (3 of 13) (Fisher's exact two-probability test, $p = .10$).

Sexual behavior with tethered sexually active male. The lordosis data are shown in Figure 4. In Tests 8 - 17 (Weeks 50-61), the lordosis responses of the males to mounting by the tethered stimulus male were recorded during partner preference testing. In Test 18 (62 wks of age), the males were pair-tested with a freely moving stimulus male. During E_2 treatment, 5 out of 13 ATD males showed lordosis, but none of the controls did (Fisher's exact one-tailed probability test, $p = .15$). During combined E_2 and DHT treatment, no alterations in the lordosis responses of ATD males were observed. In the pair test with the stimulus male (Week 62), 12 out of 13 ATD males and 2 out of 5 control males showed lordosis in response to mounting by the stimulus male (Fisher's exact one-tailed probability test, $p = .04$).

Hormone data

The mean levels of E_2 and DHT after hormone treatment are presented in Table 1. For Group A, only DHT levels were measured. ANOVA showed no group difference between ATD and control males for either group (A and B). A significant decrease was observed in the E_2 and DHT levels in the weeks following implantation: for E_2, $F(2,32) = 19.11, p < .001$, and for DHT, $F(1,16) = 15.61, p = .001$. Normal male levels of E_2 and DHT are approximately 7 pmol/l and 1 nmol/l (de Jong, Hey, & van der Molen, 1973), respectively. Thus, DHT treatment seemed to be adequate, whereas E_2 treatment resulted in above-normal levels.

DISCUSSION

From this study, it is clear that adult partner preference behavior of male rats is neonatally organized, most likely by estradiol derived from endogenous testosterone. Neonatally ATD-treated males, when tested as (intact) adults, seem to prefer the presence of an estrous female over an active male, albeit very significantly less than did control males. During

Table 1
Blood Serum E_2 and DHT Levels in Castrated ATD-Treated and Control Rats

	Controls		ATD rats	
Hormone	M	SE_M	M	SE_M
	Group A[a]			
DHT (nmol/l)				
5 weeks	3.7	0.3	4.2	0.2
Group B[b]				
E_2 (pmol/l)				
3 weeks	335	27	406	34
6 weeks	237	51	248	11
10 weeks	196	41	255	19
DHT (nmol/l)				
3 weeks	3.2	0.1	3.0	0.1
7 weeks	2.5	0.1	2.5	0.1

Note. E_2 and DHT were administered via the implantation of silastic capsules, and blood samples were collected at various weeks after implantation. E_2 = estradiol; DHT = dihydrotestosterone; ATD = 1,4,6-androstatriene-3,17-dione.
[a]For Controls, $n = 7$; for ATD rats, $n = 14$. [b]For controls, $n = 7$; for ATD rats, $n = 13$.

partner preference testing (before castration), ATD males readily interacted sexually with the tethered estrous female by mounting and intromitting her (but not ejaculating), and they interacted with the tethered active male by showing estrous behaviors (hopping and darting, presenting, earwiggling and lordosis in response to being mounted). Thus, one could say that these ATD males were masculinized but not defeminized. This corroborates earlier work in which it was shown that perinatal ATD treatment prevented defeminization in male rats (Beatty, 1992; Brand & Slob, 1991; Brand et al., 1991; Brand et al., 1993; Baum, 1979; Davis, Chaptel, & McEwen, 1979; Fadem & Barfield, 1981) such that ATD-treated males could readily display lordosis behavior.

Another finding from the present study is that testicular hormones are required for the partner preference behavior to occur. Castration abolished partner preference in both ATD and control males.

Both metabolites of testosterone - DHT and E_2 - had to be present to restore partner preference behavior to precastration levels. In this respect, partner preference behavior is very similar to male sexual behavior because both need the same activational hormones (e.g., Baum, 1979; Goy & McEwen, 1980).

A very intruiging result was obtained when only one of the two testosterone metabolites was administered. With DHT treatment alone, partner preference behavior was not significantly affected in ATD and

control males. When E_2 treatment was initiated, this clearly and very effectively caused the ATD males to prefer the male partner, whereas the control males preferred the female partner. During E_2 treatment the ATD males also visited the estrous female partner, but the increase in masculine sexual behavior was not statistically significant; in contrast, the control males showed a significant increase in mounts and intromissions but not in ejaculations. The different activational effects of E_2 and DHT on partner preference behavior are in line with earlier findings from our laboratory. In adult male rats castrated on the day of birth, E_2 was effective, but DHT was not, in activating a preference for an estrous female over a nonestrous female (Merkx, 1984). Similar findings were obtained for adult ovariectomized female rats treated with E_2 or DHT (Merkx, Slob, & van der Werff ten Bosch, 1989). It should be kept in mind, however, that the present E_2 treatment resulted in blood levels of E_2 that were above normal for male rats. DHT treatment resulted in normal DHT blood levels in males. One cannot rule out the possibility that this difference is responsible for the different activational effects of E_2 and DHT. We are currently studying the behavioral effects of lower E_2 dosages on partner preference behavior of long-term castrated male rats.

From the present study it is also clear that ATD males are somewhat less social, at least as judged from the time spent with the stimulus animals. When gonadally intact, they spent significantly more time in the empty middle compartment than did the control males. After castration this difference disappeared, primarily because control males increased their time in the middle compartment. The combined treatment (DHT + E_2) brought back the precastration difference: ATD males spent more time in the middle compartment than did the control males. DHT treatment alone had no striking effect, but E_2 treatment alone made ATD males more social; that is, they spent significantly less time in the middle compartment (and thus more time with the stimulus animals) than did the control males. From the data presented in the figures, it seems that the controls were more sensitive to hormonal changes than were the ATD males. The latter continued to spend approximately the same amount of time in the middle compartment, whereas the controls fluctuated more depending on the hormone treatment received.

ATD males almost never ejaculated during partner preference testing, although they readily mounted and intromitted the estrous female. This could mean that neonatal ATD treatment interfered with their masculinization process. This is in line with ideas expressed previously that the sensitive periods for the hormonal organization of mounting, intromission and ejaculation occur at slightly different times in development (see Ward & Ward, 1985; Beatty, 1992). Moreover, ATD-treated

males are well capable of displaying ejaculatory behavior (Brand et al., 1993), for example, when treated with 8OH-DPAT (a serotonin agonist; see Ahlenius et al., 1981; Haensel, Mos, Olivier, & Slob, 1991) or with Yohimbine (an 2 receptor blocker; see Clark, Smith, & Davidson, 1985).

A final comment should be made about the possible biological substrates that underlie the behavioral differences between ATD and control males. Recently we have found that the volume of the sex dimorphic nucleus of the preoptic area, a structure smaller in females than in males (Gorski, Harlan, Jacobson, Shryne, & Southam, 1980), is significantly smaller in ATD males compared to controls (Brand et al., 1993; Houtsmuller et al., 1993). Whether there is a causal relationship between the volume of this hypothalamic nucleus and partner preference and sexual behavior remains to be investigated.

One could also assume that ATD males and control males differ in their endogenous steroid hormone levels, which could explain differences in behavior. For example, ATD males could have lower endogenous testosterone levels or $\dot{E}_2$ levels, which might have influenced them to behave differently. However, this is not the case. In a recent study we showed that endogenous testosterone levels either did not differ or were higher in ATD males; E_2 levels did not differ significantly in ATD and control males (Brand et al., 1993).

In conclusion, partner preference behavior in male rats seems to be organized neonatally by E_2 and activated in adulthood by endogenous testosterone or by both its metabolites, DHT and E_2. Male rats treated with ATD immediately after birht showed much lower preference for the female partner. Furthermore, there was a differential activating effect of DHT and E_2 on partner preference behavior of male rats. DHT did not affect partner preference behavior, wheras E_2 clearly did. With the latter treatment, ATD males showed a clear preference for the male partner, whereas control males preferred the female partner.

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Chapter IV

POSTWEANING HOUSING CONDITIONS AND PARTNER PREFERENCE AND SEXUAL BEHAVIOR OF NEONATALLY ATD-TREATED MALE RATS

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SUMMARY

Male rats were neonatally treated with cholesterol or a substance that blocks the aromatization of testosterone to estradiol (1,4,6-androstatriene-3,17-dione: ATD). At weaning (21 days) they were either housed alone or in small groups (2-3 animals) and tested for partner preference behavior (PPB) in adulthood. Choice was between an estrous female and an active male (Part I) and between an estrous female and an ATD-male (Part II). Tests were carried out in a 3-compartment box. Social isolation did not have major effects on PPB except when sexual interaction with the stimulus animals was prevented (Part I) In this case, isolates (ATD and control) showed higher preference scores (PS) for the estrous female and spent less time in the empty middle compartment. When the choice was between an estrous female and an ATD-male, partner PS decreased in all males, most clearly in ATD-males. The latter animals spent more time with the stimulus ATD-male than they had done in previous PPB tests with the normal stimulus male. In contrast to partner preference behaviors, sexual behavior was clearly affected by social isolation. Isolates (ATD and control) displayed lower frequencies of mounts and intromissions. These effects persisted over consecutive tests. Ejaculation was not affected. In conclusion, the present results confirm earlier findings about the significance of neonatal endocrine conditions for the organization of adult PPB in male rats. The presence or absence of social conspecifics after weaning appears to have little influence on adult PPB.

INTRODUCTION

The two major factors known to affect adult sexual and related behaviors in male rats are the relative amounts of estradiol present during a critical or sensitive perinatal period (Bakker et al., 1993a; Baum, 1979; Brand et al., 1991; Vega Matuszczyk, 1993; Ward & Reed, 1985) and social stimulation during prepuberal development (Gerall et al., 1967; Spevak et al., 1973; Ward & Reed, 1985; Zimbardo, 1958).

Adult gonadally intact male rats treated shortly after birth with ATD (1,4,6-androstatriene-3,17-dione), which inhibits the aromatization of testosterone (T) to estradiol (E_2) (Brodie et al., 1979; Kaplan & McGinnis, 1989; Lieberburg et al., 1977), readily display 'masculine' sexual behaviors (i.e., mounts and intromissions) but no or few ejaculations when paired with an estrous female. Such ATD-males also easily show 'feminine' sexual behaviors (i.e., proceptive behaviors and lordosis) when paired with an active male (e.g., Bakker et al., 1993b; Brand et al., 1991; Davis et al., 1979; Vreeburg et al., 1977). Thus, these ATD-males are behaviorally less masculinized and less defeminized than normal male rats. Comparable results with ATD were obtained in ferrets (Baum & Tobet, 1986; Tobet & Baum, 1987). ATD-males also show a significantly lower partner preference (choice: estrous female vs. active male) for the estrous female than control males (Bakker et al., 1993a, 1993b; Brand et al., 1991). Additionally, partner preference behavior (PPB) and sexual behavior of ATD-males but not of control males, show a nocturnal rhythm. In the late part of the dark phase of the LD cycle the partner preference for the estrous female and frequencies of masculine sexual behaviors, including ejaculations, are significantly higher than in the early part of the dark phase. Feminine sexual behaviors (proceptive and receptive behaviors) also showed a nocturnal rhythmicity, with highest frequencies early in the dark phase (Bakker et al., 1993b).

It has been reported that housing conditions can affect the development of adult sexual behavior of the rat (e.g., Gerall et al., 1967; Spevak et al., 1973). Male rats singly housed from weaning (age 14 days) were at 90 days of age significantly less sexually active, with no or low intromission frequencies than males housed in groups (Gerall et al., 1967). Male isolates were highly interested in the estrous female, but they failed to mount and clasp the posterior region of the female. This effect was persistent over nine consecutive tests. Only living with females for at least 3 weeks somewhat improved the copulatory performance of isolated males in pair tests. Compared with group-housed male rats, these singly housed (from weaning onward) males also showed in adulthood higher levels of aberrent responses, that is, improper orientation towards the female, and hyperexcitable responses such as headshaking, leaping,

and tunneling (Gerall et al., 1967). It has been proposed that play activities during the juvenile and puberal phases are essential for the development of normal adult sexual behavior (Gerall et al., 1967; Gruendel & Arnold, 1969; Spevak et al., 1973). If, because of isolation during development, these play activities are prevented from occurring, they tend to reappear with greater intensity in adulthood when isolates are tested for sexual behavior. Thus, it was hypothesized that when isolated males are confronted with a social stimulus such as an estrous female in a mating test, play responses would be dominant over sexual ones, resulting in inadequate mating (Gerall et al., 1967; Gruendel & Arnold., 1969; Spevak et al., 1973).

Beach (1942, 1958) found no significant differences in adult sexual behavior between animals housed in isolation or in groups from weaning either at the age of 14 days or at 21 days. Kagan and Beach (1953) even reported an enhancing effect of social isolation on adult sexual behaviors (increase in intromission and ejaculation frequencies, shorter latencies to onset of copulation). However, the design and conclusions of this latter study were criticized by Larsson (1956). Other studies investigating the effects of different pre- and postweaning housing conditions on sexual behavior have given rather consistent results, that is, decrease in frequencies of sexual behaviors (Gerall et al., 1967; Gruendel & Arnold, 1969; Spevak et al., 1973; Zimbardo, 1958). Most studies were undertaken to investigate the effects of housing conditions on sexual performance only, and not on motivational aspects of sexual behavior such as partner preference behavior. Only Zimbardo (1958) investigated a possible interaction between the effects of shocking a young male rat for approaching a receptive female (early avoidance training) and housing conditions. He did not find any interaction between early avoidance training and housing conditions.

Partner preference can be defined as the preference of an animal to spend more time and/ or to interact sexually more with one animal than with another when given the choice between two stimulus animals (e.g., an estrous female and a sexually active male) (Adkins-Regan, 1988; Bakker et al., 1993a; Slob et al., 1987). Adult ATD-males housed together readily mount each other and display lordosis behavior in response; they also show various proceptive behaviors (hop & dart, presenting, earwiggling). Especially the frequent display of feminine sexual behaviors of these ATD-males is quite different from what can be observed in cages with normal intact male rats. Therefore, it could be hypothesized that sexual experiences of socially housed ATD-males could affect their partner preference behavior differently from singly housed ATD-males and control males.

This hypothesis, that is, the possible interaction between neonatal

hormone treatment and housing conditions from weaning onward, was investigated in the present study (Part I). Adult partner preference behavior and sexual behaviors were studied in neonatally ATD-treated males and control males, caged either alone (isolates) or in small groups (2-3 animals).

When no effects were found of housing conditions, we changed the stimulus animals in the partner preference tests (Part II): an ATD-male (instead of a 'normal' male), and an estrous female. This was done to investigate whether ATD-males housed in groups would prefer, compared to ATD isolates, the stimulus ATD-male over a normal stimulus male.

METHODS

Animals

Experimental animals were Wistar albino rats obtained from a commercial breeder (Harlan Sprague-Dawley, Zeist, The Netherlands). The male stimulus animals were F1 hybrids of two inbred Wistar strains (R x U), the female stimulus animals were Wistar albino rats. Food and water were available ad lib. The animals were kept in a 14:10 h light:dark cycle (lights on from 1745 to 0745h). The temperature in the animal room ranged from 20 to 22°C.

Treatments

Female rats were time-mated. Parturition occurred 22 days later (day of birth = Day 0). Within 2-4 h after birth, the newborn males received subcutaneously (SC) a silastic capsule (SR3: inner diameter 1.5 mm, outer diameter 2.1 mm, length 5 mm) containing ATD or cholesterol. The implants were removed at weaning, 21 days of age. In the present experiments 21 ATD-males (out of 10 litters) and 9 control males (out of 4 litters) were used.

At weaning the animals were randomly divided into two groups: 4 control males and 10 ATD-males were housed singly; 5 control males and 11 ATD-males were caged in groups, i.e. 2-3 rats to a cage of the same treatment. The males were left undisturbed until the onset of behavioral testing at the age of approximately 3 mo.

Stimulus animals, tested repeatedly, were sexually active males and estrous females. The stimulus females were ovariectomized and brought into behavioral estrous by injecting 20 g estradiol benzoate (EB), 24-48 h prior to testing, followed by 1.0 mg progesterone (P) 3-4 h before testing. These hormones were dissolved in olive oil and injected SC in the neck.

Behavioral testing

Three-compartment partner preference test. A test box of gray Perspex with a transparent front was used. It was divided in three compartments (60 x 30 x 40 cm each) with a small opening (13 x 12 cm) in both partitions near the front window (Slob et al., 1987). These openings could be closed by a sliding door. Stimulus animals were either tethered with a rope to the rear of one of the lateral compartments or could freely move behind a wire mesh separation halfway down the lateral compartment. The tethered animals thus had a limited action radius. When physical interaction was prevented by wire mesh, the experimental animal could only see, smell, or hear the stimulus animals.

Before testing, two stimulus animals and the experimental animal were put in the test apparatus, one in each compartment, with the sliding doors closed, for 15-20 min adaptation. At the beginning of the test (which lasted 15 min), the sliding doors were removed and the experimental animal could freely move around and interact with the stimulus animals or stay before the wire mesh separation. The time spent in each compartment was recorded. Various sexual behaviors with the tethered stimulus animals were scored. Masculine sexual behaviors was measured by the number of mounts plus intromissions and ejaculation frequencies.

To quantify partner preference behavior, a preference score was calculated for each test by subtracting the time spent in the compartment with the sexually active male (or the ATD-male in Part II) from the time spent in the compartment with the estrous female (Edwards & Pfeifle, 1983). Thus, a positive score indicates a preference for the estrous female; a negative score indicates a preference for the sexually active male (Slob et al., 1987).

Pair-test with sexually active male. After 5 min of adaptation of the stimulus male, an experimental male was introduced in a semicircular cage. The lordosis responses of the experimental male to the mounting of the stimulus male were recorded. The test lasted until the experimental animal had received 10 mounts or 10 min had elapsed. In the data analyses, the lordosis quotients of all males who received 4 or more mounts by the stimulus male are included.

Test procedure

Behavioral tests started when the males were approximately 3 mo old. Firstly, four tests were performed twice weekly without sexual interaction possible (stimulus animals behind wire mesh separation; Tests 1-4). Then six tests were carried out twice weekly with sexual interaction possible (tethered stimulus animals; Tests 5-10). All tests were performed in the early part of the dark phase (between 0830-1100h) of the LD cycle, except Test 9 which was carried out in the late part of the dark

phase (between 1400-1630h). Following the last partner preference test (Test 10), the males were tested for feminine sexual behavior, that is, lordosis in a pair-test with a sexually active male.

Part II started 1 week after Test 10. Males were subjected to three weekly partner preference tests with an estrous female and a gonadally intact sexually experienced ATD-male as stimulus animals (Tests 11-13). In Test 12, the females received a vaginal mask to prevent intromissions.

Statistics

The behavioral data were subjected to MANOVA (multivariate analysis of variance), followed by the least significant difference (LSD)-procedure (Kirk, 1968). Only significant F values are presented. The lordosis data were analyzed using the Fisher's exact one-tailed probability test (number of animals showing lordosis behavior) and using the Student's t-test (the lordosis quotients). The $p < .05$ level was used as the upper limit for statistical significance.

RESULTS

Part I: Partner Preference Behavior With an Estrous female and an Active male as Stimulus Animals

Partner preference behavior. Partner preference behavior data are shown in Fig. 1. MANOVA on the preference scores without sexual interaction possible (stimulus animals behind wire mesh; see Fig. 1A) showed a significant effect of housing conditions, $F(1,26) = 4.52$, $p = .043$, and a significant effect of testing, $F(3,78) = 3.33$, $p = .024$. Overall, male rats housed in isolation showed a significantly higher partner preference for the estrous female than male rats housed socially. The preference for the female partner increased significantly in all males over the tests. In the fourth test, isolates (ATD and controls) showed a low preference for the estrous female (mean 338 ± 33 s), whereas socially housed males (ATD and controls) showed no clear partner preference (mean 146 ± 83 s). There were no significant differences between ATD and control males in partner preference behavior without sexual interaction possible. MANOVA on the time spent in the empty middle compartment (see Fig. 1B) showed a significant effect of housing conditions, $F(1,26) = 5.11$, $p = .032$, and a significant group effect, $F(1,26) = 4.35$, $p = .047$. Overall, isolates
(ATD and controls) spent significantly less time in the empty middle

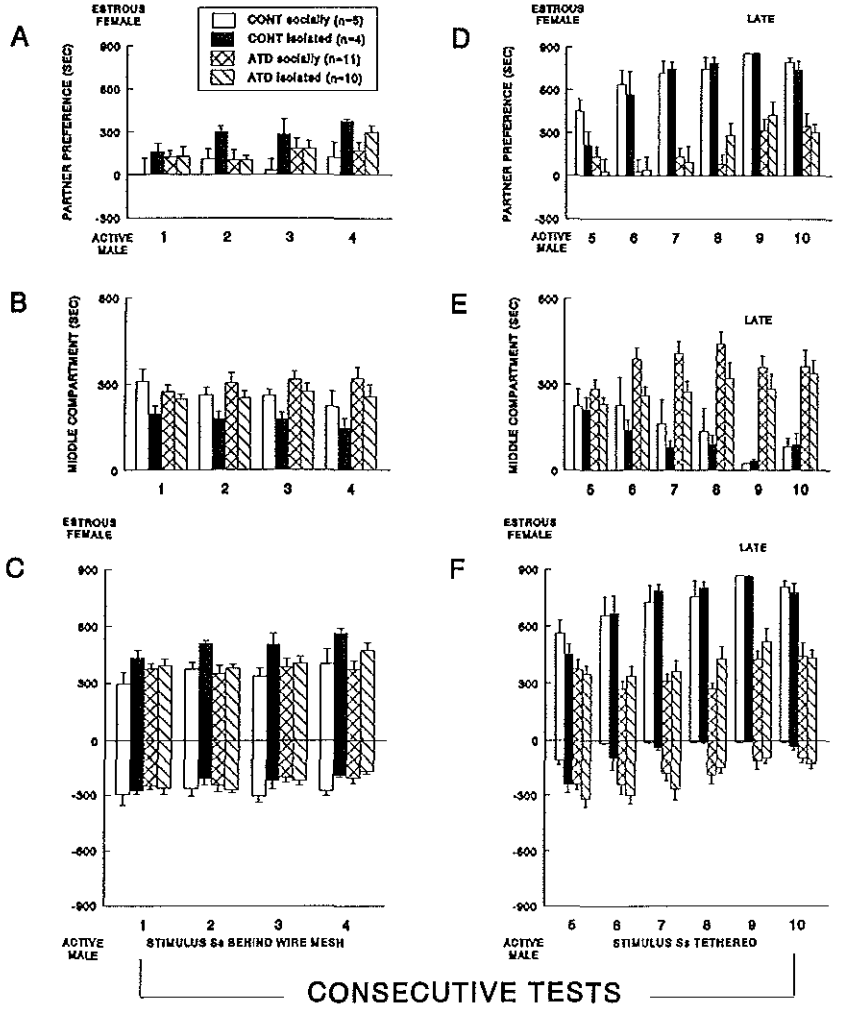

FIG. 1. Partner preference behavior in a three-compartment box (3CB) of adult, gonadally intact male rats, neonatally treated with the aromatization blocker ATD or cholesterol (controls). Male rats (ATD and control) were either housed alone (isolated) or housed in small groups (socially). In Tests 1-4 (A-C) the stimulus animals (an estrous female and an active male) were behind a wire mesh separation; in Tests 5-10 (D-F) the stimulus animals were tethered. Tests 1-8 and 10 were carried out in the early part of the dark phase; Test 9 in the late part of the dark phase. (A) shows partner preference behavior; (B) shows the time spent in the empty middle compartment; and (C) shows the time spent with the estrous female and the time spent with the active male. (D) shows partner preference behaviorl; (E) shows the time spent in the empty middle compartment; and (F) shows the time spent with the estrous female and the time spent with the active male. Values are means and SEM.

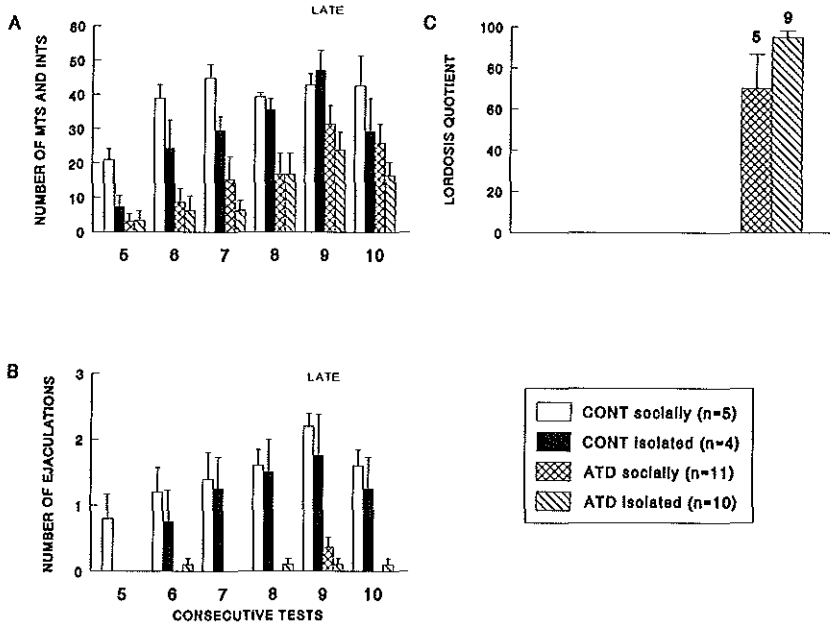

FIG. 2. Sexual behaviors of isolated and socially housed ATD and control male rats with the tethered stimulus female during partner preference testing (A, B) and lordosis behavior in a pair-test with an active male (C). Tests 5-8 and 10 were performed in the early part of the dark phase; Test 9 in the late part of the dark phase. (A) shows the total number of mounts plus intromissions with the estrous female. (B) shows the number of ejaculations. (C) shows the lordosis quotients of adult male rats and the number of ATD male rats mounted by the active male. Values are means and SEM.

compartment than socially housed males (ATD and controls). Group comparisons revealed that control males spent significantly less time in the empty middle compartment than ATD-males. In Fig. 1C the time spent with the estrous female and the time spent with the active male is shown. From looking at this figure, it is clear that isolates (ATD and controls) spent more time with the stimulus animals than males socially housed (ATD and controls).

MANOVA on the partner preference data with sexual interaction possible (stimulus animals tethered; Fig. 1D) showed a significant effect of groups, $F(1,26) = 54.48$, $p < .001$, and a significant Group by Test interaction, $F(5,130) = 2.46$, $p = .036$. Further analysis with LSD (5% = 240 s) showed that the preference for the female partner increased significantly in all males, with ATD-males showing a significantly lower preference for the estrous female partner than control males. There were no differences in partner preference behavior with sexual interaction between isolated males and socially housed males. When the males were tested in the late part of the dark phase (Test 9), the preference for the

female partner increased almost significantly in ATD-males [LSD(5%) = 240 s].

MANOVA on the time spent in the empty middle compartment (Fig. 1E) revealed an effect of groups, $F(1,26) = 24.04$, $p < .001$, and a significant Group by Test interaction, $F(5,130) = 5.10$, $p < .001$. Overall, ATD-males spent significantly more time in the empty middle compartment than control males. Further analysis with LSD (5% = 120 s) showed that the time spent in the middle compartment significantly decreased over tests in control males. The time spent with the estrous female and the time spent with the active male is shown in Fig. 1F. From looking at this picture, it is clear that ATD-males spent more time with the active male and less time with the estrous female than control males. No clearcut differences between singly and socially housed males, ATD as well as controls, can be seen. In the course of testing, the preference for the estrous female became more and more obvious.

Sexual behavior with the tethered estrous female during partner preference testing. The mean number of mounts plus intromissions are depicted in Fig. 2A. MANOVA indicated a significant effect of housing conditions, $F(1,26) = 7.06$, $p = .013$, a significant group effect, $F(1,26) = 51.57$, $p < .001$, a significant effect of testing, $F(5,130) = 32.29$, $p < .001$, and a significant Group by Test interaction, $F(5,130) = 3.11$, $p = .011$. Isolates (ATD and control) displayed significantly fewer mounts and intromissions than socially housed males (ATD and control). Furthermore, ATD-males showed lower frequencies of masculine sexual behaviors (mounts and intromissions) than control males. Overall, the number of mounts and intromissions increased significantly in all males over the tests.

Ejaculation frequencies are shown in Fig. 2B. ATD-males almost never ejaculated when tested early in the dark phase. Late in the dark phase (Test 9) social ATD-males started to ejaculate (4 of 11 vs. 0 of 11 in all 'early' tests). ANOVA on the ejaculation frequencies of control males revealed only a significant effect of tests, $F(5,35) = 6.76$, $p < .001$. The number of ejaculations increased in both control groups between Tests 5 and 10. None of the control isolates ejaculated in the first test (Test 5), whereas 3 of 5 socially housed controls ejaculated. In the second test (Test 6) 2 of 4 isolates ejaculated vs. 4 of 5 social males. In the third test the differences had disappeared: 3 of 4 isolates vs. 4 of 5 social control males. By the end of the partner preference testing (Test 10) all control males ejaculated, with the exception of one singly housed control male.

Sexual pair test with active male. The lordosis data are shown in Fig. 2C. Control males did not display any lordosis behavior. Significantly

more singly housed than socially housed ATD-males were mounted by the stimulus male: 9 of 11 isolates vs. 5 of 11 socials (Fisher's exact one-tailed probability test p =.04). The lordosis quotients did not differ significantly between the two ATD-groups.

Part II: Partner Preference Behavior With an Estrous Female and an ATD-Male as Stimulus Animals

Partner preference behavior. The preference scores are depicted in Fig. 3. For reasons of comparison the results of Test 10 (last test with choice between estrous female and active male) are also shown and included in the statistical analysis. MANOVA on the data of Tests 10-13 (Fig. 3A) revealed a significant effect of groups, $F(1,26) = 48.08$, p <.001, and a significant effect of testing, $F(3,78) = 4.28$, p =.008. The preference for the estrous female decreased significantly in all males in Test 11-13 with an ATD-male as stimulus male compared to Test 10 with an active male as stimulus male.

MANOVA on the middle compartment data (Fig. 3B) showed a significant effect of groups, $F(1,26) = 14.3$, p =.001, and a significant effect of tests, $F(3,78) = 3.56$, p =.018. The time spent in the empty middle compartment increased in all males over tests. In Fig. 3C, the time spent with the estrous female and the time spent with the stimulus male is depicted. From this figure, it is clear that both ATD-male groups spent more time with the stimulus ATD-male than with the active male.

Sexual behavior with the tethered estrous female during partner preference testing. MANOVA on the total number of mounts and intromissions (Fig. 3D) revealed a significant effect of housing conditions, $F(1,26) = 7.26$, p =.012, a significant group effect, $F(1,26) = 35.92$, p <.001, a significant effect of tests, $F(3,78) = 10.49$, p <.001, and a significant Group by Test interaction, $F(3,78) = 2.79$, p =.046. Overall, isolates (ATD and control) displayed fewer mounts and intromissions than socials (ATD and control). Subsequent LSD analysis (5% = 9.5) of the group by test interaction showed that the number of mounts and intromissions in control males fluctuated over tests, with the highest frequencies in Test 12 when intromissions were prevented by a vaginal mask of the stimulus female. The mount and intromission frequencies did not change in ATD-males over tests.

Ejaculation frequencies are shown in Fig. 3E. ANOVA on the data of control males in Tests 10, 11 and 13 (Test 12 is excluded from statistical analysis because intromissions and ejaculations were prevented by vaginal occlusion of stimulus females) showed a significant effect of testing, $F(2,14) = 17.39$, p <.001. The ejaculation frequencies increased significantly between Tests 10 and 13 in control males.

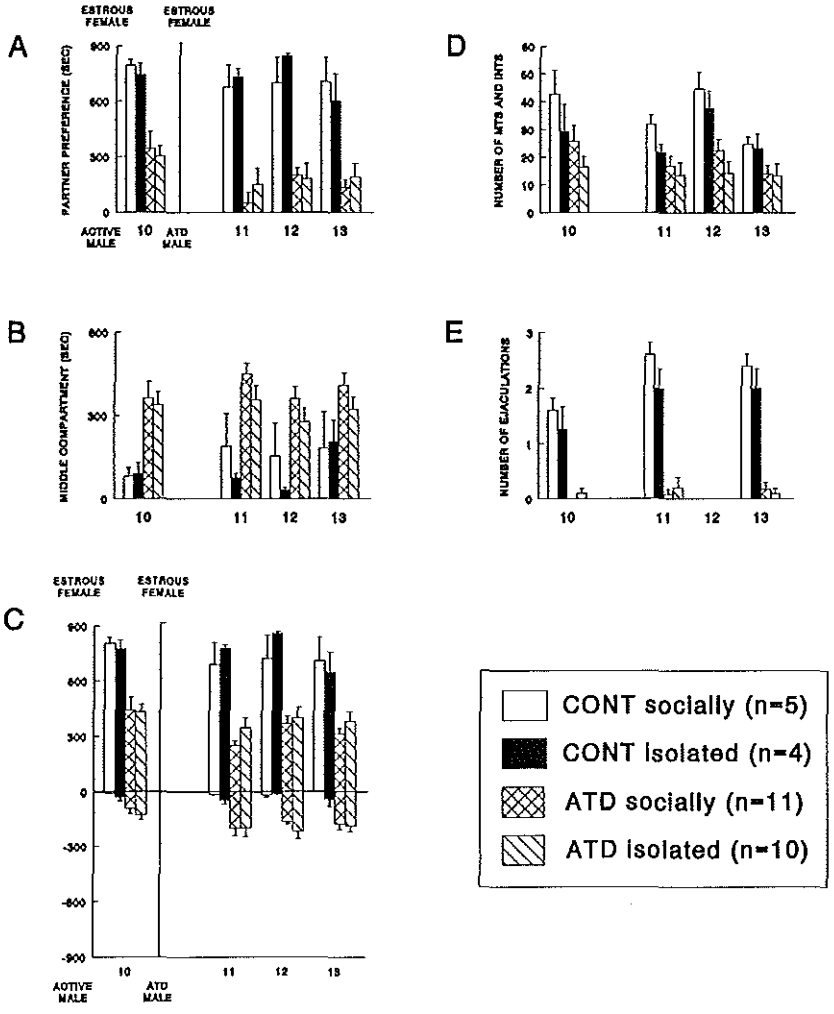

FIG. 3. Partner preference behavior in the three-compartment box of adult, gonadally intact male rats neonatally treated with the aromatization blocker ATD or cholesterol (controls). Male rats were either housed alone (isolated) or housed in small groups (socially). For reasons of comparison the results of Test 10 (see Fig. 1; last test with choice between estrous female and active male) are also shown. In Test 12 intromissions were prevented by vaginal occlusion of the stimulus females. (A) shows partner preference behavior; (B) shows the time spent in the empty middle compartment; and (C) shows the time spent with the estrous female and the time spent with the ATD-male. (D) shows the total number of mounts plus intromissions with the estrous female during partner preference testing. (E) shows the number of ejaculations. Values are means and SEM.

DISCUSSION

From the present experiments, it is clear that postweaning social conditions did not have major effects on adult partner preference behavior of gonadally intact ATD and control male rats. Housing conditions only affected partner preference behavior when no social interaction with the stimulus animals was possible. Then, the isolated male rats (ATD and control) showed a higher partner preference for the estrous female and spent less time in the empty middle compartment. When social interaction with the stimulus animals was possible, no differences were found between isolated and socially housed male rats (ATD and control).

Partner preference behavior was clearly affected by neonatal treatment with ATD, which corroborates earlier results from our laboratory (Bakker et al., 1993a; 1993b; Brand et al., 1991). When the animals were offered a choice between an estrous female and an ATD-male (instead of a 'normal' male) partner, preference scores decreased in all males, but most clearly in the experimental ATD-males. The latter males spent less time with the estrous female, and more time with the stimulus ATD-male. The hypothesis put forward in the Introduction could not be supported: social ATD-males compared with ATD isolates, did *not* show a higher preference for the stimulus ATD-male. Furthermore, ATD-males spent more time with the stimulus ATD-male than in previous PPB tests with the normal stimulus male (estrous female vs. normal male).

The stimulus ATD-males were regularly mounted by the experimental males (both ATD and control males) and showed high frequencies of proceptive and receptive behaviors. The stimulus ATD-males did not mount the experimental males. Presumably, being tethered rendered the stimulus ATD-males more likely to display their 'feminine' behaviors than to show their 'masculine' behaviors. Thus, the experimental male could mount and intromit the estrous female or mount the stimulus ATD-male. The clear preference for the estrous female over the stimulus ATD-male displayed by control males, could be explained by the fact that with the estrous female intromissions and ejaculations were possible, whereas the stimulus ATD-male could only be mounted. However, preventing intromissions and ejaculations by vaginal occlusion of the estrous female (albeit in only one test), did not change the partner preference behavior of the control males. Maybe, other factors than penile stimulation by intromission play an important role in determining the preference for the estrous female in control males. Further research should address this possibility.

Although partner preference behavior was not changed by postweaning social conditions, sexual behaviors during partner preference testing

clearly were affected. Isolated males displayed fewer mounts and intromissions than socially housed males. These effects were persistent over tests. Ejaculatory behaviors were not affected.

The question why social isolation causes a permanent detrimental effect on adult sexual behavior in male rats needs to be addressed. It was postulated that a lack of play behavior in the early isolated males makes them more 'playfull' and less 'sexual' when tested in adulthood with an estrous female rat (see also Introduction; Gerall et al., 1967). In the present study we did not observe that the isolated males showed more play behaviors during partner preference behavior testing than the socially housed males. Others postulated that the 'deprivation of opportunities of practicing climbing during infancy' (Hård & Larsson, 1968) resulted in retarded sexual development. Once a normal mount was performed, a normal mating pattern followed (Hård & Larsson, 1968). The latter authors showed that most isolated males 'overcame' their isolation effect and became normal copulators, whereas a minority of their isolated males never started to copulate. In the present study, we did not find such a phenomenon: all isolated control and ATD-male rats (with one exception) displayed mounting and intromission behaviors, albeit with lower frequencies than the socially housed males.

Finally, a new finding emerged from the present investigation. Although the lordosis quotients of ATD-male rats did not differ between isolates and socials, the isolated ATD-male rats seemed to be more sexually attractive. When pair-tested with a normal stimulus male, significantly more isolated ATD-males (9 of 10) were mounted than socially housed ATD-males (5 of 11). We have no explanation for this finding and future research is needed.

As was hypothetized in the introduction, we contemplated an effect of housing conditions on partner preference behavior. Especially in ATD-male rats, we assumed that the frequent occurrence of mounting and lordosing in the homecage could affect their partner preference behavior, that is, induce a more male-oriented preference. The present results do not support this idea.

In conclusion, the present results emphasize the importance of neonatal endocrine conditions for the organization of adult partner preference behavior in the male rat. Apparently, the presence or absence of social conspecifics from weaning onward plays no role in determining adult partner preference behavior. Future research with the ATD-male exposed to various choices of sex partners are needed to shed more light on the interesting phenomenon of sexual partner preference behavior.

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Chapter V

SEXUAL DIFFERENTIATION OF ODOR AND PARTNER PREFERENCE IN THE RAT

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SUMMARY

Bakker J., J. van Ophemert and A.K. Slob. Sexual differentiation of odor and partner preference in the rat.

Previous studies have shown that adult male rats, in which brain estrogen formation was inhibited neonatally by s.c. administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD), show an altered sexual partner preference: when tested in a three compartment box, such gonadally intact ATD males approach and mate both with the estrous female and the sexually active male, whereas normal males prefer to approach and mate with the estrous female while avoiding the stimulus male. After castration in adulthood and estradiol-treated, ATD males prefer sexually active males. Like-wise treated normal males prefer estrous females, and estrous females prefer to mate with males. In the present study, we asked what stimulus characteristics of active males versus estrous females determined the different sexual preferences of males, ATD males, and of females: chemosensory cues or more distal cues like actually seeing and hearing the stimulus animals or the reward of sexual activity with the stimulus animals?

Sex differences in preference were evident when given a choice between soiled bedding from estrous females and from sexually active males. ATD and control males preferred to sit significantly more on soiled bedding from estrous females than on soiled bedding from sexually active males. Control females preferrred to sit significantly more on soiled bedding from sexually active males than on soiled bedding from estrous females. More distal cues like seeing and hearing the stimulus animals revealed differences in preference between control males and females, but not between ATD and control males. Physical interaction with the stimulus animals was a

prerequisite for revealing differences in preference between ATD and control males. Then, ATD males were clearly intermediate between the extremes of the male and female control group.

INTRODUCTION

During a critical perinatal period, androgens secreted by the testes act on the developing central nervous system by permanently altering its structure and function, i.e. masculinizing and defeminizing brain structures that control gonadotropin secretion and sexual behavior (e.g. 7,24,30,31,35). In the rat, masculinization results in the display of male-typical sexual behaviors in adulthood (mounts, intromissions and ejaculations). Defeminization results in a loss of a cyclic release of gonadotropins necessary for ovulation and female-typical sexual behaviors (proceptive and receptive behavior) (30). Thus, gonadally intact male rats display the complete pattern of masculine sexual behavior and no feminine sexual behavior in adulthood. Not only coital behavior, but also motivational aspects of sexual behavior are sexually differentiated under the influence of androgens during early development (1,16,19,20,40). In adulthood, gonadally intact males prefer estrous females over males, and gonadally intact females prefer males over females (25,33).

Many facets of forebrain sexual differentiation reflect the action of estradiol, derived from neural aromatization of testosterone, in the male. Male rats in which brain estrogen formation has been inhibited perinatally by administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD), can readily show masculine as well as feminine sexual behaviors (1,16,19,41). Their sexual partner preference is also incompletely sexually differentiated: when given free access to a sexually active male and an estrous female, neonatally estrogen-deprived males do not show a clear partner preference, i.e. they approach and mate with the estrous female and they approach the sexually active male, to whom they show lordosis and proceptive behavior. If gonadally intact, ATD males' partner preference fluctuates between a low preference for a sexually active male and a low preference for an estrous female (1,2,6). Control males prefer the estrous female, i.e. they frequently visit and mate with the estrous female and rarely approach the sexually active male (1,6,16). The most striking differences between neonatally estrogen-deprived males and normal males were obtained when the subjects were castrated in adulthood and subsequently treated with estradiol (4). ATD males showed a clearcut preference for the sexually active male, whereas control males preferred to mate with the estrous female.

In rodent species, reproductively relevant pheromonal cues are detected by receptors in the vomeronasal organ, which in turn transmit this information centrally via the accessory olfactory bulb, the medial amygdala, the bed nucleus of the stria terminalis and the medial preoptic area (29). This vomeronasal projection pathway is a sexually dimorphic network with males having more vomeronasal receptors and a larger number of neurons throughout this chemosensitive pathway (reviewed in 36). The sexual differentiation of the vomeronasal system is under the influence of perinatal androgens, acting via aromatization to estrogens (36). However, we have recently found that the neuronal responsiveness, as measured by the induction of c-*Fos* expression, of the vomeronasal projection circuit to odors of estrous females is independent of neonatal estrogens (3). The distribution and intensity of increments in neuronal *Fos*-immunoreactivity following exposure to soiled bedding from estrous females were very similar in both neonatally ATD treated males and normal males. Additionally, Bressler and Baum (17) have found that male and female rats had similar increments in FOS-immunoreactivity after exposure to soiled bedding from either estrous or anestrous females at each level of the vomeronasal projection system. However, it should be noted that normal female rats are exposed to rather high levels of androgens derived from the placenta during embryonic development (11,23,41). Therefore, the female brain is to some extent masculinized: female rats, ovariectomized in adulthood and chronically treated with high doses of either testosterone-propionate or estradiol benzoate can show all the elements of masculine sexual behavior, including mounts as well as intromissive and ejaculatory behavior (10,13,21).

By contrast, the neuronal responsiveness to odors of sexually active males is clearly dependent on the neonatal action of estrogens (3). Neonatally ATD treated males show a female-like responsiveness, i.e. *Fos* responses were evident at each level of the vomeronasal system, in similar numbers as seen in females, whereas normal males only showed Fos responses at proximal levels of the vomeronasal system. Thus, neonatally ATD treated males showed a male-like responsiveness of the vomeronasal projection pathway to odors of estrous females and a female-like responsiveness of the vomeronasal projection pathway to odors of sexually active males (3). Interestingly, female rats also showed a neuronal responsiveness of the vomeronasal projection pathway to odors of estrous females (17) and to odors of sexually active males (3). These findings suggest that there are clear analogies between ATD males and normal females in their neuronal responsivess, emphasizing the pivotal role of neonatal estrogens in the sexual differentiation of the brain. However, we don't know to what extent ATD males' brains have

remained 'feminine', because, to date, sexual behavior and sexual partner preference of ATD males have always been compared to normal males and never to normal females (1,2,4,6,16).

In the present study, we asked what stimulus characteristics of active males versus estrous females were responsible for the previous observed preference of adult castrated males, treated with estradiol, for estrous females, and of adult like-wise treated females and ATD males for males? Are chemosensory cues, i.e. soiled bedding from the stimulus animals, or more distal cues like actually seeing and hearing the stimulus animals or the reward of sexual activity with the stimulus animals responsible for the observed differences in partner preference? To that end, ATD males, normal males and females were first subjected to preference tests, in which they were offered a choice between soiled bedding from estrous females and that from sexually active males. After these tests, they were given a preference test, in which they were given a choice between an estrous female and a sexually active male behind a wire mesh separation. Finally, they were subjected to preference tests between a tethered estrous female and a tethered sexually active male. All experimental animals were gonadectomized in adulthood and treated with estradiol. We choose estradiol treatment, because ATD males differed most dramatically from control males when tested for partner preference under this hormonal condition (4).

GENERAL METHODS

Animals

The experimental males and the stimulus males were Wistar albino rats (Wu-strain, outbred), commercially obtained (Harlan, Zeist). The experimental females and stimulus females were Wistar albino rats (RP-strain, inbred) bred in our laboratory. Two to four rats of the same sex were housed in a cage with food and water available ad libitum. All rats were kept in a reversed 14-hr/10-hr light (L)/dark (D) cycle (lights off from 7:45 a.m. to 5:45 p.m.).

Treatments

Female rats were time mated, and parturition occurred 22 days later. Within 2-4 hr after birth, newborn male pups received small silastic capsules (inner diameter 1.5 mm; outer diameter 2.1 mm; length 5 mm) containing crystalline ATD subcutaneously in the back under ice anesthesia. Control males received empty silastic capsules sc in the back (one treatment per litter). The implants were removed when the pups

were 21 days of age. In the present study, 9 ATD males (out of 3 litters) and 10 control males (out of 4 litters) were used.

The pups were weaned at 21 days of age and then housed in a cage with 2-3 conspecifics of the same sex and treatment. Animals were left undisturbed until the onset of behavioral testing. At the age of approximately 6 months, the males were tested for partner preference and sexual behavior in our laboratory (unpublished). The stimulus females, all ovariectomized, of that particular experiment were used as control females in the present study. Thus, all experimental animals were sexually experienced and were familiar with the three compartment box used for partner preference testing. At the age of approximately 10 months, ATD and control males were castrated through a midline abdominal incision under ether anesthesia. Additionally, all animals received subcutaneously a silastic capsule (inner diameter 0.5 mm; outer diameter 1.0 mm; length 2.5 mm) containing crystalline estradiol.

The stimulus animals were sexually active, gonadally intact, males and estrous females. The latter were ovariectomized and brought into behavioral estrus with an injection of 20 g of estradiol benzoate (EB) 24-48 hr prior to testing, followed by an injection of 1.0 mg progesterone (P) 3-4 hr before testing. These hormones were dissolved in olive oil and injected s.c. in the neck.

Test procedure

Preference test in three compartment box. A test box made of gray perspex with a transparent front was used (5): it has three compartments (60 x 30 x 40 cm) with a small opening opening (13 x 12 cm) in both partitions near the back. These openings can be closed by a sliding door. In each opening, position sensitive tilt-platforms are situated in order to register movement of the experimental animal from one compartment to another. The position of the tilt-platform is determined using a magnetic field (5).

Odor preference

Six groups of ovariectomized females (3 per group) were injected subcutaneously with estradiol benzoate (20 g) 24-48 hr prior to a progesterone (1.0 mg) injection. Following the progesterone injection, all females were placed in clean cages which contained fresh sawdust. Concomitantly, six groups of sexually active males (3 per group) were placed in clean cages with fresh sawdust. Bedding was collected 14 hr later and immediately used in the experiment. The soiled bedding from the sexually active males was put in the left compartment and the soiled bedding from the estrous females was put in the right compartment of the three compartment box in Tests 1 and 2. In Tests 3 and 4, the soiled bedding from estrous females was put in the left compartment and the

soiled bedding from sexually active males in the right compartment. The middle compartment always contained clean bedding.

Before testing, the experimental animal was placed in the middle compartment, with the sliding doors closed, for a 15 min adaptation period. At the beginning of the test (which lasted 15 min), the sliding doors were removed, and the experimental animal could freely move around. The time spent in each compartment and the number of visits to each compartment were recorded automatically (for further details see 5). At the end of each test, an odor preference score was calculated by subtracting the time spent in the compartment with soiled bedding from sexually active males from the time spent in the compartment with soiled bedding from estrous females. Thus, a positive score indicates a preference for estrous females' odors, a negative score for the sexually active males' odors.

Partner preference

In the partner preference tests, stimulus animals (a sexually active male and an estrous female) were placed in the lateral compartments of the three compartment box. In Test 5, stimulus animals were placed behind a wire mesh separation halfway down the lateral compartment to prevent physical interaction. In Tests 6 and 7, stimulus animals were tethered with a rope to the front of the lateral compartments. In all tests, the position of the stimulus animals was at random for the experimental subjects. Before testing, the experimental animal was put in the test apparatus, with the sliding doors closed, for a 15 min adaptation period. At the beginning of the test (which lasted 15 min), the sliding doors were removed, and the experimental animal could freely move around. When sexual interaction was possible (Tests 6 and 7), various sexual behaviors with the stimulus animals were scored manually. Following each test, a partner preference score was calculated by subtracting the time spent with the sexually active male from the time spent with the estrous female. Thus, a positive score indicates a preference for the estrous female, a negative score a preference for the sexually active male. All tests (1-7) were carried out early in the dark phase (between 8:30-11:30 am) of the light-dark cycle.

Statistics

Data were subjected to one- or two-way analyses of variance (ANOVA), followed by the least significant difference procedure (28). The $p=0.05$ level is used as the upper limit for statistical significance.

RESULTS

Odor preference

When given free access to soiled bedding from sexually active males and soiled bedding from estrous females, there were clear sex differences in odor preference: ATD and control males preferred soiled bedding from estrous females, whereas control females preferred soiled bedding from sexually active males (Fig 1A).

ANOVA on the preference scores revealed significant group differences ($F(2/25)= 13.3$, $p< 0.001$). Post-hoc analysis with least significant difference (5%= 150 sec) showed that control females had significantly lower preference scores than ATD and control males. ANOVA showed significant differences between the groups in the time spent at the active males' bedding ($F(2/25)= 10.9$, $p< 0.001$) and in the time spent at the estrous females' bedding ($F(2/25)= 5.8$, $p= 0.008$). Post-hoc analysis of the time spent at the active males' bedding with least significant difference (5%= 136 sec) indicated that control females spent significantly more time at soiled bedding from sexually active males than ATD and control males. Post-hoc analysis of the time spent at the estrous females' bedding with least significant difference (5%= 97 sec) showed that control females spent significantly less time at soiled bedding from estrous females than ATD and control males.

The total number of excursions, i.e. the sum of the number of excursions to soiled bedding from sexually active males, to soiled bedding from estrous females and to clean bedding is shown in Fig 2. ANOVA revealed significant group differences ($F(2/25)= 8.5$, $p= 0.002$). Post-hoc analysis with least significant difference (5%= 7) indicated that ATD males were more active, i.e. they paid more visits to each compartment than control males and females.

Partner preference with no physical interaction possible

When stimulus animals were separated by wire mesh to prevent physical interaction, both males and females preferred the compartment with the sexually active male with control females showing a greater preference for the sexually active male than males (Fig 1A). ANOVA did not show significant group differences ($F(2/25)= 2.7$, $p= 0.09$). However, post-hoc analysis with least significant difference (5%= 160 sec) revealed that the preference scores of ATD and control males differed significantly from those of control females, i.e. control females showed a greater preference for the sexually active male than ATD and control males.

ANOVA on the total number of excursions (Fig 2) did not reveal significant group differences ($F(2/25)= 2.7$, $p= 0.09$). However, post-hoc

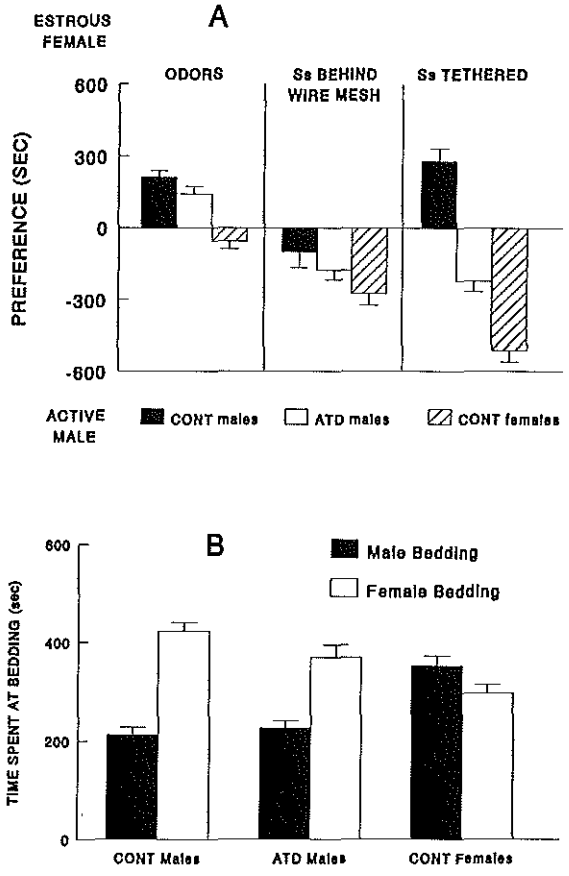

Figure 1. Partner preference of adult male rats, which received on postnatal days 0-10 silastic capsules containing the aromatase inhibitor ATD (ATD males) or received empty silastic capsules (control males). Control females were not treated neonatally. All males and females were gonadectomized in adulthood and subsequently treated with estradiol. A Preference scores. Left panel: preference scores when given a choice between soiled bedding from estrous females and from sexually active males (values are mean + SEM of 4 tests); middle panel: preference scores when given a choice between an estrous female and a sexually active male behind a wire mesh separation (values are mean + SEM); right panel: preference scores when given a choice between a tethered estrous females and a tethered sexually active male (values are means ± SEM of 2 tests). B Time spent at soiled bedding from estrous females and time spent at soiled bedding from sexually active males. Values are mean + SEM of 4 tests.

analysis with least significant difference (5%= 10) indicated that ATD males had more excursions than control males. Control females were intermediate between ATD and control males: statistically, they did neither differ from ATD males nor from control males.

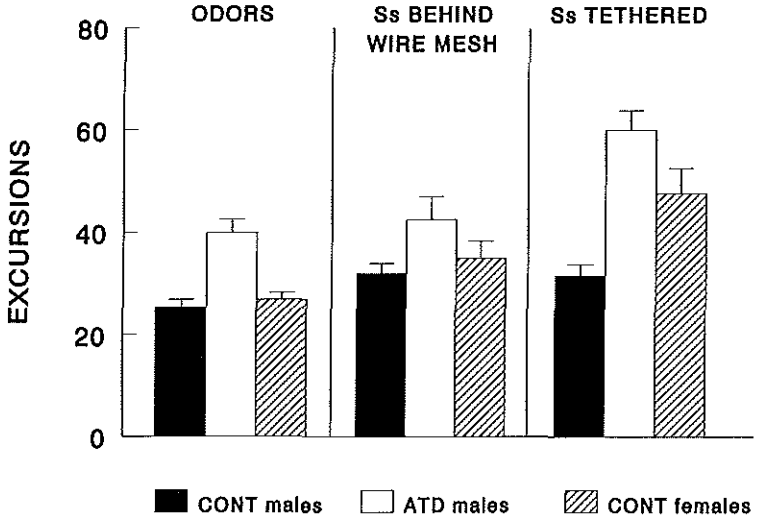

Figure 2: total number of excursions, i.e. the sum of excursions to each compartment, of adult male rats, which received on postnatal days 0-10 silastic capsules containing the aromatase inhibitor ATD (ATD males) or received empty silastic capsules (control males). Control female rats were not treated neonatally. All males and females were gonadectomized in adulthood and subsequently treated with estradiol. Left panel: total number of excursions when given a choice between soiled bedding from estrous females and from active males (values are mean + SEM of 4 tests); middle panel: total number of excursions when given a choice between an estrous female and a sexually active male behind a wire mesh separation (values are mean + SEM); right panel: total number of excursions when given a choice between a tethered estrous female and a tethered sexually active male (values are mean + SEM of 2 tests).

Partner preference with physical interaction possible

When stimulus animals were tethered and physical interaction was possible, sex differences were clearly visible (Fig 1A): control males preferred the estrous female, whereas control females showed a distinct preference for the sexually active male. ATD males showed a clear preference for the sexually active male, albeit somewhat lower than control females (Fig 1A). ANOVA on preference scores indicated significant group differences ($F(2/25)= 52$, $p< 0.001$). Post-hoc analysis with least significant difference (5%= 232 sec) revealed that control males differed in preference from ATD males and control females. Furthermore, control females showed a greater preference for the sexually active male than ATD males.

The total number of excursions are depicted in Fig 2. ANOVA indicated significant group differences ($F(2/25)= 12.4$, $p< 0.001$). Post-hoc analysis with least significant difference (5%= 17) showed that ATD males and control females had more excursions than control males. ATD

Table 1: Sexual behavior with stimulus animals (estrous female and sexually active male) during partner preference testing of adult male rats treated neonatally with the aromatase inhibitor ATD (ATD males) or with cholesterol (control males). Control females were not treated neonatally. All males and females were gonadectomized in adulthood and subsequently treated with estradiol through silastic capsules. Values (means ± SEM of 2 tests) represent frequencies. The numbers in brackets represent the number of animals displaying the behavior.

	Males		Females
	control (n=10)	ATD (n=9)	control (n=9)
masculine sexual behavior			
mounts plus intromissions	19.9 ± 1.7*(10)	3.9 ± 1.2 (6)	0 ± 0 (0)
ejaculations	0.8 ± 0.3 (4)	0.1 ± 0.1 (1)	0 ± 0 (0)
feminine sexual behavior			
receptive (lordosis)	0 ± 0 (0)	12.3 ± 2.3 (10)	11.5 ± 2.0 (10)
proceptive	0 ± 0 (0)	53.7 ± 7.2 (10)	55.9 ± 5.9 (10)

* significantly higher ($p<0.05$) than ATD males.

males did not differ from control females in the total number of excursions.

Sexual behavior with the tethered estrous female

Frequencies of mounts plus intromissions with the estrous female are shown in Table 1. Control females did not show any masculine sexual behaviors and were excluded from statistical analysis. ANOVA revealed significant group differences ($F(1/17)= 31.5$, $p< 0.001$). Post-hoc analysis with least significant difference (5%= 8.5) indicated that control males displayed more mounting and intromissive behavior than ATD males. Ejaculation frequencies are shown in Table 1. Only a small portion of the males ejaculated frequently: 40% of the control males and only 11% of the ATD males.

Sexual behavior with the tethered sexually active male

Frequencies of feminine sexual behaviors, i.e. proceptive (the sum of presenting, earwiggling and hopping & darting) and receptive (lordosis) behaviors, are shown in Table 1. Control males did not show any feminine sexual behaviors and were excluded from statistical analysis. Frequencies of proceptive and receptive behaviors were similar in control females and ATD males. Although lordosis quotients are not presented here, ATD males and control females showed lordosis quotients higher than 100%, i.e. were higly receptive. Lordosis behavior was not only elicited by mounting of the stimulus male, but also by small tactile stimuli, such as sniffing of the perianal region by the stimulus male.

DISCUSSION

The present study clearly showed that given free access to soiled bedding from estrous females and that from sexually active males was sufficient to reveal a sex difference in preference. ATD and control males preferred to sit significantly more on soiled bedding from estrous females than on soiled bedding from sexually active males, whereas control females preferred to sit significantly more on soiled bedding from sexually active males than on soiled bedding from estrous females.

These functional sex differences in odor preference might be related to morphological sex differences in the vomeronasal projection pathway. Recently, it has been found that female rats showed a neuronal responsiveness in the vomeronasal projection pathway both to odors of estrous females (17) and to odors of sexually active males (3). Control males showed a neuronal reponsiveness to odors of estrous females, but not to odors of sexually active males (3). This sex dimorphism in functional activity of the vomeronasal system to odors of sexually active males is clearly dependent on the action of neonatal estrogens. ATD males showed a neuronal responsiveness of the vomeronasal system both to odors of estrous females and to odors of sexually active males. However, when given a choice between odors of estrous females and those of sexually active males, control females preferred the odors of sexually active males, whereas ATD males preferred the odors of estrous females. Apparently, neural reponses of the vomeronasal system do not predict odor preference.

The observed differences in partner preference between ATD and normal males, are not primarily induced by chemosensory cues of the stimulus animals. More distal cues like seeing and hearing the stimulus animals, revealed a difference in preference between control males and females, but not between ATD and control males. Males (ATD and control) and females showed a preference for the sexually active male. However, control females showed a greater preference for the stimulus male than control males. ATD males were intermediate between the extremes of the male and female control group. Statistically, they did neither differ from control males nor from control females. Rather unexpectedly, control males showed a low preference for the sexually active male. Presumably, the preference for the sexually active male has been caused by nonsexual factors such as social affiliation with the stimulus male or curiosity evoked by the novelty (32).

When physical interaction with the stimulus animals was allowed, the sex differences in partner preference became most explicit. Control

males preferred to mate with the estrous female, whereas control females showed a clearcut preference for the sexually active male. ATD males showed a preference for the sexually active male, albeit significantly lower compared to females. ATD males showed high levels of feminine sexual behaviors toward the sexually active male and were in that respect indistinguishable from females. In contrast to females, ATD males also showed some masculine sexual behaviors towards the estrous female, albeit much less compared to normal males. Females did not show any masculine sexual behaviors in spite of high levels of proceptive behaviors displayed by the stimulus females. As described in the introduction, adult ovariectomized and estradiol-treated females can display all elements of masculine sexual behavior when tested with other females in a pair test situation (10). Presumably, this discrepancy can be explained by the different test situation in the present study. Pair tests do not allow the experimental female to choose whether or not to copulate with the stimulus female, whereas in partner preference tests the female can freely choise her sex partner and consequently prefers to mate with the stimulus male rather than with the stimulus female.

Neonatally ATD-treated males were clearly in-between control males and females in partner preference and masculine sexual behavior. In the display of feminine sexual behavior ATD males were indistinguishable from females. It has been postulated that masculinization and defeminization of sexual behavior are two separable processes that are differentially affected by the organizational actions of testosterone and its metabolites (e.g. 7). Defeminization depends primarily on aromatization of testosterone into estradiol (7,9,14) and masculinization may be achieved partly through estrogens, but also through androgens (19). Our findings in ATD males support this supposition. Although the ATD males in the present study showed a somewhat affected masculine sexual behavior, i.e. they mounted and intromitted the stimulus female, but hardly ejaculated (only 1 out of 9 ATD males), they certainly are capable of displaying all the elements of masculine sexual behavior. A complex phenomenon is that ATD males show a time-dependency in their partner preference and sexual behavior. Early in the dark phase of the light/dark cycle, ATD males show high levels of feminine sexual behavior and impaired ejaculatory behavior, together with no preference or a preference for a sexually active male. Late in the dark phase, ATD males show ejaculatory behavior and low levels of feminine sexual behavior together with a low preference for the estrous female (1,6). Thus, ATD males are masculinized, but obviously not defeminized. It should also be taken into account that ATD males were only deprived of estrogens postnatally. This means that the normal sex differences in testosterone in fetal (11)

and immediate postpartum (12,37) existed for these males. Thus, ATD males' brains could have been already partly organized in a male direction perinatally. These results suggest that defeminization of the brain occurs primarily postnatally under the influence of estrogens and that masculinization of the brain takes place ma0inly prenatally and immediately after birth under the influence of androgens/estrogens. Prenatally ATD-treated males only showed slighlty impaired ejaculatory behavior suggesting that the masculinization of the brain requires the action of both testosterone and estradiol (16).

The control males in the present study also showed lower ejaculation frequencies compared to previous studies (1,6). All animals in the present study were gonadectomized and treated with estradiol. It has be shown that estradiol was very effective in activating mating behavior in gonadectomized male rats (18,38), although estradiol treatment together with dihydrotestosterone (DHT) treatment could restore mating behavior to the level of gonadally intact males (22) or testosterone treated males (8). In general, it is thought that estradiol affects the central nervous system to promote sexual behavior, whereas DHT is important to restore penile tactile sensitivity to precastration levels (32). In ferret studies, it has been shown that the most robust sex differences in partner preference were seen when gonadectomized in adulthood and treated with estradiol instead of testosterone (39). When tested in a T-maze, female ferrets prefer to approach and receive neck grips from a stimulus male whereas males prefer to approach and neck grip an estrous female (34,39). Similar findings have been found in the present study. Clear sex differences in partner preference were seen in rats gonadectomized and treated with estradiol. Males prefer to mount and intromit with estrous females, females prefer to approach and receive mounts and intromissions by the stimulus male. Moreover, the most robust differences between ATD males and control males in partner preference have been obtained when castrated in adulthood and treated with estradiol (4). Thus, estradiol treatment in adulthood is very effective in revealing sex differences in partner preference.

Interestingly, ATD males had more excursions to the three compartments than normal males and females, independently of whether or not stimulus animals were present. This running of ATD males from one side to the other could presumably reflect a conflict situation, i.e. ATD males have difficulty in making a choice. Especially when physical interaction with the stimulus animals is possible, ATD males receive the reward of sexual activity at both sides of the three compartment box, which makes them run even more. Additionally, clear sex differences have been reported in locomotor activity, i.e. wheel running or the open field test.

On both measures, females are more active than males (for overview see 15). Recently, we found that ATD males showed a higher locomotor activity in the open field test than control males (unpublished results). This suggest that the higher locomotor activity of ATD males might be a manifestation of their incomplete defeminization process. However, in the present study, control females only differed in the total number of excursions from control males when tethered stimulus animals were used. Presumably, the greater number of excursions of the control females is linked to the presence of tethered stimulus animals in the three compartment box rather than a higher locomotor activity.

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Chapter VI

ORGANIZATION OF PARTNER PREFERENCE AND SEXUAL BEHAVIOR AND ITS NOCTURNAL RHYTHMICITY IN MALE RATS

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SUMMARY

Male rats were neonatally treated with 1,4,6-androstatriene-3,17-dione (ATD), which blocks aromatization of testosterone (T) to estradiol (E_2), from Days 0, 2, or 5 through 14. Adult partner preference behavior (PPB; choice between estrous female rat [F] and active male rat [M]) was studied in the early part of the dark phase of the light-dark (LD) cycle. ATD Day 0 Ms showed a preference for the stimulus M or showed no preference for either of the stimulus Ss. Controls preferred the estrous F. ATD Days 2 and 5 Ms showed PPB intermediate between ATD Day 0 Ms and controls. Thus the neonatally sensitive period for the organization of adult PPB extends beyond Day 5. Furthermore, PPB showed a nocturnal rhythmicity in ATD Ms but not in controls. In the late part of the dark phase, all Ms showed a preference for the stimulus F. ATD Days 2 and 5 Ms and control Ms were no longer different in PPB, but ATD Day 0 Ms still showed significantly lower preference scores for the F than all other Ms. Thus the E_2 metabolite of T suppresses organization of an adult nocturnal rhythm in PPB.

INTRODUCTION

During a critical or sensitive perinatal period, the estrogenic metabolite of testosterone, estradiol (E_2), is involved not only in the processes of masculinization and defeminization of sexual behavior (e.g., Baum, 1979), but also in the organization of partner preference behavior in the male rat (Bakker, Brand, van Ophemert, & Slob, 1993; Brand, Kroonen,

Mos, & Slob, 1991). Exposure to endogenous E_2 'organizes' the developing brain mechanisms that later control partner preference behavior and sexual behavior (e.g., Beatty, 1992; Brand & Slob, 1991; Goy & McEwen, 1980).

Male rats castrated on Day 0 (day of birth = Day 0), or on Day 4, or on Day 6 after birth, showed in adulthood following treatment with E_2 and progesterone higher levels of female sexual behavior (lordosis) than male rats castrated on Day 10 after birth (Booth, 1979; Grady, Phoenix, & Young, 1965). Following treatment with testosterone, such neonatally castrated males showed lower levels of male sexual behavior, that is, fewer ejaculations than controls (Grady et al., 1965; Hart, 1972). This suggests that the sensitive period for masculinization and defeminization of sexual behavior of the male rat has ended around day 10 of postnatal life (Young, Goy, & Phoenix, 1964).

Relatively little is known about the duration of the sensitive period for the organization of partner preference behavior. Male rats treated from very shortly after birth (within 2-4 hr on Day 0) with ATD (1,4,6-androstatriene-3,17-dione), which blocks the aromatization of testosterone to E_2, showed high levels of male sexual behavior (mounts and intromissions, but no ejaculations) when paired with an estrous female rat, as well as high levels of female sexual behavior (lordosis) when paired with an active male rat (Brand et al., 1991). It should be stressed that these males were gonadally intact. When such ATD male rats were tested for partner preference behavior in adulthood (choice: estrous female vs. active male), they showed no clear preference for either partner or they showed a very low but significant preference for the female rat but they differed significantly from control male rats, which showed higher preference scores for the female rat. Davis, Chaptal, and McEwen (1979) reported that male rats treated with ATD from Day 1 till Day 10 after birth did not differ from control males in two partner preference behavior tests: All male rats significantly preferred the female partner. This could indicate that the sensitive period for the organization of adult partner preference behavior extends to only Day 1 after birth and is thus much shorter than the sensitive period for sexual behavior organization.

Södersten & coworkers found a diurnal rhythm in the display of sexual behavior by male and female rats. Female rats which were ovariectomized and treated with E_2-filled Silastic implants showed low levels of lordosis behavior during the light phase (L) and high levels during the dark phase (D) of the LD-cycle (Hansen, Södersten, & Srebro, 1978). However, such a diurnal rhythm was not found in female rats by other investigators (Erskine, Marcus, & Baum, 1980; Harlan, Shivers, Moss, Shryne, & Gorski, 1980). Male rats displayed more mounting behavior and showed

shorter latencies to first ejaculation as the dark period progressed (Dewsbury, 1968; Harlan et al., 1980; Södersten & Eneroth, 1980). In intact male rats, neonatally treated with the antiestrogen MER-25, this diurnal rhythm in sexual behavior was more pronounced than in control male rats (Södersten & Eneroth, 1980). Male rats neonatally castrated (Day 1) and E_2-treated in adulthood displayed a diurnal rhythm in lordosis behavior similar to female rats (Hansen, Södersten, Eneroth, Srebro, & Hole, 1979). This suggests that in male rats endogenous neonatal E_2 suppresses to a certain extent the organization of a diurnal rhythm mechanism. The possible existence of a rhythm in partner preference behavior of male rats does not appear to have been studied. In our department, male rats' partner preference behavior has virtually always been tested during the early portion of the dark phase of the LD cycle (e.g., Brand et al., 1991). Accidently we once encountered nocturnal behavioral differences that prompted us to study nocturnal rhythmicity in ATD male rats' sexual behavior. When rats were tested early and late in the dark phase of the LD cycle, it was found that the ejaculation frequencies of ATD males (ATD from Days 0 till 21; $n = 11$) were significantly higher during the late portion of the dark phase ($M = 1.6 \pm 0.2$ [SE_M]) than during the early portion ($M = 0.6 \pm 0.2$), $F(1,10) = 34.57$, $p < .001$. The ejaculation frequencies of control males ($n = 10$) did not vary over the dark phase (1.3 ± 0.7 vs. 1.5 ± 0.7 for late v.s early, respectively).

In the present study, we attempted to determine the duration of the sensitive period for the organization of adult partner preference behavior, and we investigated the possible existence of a nocturnal rhythm in partner preference behavior and sexual behavior. Male rats neonatally received ATD, beginning either directly after birth or on Day 2 or Day 5 after birth. In adulthood the partner preference and sexual behavior of such male rats were studied during the dark phase of the LD cycle.

METHOD

Animals

Experimental animals were Wistar albino rats (R strain) bred in our laboratory. The male stimulus animals were F1 hybrids of two inbred Wistar strains (R x U). The female stimulus animals were Wistar albino rats (R strain). Two to four rats were housed in a cage with food and water available ad libitum. All rats were kept on a reversed 14:10 hr light-dark (LD) cycle (lights off from 7:45 a.m. to 5:45 p.m.). The temperature in the animal room ranged from 20°C to 22°C.

Treatments

Female rats were time mated, and parturition occurred 22 days later (day of birth = Day 0). The newborn male pups were divided into three groups on the basis of age of neonatal treatment. All male rats received a small Silastic capsule (inner diameter = 1.5 mm; outer diameter = 2.1 mm; length = 5 mm) containing ATD or cholesterol, which was implanted subcutaneously (SC) in the back under ice anesthesia (one treatment per litter). ATD Day 0 male rats were implanted between 2 and 4 hr after birth, ATD Day 2 male rats and ATD Day 5 male rats received their implants on Days 2 and 5, respectively. Each ATD group had a corresponding control group that received a Silastic capsule with cholesterol (Day 0, Day 2 and Day 5). In the present experiment, 7 ATD Day 0 male rats (out of 5 litters), 9 ATD Day 2 male rats (out of 4 litters), 17 ATD Day 5 male rats (out of 4 litters) and 23 control male rats (out of 10 litters) were used.

The implants were removed when the pups were 14 days of age. The pups were weaned at 21 days of age and housed in a cage with 2-3 other rats that had received the same treatment. To assess the onset of puberty, from Day 30 onward we inspected the rats daily for cleavage of the balano-preputial skinfold (the method used by de Jong & van der Schoot, 1979; van der Schoot & Baumgarten, 1990).

Stimulus animals were sexually active male and estrous female rats. The latter were ovariectomized and brought into behavioral estrus by injecting 20 g of estradiol benzoate 24-48 hr before testing followed by 1.0 mg progesterone 3 to 4 hr before testing. These hormones were dissolved in olive oil and injected sc in the neck.

Behavioral testing

Three-compartment partner preference test. We used a test box made of gray Perspex with a transparent front; it 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 (Slob, de Klerk, & Brand, 1987). These openings could be closed by a sliding door. Stimulus animals either were tethered with a rope to the rear of one of the lateral compartments or could freely move behind a wire mesh separation halfway down the lateral compartment. The tethered animals thus had a limited action radius. When physical interaction was prevented by wire mesh, the experimental animal could only see, smell, or hear the stimulus animals.

Before testing, 2 stimulus animals and the experimental animal were put in the test apparatus, one in each compartment, with the sliding doors closed, for 15-20- min of adaptation. At the beginning of the test (which lasted 15 min), 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. The time spent in each compartment was recorded. Various sexual behaviors with the tethered stimulus animals were scored. Masculine sexual behaviors presented are frequencies of mounts and intromissions and the number of ejaculations. Feminine sexual behaviors comprise the sum of proceptive behaviors (i.e., hop and dart, presenting and earwiggling).

To quantify partner preference, we calculated a preference score for each test by subtracting the time spent in the compartment containing the sexually active male rat from the time spent in the compartment containing the estrous female rat (the method used by Edwards & Pfeifle, 1983). Thus, a positive score indicates a preference for the estrous female rat; a negative score indicates a preference for the sexually active male rat.

Pair test with estrous female. Semicircular cages measuring 62 x 40 x 36 cm were used. Before testing, we introduced the experimental animal into the cage for a 15-min adaptation period. At the beginning of the test (which lasted 15 min), an estrous female rat was put in the cage. The following sexual behaviors were scored: the number of mounts (with pelvic thrusting), the number of intromissions and the number of ejaculations, and ejaculation latency (i.e., the time from the introduction of the female to the first ejaculation). When male rats did not ejaculate within 15 min, the total test time (i.e., 900 s) was taken as ejaculation latency. A mean interintromission Interval was calculated for each animal, that is, the mean interval between the intromissions within one or more ejaculatory series. An ejaculatory series comprised a number of mounts and intromissions prior to an ejaculation (Beach & Jordan, 1956).

Pair test with sexually active male. After 5 min of adaptation of the stimulus animal, an experimental animal was introduced into the semicircular cage. The lordosis responses of the experimental male rat to the mounting of the stimulus male rat were recorded. The test lasted until the experimental male rat had received 10 mounts or 10 min had elapsed. Because many males were not readily mounted, we included in the data analyses the lordosis quotients of all male rats who received four or more mounts by the stimulus male rat. Thus, 1 ATD Day 2 male and 5 ATD Day 5 male rats were excluded.

Test procedure

Behavioral tests commenced around the age of 98 days. First the males were subjected to four consecutive weekly partner preference tests without sexual interaction (stimulus animals behind wire mesh; Tests 1-4). Then seven partner preference tests with sexual interaction possible (tethered stimulus animals; Tests 5-11) were carried out on a once weekly basis. Tests 1-7, 9 and 10 were done in the early portion of the dark phase

(between 8:30 a.m. and 11:00 a.m.). Tests 8 and 11 were executed in the late portion of the dark phase (between 2:00 p.m. and 4:30 p.m.).

After partner preference testing, pair tests for sexual behavior with an estrous female rat were carried out twice weekly for a total of six pair tests. Three tests were done early in the dark phase and three tests late in the dark phase. After the pair tests with the estrous female rat (for masculine sexual behavior), the male rats received twice weekly pair tests with a sexually active male for feminine sexual behavior (lordosis) for a total of five pair tests. Three tests occurred early and two tests late in the dark phase.

At the end of behavioral testing (approximately 270 days of age), all males were castrated under ether anesthesia through a midline abdominal incision. Both testes were weighed.

Blood collection and hormone assay

Blood was collected in the early portion of the dark phase (between 10:00 a.m. and 11:00 a.m.) from the orbital plexus under light ether anesthesia at the age of approximately 168 days. Testosterone levels were estimated in serum by radioimmunoassay (Verjans, Cooke, de Jong, de Jong, & van der Molen, 1973). The interassay and intra-assay coefficients of variation were 13.4% and 5.6%, respectively.

Statistics

The behavioral data were subjected to two-way analysis of variance (ANOVA), followed by the least significant difference (LSD) procedure (Kirk, 1968). Only significant F values are presented. The ejaculation data obtained during partner preference testing were also analyzed with Fisher's exact two-tailed probability test (Perlman, 1986). No significant group differences were observed between the three control groups (Day 0, Day 2, and Day 5). Therefore, all control male rats were considered as one group. The p =.05 level is used as the upper limit for statistical significance.

RESULTS

Somatic Data

Various somatic data are shown in Table 1. The mean (± SE_M) age at which the balano-preputial skinfold separated from the glans penis was 51.7 (± 0.1) days. There were no significant differences between ATD male and control male rats in day of cleavage. ANOVA on the blood serum levels of testosterone revealed a significant group effect, $F(3,52) = 2.83, p = .05$. Subsequent LSD analysis (5% = 1.84) revealed that ATD Day 0 male and ATD Day 2 male rats had significantly lower testosterone

Table 1
Somatic Parameters of Neonatally ATD-Treated and Control Male Rats

						Testes weights[c]			
		Day of cleavage[a]		Testosterone (nmol/L)[b]		g		g/100 g of bodyweight	
Group	n	M	SE_M	M	SE_M	M	SE_M	M	SE_M
ATD Day 0	7	51.9	0.4	3.79*	0.45	2.98	0.04	0.76	0.01
ATD Day 2	9	51.9	0.6	4.44*	0.48	2.98	0.06	0.74	0.02
ATD Day 5	13	51.3	0.4	5.89	0.72	2.64	0.13	0.73	0.03
Control	23	51.7	0.9	6.04	0.39	2.82	0.11	0.70	0.03

Note. ATD = 1,4,6-androstatriene-3,17-dione.
[a]Day of cleavage of the balano-preputial skinfold of the glans penis. [b]Endogenous blood serum testosterone levels at the age of approximately 168 days (1 week after partner preference testing). [c]Testes weights (sum of two testes) at time of castration, approximately 270 days of age.
*Significantly different from ATD Day 5 and control male rats, $p < .05$.

levels than ATD Day 5 male and control male rats. The relative testes weights (per 100 g of body weight) did not differ between the groups. There was no significant correlation between blood testosterone levels and relative and absolute testes weights (Pearson correlation coefficient $r = .25$).

Partner Preference Behavior

Mean partner preference behavior data are depicted in Figure 1. The data of Tests 1-4, in which the stimulus animals behind wire mesh, were analyzed separately from those of Tests 5-11, in which the stimulus animals were tethered. ANOVA of Tests 1-4 (see Figure 1A) showed no significant partner preferences in any of the male groups; neither was there a significant effect of testing. ANOVA on the time spent in the empty middle compartment in Tests 1-4 (see Figure 1C) showed an almost significant effect of groups, $F(3,52) = 2.67$, $p = .057$, a significant effect of testing, $F(3,156) = 24.38$, $p < .001$, and no interaction. For all male groups, the time spent in the empty middle compartment increased significantly over tests (LSD, 5% = 72 s). Overall, ATD Day 0 male and ATD Day 2 male rats seemed to spend more time in the empty middle compartment than ATD Day 5 male and control male rats, but this difference was not statistically significant.

ANOVA on the data of Tests 5-11 (see Figure 1B) revealed a significant group effect, $F(3,52) = 7.60$, $p < .001$, a significant effect of testing, $F(6,312) = 39.19$, $p < .001$, and a significant Group x Test interaction, $F(18,312) = 2.08$, $p = .007$. LSD analysis of this interaction (5% = 182 s) indicated that when tested early in the dark phase, ATD Day 0 male rats

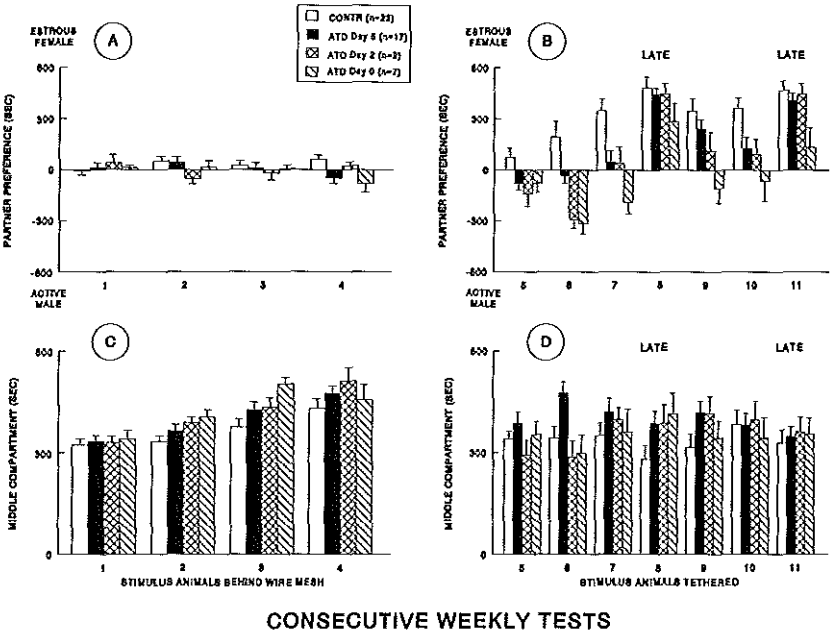

Figure 1. Partner preference behavior of adult gonadally intact male rats, neonatally treated with the aromatization blocker ATD from Day 0 (striped bars), Day 2 (grey bars), or from Day 5 (black bars); controls (CONTR) were treated with cholesterol (open bars). (Tests 1-7 and 9-10 were executed early in the dark phase; Tests 8 and 11 late in the dark phase. Panel A shows the preference for an estrous female over a sexually active male with the stimulus animals behind a wire mesh separation. Panel B shows the preference for an estrous female rat over a sexually active male rat with tethered stimulus animals. Panel C shows the time spent in the empty middle compartment of the three compartment box [3CB] with stimulus animals behind a wire mesh separation. Panel D shows the time spent in the empty middle compartment of the 3CB with tethered stimulus animals. Values are means and SE_M. ATD = 1,4,6-androstatriene-3,17-dione.)

either significantly preferred the sexually active male rat (Tests 6-7) or showed no partner preference (Tests 5 and 9-10). Partner preference behavior of ATD Day 2 male rats changed significantly from a preference for the male partner (Tests 5-6) to no preference for either partner (Tests 7 and 9-10). ATD Day 5 male rats showed no partner preference behavior in the first three tests (Tests 5-7) and a preference for the female rat in Tests 9 and 10. Control males always preferred the female partner, except during the first test (Test 5). When the male rats were tested late in the dark phase (Tests 8 and 11), a preference for the estrous female rat emerged for all ATD male groups, with ATD Day 0 male rats showing a significantly lower preference for the female compared with ATD Day 2 male and ATD Day 5 male rats (LSD,5% = 182 s). Controls showed no significant nocturnal rhythm in partner preference behavior. ANOVA on the time spent in the empty middle compartment during Tests 5-11 (see

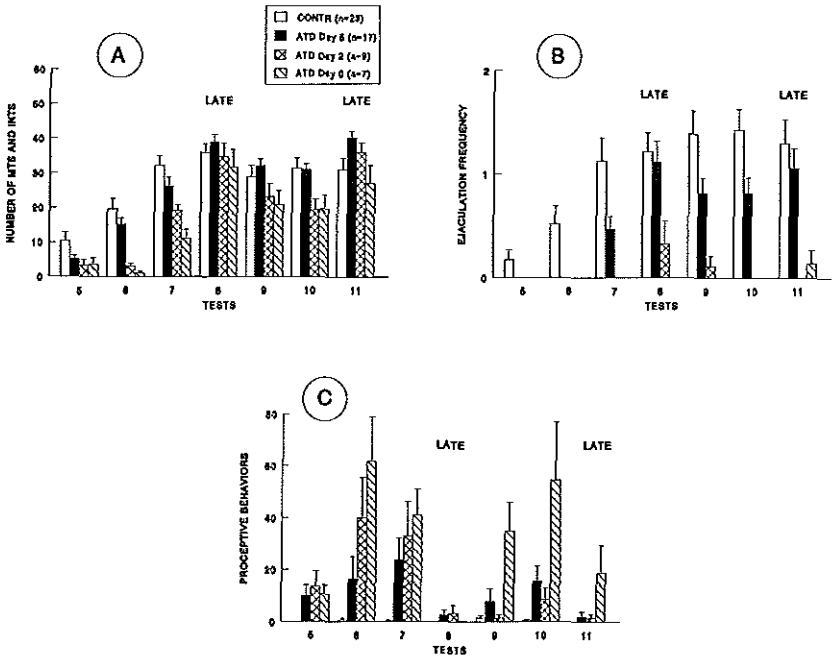

Figure 2. Sexual behaviors of neonatally ATD-treated and control (CONTR) male rats with the tethered stimulus animals during partner preference testing. (Tests 8 and 11 were performed late in the dark phase. All male rats were gonadally intact. Panel A shows the number of mounts [MTS] and intromissions [INTS] with the estrous female rat. Panel B shows the ejaculation frequency. Panel C shows the frequency of proceptive behaviors [total sum of hop and dart, presenting and earwiggling]. Values are means and SE_M. ATD = 1,4,6-androstatriene-3,17-dione.)

Figure 1D) revealed no significant differences between groups and no effect of testing.

Sexual Behavior With the Tethered Estrous Female During Partner Preference Testing

The mean numbers of mounts and intromissions are presented in Figure 2A. ANOVA indicated a significant group effect, $F(3,52) = 5.61$, $p = .002$, a significant effect of testing, $F(6,312) = 68.83$, $p < .001$, and a significant interaction, $F(18,312) = 2.38$, $p = .001$. Further analysis (LSD, 5% = 8) revealed that the number of mounts and intromissions significantly increased in all male groups from Tests 5 to 7. Group comparisons of male rats tested early in the dark phase (Tests 5-7 and 9-10), showed that ATD Day 0 male and ATD Day 2 male rats displayed significantly fewer mounts and intromissions than ATD Day 5 male and control male rats. When the animals were tested late in the dark phase (Tests 8 and 11), the number of mounts and intromissions increased significantly in all

ATD male groups, with no significant differences between the groups (LSD, 5% = 8). There was no difference in sexual activity between ATD Day 5 male and control male rats, except on Test 11. During this last late test, ATD Day 5 male rats showed significantly more mounts and intromissions than control male rats (LSD, 5% = 8).

Ejaculation behavior is shown in Figure 2B. ATD Day 0 male and ATD Day 2 male rats almost never ejaculated, whereas ATD Day 5 male and control male rats did ejaculate frequently, except during the first two tests (5 and 6), in which ATD Day 5 male rats did not ejaculate. Overall, there was no difference in the number of male rats ejaculating between the two groups: 12 out of 17 ATD Day 5 male rats ejaculated at least once versus 16 out of 23 control male rats (Fisher's exact two-tailed probability testing, $p = 1.00$). ANOVA on the ejaculation frequencies of ATD Day 5 male and control male rats (Tests 7-11) indicated a significant effect of testing, $F(4,152) = 3.65$, $p = .007$, and a significant Group x Test interaction, $F(4,152) = 2.57$, $p = .04$. Subsequent LSD analysis (5% = 0.30) revealed that early in the dark phase, ATD Day 5 male rats ejaculated significantly less frequently than controls. However, when the animals were tested late in the dark phase, these differences in ejaculation frequencies between ATD Day 5 male and control male rats had disappeared (see Figure 2B).

Sexual Behavior With the Tethered Male During Partner Preference Testing

Frequencies of proceptive behaviors are depicted in Figure 2C. ANOVA revealed a significant effect of group, $F(2,30) = 4.69$, $p = .017$, a significant effect of time of testing, $F(1,30) = 44.63$, $p < .001$, and a significant Group x Time interaction, $F(2,30) = 3.70$, $p = .037$. Subsequent LSD analysis (5% = 10) showed that ATD Day 0 males displayed significantly more proceptive behaviors than ATD Day 2 male and ATD Day 5 male rats. Control male rats rarely showed proceptive behaviors. Furthermore, proceptive behaviors were displayed in very low frequencies late in the dark phase compared with early in the dark phase.

Sexual Pair Tests With Estrous Female

The results can be seen in Figure 3. The data are presented as means over three early tests and three late tests. ANOVA on the number of mounts and intromissions (see Figure 3A) revealed a significant effect of testing, $F(1,52) = 13.22$, $p = .001$, and a significant Group x Time interaction, $F(3,52) = 14.74$, $p < .001$. Subsequent LSD analysis (5% = 4.1) revealed that ATD Day 0 male and ATD Day 2 male rats displayed a clear nocturnal rhythm in sexual activity, with ATD Day 0 male rats showing a more pronounced rhythmicity ($p = .001$) than the ATD Day 2

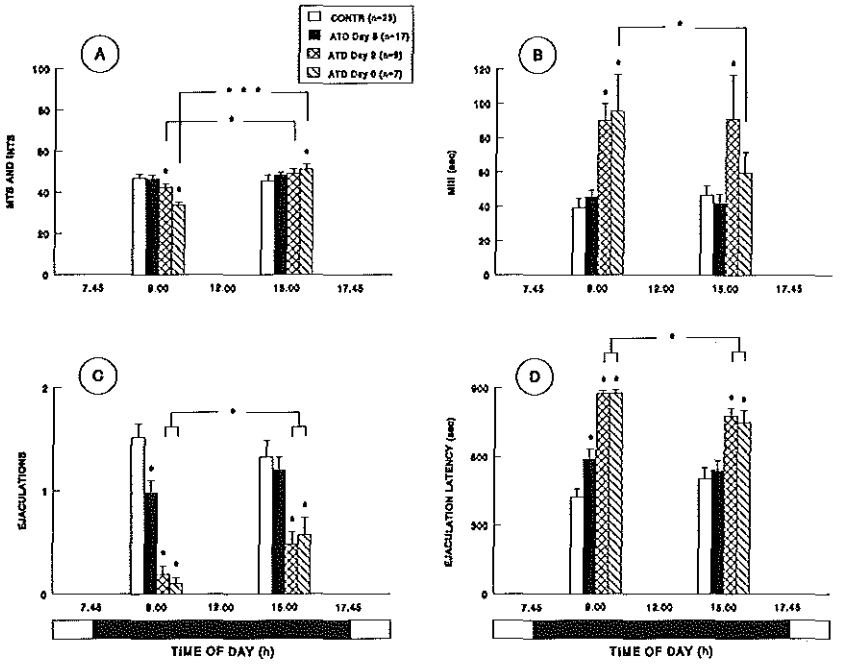

Figure 3. Various sexual behavior parameters during pair tests (15 min) with estrous female rat of ATD-treated and control (CONTR) male rats when tested early and late in the dark phase (all male rats gonadally intact). (Values are means and SE_M of three early and of three late tests. Panel A shows the number of mounts [MTS] and intromissions [INTS]. Panel B shows the mean interintromission intervals [MIII]. Panel C shows the ejaculation frequency. Panel D shows the mean ejaculation latency. * p <.05 compared with control male rats and early vs. late; *** p <.001 early vs. late. ATD = 1,4,6-androstatriene-3,17-dione.)

male rats (p =.05). In other words, the male rats of these two groups displayed more mounting and intromission behavior late in the dark phase than early in the dark phase. The number of mounts and intromissions of ATD Day 5 male and control male rats did not show any nocturnal rhythmicity.

ANOVA on the mean interintromission intervals (see Figure 3B) revealed a significant group difference, $F(3,51) = 8.42$, p <.001. Further analysis of this group effect (LSD, 5% = 34 s) showed that when tested early, ATD Day 0 male and ATD Day 2 male rats had significantly longer mean interintromission intervals than ATD Day 5 male and control male rats. When tested late, these ATD Day 0 male rats no longer differed from ATD Day 5 and control males. The mean interintromission intervals of ATD Day 2 male rats did not vary over the dark phase.

Ejaculation behavior is shown in Figure 3C. ANOVA showed a significant group effect, $F(3,52)$ = 12.96, p <.001, and a significant Group x Time interaction, $F(3,52)$ = 6.61, p =.001. LSD (5% = 0.25)

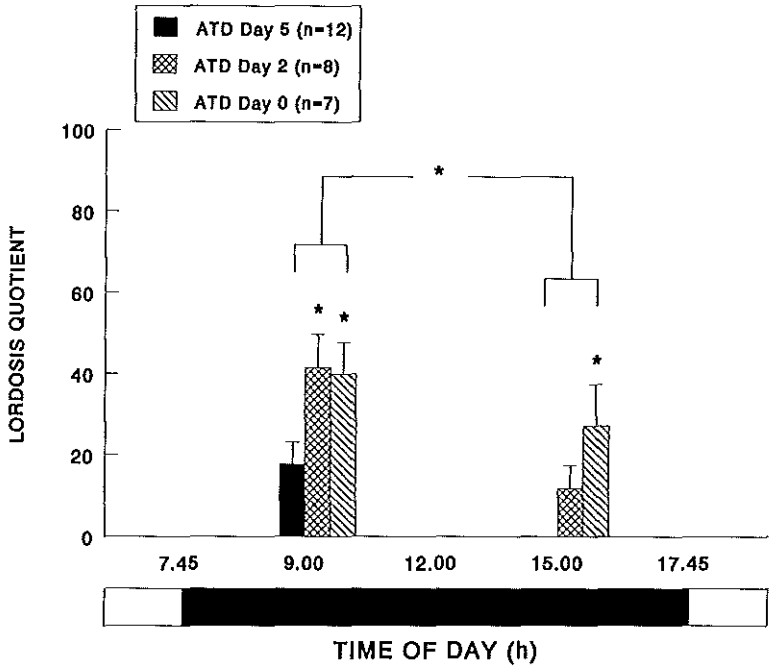

Figure 4. Lordosis quotients of adult male rats, gonadally intact and treated neonatally with ATD, during pair tests with a sexually active male rat when tested early and late in the dark phase. (Values are means and SE_M of three early and two late tests. The lordosis quotients of all male rats that received four or more mounts by the stimulus male, are presented. * $p < .05$ compared with ATD Day 5 male rats and early vs. late. ATD = 1,4,6-androstatriene-3,17-dione.)

revealed that early in the dark phase all ATD male rats showed significantly lower ejaculation frequencies than control male rats. Furthermore, the ejaculation frequencies of ATD Day 0 male and ATD Day 2 male rats were significantly higher late in the dark phase ($p = .05$).

ANOVA on the ejaculation latencies (Figure 3D) revealed a significant group effect, $F(3,52) = 15.02$, $p < .001$, and a significant Group x Time interaction, $F(3,52) = 6.96$, $p = .001$. Further analysis (LSD, 5% = 80 s) showed that early in the dark phase the ejaculation latencies of all ATD male groups were significantly longer than those of the control male rats. Ejaculation latencies of ATD Day 0 male and ATD Day 2 male rats decreased significantly in the late dark phase, although they remained significantly longer than those of ATD Day 5 male and control male rats.

Sexual Pair Tests With Active Male

The lordosis data of the different ATD male groups can be seen in Figure 4 (control males did not display lordosis behavior). ANOVA

revealed a significant effect of group, $F(2,24) = 8.14$, $p = .002$, and a significant effect of time of testing, $F(1,24) = 13.52$, $p = .001$. LSD analysis (5% = 20 s) showed a significant decrease in lordosis behavior in all ATD male groups in the late portion of the dark phase. Also, ATD Day 0 male and ATD Day 2 male rats displayed significantly more lordosis behavior than ATD Day 5 male rats.

DISCUSSION

Neonatal treatment with ATD clearly affected adult partner preference behavior when behavioral interaction with the stimulus animals was possible. This effect was most obvious in male rats that received ATD immediately after birth (ATD Day 0 male rats). Such male rats showed a preference for the male or no preference for either stimulus animal. The control male rats clearly preferred the vicinity of and behavioral interaction with the estrous female partner. Male rats that had received ATD beginning on Day 2 or 5 and through Day 14, showed partner preference scores intermediate between those of ATD Day 0 male and control male rats. Thus, inhibiting the aromatization of testosterone between Days 5 and 14 still affects adult behaviors. From this latter finding it can be concluded that the neonatally sensitive period for the organization of adult partner preference behavior extends beyond Day 5 after birth. The end of the sensitive period cannot be determined from the present data. The most significant behavioral effects, however, were obtained when the aromatization of testosterone was blocked from immediately after birth onward.

The age of cleavage of the balano-preputial skinfold from the glans penis can be used as a parameter for the onset of puberty, that is, the pubertal rise in androgen secretion. In the absence of testicular androgens, this cleavage does not occur (de Jong & van der Schoot, 1979). In the present study, there were no differences between ATD and control male rats for this pubertal parameter. Furthermore, there were no differences in relative testes weights among the four groups. This indicates that there are no differences in testicular development between ATD male and control male rats. Thus, ATD treatment does not interfere with the process of testicular development.

In adulthood, testosterone levels were significantly lower in ATD Day 0 male and ATD Day 2 male rats than in ATD Day 5 male and control male rats. This is in contrast with earlier findings in our department (Brand et al., 1991; Brand, Houtsmuller, & Slob, 1993) in which endogenous testosterone levels did not differ between adult ATD male and control male rats. We have no explanation for this difference. It

should be noted, however, that the circulating blood testosterone levels are much higher in all male rats than needed to maintain male sexual behavior (Damassa, Smith, Tennent, & Davidson, 1977).

An interesting new finding that emerged from the present study was a nocturnal rhythmicity in partner preference behavior. When tested late in the dark phase, all male rats showed a preference for the stimulus female rat, and ATD Day 2 male, ATD Day 5 male, and control male rats were not different in partner preference behavior. Only the ATD Day 0 male rats showed a significantly lower mean preference score for the estrous female rat compared with all other male rats. When tested early in the dark phase, all ATD male rats showed a lower preference for the female rat and differed significantly from control male rats in partner preference behavior. This nocturnal rhythmicity was statistically significant in all ATD-treated male rats but not in controls.

Masculine sexual behaviors during partner preference behavior testing were affected differently by neonatal ATD treatment and time of testing. Frequencies of mounts and intromissions were similar for ATD Day 5 male and control male rats and were significantly higher than those of ATD Day 2 male and ATD Day 0 male rats. The latter two groups did not differ from each other in mount and intromission frequencies. The highest ejaculation frequencies were observed in control male rats, followed by those in ATD Day 5 male rats; ATD Day 2 male and ATD Day 0 male rats hardly ever ejaculated. Differences between the male rats were smaller or no longer statistically significant when tested late in the dark phase of the LD cycle.

Neonatal ATD treatment and time of testing also clearly affected the display of feminine sexual behaviors during partner preference testing. ATD Day 0 male rats showed significantly higher frequencies of proceptive behaviors (i.e., hopping and darting, presenting and earwiggling) than ATD Day 2 male and ATD Day 5 male rats. Thus, ATD Day 0 male rats are clearly less defeminized than ATD Day 2 male and ATD Day 5 male rats. Proceptive behaviors were displayed in very low frequencies late compared with early in the dark phase.

This nocturnal rhythmicity phenomenon in sexual behavior was investigated in more detail in pair tests. Then it appeared that ATD Day 0 male and ATD Day 2 male rats showed a clear rhythmicity in masculine behaviors, with the highest frequencies occurring late in the dark phase; ATD Day 5 male and control male rats did not show such rhythmicity. The latter two groups were overall sexually more active and ejaculated more frequently during testing than did ATD Day 0 male and ATD Day 2 male rats. Furthermore, ATD Day 5 male and control male rats showed significantly shorter mean intervals between the intromissions than did ATD Day 0 male and ATD Day 2 male rats. Late in the dark phase,

however, ATD Day 0 male rats showed shortened mean interintromission intervals and were no longer different from ATD Day 5 male and control male rats. At the same time, ATD Day 0 male rats started to ejaculate. One could hypothesize that the mean length of the intervals between the intromissions could be a critical factor in triggering the ejaculation response.

Feminine behavior, that is, lordosis, also showed a nocturnal rhythmicity in all ATD male rats, with the highest frequencies occurring early in the dark phase. Control male rats did not display lordosis behavior.

It is of interest to see that these gonadally intact ATD males are behaviorally less 'masculine' and more 'feminine' early in the dark phase, whereas they become more 'masculine' and less 'feminine' toward the end of the dark phase. Could this be explained by changes in endogenous hormone levels, that is, in steroid hormones from testes or adrenals? Future research should address this question.

The partner preference data obtained in ATD-treated males are in line with earlier results from our laboratory (e.g., Brand et al., 1991). They differ from the findings reported by Davis et al. (1979), who did not discern a difference in partner preference behavior and masculine behaviors between ATD Day 1 male and control male rats. It was earlier postulated that the onset of ATD treatment at Day 1 was too late (Brand et al., 1991). In light of the present results, however, with the ATD Day 2 males clearly affected in partner preference and masculine behavior, we no longer support this supposition. Through the present results, that is, the finding of a nocturnal rhythmicity, another explanation comes forward. Davis et al. (1979) tested their males for masculine behavior and presumably also for partner preference behavior late in the dark period. From the present study it is clear that the differences between ATD Day 2 male, ATD Day 5 male, and control male rats for partner preference behavior and masculine behavior have disappeared or become much smaller in the late portion of the dark phase. This could explain why Davis et al. (1979) failed to find significant effects of neonatal ATD treatment on masculine and partner preference behavior.

In the present study male rats did not show any partner preference when behavioral interaction was prevented (see Figure 1A). This finding differs from an earlier study (Brand et al., 1991) in which ATD male and control male rats preferred the vicinity of the female stimulus animal behind a wire mesh separation, albeit the ATD male rats showed significantly lower preference scores than the control male rats. We have no explanation for this discrepancy, other than a difference in the substrains of rats used; the present study used Wistar R strain (locally bred) rats, and the previous study used Wistar RP strain (commercially bred) rats.

The time spent in the empty middle compartment increased significantly in all male groups during partner preference testing with stimulus animals behind a wire mesh separation. Without the possibility of behavioral interaction the males became somewhat less 'social'. This seemed to be more apparent in ATD male than in control male rats. With behavioral interaction possible, the significant differences between ATD male and control male rats disappeared.

The nocturnal rhythmicity findings of the present experiment warrant some discussion. It was earlier suggested that a sex difference existed in a diurnal rhythm in rat sexual behaviors (Hansen et al., 1979; Södersten & Eneroth, 1980). This diurnal rhythm was presumably organized neonatally by presence or absence of testicular androgens, because neonatal castration made adult male rats show this rhythm whereas intact males did not (Hansen et al., 1979). Neonatal testosterone treatment abolished this diurnal rhythm in female rats (Hansen et al., 1979). In our male rats (the controls in the present study) there was no significant nocturnal rhythmicity in partner preference and sexual behavior, in contrast to findings of other studies (Dewsbury, 1968; Harlan et al., 1980; Södersten & Eneroth, 1980). When treated with ATD neonatally, such male rats show a nocturnal rhythm in adulthood. The earlier the ATD treatment commences, the more outspoken this rhythm is. This strongly suggests that in normal male rats the endogenous E_2 metabolite of testosterone suppresses the organization of an adult nocturnal rhythm as was also suggested for a diurnal rhythm by Södersten and Eneroth (1980). Another way of interpreting the data is to assume that neonatal E_2 organizes males to behave sexually more constantly throughout the nocturnal period.

As was discussed earlier, ATD Day 0 male and Day 2 male rats had significantly lower blood testosterone levels. One could hypothesize that the nocturnal rhythmicity in these males reflects rhythms in circulating testosterone levels, which indicates an activational effect. This seems unlikely, because the highest testosterone concentrations were reported during the late portion of the light phase (when sex behaviors were reported to be lowest) and the lowest testosterone concentrations occurred during the late portion of the dark phase (when sex behaviors were reported to be highest) (Harlan et al., 1980; Södersten, Eneroth, & Ekberg, 1980). This makes it more likely that the rhythmicity reflects an organizational rather than an activational effect of neonatal ATD treatment on the eventual expression of behaviors.

We can conclude that ATD males are less masculinized and less defeminized not only with respect to partner preference behavior and sexual behavior, but also with regard to the nocturnal rhythmicity phenomenon. This latter manifestation can be added to the list of

behaviors in the male rat that are organized neonatally by the presence or absence of the endogenous estradiol metabolite of testosterone.

Finally, the earlier the ATD treatment starts after birth, the more 'bisexual' these male rats remain. Such males grow up to be adults which can readily display complementary sexual behaviors depending on the mating partner. One could say that these intact males have kept their bisexual potency (Beach, 1979; Goy & Goldfoot, 1975).

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Chapter VII

ENDOGENOUS REPRODUCTIVE HORMONES AND NOCTURNAL RHYTHMS IN PARTNER PREFERENCE AND SEXUAL BEHAVIOR OF ATD-TREATED MALE RATS

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ABSTRACT

Male rats received subcutaneously silastic capsules, containing the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD), shortly after birth. Control males were given silastic capsules containing cholesterol. The capsules were removed at the age of 21 days. In adulthood, blood serum was collected early and late in the dark phase of the light/dark cycle (experiment I). Testosterone and luteinizing hormone (LH) and follicle stimulating hormone (FSH) fluctuated nocturnally, both in ATD and control males, with highest levels late in the dark phase. FSH levels were significantly higher in ATD males. Nocturnal levels of inhibin, a selective suppressor of pituitary FSH secretion also fluctuated in both ATD and control males, with lowest levels late in the dark phase. In experiment II, ATD and control males were tested for partner preference behavior in a three-compartment box (choice: sexually active male vs. estrous female) early and late in the dark phase. When gonadally intact, ATD males, but not controls, showed a clear nocturnal rhythmicity in partner preference behavior and sexual behavior. Early in the dark phase, such ATD males preferred the vicinity of and interaction with a sexually active male. Late in the dark phase, this preference for the active male shifted to a preference for the estrous female. Control males preferred the estrous female. After castration and subsequent treatment with testosterone via silastic capsules, which ensured constant blood serum levels, ATD males continued to show their nocturnal rhythms in partner preference behavior and in sexual behavior. Thus, the underlying mechanism of the nocturnal rhythmicity phenomenon is an organizational effect of neonatal ATD treat-

ment rather than an activational effect of fluctuating serum hormone levels.

INTRODUCTION

Neonatal treatment of male rats with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) interferes with the sexual differentiation of the Central Nervous System (CNS), but not with the somatic development [1]. In adulthood, such ATD males display male sexual behavior when pair-tested with an estrous female and female sexual behavior when pair-tested with a sexually active male [1-4]. Neonatal ATD treatment also affects adult partner preference behavior: gonadally intact adult ATD males show a significantly lower preference for the estrous female partner than control males, when tested for partner preference behavior in a three compartment box (choice: estrous female vs active male) [4,5].

Recently we found that gonadally intact ATD males, but not control males, show a nocturnal rhythmicity in sexual partner preference and related behaviors [3]. Early in the dark phase of the light-dark (LD) cycle, ATD males show no clear preference: they interact frequently with the active male, readily showing female sexual behaviors, but they also mount and intromit the estrous female partner, without ejaculating, however. Late in the dark phase they resemble control males: they prefer the vicinity of the estrous female partner and readily copulate, including ejaculating, but they do not interact actively with the male partner [3]. It seems that these ATD males are early in the dark phase behaviorally more 'feminine' and less 'masculine', while they behave more 'masculine' and less 'feminine' towards the end of the dark phase. This nocturnal behavioral rhythmicity was most outspoken in ATD males treated from directly after birth compared to males treated with ATD from Days 2 or 5 onward [3]. These results indicate that in normal male rats neonatal estradiol, derived from endogenous testosterone, suppresses the 'organization' of an adult nocturnal rhythm in sexual and partner preference behavior, as was suggested earlier for a diurnal rhythm in sexual behavior [6].

We reported earlier that adult endogenous blood testosterone (T) levels were lower in ATD males than in controls [3], but were still within the range needed for the activation of normal male sexual behavior [7]. Blood was collected early in the dark phase, which is the phase when ATD males normally exhibit low levels of masculine sexual behavior [3]. We suggested that the nocturnal rhythmicity in partner preference and sexual behavior, as shown by gonadally intact ATD males, could reflect rhythms in circulating endogenous testosterone, and possibly

gonadotrophins, i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH).

In the present study, therefore, we investigated whether or not the nocturnal rhythmicity in adult partner preference behavior of ATD males, parallels rhythmic changes in endogenous reproductive hormones i.e. testosterone, FSH and LH. To that end, blood was collected early and late in the dark phase in adult ATD and control males which were not exposed to behavioral tests (experiment I). Another group of adult ATD and control males were tested for partner preference behavior before castration and after castration with subsequent testosterone treatment using silastic capsules (experiment II). The latter treatment ensures a constant blood testosterone level, presumably eliminating any rhythm in testosterone and gonadotrophins.

METHODS

Animals

Experimental animals and stimulus females were Wistar albino rats (RP strain, inbred) bred in our laboratory. The male stimulus animals were commercially obtained (Harlan, Zeist) Wistar albino rats (Wu strain, outbred). Two to four rats of the same sex and treatment were housed in a cage with food and water available ad libitum. All rats were kept in a reversed 14-hour/10-hour light (L)/dark (D) cycle (lights off from 07.45 to 17.45). The temperature in the animal room ranged from 20 to 22°C.

Treatments

Female rats were time mated and parturition occurred 22 days later. Within 2-4 h after birth (the period of high endogenous testosterone levels in the blood [8,9]), small silastic capsules (inner diameter 1.5 mm; outer diameter 2.1 mm; length 5 mm) containing ATD or cholesterol were implanted s.c. in the back of the newborn males under ice anesthesia (one treatment per litter). The implants were removed at 21 days of age under light ether anesthesia. In experiment I, 9 ATD (out of 4 litters) and 11 control males (out of 4 litters) were used. In the behavioral experiment (experiment II), 9 ATD (out of 3 litters) and 9 control (out of 3 litters) males were used.

The pups were weaned at 21 days of age and then housed in a cage with 2-3 conspecifics of the same treatment. They were left undisturbed until the onset of behavioral testing, at about 6 months of age (experiment II).

Following 8 behavioral tests, at the age of approximately 7 months, the males were castrated under ether anesthesia through a midline abdominal incision. At the same time, silastic capsules (inner diameter 1.5 mm;

outer diameter 2.1 mm; length 7 mm) containing testosterone were implanted s.c.

The stimulus animals were sexually active males and estrous females. The latter were ovariectomized and brought into behavioral estrus with an injection of 20 g of estradiol benzoate (EB) 24-48 h prior to testing, followed by an injection of 1.0 mg progesterone (P) 3-4 h before testing. These hormones were dissolved in olive oil and injected s.c. in the neck. Stimulus animals were used once a week.

Behavioral testing

In the partner preference behavior test, a test box made of gray perspex with a transparent front was used [10]: it had three compartments (60 x 30 x 40 cm) with a small opening (13 x 12 cm) in both partitions near the back. These openings could be closed by a sliding door. Stimulus animals were tethered with a rope to the front of one of the lateral compartments. The tethered animals thus had a limited action radius.

Before testing, 2 stimulus animals and the experimental animal were put in the test apparatus, one in each compartment, with the sliding doors closed, for a 15-min adaptation period. At the beginning of the test (which lasted 15 min), the sliding doors were removed, and the experimental animal could freely move around and interact with the stimulus animals. The time that the experimental animal spent in each compartment was recorded automatically. Movement of the experimental animal from one compartment to another, was registered with position-sensitive tilt platforms situated in each opening. The position of the tilt-platform was determined using a magnetic field [for further details, see 10]. Software automatically calculated a preference score, and counted the number of visits the freely moving animal paid to each compartment. Various sexual behaviors with the stimulus animals were scored manually by 3 trained observers, of which 1 'supervised' the other 2.

Preference scores were calculated by subtracting the time spent in the compartment containing the active male from the time spent in the compartment containing the estrous female. Thus, a positive score indicates a preference for the estrous female, a negative score for the active male.

Test procedures

Behavioral tests started at the age of approximately 6 months (experiment II). In order to get sexual experience, the males were first subjected to two pair tests with an estrous female, one early and one late in the dark phase (15-min duration; all males displayed intromission behavior) and two pair tests with an active male: early and late in the dark phase (5 of 9 ATD males, but none of the control males, showed lordosis behavior).

After these pair-tests, the males were subjected to a total of 8 partner preference tests twice weekly. Tests 1E (E='early'), 3E, 6E, and 8E were carried out in the early part of the dark phase (between 09.00-11.00 h). Tests 2L (L='late'), 4L, 5L, and 7L were performed in the late part of the dark phase (between 14.00-16.00 h).

After castration and subsequent testosterone treatment, the males again received a total of 8 partner preference tests on a twice weekly basis. Tests 9E, 11E, 14E and 16E were executed early in the dark phase, tests 10L, 12L, 13L and 15L late in the dark phase.

Blood collection and hormone assay

Blood was collected from the orbital plexus under light ether anesthesia from 9 gonadally intact ATD and 11 control males at the age of approximately 9 months (experiment I; homecage animals: no concomitant behavioral tests). Blood collection took place on two different occasions early in the dark phase (between 9.00-10.00 h), and twice late in the dark phase (between 15.00-16.00 h). From the 9 ATD and 9 control males (at the age of 6-7 months) that were subjected to behavioral testing (experiment II), blood was sampled before castration (early and late in the dark phase), and at 2, 4 and 8 weeks after castration and subsequent testosterone treatment. Blood collection took place at least 24 h after behavioral testing. Blood serum testosterone levels were estimated by radioimmunoassay [11]. The serum was first extracted with n-hexane-ether before it was used in the assay. The antibodies raised against testosterone-3-(0-carboxymethyl)-oxime-bovine serum albumin in female rabbits, had a cross reactivity with dihydrotestosterone of 56% [for more details see 11]. The interassay and intra-assay coefficients of variation were 13.4 and 8%, respectively. FSH and LH were measured by radioimmunoassay as described by Grootenhuis et al. [12], using the antibodies developed by Welschen et al. [13]. All results are expressed in terms of NIAADDK-rat FSH-RP-3 and NIAADDK-rat LH-RP-2. The intra-assay coefficients for FSH and LH were 17.9 and 11.4%, respectively. Inhibin was measured by radioimmunoassay as described by Robertson et al. [14], using an antiserum against purified 32kDa bovine follicular fluid (bFF) inhibin and iodinated 32 kDa bFF inhibin. These materials were purchased from Drs Robertson and de Kretser, Department of Anatomy, Monash University, Melbourne, Australia. A sample of bovine follicular fluid with an arbitrary potency of 1U/g protein [12] was used as a standard. The intra-assay coefficient was 18.3%.

Statistics

The data were subjected to 2- or 3-way analyses of variance (ANOVA) for repeated measures, followed by the least significant difference

procedure when significant overall differences were found [15]. Only significant F values are presented. The $p = 0.05$ level is used as the upper limit for statistical significance.

RESULTS

Experiment I

Various reproductive hormone data of gonadally intact ATD and control males, which were not concomitantly exposed to behavioral testing, are shown in Table 1. Statistical analyses (two-way ANOVA with variables groups and time of blood collection) revealed a significant effect of time of blood collection for testosterone ($F(1/18) = 105.3$, $p < 0.001$), FSH ($F(1/18) = 36.7$, $p < 0.001$), LH ($F(1/18) = 66.3$, $p < 0.001$) and Inhibin ($F(1/18) = 16.2$, $p < 0.001$). For FSH levels there was also a significant group difference ($F(1/18) = 12.0$, $p = 0.003$). Thus, there appeared to be a nocturnal rhythm in testosterone, FSH and LH levels in both ATD and control males with highest levels late in the dark phase. Inhibin also fluctuated nocturnally with highest levels early in the dark

Table 1: Various adult endogenous blood serum reproductive hormone levels (mean ± SEM) of neonatally ATD-treated and control male rats. The males were gonadally intact and not exposed to behavioral testing (homecage animals). T=testosterone, FSH= follicle stimulating hormone, LH= luteotropic hormone. Animals were maintained on a 14-hr/10-hr light/dark cycle (lights off from 07.45 to 17.45). Early: blood-samplings (2 occassions, 1 week apart) took place between 09.00-10.00; Late: blood collections (2 occassions, 1 week apart) took place between 15.00-16.00.

Hormone	Controls (n=11)	ATD males (n=9)	Statistics 2-way ANOVA
T (nmol/l)			
early	5.0 ± 0.6	5.0 ± 0.5	groups: ns
late	9.7 ± 0.7	9.2 ± 0.4	time: p<0.001
			gr x ti: ns
FSH (ng/ml)			
early	9.7 ± 0.2	11.9 ± 0.5	groups: p=0.003
late	12.0 ± 0.4	13.7 ± 0.7	time: p<0.001
			gr x ti: ns
LH (ng/ml)			
early	0.8 ± 0.1	1.0 ± 0.1	groups: ns
late	2.8 ± 0.3	2.5 ± 0.3	time: p<0.001
			gr x ti: ns
Inhibin (U/ml)			
early	2.5 ± 0.2	2.7 ± 0.3	groups: ns
late	1.6 ± 0.1	1.9 ± 0.2	time: p<0.001
			gr x ti: ns

phase. ATD and control males showed similar levels of endogenous testosterone, LH and Inhibin. FSH levels differed: in ATD males these were significantly higher than in control males.

Experiment II

Hormone Data

Table 2 shows blood T levels of ATD and control males, exposed to partner preference testing, before castration and after castration with subsequent testosterone treatment. In gonadally intact animals, endogenous T levels fluctuated nocturnally (two-way ANOVA, time of blood collection: $F(1/16) = 5.7$, $p = 0.03$): late in the dark phase testosterone levels were higher than early in the dark phase. Following castration and implantation of testosterone implants, there was a significant decrease in testosterone levels in the weeks following implantation in both ATD and control males [two-way ANOVA, tests: $F(2/32) = 23.0$, $p<0.001$; least significant difference (5%) = 0.63]. There were no nocturnal fluctuations in testosterone levels.

Partner Preference Behavior before Castration

Partner preference behavior data are depicted in figure 1. Three-way ANOVA (variables: groups, tests, time of testing) of the preference scores (fig. 1a) revealed a significant group difference ($F(1/16) = 18.9$, $p < 0.001$), a significant effect of testing ($F(3/48) = 6.0$, $p = 0.001$), a significant effect of time of testing ($F(1/16) = 33.0$, $p < 0.001$) and an almost significant group x time interaction ($F(1/16) = 4$, $p = 0.06$). Overall, ATD males showed significantly lower preference scores than control males. Furthermore, the preference for the estrous female decreased significantly in all males over tests. Subsequent least significant difference analysis (5% = 216 s) of the group x time interaction, revealed that there was a significant nocturnal rhythmicity in partner preference behavior in ATD males, but not in control males. In the early part of the dark phase: the ATD males showed a preference for the stimulus male, in the late part of the dark phase they showed a preference for the stimulus female. In figure 1a, total means and SEM of the 4 'early' and of the 4 'late' tests are also shown for each group.

The time spent in the empty middle compartment is shown in figure 1b. Three-way ANOVA (variables: groups, tests, time of testing, time in middle compartment) showed only a significant group x test interaction ($F(3/48) = 3.2$, $p = 0.03$). Subsequent least significant difference (5% = 125 s) analysis of this interaction revealed that the time spent in the middle compartment decreased over tests in ATD males, but not in control males. In figure 1b, total means and SEM of the 4 'early' and of

Table 2: Blood serum testosterone levels (mean ± SEM) of neonatally ATD-treated and control male rats before castration (blood was collected early and late in the dark phase) and after castration and subsequent testosterone treatment through silastic capsules.

Group	n	T, nmol/l				
		before castration		after castration + T treatment		
		early	late	early (2 weeks)	late (4 weeks)	early (8 weeks)
ATD	9	8.3 ± 1.6	9.3 ± 0.9*	6.0 ± 0.4	4.3 ± 0.3+	4.9 ± 0.3+
Cont	9	5.9 ± 0.7	9.9 ± 1.2*	5.1 ± 0.3	3.9 ± 0.2+	4.2 ± 0.3+

Blood samples were collected at various weeks after onset of testosterone (T) treatment. Blood was collected approximately 24 h following behavioral testing.
* Significantly different from 'early' ($p = 0.03$)
+ Significantly different from 'early (2 weeks)' ($p < 0.05$).

the 4 'late' tests are shown for each group.

The total number of excursions, i.e. the sum of the number of excursions to the stimulus male, to the stimulus female and to the empty middle compartment, are depicted in figure 1c. Three-way ANOVA (variables: groups, tests, time of testing) on the total number of excursions revealed a significant group difference ($F(1/16) = 113.6$, $p < 0.001$), i.e. ATD males were much more active than controls. Three-way ANOVA on the number of excursions to the stimulus male (black bars in fig. 1c) showed a significant group difference ($F(1/16) = 56.1$, $p < 0.001$), a significant effect of time of testing ($F(1/16) = 10.3$, $p = 0.005$), an almost significant group x time interaction ($F(1/16) = 4.2$, $p = 0.06$) and a significant test x time interaction ($F(3/48) = 3.7$, $p = 0.02$). ATD males paid significantly more visits to the compartment containing the stimulus male than control males. When tested in the late part of the dark phase, the number of visits to the stimulus male decreased significantly in ATD males, but not in control males (least significant difference (5% = 5).

Three-way ANOVA on the number of excursions to the stimulus female (hatched bars in fig. 1c) showed a significant group difference ($F(1/16) = 48.5$, $p < 0.001$) and a significant effect of time of testing ($F(1/16) = 10.8$, $p = 0.005$). ATD males paid more visits to the stimulus female than control males. When tested late in the dark phase, the number of excursions to the stimulus female increased in both groups of males. Three-way ANOVA on the number of excursions to the empty middle compartment (open bars in fig. 1c) revealed only a significant overall group difference ($F(1/16) = 114.2$, $p < 0.001$), i.e. ATD males paid more visits to the empty middle compartment than control males.

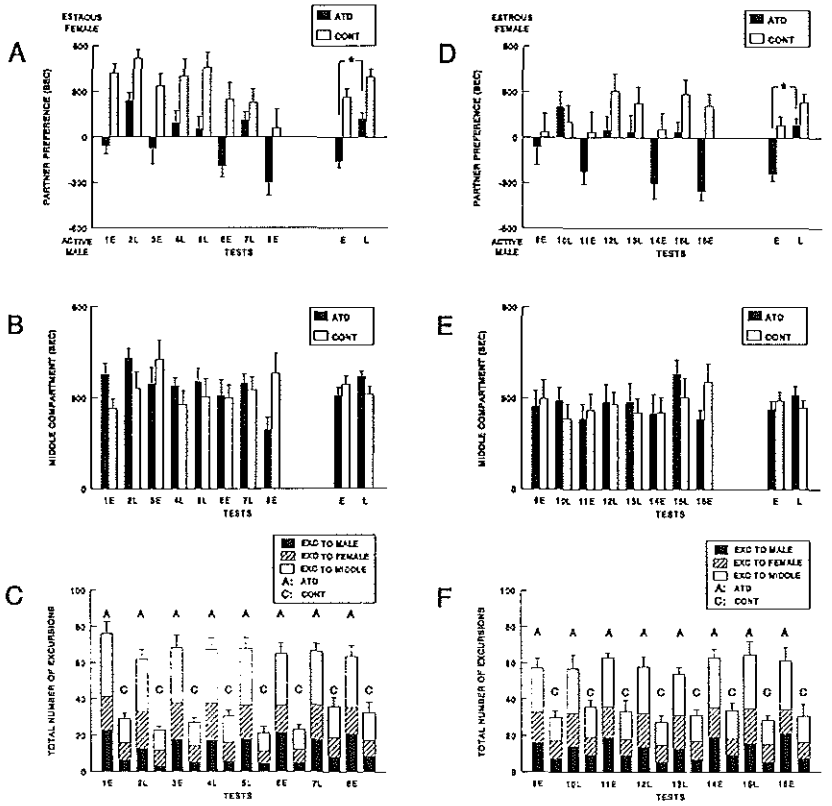

Fig. 1. Partner preference behavior in a three-compartment box (3CB) of adult male rats, neonatally treated with the aromatization blocker ATD or cholesterol (controls, C), before (tests 1-8; **a-c**) and after castration with subsequent testosterone (T) treatment (tests 9-16; **d-f**). Tests 1E, 3E, 6E, 8E, 9E, 11E, 14E and 16E were carried out early in the dark phase of the light/dark cycle; tests 2L, 4L, 5L, 7L, 10L, 12L, 13L, 15L late in the dark phase. **a** Partner preference behavior before castration. **b** Time spent in the empty middle compartment. **c** Total number of excursions, i.e. the number of excursions to the stimulus male, to the stimulus female and to the empty middle compartment. **d** Partner preference behavior after castration with subsequent T treatment. **e** Time spent in the empty middle compartment. **f** Total number of excursions, i.e. the number of excursions to the stimulus male, to the stimulus female and to the empty middle compartment. **a,b,d,e** Total means (+ SEM) of the 4 'early' (E) and 4 'late' (L) tests are shown (right-hand side).

Sexual Behavior with the Stimulus Female during Partner Preference Testing

The mean (+ SEM) number of mounts plus intromissions is presented in figure 2a. Three-way ANOVA (variables: groups, tests, time of testing, number of mounts and intromissions) indicated a significant effect of time of testing ($F(1/16) = 14.1$, $p = 0.002$), and a significant group x time interaction ($F(1/16) = 15.2$, $p = 0.001$). Further (least significant difference (5%) = 8) analysis revealed that there was a significant nocturnal rhythmicity in the number of mounts and

intromissions in ATD males, but not in control males. Late in the dark phase, ATD males displayed more mounting and intromission behavior than early in the dark phase. In figure 2a, total means and SEM of the 4 'early' and of the 4 'late' tests are also shown for each group.

Ejaculation frequency is shown in figure 2b. Three-way ANOVA (variables: groups, tests, time of testing, ejaculation frequencies) revealed a significant group difference ($F(1/16) = 40.7$, $p < 0.001$), a significant effect of testing ($F(3/48) = 9.9$, $p < 0.001$), a significant group x test interaction ($F(3/48) = 5.7$, $p = 0.002$), a significant effect of time of testing ($F(1/16) = 4.5$, $p = 0.05$), a significant group x time interaction ($F(1/16) = 26.4$, $p < 0.001$) and a significant test x time interaction ($F(3/48) = 5.0$, $p = 0.004$). Overall, ATD males ejaculated less frequently than control males. Further analysis (least significant difference (5%) = 0.5) of the group x test interaction showed that the number of ejaculations increased significantly in control males over tests, but not in ATD males. Least significant difference (5% = 0.6) analysis of the group x time interaction revealed a clear nocturnal rhythm in ejaculation frequencies of ATD males, but not in controls. Late in the dark phase, ATD males ejaculated more frequently than early in the dark phase. In figure 2b, total means and SEM of the 4 'early' and of the 4 'late' tests are also shown for each group.

Sexual Behavior with Stimulus Male during Partner Preference Testing

Estrous behaviors i.e. the sum of proceptive (presenting, earwiggling and hopping and darting) and receptive (lordosis) behaviors, are shown in figure 2c. Only ATD males showed these feminine sexual behaviors. Two-way ANOVA (variables: tests, time of testing; estrous behaviors) indicated a significant effect of time of testing ($F(1/8) = 10.2$, $p = 0.01$). Estrous behaviors were displayed in much lower frequencies late in the dark phase than early in the dark phase. In figure 2c, total means of the 4 'early' and of the 4 'late' tests are also shown.

Effects of Castration and Subsequent Treatment with Testosterone

Partner preference data after castration and subsequent testosterone treatment are shown in figure 1d. Three-way ANOVA (variables: groups, tests, time of testing) on the preference scores (see fig. 1d) indicated an almost significant group effect ($F(1/16) = 4.1$, $p = 0.06$), a significant group x test interaction ($F(3/48) = 3.6$, $p = 0.02$), a significant effect of time of testing ($F(1/16) = 41.3$, $p < 0.001$), and a significant group x time interaction ($F(1/16) = 5.3$, $p = 0.04$). Further least significant difference (5% = 254 s) analysis of the group x test interaction revealed that in ATD males the preference for the stimulus male increased over tests, whereas in control males the preference for the stimulus female increased over

tests. Furthermore, ATD males, but not control males, showed a clear nocturnal rhythmicity in partner preference behavior. Early in the dark phase they preferred a sexually active male, late in the dark phase, they showed no clear preference or a very low preference for an estrous female. In figure 1d, total means and SEM of the 4 'early' and of the 4 'late' tests are also shown for each group.

The time spent in the empty middle compartment (fig. 1e) did not differ between ATD and control males nor was there an effect of time of testing (three-way ANOVA). In figure 1e, means and SEM of the 4 'early' and of the 4 'late' tests are shown for each group.

Three-way ANOVA (variables: groups, tests, time of testing) on the total number of excursions, i.e the sum of the number of excursions to the stimulus male, to the stimulus female and to the empty middle compartment (fig. 1f), indicated a significant group effect ($F(1/16) = 42.4$, $p < 0.001$). ATD males were more active than controls. Three-way ANOVA on the number of excursions to the stimulus male (black bars in fig. 1f) indicated a significant group difference ($F(1/16) = 17.8$, $p = 0.001$) and a significant effect of time of testing ($F(1/16) = 10.3$, $p = 0.006$). ATD males paid more visits to the compartment containing the stimulus male than control males. Furthermore, in late tests compared to early tests, the number of excursions to the stimulus male were lowest in all males.

Three-way ANOVA on the number of excursions to the stimulus female (hatched bars in fig. 1f) revealed significant group differences ($F(1/16) = 50.8$, $p < 0.001$), a significant effect of time of testing ($F(1/16) = 18.1$, $p = 0.001$) and a significant group x time interaction ($F(1/16) = 7.1$, $p = 0.017$). Subsequent least significant difference ($5\% = 2$) analysis of the group x time interaction showed that late in the dark phase, the number of excursions to the estrous female increased significantly in ATD males, but not in control males. ATD males paid more visits to the compartment with the estrous female compared to controls. Three-way ANOVA on the number of excursions to the middle compartment (open bars in fig. 1f) showed a significant group effect ($F(1/16) = 36.7$, $p < 0.001$) i.e. ATD males visited the empty middle compartment more frequently than controls.

Sexual Behavior with the Stimulus Female during Partner Preference Testing

The mean (+SEM) total number of mounts plus intromissions can be seen in figure 2d. Three-way ANOVA (variables: groups, tests, time of testing, number of mounts and intromissions) indicated a significant effect of time of testing ($F(1/16) = 46.5$, $p < 0.001$) and a significant group x time interaction ($F(1/16) = 33.2$, $p < 0.001$). Least significant difference ($5\% = 6$) analysis of this interaction revealed a clear nocturnal rhythmicity

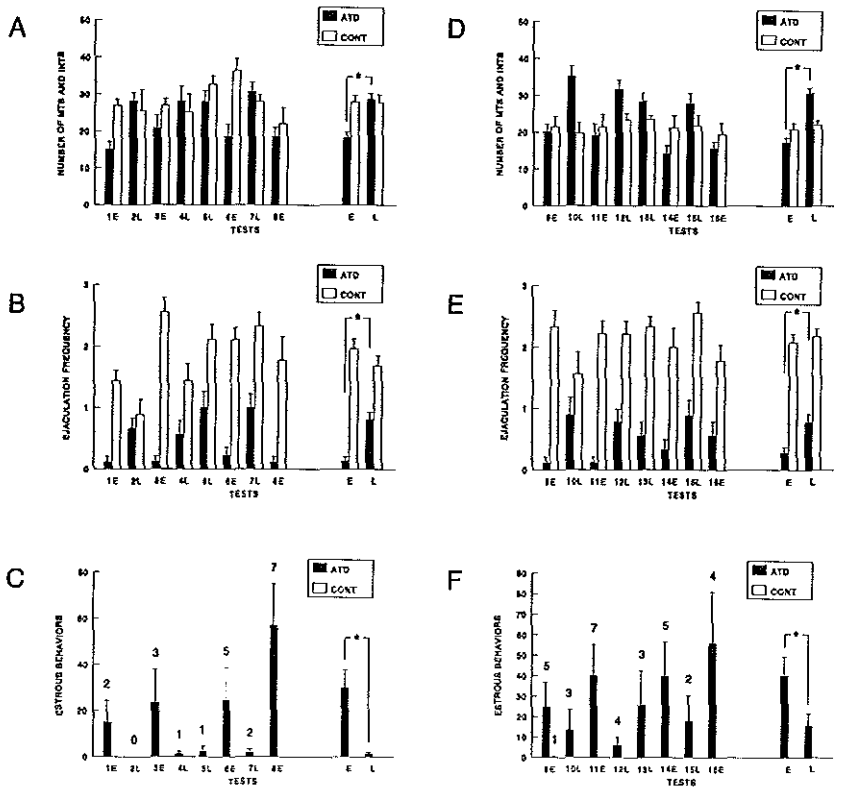

Fig. 2. Sexual behavior of neonatally ATD-treated and control (C) male rats with the tethered stimulus animals during partner preference testing before (tests 1-8; a-c) and after castration with subsequent testosterone (T) treatment (tests 9-16; d-f). Tests 1E, 3E, 6E, 8E, 9E, 11E, 14E and 16E were performed early in the dark phase; tests 2L, 4L, 5L, 7L, 10L, 12L, 13L and 15L late in the dark phase. a Total number of mounts plus intromissions with the estrous female before castration. b Number of ejaculations. c Estrous behaviors, i.e. the sum of proceptive and receptive behaviors, displayed by ATD males in the compartment with the stimulus male. d Total number of mounts plus intromissions with the estrous female after castration with subsequent T treatment. e Number of ejaculations. f Estrous behaviors, i.e. the sum of proceptive and receptive behaviors, displayed by ATD males in the compartment with the stimulus male. a,b,d,e Total means (+ SEM) of the 4 'early' (E) and 4 'late' (L) tests are shown (right-hand side). The numbers above the columns (c, f) represent the number of animals displaying estrous behaviors.

in number of mounts and intromissions in ATD males, but not in control males. Late in the dark phase, ATD males displayed significantly more mounting and intromission behavior than early in the dark phase. In figure 2d, total means of the 4 'early' and of the 4 'late' tests are also shown for each group.

Three-way ANOVA (variables: groups, tests, time of testing) on the number of ejaculations (fig. 2e) showed a significant group effect ($F(1/16) = 70.16$, $p < 0.001$), a significant effect of time of testing ($F(1/16) =$

7, p = 0.018) and a significant group x test x time interaction (F(3/48) = 4.1, p = 0.012). Subsequent least significant difference (5% = 0.6) analysis of this latter interaction showed nocturnal fluctuations in ejaculation frequencies in ATD males between Tests 9-12 with highest frequencies late in the dark phase. Furthermore, the number of ejaculations increased significantly in ATD males over tests. For control males, the number of ejaculations decreased significantly in Test 10L compared to 9E and in Test 16E compared to 15L. In figure 2e, total means of the 4 'early' and of the 4 'late' tests are also shown for each group.

Sexual Behavior with the Stimulus Male during Partner Preference Testing

Estrous behaviors, i.e. the sum of proceptive (presenting, earwiggling and hopping and darting) and receptive (lordosis), are shown in figure 2f. Only ATD males showed regularly feminine sexual behaviors. Two-way ANOVA (variables: tests, time of testing, estrous behaviors) indicated a significant effect of time of testing (F(1/8) = 5.8, p = 0.043) i.e. highest frequencies of estrous behaviors were shown in the early part of the dark period.

DISCUSSION

Neonatal treatment in the male rat with the aromatase inhibitor ATD clearly interfered not only with the processes of masculinization and defeminization of sexual behavior and with the organization of partner preference behavior, but also with the suppression of a nocturnal rhythmicity in partner preference and sexual behavior. ATD males, but not control males, showed behavioral differences over the dark phase. Early in the dark phase, ATD males showed a preference for the stimulus male, displayed low levels of masculine sexual behavior with the estrous female and high levels of feminine sexual behavior with the stimulus male. Late in the dark phase, they showed either no preference or a low preference for the estrous female and displayed concomitantly high levels of masculine sexual behaviors and low levels of feminine sexual behaviors. After castration and subsequent testosterone treatment through silastic capsules, similar results were obtained: ATD males continued to show a clear nocturnal rhythmicity in partner preference and sexual behavior.

Endogenous reproductive hormones, i.e. testosterone, FSH and LH, showed nocturnal fluctuations with highest levels late in the dark phase. Inhibin, a selective suppressor of pituitary FSH secretion, also showed a nocturnal rhythmicity with highest levels early in the dark phase. ATD

and control males showed similar levels of testosterone, LH and inhibin. FSH levels differed: ATD males had significantly higher levels than control males. The present testosterone data, i.e. similar levels in controls and ATD males, are at variance with our earlier data [3], i.e. lower levels in ATD males compared to control males. We have no explanation for this discrepancy but are inclined to believe that the present data are more reliable since blood collection took place on a total of four different occassions, whereas in our earlier experiment blood was collected only once. Furthermore, Södersten [16] also found no effect on adult testosterone levels in male rats neonatally treated with the antiestrogen MER-25.

We had earlier suggested that the nocturnal rhythmicity in partner preference behavior and sexual behavior of gonadally intact ATD males, could reflect rhythms in circulating endogenous testosterone [3], and possibly gonadotrophins. However, after castration and subsequent testosterone treatment, which resulted in constant levels of testosterone, ATD males continued to show a nocturnal rhythmiticy. Therefore, it can be concluded that the underlying mechanism of the nocturnal rhythmicity phenomenon is an organizational effect of neonatal ATD treatment and not an activational effect of fluctuating endogenous blood hormone levels. Presumably, neonatal ATD treatment has irreversibly altered structures and/or functions in the CNS which are involved in expressing a nocturnal rhythmicity in sexual and related behaviors. This conclusion is supported by the finding that hormonal rhythmicity was equal in both groups, whereas behavioral rhythmicity differed.

One could speculate that repeated blood collection might influence behavior and vice versa. We do not believe so. The testosterone data in tables 1 (males without behavioral testing) and 2 (males with behavioral testing) show nocturnal rhythmicity and rather similar concentrations. We always ensured that at least 24 h lay between blood collection and behavioral testing. Furthermore, the behavioral data of the males in the present study resemble those of an earlier study in which the males were not concomitantly exposed to blood collection [e.g. 3].

Over recent years, evidence has been accumulating that the CNS of ATD males differs from that of control males, with respect to anatomical and neuroimmunocytochemical criteria. The volume of the sexually dimorphic nucleus (SDN) in the medial preoptic area (mPOA), which is normally severalfold larger in male than in female rats [17,18], was reduced in prenatally and pre- and neonatally ATD-treated males, with the largest reduction in the latter group [19]. Besides a reduced SDN-mPOA volume, it was found that the suprachiasmatic nucleus (SCN) contained more vasopressin-active neurons in pre- and neonatally ATD-treated males than in prenatally ATD-treated males and control males

[20]. The contribution of the SCN for ATD male behaviors (nocturnal rhythmicity in partner preference and sexual behavior) is currently being investigated through lesions of the SCN. Södersten et al. [21] have found that lesions of the SCN disrupted the daily rhythmicity in mounting and lordosis behavior of male rats treated neonatally with the antiestrogen MER-25. We are interested in the effects of SCN lesions on the nocturnal rhythmicity in partner preference behavior of ATD males: what type of sexual behavior, i.e. significant 'masculine (typical for the late dark phase) or 'feminine' (typical for the early dark phase), or partner preference will ATD males show after disruption of their nocturnal rhythmicity?

A recent finding of altered CNS function in ATD males appeared in a significantly higher expression of the proto-oncogene c-fos, a marker for neuronal activity [e.g. 22], in the mPOA after equal amounts of mating stimulation, i.e. 8 intromissions with an estrous female [unpubl. data].

An interesting new finding of the present study were the elevated FSH levels in ATD males both early and late in the dark phase. Inhibin was unaffected by neonatal ATD treatment. Interestingly, inhibin levels were lowest when FSH levels were highest (late in the dark phase). Higher FSH levels have also been found in adult male rats treated neonatally with dihydrotestosterone propianate (DHTP) on postnatal Days 0-10 [unpubl. data]. Because ATD treatment blocks the aromatization of testosterone to estradiol, one could hypothesize that testosterone levels and/or dihydrotestosterone (DHT) levels are elevated in ATD males on postnatal days 0-21. These presumably elevated testosterone and/or DHT levels could affect the development of pituitary feedback systems resulting in elevated FSH levels (and suppressed LH levels in neonatally DHTP-treated male rats) in adulthood.

Our findings that endogenous blood testosterone levels were significantly higher in the late part of the dark phase than in the early part of the dark phase, are not in line with the results of Södersten et al. [23]. They reported that highest testosterone levels were measured late in the light phase (when sex behaviors were reported to be lowest) and lowest testosterone levels late in the dark phase (when sex behaviors were reported to be highest). We have no explanation for this discrepancy. It has been suggested that LH and T are secreted episodically [24,25]. This means that there could not be a rhythmicity in serum testosterone. If this is true, then T levels would be similar early and late in the dark phase. Therefore, we believe that the higher testosterone levels in the late dark phase has presumably been caused by a higher pulsatility of LH.

Neonatal treatment also affected locomotor activity, as determined by the number of excursions to each compartment. ATD males visited both the stimulus animals and the middle compartment more frequently than

controls. An explanation for the higher locomotor activity of ATD males, is that, because ATD males have kept their 'bisexual' potency [26,27], both stimulus animals are sexually attractive. This makes them run back and forth between the lateral compartments of the three-compartment box. To control males only the stimulus female is sexually attractive, which causes them to pay less (or no) visits to other compartments of the test box.

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Chapter VIII

LESIONS OF THE SUPRACHIASMATIC NUCLEUS DISRUPT THE NOCTURNAL FLUCTUATIONS IN PARTNER PREFERENCE OF NEONATALLY ESTROGEN-DEPRIVED MALE RATS

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ABSTRACT

Previous studies have shown that partner preference of adult male rats, in which the neural aromatization of testosterone to estradiol was inhibited neonatally by administration of ATD fluctuated nocturnally: when tested early in the dark phase of the light/dark cycle, ATD males showed a much smaller preference for an estrous female over a sexually active male than when tested late in the dark phase. In the present study, we investigated the question whether the nocturnal fluctuations in ATD males' partner preference would be disrupted after lesioning the suprachiasmatic nucleus (SCN), i.e. the biological clock.

SCN lesions disrupted the nocturnal fluctuations in partner preference and dramatically increased ATD males' preference for the estrous female: the SCN-lesioned ATD males no longer differed from control males in partner preference. The present data suggest that the SCN plays a role in the display of partner preference in ATD males.

INTRODUCTION

We have previously found that partner preference of male rats, in which brain estrogen formation was inhibited neonatally by the administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD)

fluctuated over the dark phase. When tested early in the dark phase of the light/dark cycle, such neonatally ATD-treated male rats showed a much smaller preference for an estrous female over a sexually active male than when tested late in the dark phase (Bakker, van Ophemert, and Slob, 1993; Bakker, van Ophemert, Timmerman, de Jong, and Slob, 1995). Coital behavior also fluctuated over the dark phase: when tested early in the dark phase, ATD males showed lower levels of male-typical sexual behavior (mounts, intromissions, ejaculations) and higher levels of female-typical sexual behavior (lordosis, presenting, earwiggling, hop & dart) than when tested late in the dark phase (Bakker, van Ophemert, and Slob, 1993; Bakker, van Ophemert, Timmerman, de Jong, and Slob, 1995). These nocturnal fluctuations were absent in control males. The latter males showed a clear preference for an estrous female and constant levels of male-typical sexual behavior throughout the dark phase. The nocturnal fluctuations in ATD males' sexual behavior appeared to be the consequence of the absence of neonatal estradiol and not a reflection of fluctuating testosterone levels. After castration and subsequent testosterone treatment through silastic capsules, which resulted in constant testosterone levels, ATD males' sexual behavior still fluctuated nocturnally (Bakker, van Ophemert, Timmerman, de Jong, and Slob, 1995).

A functional role for the suprachiasmatic nucleus (SCN), the biological clock, was assumed in the nocturnal fluctuations of ATD males' sexual behavior. Recently, it was found that the SCN of male rats which were treated with ATD both pre- and neonatally contained more vasopressin-expressing neurons than those of male rats which were treated with ATD prenatally, and control males. The latter two groups did not differ (Swaab, Slob, Houtsmuller, Brand, and Zhou, 1995). These findings suggest that perinatal estradiol is involved in the development of the vasopressin-expressing neurons in the SCN. The nocturnal fluctuations in partner preference might be generated by the SCN and transmitted by its vasopressinergic projections into the brain.

In the present study, we investigated the effects of SCN lesions on the nocturnal fluctuations in partner preference and coital behavior of neonatally ATD treated and control males. Södersten et al (1981) had found that lesions of the SCN disrupted the daily rhythmicity in mounting and lordosis behavior of male rats treated neonatally with the antiestrogen MER-25. In the present study, we asked the question whether ATD males' partner preference would alter after SCN lesions.

GENERAL METHODS

Animals

The experimental males and the stimulus males and females were Wistar albino rats (Wu-strain, outbred), commercially obtained (Harlan, Zeist). Two to four rats of the same sex were housed in a cage with food and water available ad libitum. All rats were kept in a reversed 14-hr/10-hr light/dark cycle (lights off from 7:45 a.m. to 5:45 p.m.).

Treatments

Female rats were time mated and parturition occurred 22 days later. Within 2-4 hr after birth, newborn male pups received subcutaneously in the back under ice anesthesia small silastic capsules (inner diameter 1.5 mm; outer diameter 2.1 mm; length 5 mm) containing crystalline ATD or received empty capsules (control males) (one treatment per litter). The implants were removed when the pups were 11 days of age. In the present study, 20 ATD males (out of 5 litters) and 18 control males (out of 5 litters) were used.

The pups were weaned at 21 days of age and then housed in a cage with 2-3 other rats of the same sex and treatment. They were left undisturbed until the onset of behavioral testing. The stimulus animals were sexually active males and estrous females. The estrous females were ovariectomized and brought into behavioral estrus with an injection of 20 g of estradiol benzoate 24-48 hr prior to testing, followed by an injection of 1.0 mg progesterone 3-4 hr before testing. These hormones were dissolved in olive oil and injected sc in the neck.

Test procedure

Preference test in three compartment box. A test box made of gray perspex with a transparent front was used (Bakker, van Ophemert, Eijskoot, and Slob, 1994): it had three compartments (60 x 30 x 40 cm) with a small opening (13 x 12 cm) in both partitions near the back. These openings could be closed by a sliding door. In each opening, position sensitive tilt-platforms were situated in order to register movement of the experimental animal from one compartment to another. The position of the tilt-platform was determined using a magnetic field (Bakker, van Ophemert, Eijskoot, and Slob, 1994). In all tests, stimulus animals were tethered with a rope to the front of one of the lateral compartments. The tethered stimulus animals thus had a limited action radius. Before testing, the stimulus animals and the experimental animal were put in the test apparatus, one in each compartment, with the sliding doors closed for a 15 min adaptation period. At the beginning of the test (which lasted 15 min), the sliding doors were removed, and the experimental animal could

freely move around and interact with the stimulus animals. The time that the animal spent in each compartment and the number of excursions to each compartment were recorded automatically. At the end of each test, a preference score was calculated by subtracting the time spent in the compartment with the sexually active male from the time spent in the compartment with the estrous female. Thus, a positive score indicates a preference for an estrous female, a negative score for a sexually active male. Various sexual behaviors with the stimulus animals were scored manually.

Lordosis test. A semicircular cage with a wired floor was used. After a 5 min adaptation period of the stimulus male, an experimental animal was put in the cage. The lordosis responses of the experimental male to the mounting of the stimulus male were recorded. The test lasted until the experimental male had received 10 mounts or 10 min had elapsed.

Test procedure

Behavioral testing started at the age of 2.5 months. To obtain sexual experience, all males were first tested for masculine sexual behavior in a pair-test with an estrous female and for feminine sexual behavior in a pair-test with a sexually active male. Then, the males were subjected to a total of 4 partner preference tests twice weekly. Tests 1 and 3 were performed early in the dark phase (between 8:30 and 11:30 a.m.), Tests 2 and 4 late in the dark phase (between 2.00 and 4:30 p.m.). After these tests, the males were tested for lordosis behavior in the pair-test with a sexually active male. This test was performed early in the dark phase.

Two weeks after receiving lesions in the SCN, the males were subjected to a total of 8 partner preference tests twice weekly. Tests 5, 7, 10, and 12 in the early dark phase; Tests 6, 8, 9, and 11 late in the dark phase. After these tests, the males were pair-tested with a sexually active male for lordosis behavior, early in the dark phase.

Surgery

At the age of 3 months, SCN lesions were made under anaesthesia (Hypnorm 0.1 ml/100gr and Nembutal 0.1 ml/300 gr). A stainless steel electrode (diameter 0.4 mm) was inserted bilaterally in the SCN with the aid of a David Kopf stereotaxis apparatus. The coordinates used for lesions were: 1.4 mm anterior from bregma, +/- 0.35 mm lateral from midline, and 9.4 mm ventral below the dura, according to the atlas of Pellegrino et al (1985). Lesions were made by leading a 1.5 mA anodal DC for 10 sec through the electrode (16 ATD and 14 control males). Sham-lesioned animals (4 ATD and 4 control males) were subjected to the same surgery except for the actual electrolytic lesioning.

Protocol for circadian rhythmicity

After behavioral testing was finished, all males (lesioned and sham-lesioned) were transported to another laboratory. To ascertain the completeness of the SCN lesions, free-running rhythmicity or arrhythmicity in drinking behavior was used by continuously monitoring drinking nipple contacts for at least 4 weeks under constant red light conditions in the Department of Physiology of the University of Leiden. The number of nipple contacts for each recording period of 30 min was calculated by computer (for details see Griffioen, Duindam, Van der Woude, Rietveld, and Boer, 1993). This procedure permitted the chi-square periodogram analysis (Sokolove and Bushel, 1978) to be performed with relatively high resolutions by suppressing the occurrence of multiple peak values. The level of significance for circadian rhythms was defined at p 0.01. Lesions were considered to be functionally complete if no circadian periods were visible (observation with the naked eye) in the drinking behavior actograms and the chi-square periodogram analysis did not reveal a significant rhythmicity. Evaluating the drinking behavior data, it appeared that no rhythmicity in drinking behavior could be found in 3 ATD and 3 control males and that a statistically significant rhythmicity was visible in 8 ATD and 6 control males. Furthermore, 5 ATD and 5 control males were clearly intermediate between the arrhythmic and rhythmic males. Such males showed a weak circadian rhythmicity, i.e. in some males, but not in others, it was statistically significant. However, looking more carefully at their drinking behavior actograms, a free-running rhythmicity, albeit weak, could be distinguished in all males. Therefore, these males were considered as a separate group.

Immunocytochemistry

The location and the extent of the lesions were verified on the basis of staining for vasopressin (VP)- and vasoactive intestinal peptide (VIP)-immunoreactivity (IR). At the end of the experiment, all males were deeply anesthesized (Hypnorm 0.1 ml/100 gr) and perfused with freshly made 4% paraformaldehyde in phosphate buffer (pH= 7.0). Brains were removed and postfixed for 24 hours in 4% paraformaldehyde in phosphate buffer and placed in 0.1 M phosphate-buffered saline (PBS; pH= 7.3). Males were selected for VP- and VIP-immunocytochemistry on the basis of their rhythmicity in drinking behavior. Sections of 50 m were cut on a Vibratome. Tris-buffered saline (TBS; 0.05 M, 0.9% NaCl, pH= 7.6) was used to wash the sections for at least 1 hour (three washes with TBS) between all steps of the immunocytochemical procedure. All antibodies were diluted in 0.05 M TBS containing 0.25% gelatine and 0.5% Triton-X100. For VP-IR and VIP-IR, the antibodies Truus (1:2000; Netherlands Institute for Brain Research) and Viper (1:200; Netherlands Institute for

Brain Research) were used, respectively. Freefloating sections were incubated with primary antiserum and left for 1 hr at room temperature and subsequently at 4°C overnight. Goat-anti-Rabbit (Betsy; 1:100; Netherlands Institute for Brain Research) was applied for 1 hr at room temperature followed by peroxidase-anti-peroxidase (PAP; 1:1000; Netherlands Institute for Brain Research) for 1 hr. All incubations were done on a rocking table. Then the sections were stained with 3,3' diaminobenzidinetetrahydrochloride (DAB; 0.5 mg/ml) containing 0.01% H_2O_2 and 0.2% nickel-ammonium sulphate in 0.05 M TBS (pH= 7.2) for 10 min.

After scoring the lesions with VP- and VIP-immunoreactivity, it turned out that males which were rhythmic in drinking behavior, were clearly incompletely lesioned: either only 1 SCN was lesioned or the lesions were placed too rostral. Males, which were arrhythmic in drinking behavior, showed no or insignificant VP- or VIP-immunoreactivity, i.e. the SCN was completely lesioned. Males which were intermediate between the arrhythmic and rhythmic groups had almost complete SCN lesions, although small parts of the SCN (rostral and/or caudal) were still present.

Analysis partner preference data

All partner preference data before SCN lesioning were subjected to two-way analyses of variance (ANOVA; variables: neonatal treatment x testing) followed by the least significant difference procedure when overall significant differences were found. After SCN lesioning, all males were divided into groups on the basis of neonatal treatment (ATD, control) and of completeness of the lesions, i.e. completely lesioned, almost completely lesioned, incompletely lesioned, or sham-lesioned. Mean values were calculated of 4 partner preference tests performed early in the dark phase and of 4 partner preference tests performed late in the dark phase. Subsequently, the mean values were subjected to two-way analyses of variance (ANOVA; variables: group (neonatal treatment and lesion) x time of testing) followed by the least significant difference procedure when overall significant differences were found. Only significant F values are presented. The $p= 0.05$ level is used as the upper limit for statistical significance.

RESULTS

Partner preference

Partner preference before SCN lesioning

When given free access to an estrous female and a sexually active male, neonatally ATD-treated males showed a much lower preference for an estrous female than control males (Fig 1A). Furthermore, partner preference of ATD males, but not that of control males, fluctuated over the dark phase: when tested late in the dark phase, ATD males showed a greater preference for the estrous female (Fig 1A) than when tested early in the dark phase. Furthermore, ATD males had more excursions than control males (Fig 1B).

Two-way ANOVA on preference scores (Fig 1A) revealed a significant effect of neonatal treatment ($F(1/36)= 84.6$, $p< 0.001$), a significant effect of testing ($F(3/108)= 15.8$, $p< 0.001$) and no significant group x test interaction. Post-hoc analysis of the effect of neonatal treatment on preference scores with least significant difference (5%= 192 sec) showed that neonatally ATD-treated males had significantly lower preference scores than control males, independently of time of testing. Post-hoc analysis of the effect of testing with least significant difference (5%= 141 sec) indicated that ATD males' partner preference fluctuated nocturnally: late in the dark phase, preference scores were significantly higher than early in the dark phase. Furthermore, there was a clear effect of testing on control males' partner preference. The preference for the estrous female of control males was lowest in the first partner preference test.

Two-way ANOVA on the total number of excursions (Fig 1B), i.e. the sum of excursions to the compartment with the estrous female, to the compartment with the sexually active male, and to the empty middle compartment, showed a significant effect of neonatal treatment ($F(1/36)= 125.9$, $p< 0.001$). Post-hoc analysis with least significant difference (5%= 15) indicated that in all partner preference tests ATD males paid more visits to each compartment than control males, early and late in the dark phase.

Partner preference after SCN lesioning

In general, the SCN-lesioned ATD males, regardless of completeness of the lesions, showed a greater preference for the estrous female than before lesioning the SCN (Fig 2A compared to Fig 1A). In fact, the SCN lesioned ATD males did no longer differ from control males in partner preference. Moreover, partner preference of the SCN-lesioned ATD males did no longer fluctuate over the dark phase. As was to be expected, sham-lesioned ATD males showed a much lower preference for the

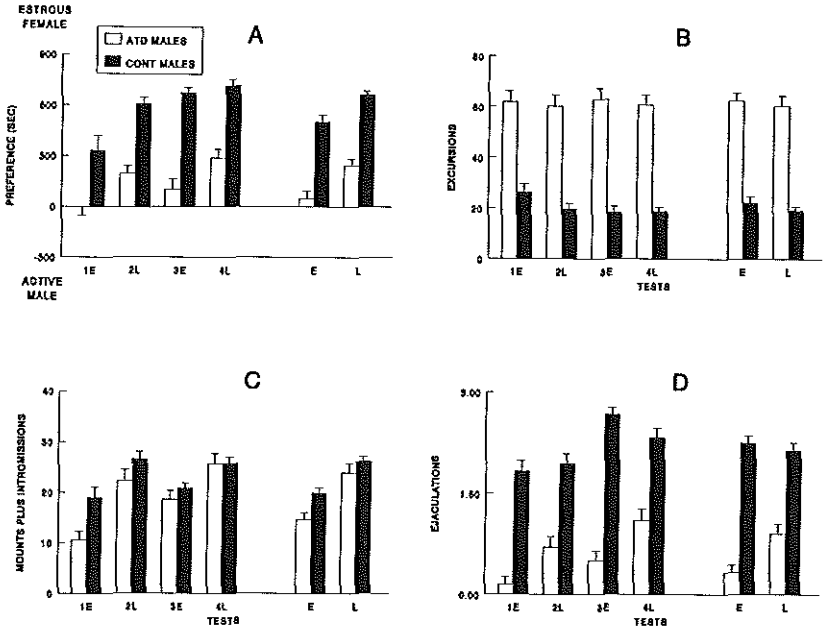

Figure 1: Partner preference and masculine sexual behavior of male rats (n= 20), which received directly after birth silastic capsules containing the aromatase inhibitor ATD. Control males (n= 18) received directly after birth empty silastic capsules. Tests 1E and 3E were carried out early in the dark phase of the light/dark cycle; Tests 2L and 4L late in the dark phase. A preference for an estrous female over a sexually active male, B total number of excursions, i.e. the number of excursions to the stimulus male, to the stimulus female, and to the empty middle compartment, C number of mounts and intromissions with the estrous female, D number of ejaculations. Panel A-D Total means (+ SEM) of the 2 tests early in the dark phase and 4 tests late in the dark phase are shown (right hand-side).

estrous female than control males (Fig 2, panel A). However, partner preference of sham-lesioned ATD males was also constant over the dark phase.

Two-way ANOVA on the preference scores indicated significant group differences only ($F(7/30)= 3.2$, $p= 0.011$). Post-hoc analysis with least significant difference (5%= 392 sec) revealed that sham-lesioned ATD males had significantly lower preference scores than sham-lesioned and SCN-lesioned control males. SCN-lesioned ATD males did not differ significantly in partner preference from sham-lesioned and SCN-lesioned control males. Furthermore, partner preference of sham-lesioned and SCN-lesioned ATD males did not show nocturnal fluctuations.

Two-way ANOVA on the total number of excursions (Fig 2B) showed significant group differences ($F(7/30)= 5.2$, $p= 0.001$), a significant effect of time of testing ($F(1/30)= 5.0$, $p= 0.033$) and an almost significant group x time of testing interaction ($F(7/30)= 2.3$, $p= 0.06$). Post-hoc

analysis of the group differences with least significant difference (5%= 27) revealed that ATD males had more excursions than control males. Post-hoc analysis of the effect of time of testing with least significant difference (5%= 10) showed that excursions fluctuated nocturnally in sham-lesioned ATD males: early in the dark phase, they paid more visits to each compartment than late in the dark phase.

Masculine sexual behavior with the estrous female during partner preference testing

Masculine sexual behavior before SCN lesioning

The total number of mounts plus intromissions are depicted in Fig 1C. Two-way ANOVA revealed a significant effect of neonatal treatment ($F(1/36)= 4.2$, $p= 0.05$), a significant effect of testing ($F(3/108)= 23.8$, $p< 0.001$) and a significant neonatal treatment x test interaction ($F(3/108)= 2.9$, $p= 0.04$). Post-hoc analysis with least significant difference (5%= 4) revealed that both ATD and control males showed nocturnal fluctuations in mounting and intromissive behavior: late in the dark phase, ATD and control males displayed significantly more mounts and intromissions than early in the dark phase. Furthermore, ATD males had lower mount and intromission frequencies in Tests 1-2 than control males.

Ejaculation behavior is shown in Fig 1D. Two-way ANOVA revealed a significant effect of neonatal treatment ($F(1/36)= 103.4$, $p< 0.001$), a significant effect of testing ($F(3/108)= 15.1$, $p< 0.001$) and a significant neonatal treatment x test interaction ($F(3/108)= 7.2$, $p< 0.001$). Post-hoc analysis with least significant difference (5%= 0.3) showed that in all tests ATD males ejaculated less frequently than control males. Moreover, ejaculation behavior of ATD males, but not that of control males, fluctuated nocturnally: late in the dark phase, ATD males ejaculated more frequently than early in the dark phase.

Masculine sexual behavior after SCN lesions

In general, SCN lesions did not affect the number of mounts plus intromissions in both ATD and control males. However, ejaculation behavior of control males was affected by lesioning the SCN. The SCN-lesioned control males had lower ejaculation frequencies than before the SCN was lesioned.

Two-way ANOVA on the total number of mounts and intromissions (Fig 2C) indicated a significant effect of time of testing ($F(1/30)= 7.2$, $p= 0.011$). Post-hoc analysis with least significant difference (5%= 5) showed that completely lesioned control males displayed significantly more mounting and intromissive behavior late in the dark phase.

Ejaculation behavior is presented in Fig 2D. Two-way ANOVA on ejaculation frequencies revealed significant group differences ($F(7/30)=$

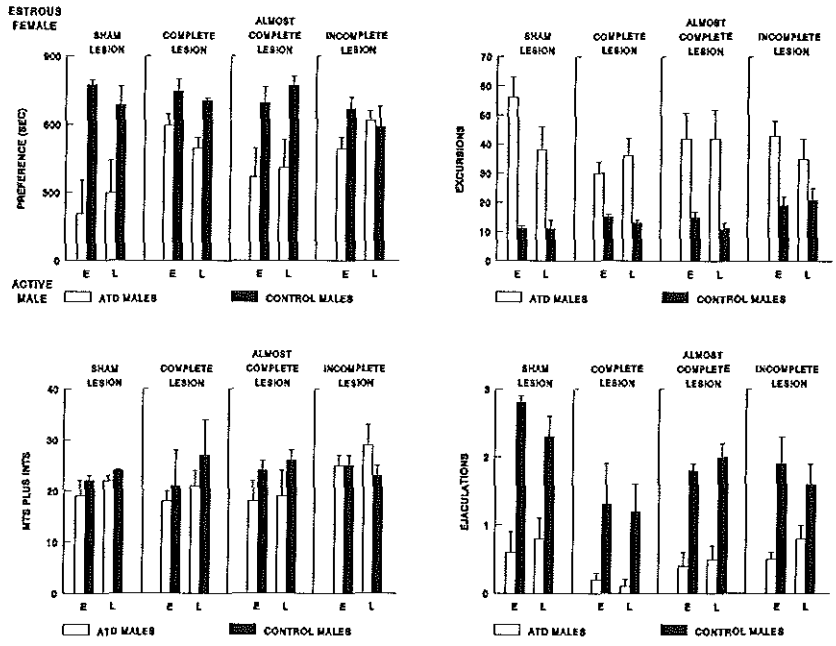

Figure 2: Partner preference and masculine coital behavior of neonatally ATD-treated and control males after receiving electrolytic or sham lesions in the SCN. A partner preference, B total number of excursions C total number of mounts plus intromissions, D ejaculation frequencies. Values are means (+ SEM) of the 4 tests early in the dark phase and of the 4 tests late in the dark phase.

8.5, $p < 0.001$). Post-hoc analysis with least significant difference (5%= 1.2) showed that ATD males ejaculated less frequently than control males. Furthermore, completely SCN lesioned control males had lower ejaculation frequencies than sham-lesioned control males (Fig 2D).

Feminine sexual behavior with the sexually active male during partner preference testing

Feminine sexual behavior before SCN lesions

Only ATD males showed feminine sexual behaviors, i.e. proceptive (presenting, earwiggling, hopping and darting) and receptive (lordosis) behavior. Before lesions were made in the SCN, 13 out of 20 ATD males regularly showed feminine sexual behaviors, albeit in rather low frequencies. There was no effect of time of testing on the display of feminine sexual behaviors (early in the dark phase: mean (± SEM)= 9.0 ± 2.6; late in the dark phase: mean (± SEM): 8.5 ± 2.9).

Feminine sexual behavior after SCN lesions

Two-way ANOVA on frequencies of feminine sexual behavior (Fig 3) revealed significant group differences ($F(3/16)=4.7$, $p=0.015$), a significant effect of time of testing ($F(1/16)= 5.6$, $p= 0.031$) and a significant

group x time of testing interaction ($F(3/16)= 5.6$, $p= 0.008$). Post-hoc analysis of this interaction with least significant group difference (5%= 8.2) showed that sham-lesioned ATD males had higher frequencies of feminine sexual behavior than SCN-lesioned ATD males. Furthermore, sham-lesioned ATD males displayed less feminine sexual behavior late in the dark phase.

Lordosis behavior in pair-test with sexually active male

Two-way ANOVA on lordosis quotients of ATD males pair-tested with a sexually active male before and after SCN lesioning (Table 1) revealed a significant effect of SCN lesioning ($F(1/16)= 11.4$, $p= 0.004$). Post-hoc analysis with least significant difference (5%= 40) showed that (almost) completely lesioned ATD males had lower lordosis quotients after SCN lesions. In fact, none of the completely SCN lesioned ATD males showed lordosis when mounted by the stimulus male. No significant changes in lordosis behavior were observed in incompletely SCN-lesioned and sham-lesioned ATD males.

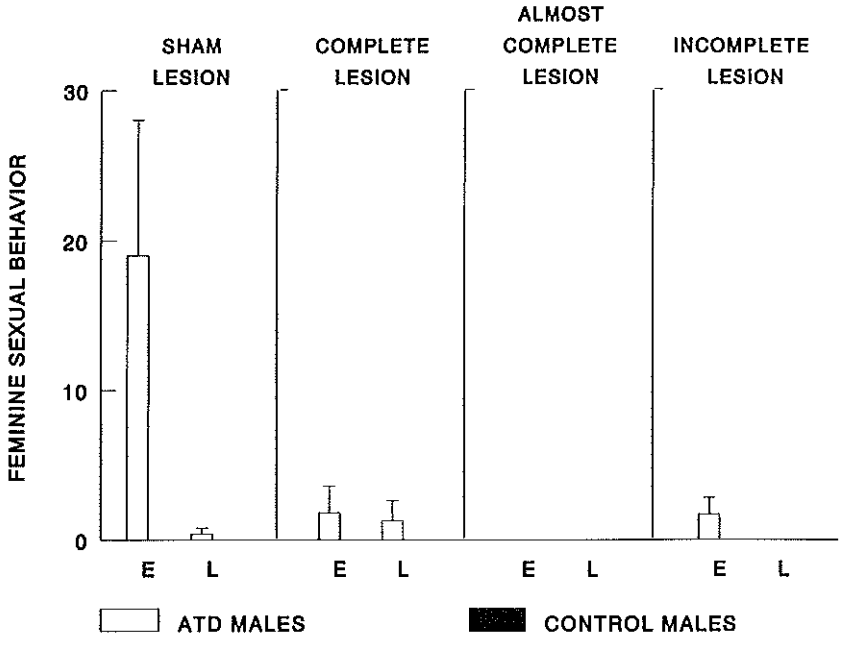

Figure 3: Feminine sexual behavior (the sum of proceptive and receptive behavior) with the sexually active male during partner preference testing of neonatally ATD-treated and control males after receiving electrolytic or sham lesions in the SCN. Values are means (+ SEM) of the 4 tests early in the dark phase and of the 4 tests late in the dark phase.

Table 1: Lordosis quotients of neonatally ATD-treated male rats before and after receiving lesions in the suprachiasmatic nucleus (SCN). Lordosis behavior was tested in a total of two pair-tests with a sexually active male: 1 test was executed before lesions were made in the SCN, 1 after the SCN was lesioned. Both tests were performed early in the dark phase. None of the control males showed lordosis behavior. The numbers in brackets represent the numbers of the animals displaying lordosis.

SCN Lesion	N	Before lesions	After lesions
Sham	4	83 ± 7 (4)	69 ± 11 (4)
Complete	3	53 ± 24 (2)	0 ± 0* (0)
Almost complete	5	94 ± 2 (5)	33 ± 17* (3)
Incomplete	8	71 ± 9 (8)	61 ± 16 (6)

* significantly lower ($p < 0.05$) compared to before lesions.

DISCUSSION

The present study showed that the suprachiasmatic nucleus (SCN) is a crucial structure in controlling the nocturnal fluctuations in partner preference. Before lesions were made in the SCN, partner preference of male rats, in which the neural aromatization of testosterone to estradiol was inhibited neonatally by ATD-treatment, fluctuated nocturnally. When tested late in the dark phase of the light/dark cycle, ATD males showed a greater preference for the estrous female than when tested early in the dark phase. These nocturnal fluctuations were absent in control males' partner preference: they showed a great preference for the estrous female throughout the dark phase. However, male coital performance fluctuated over the dark phase in both ATD and control males. When tested late in the dark phase, ATD and control males displayed more mounting and intromissive behavior than when tested early in the dark phase. After SCN lesioning, ATD males' partner preference was constant throughout the dark phase. Likewise, ATD and control males' coital performance did not fluctuate any longer over the dark phase. Unexpectedly, sham-lesioned ATD males did not show nocturnal fluctuations in partner preference. They did, however, show nocturnal fluctuations in feminine sexual behavior: when tested early in the dark phase, sham-lesioned ATD males displayed more proceptive and receptive behavior than when tested late in the dark phase. It turned out that 2 out of 4 sham-lesioned ATD males showed a weak circadian rhythmicity in drinking behavior, although VP- and VIP-immunocytochemistry revealed two intact SCN's in these animals. We have no explanation for the somewhat affected rhythmicity in these sham-lesioned males. Possibly, efferents of the SCN were affected during brain surgery in these animals.

Interestingly, ATD males' partner preference was clearly altered after SCN-lesioning. SCN-lesioned ATD males, regardless the completeness of the lesions, showed a greater preference for the estrous female than before lesions were made. In fact, SCN-lesioned ATD males did no longer differ in partner preference from control males. Partner preference of control males was not affected by SCN lesions. This is in line with the findings of Kruijver et al (1993).

Since normal male rats do not show a cyclic gonadotropin release, whereas neonatally castrated males, implanted with ovaries, undergo estrous cycles and ovulate (Harris, 1964), it has been postulated that gonadal hormones perinatally suppress the hypothalamic clock essential for cyclic release of luteinizing hormone (LH) (Gorski, 1971). Presumably, neonatal estradiol is the hormone responsible for the suppression of cyclic gonadotropin release, since neonatally ATD treated males show a cyclic secretion of gonadotropins, as evidenced by the occurrence of ovulation in ovaries grafted under the kidney capsule (Vreeburg, Van der Vaart, and Van der Schoot, 1977). A similar hypothesis can be formulated for the occurrence of a diurnal rhythm (Södersten and Eneroth, 1980; Södersten, Hansen, and Srebro, 1981; Södersten, Boer, De Vries, Buijs, and Melin, 1986) in mounting and lordosis behavior and for the occurrence of nocturnal fluctuations in partner preference and sexual behavior (Bakker, van Ophemert, and Slob, 1993; Bakker, van Ophemert, Timmerman, de Jong, and Slob, 1995; present study). Presumably, neonatal estrogenic stimulation uncouples the SCN rhythm generator from the neural structures that control sexual behavior in the adult. The present findings support this hypothesis. SCN lesions disrupted the nocturnal fluctuations in partner preference and coital behavior in male rats in which these fluctuations were induced by neonatal ATD treatment. In addition, ATD males' partner preference was clearly altered after lesioning the SCN. Therefore, it appears that the SCN plays a role in the display of partner preference, in addition to generating circadian rhythms. Some support for this supposition is that, in humans, the size of the vasopressin-containing subnucleus of the SCN is correlated with sexual orientation, i.e. it is twice as large in homosexual men compared to heterosexual men (Swaab and Hofman, 1990). In addition, it has recently been found that the SCN of male rats which were treated with ATD both pre- and neonatally, contained more vasopressin-expressing neurons than those of prenatally ATD treated males and control males. The latter two groups did not differ (Swaab, Slob, Houtsmuller, Brand, and Zhou, 1995). These findings suggest a possible role for neonatal estradiol in the differentiation of vasopressin-expressing neurons in the rat SCN. To date, there is no evidence that the vasopressin-expressing neurons in the SCN and the vasopressinergic projections of the SCN are

hormonally dependent in the adult rat, i.e. castration or hormone treatment had no effect (De Vries, Buijs, Van Leeuwen, Caffé, and Swaab, 1985). Furthermore, the adult rat SCN contains hardly any steroid-binding cells (Pfaff and Keiner, 1973; Zhou, Blaustein, and De Vries, 1994), although in the adult ewe estrogen receptors can be detected in the SCN (Blanche, Batailler, and Fabre-Nys, 1994). However, the possibility of transient estrogen receptor expression during certain periods of development cannot be ruled out. For instance, estrogen receptors are transiently expressed in the layer V of the developing rat cerebral cortex. Estrogen receptor-immunoreactivity was present at postnatal days 1, 10 and 13, but disappeared at postnatal day 15 (Yokosuka, Okamura, and Hayashi, 1995). Future studies should investigate, therefore, the presence of estrogen receptors during the course of development in the rat SCN and adjacent structures. In humans, no sex difference has been found in the total number of vasopressin-expressing neurons of the SCN, but the shape of the vasopressin-containing subnucleus of the SCN was sexually dimorphic, i.e. it was elongated in women and more round in men (Swaab and Fliers, 1985).

It is not clear at present how the higher vasopressin-content of the SCN in pre- and neonatally ATD-treated male rats has to be explained. It is quite possible that perinatal estradiol influenced the vasopressin-containing neurons of the SCN via interneurons containing steroid receptors. A similar mechanism for steroid action has been postulated for the sexually dimorphic vasopressin- and oxytocin-containing nucleus (VON) in domestic pigs (Van Leeuwen, Chouham, Axelson, Swaab, and Van Eerdenburg, 1995). The VON was completely void of any nuclear estrogen receptor-immunoreactivity. However, since a cluster of estrogen-receptor immunoreactive cells was found dorsolaterally of the VON, the reported effects of gonadal steroids on the VON may be induced indirectly by interneurons located immediately adjacent to this nucleus (Van Leeuwen, Chouham, Axelson, Swaab, and Van Eerdenburg, 1995). Although the number of vasopressin-containing neurons has so far only been determined in prenatally and pre- and neonatally ATD-treated males (Swaab, Slob, Houtsmuller, Brand, and Zhou, 1995), it is presumed that the number of vasopressin-containing neurons will be increased by neonatal ATD treatment alone, since the critical period for the behavioral ATD effects is mainly postnatally. We are currently studying the number of vasopressin-containing neurons in neonatally ATD-treated males.

The SCN transfers its rhythmicity into the brain probably by its vasopressinergic projections (Kalsbeek, Buijs, Engelmann, Wotjak, and Landgraf, 1995). It has been found that the vasopressinergic projections of the SCN closely follow the projections of the SCN as established by

anterograde techniques (Berk and Finkelstein, 1981; Hoorneman and Buijs, 1982). Whether or not there is a functional link between increased numbers of vasopressin-expressing neurons in the SCN and the nocturnal fluctuations in partner preference, remains to be elucidated. Future studies might investigate ATD males' partner preference after blocking vasopressin synthesis in the SCN or vasopressin action by antisense oligonucleotides or antagonists.

There is some evidence that vasopressin might be involved in sexual behavior (Södersten, Hennning, Melin, and Lundin, 1983; Södersten, De Vries, Buijs, and Melin, 1985; Södersten, Boer, De Vries, Buijs, and Melin, 1986). For instance, it has been found that an intracerebroventricular injection of vasopressin inhibits (Södersten, Henning, Melin, and Lundin, 1983) and of a vasopressin antagonist stimulates lordosis behavior in ovariectomized female rats (Södersten, De Vries, Buijs, and Melin, 1985). These findings suggest that vasopressin has an inhibitory effect on female sexual behavior. Although it is not known by what mechanism vasopressin exerts this action, one of the putative target areas might be the sexually dimorphic innervation of the lateral septum, since various studies have demonstrated that the septal nuclei are implicated in the (predominantly) inhibitory control of feminine sexual behavior (Nance, Shryne, and Gorski, 1974; Zasorin, Malsbury, and Pfaff, 1975; McGinnis and Gorski, 1976; Gorzalka and Gray, 1981; Nance, 1982). There is, however, so far no evidence that the vasopressinergic projections from the SCN are involved in the expression of sexual behavior. The question whether or not vasopressin is involved in the central regulation of sexual behavior and if so, by which part of the vasopressinergic system, might be investigated by means of local administration of vasopressin or vasopressin antagonists in more discrete brain regions.

Numerous studies (Brown-Grant and Raisman, 1977; Raisman and Brown-Grant, 1977; Gray, Södersten, Tallentire, and Davidson, 1978; Wiegand, Terasawa, Bridson, and Goy, 1980) have implicated the SCN in the regulation of reproductive functions. Large lesions which included the SCN induced an anovulatory condition characterized by persistent vaginal cornification and polifollicular ovaries (Wiegand, Terasawa, Bridson, and Goy, 1980). Furthermore, lesions of the SCN in female rats completely eliminated LH release in ovariectomized female rats as measured by positive feedback response to estradiol and progesterone (Gray, Södersten, Tallentire, and Davidson, 1978). It has been found that the female rat brain vasoactive intestinal peptide (VIP)-containing fibers project from the SCN to gonadotropin-releasing hormone-containing cells (Van der Beek, Wiegant, and Van der Donk, 1993). Lesions of the SCN did not affect lordosis behavior in ovariectomized female rats,

treated with estradiol and progesterone (Gray, Södersten, Tallentire, and Davidson, 1978). Likewise, Södersten et al (1981) reported that lordosis behavior was not inhibited in SCN-lesioned male rats treated neonatally with the antiestrogen MER-25. In the present study, however, almost completely SCN-lesioned and completely SCN-lesioned ATD males showed less lordosis behavior than before SCN lesions. These findings are at variance with those of Gray et al (1978) and Södersten et al (1981). Differences in the extent of the lesions or testing procedures may explain these discrepancies. Our data indicate that the SCN might be implicated in lordosis behavior. It has been shown that the SCN project to several hypothalamic nuclei (Swanson and Cowan, 1975), including the ventromedial nucleus, which plays an important role in the hormonal control of lordosis in female rats (Pfaff and Schwartz-Giblin, 1988). Whether or not these projections are involved in the regulation of lordosis behavior remains to be tested.

CONCLUDING REMARKS

The present study shows that the SCN is a crucial structure in controlling the nocturnal fluctuations in partner preference in male rats, in which this rhythmicity was induced by neonatal inhibition of brain estrogen formation by treatment with the aromatase inhibitor ATD. SCN lesions disrupted these nocturnal fluctuations and at the same time dramatically increased ATD males' preference for the estrous female: the SCN-lesioned ATD males differed no longer from control males in partner preference. The present data suggest that the SCN plays a role in the display of partner preference, in addition to generating circadian rhythms.

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Chapter IX

NEONATAL INHIBITION OF BRAIN ESTROGEN SYNTHESIS ALTERS ADULT NEURAL C-FOS RESPONSES TO MATING AND PHEROMONAL STIMULATION IN THE MALE RAT

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ABSTRACT

Perinatal inhibition of brain estrogen formation by administration of the aromatase inhibitor ATD to male rats affects the sexual differentiation of the Central Nervous System. Such perinatally estrogen-deprived males possess in adulthood a sexually dimorphic nucleus of the preoptic area (SDN-POA), which is significantly reduced in volume, and show an altered sexual partner preference, i.e. when they have free access to an estrous female and a sexually active male, they show masculine sexual behaviors with the estrous female and they approach the active male, to whom they show feminine sexual behaviors. In the present study, we used *c-Fos* expression as a marker of neuronal activity to reveal possible differences between neonatally estrogen-deprived and normal males' responses to genital and chemosensory stimulation associated with sexual behavior.

Heterosexual mating, i.e. 8 intromissions with an estrous female, significantly augmented the number of Fos protein-immunoreactive (Fos-IR) neurons in the bed nucleus of the stria terminalis, the posterodorsal portion of the medial amygdala and the midbrain central tegmental field to equivalent numbers in neonatally ATD-treated and cholesterol (Chol)-treated males (Experiment 1). Mating led to a greater neuronal *c-Fos* response in the medial preoptic area of ATD males compared to the medial preoptic area of Chol males. The number of Fos-IR neurons was also augmented in the SDN-POA of both ATD and Chol males following mating stimula-

tion, with ATD males showing a greater *c-Fos* response.

The distribution and intensity of increments in neuronal Fos-IR following exposure to soiled bedding from estrous females were very similar in both ATD and Chol males (Experiment 2a). *c-Fos* Responses were evident throughout the vomeronasal pathway, i.e. the posterior medial amygdala and the medial POA. Thus, there was no effect of neonatal estrogen deprivation on male rats' later neuronal responsiveness to odors from estrous females.

There was a clear sexual dimorphism in the neuronal responsiveness as revealed by Fos-IR in the vomeronasal pathway to odors from sexually active males (Experiment 2b). At the beginning of the vomeronasal pathway, i.e. the accessory olfactory bulb and the posterior medial amygdala, there were no differences in the number of Fos-IR neurons between the sexes. At more distal levels of the vomeronasal pathway, i.e. the bed nucleus of the stria terminalis and the medial preoptic area, sex differences became evident. In female rats, the bed nucleus of the stria terminalis and the medial preoptic area were clearly responsive to odors from active males, whereas in Chol males these brain areas were not responsive. This sexual dimorphism is clearly dependent on the presence of neonatal estrogens: ATD males showed a female-like responsiveness, i.e. *c-Fos* responses were evident at each level of the vomeronasal system. These results suggest that the neuronal responsiveness of the vomeronasal projection circuit to odors from estrous females is not sexually differentiated, whereas the neuronal responsiveness to odors from sexually active males clearly is sexually differentiated.

INTRODUCTION

Perinatal administration to male rats of 1,4,6-androstatriene-3,17-dione (ATD), which blocks the aromatization of testosterone to estradiol, affects the sexual differentiation of the central nervous system[3,9,11,21,36,38]. Such gonadally intact, estrogen-deprived males show in adulthood an altered sexual partner preference, i.e. when they have free access to an estrous female and a sexually active male, they approach and mount and intromit with the estrous female and they approach the active male, to whom they show lordosis and proceptive behaviors (presenting, earwiggling, hopping and darting), dividing time between the estrous female and the active male[2,3,4,9]. However, when castrated as adults and

treated with estradiol, neonatally ATD treated males spend significantly more time with the active male than with the estrous female, whereas neonatally cholesterol (Chol) treated males spend significantly more time with the estrous female[2].

The medial preoptic area (POA) has been implicated as a site controlling the expression of male rats' masculine sexual behaviors while inhibiting males' feminine sexual responsiveness. Numerous studies[34] have shown that bilateral lesions of the medial POA/anterior hypothalamus (AH) continuum disrupt masculine coital function in male vertebrates and enhance males' proceptive and receptive responsiveness towards a stimulus male in cats[18], in rats[19,27,29], in guinea pigs[33] and induces a partner preference for a male in ferrets[30]. Within the medial POA, a sexually dimorphic nucleus (SDN-POA), which is significantly larger in males than in females, was first described in rats[16]. In rats, the sexual differentiation of the SDN-POA shows a close parallel with the sexual differentiation of behavior i.e. its larger size in males depends on the action of estrogenic metabolites of testosterone, during a critical perinatal period[12,13,22]. This correlation was emphasized by a recent study[21] in which the effect of perinatal treatment with ATD on SDN-POA volume was investigated. It was found that the SDN-POA volume was reduced in both prenatally and pre- and neonatally ATD treated males, with a larger reduction in the latter than in the former group[21]. The actual function of the SDN-POA neurons in controlling the expression of sexual behaviors is still not clear. Lesions restricted to the SDN showed either no effect or a very small effect on masculine sexual behaviors in rats[1,25] and ferrets[10], though in male gerbils lesions of the sexually dimorphic area disrupted mating[42]. A study by Hennesey et al.[19] suggested that the SDN-POA in male rats may be involved in the inhibition of feminine sexual behavior. Bilateral lesions of the medial POA/AH augmented the display of lordosis in male rats treated with estrogen and progesterone. The most effectively feminizing brain lesions were those which bilaterally destroyed a substantial portion of the mPOA encompassing the SDN.

Immunocytochemical visualization of Fos, the nuclear protein product of the immediate early gene, *c-Fos*, has provided useful information about the neural circuits which are activated following mating in male rats (e.g. [6]). In male rats, neural Fos-IR is increased after mating in several forebrain regions, including the medial POA, the bed nucleus of the stria terminalis (BNST), the posterodorsal portion of the medial amygdala (MePD), and in the midbrain central tegmental field (CTF)[6,7,32,39].

In the present study, we used *c-Fos* expression as a marker of neural activity to reveal possible differences between neonatally estrogen-deprived (i.e. ATD treated) males and normal (i.e. neonatally Chol treated) male rats' responses to genital and chemosensory stimulation

associated with sexual behavior. In Experiment 1, the number of Fos-IR neurons was compared in several brain regions of male rats treated neonatally with ATD or cholesterol and which in adulthood were killed 1 hr after equal amounts of heterosexual mating stimulation, i.e. 8 intromissions with an estrous female. All subjects were castrated as adults and treated daily with testosterone propionate. We asked whether the *c-Fos* response to intromissive stimulation would be lower in ATD males than in Chol males, in a manner corresponding to the reduced volume of the SDN-POA in such ATD males versus Chol males. Baum & Everitt[6] presented evidence suggesting that the mating-induced increment in Fos-IR in medial POA relies on ipsilateral inputs from both the medial amygdala and CTF. Therefore, we also asked whether the mating-induced Fos-IR within the two regions which project to mPOA could differ between ATD and Chol males.

In rodent species, reproductively relevant pheromonal cues are detected by receptors in the vomeronasal organ, which in turn transmits this information centrally via the accessory olfactory bulb, the medial amygdala, the bed nucleus of the stria terminalis, and the medial preoptic area[26]. In the rat, this vomeronasal projection pathway has been found to be sexually dimorphic, with males having more vomeronasal receptors and a larger number of neurons throughout this chemosensitive pathway (reviewed in [35]). Presumably, the sexual differentiation of the vomeronasal system is under influence of perinatal estrogens[17,35]. Therefore, in Experiment 2, we asked whether chemosensory cues (derived from either the urine and feces of estrous females or those of active males) may reveal differences in neural *c-Fos* responses in the vomeronasal projection to the medial POA and/or the nucleus accumbens, a brain region which has been linked to reward and (sexual) motivation[15,28,40], between neonatally estrogen-deprived males and Chol males.

EXPERIMENTAL PROCEDURES

Animals

Male and female Wistar RP rats bred in our laboratory, were housed in single-sex groups of 2 to 3. Food and water was available ad libitum. All rats were kept on a reversed 14:10 hr light-dark cycle (lights off from 7:45 am to 5:45 pm). Female rats were time-mated and parturition occurred 22 days later. Within 2-4 hours after birth, the newborn males received subcutaneously a silastic capsule (SR3: inner diameter 1.5 mm, outer diameter 2.1 mm, length 5 mm) containing crystalline ATD or cholesterol (Chol) under ice anesthesia. The implants were removed at wean-

ing, 21 days of age and the animals were housed 2-3 of the same treatment to a cage.

Experimental Protocol

Experiment 1: neural c-Fos responses to mating stimulation

At the age of approximately 3 months, 15 neonatally ATD treated and 15 Chol treated males were castrated under ether anesthesia using a midline abdominal incision and treated daily with testosterone propionate (200 g s.c. in olive oil). The ATD and Chol males were pretested 3 times (30 min/test) with an estrous female in order to provide them with heterosexual experience. All behavioral testing was conducted in semi-circular cages measuring 62 x 40 x 36 cm. Before testing, the experimental animal spent a 15-min adaptation period in the cage. The test began when a female was put in the cage. The stimulus females were ovariectomized and brought into behavioral estrus by injecting 20 g of estradiol benzoate 24-48 hr before testing followed by 1.0 mg of progesterone 3 to 4 hr before testing.

All males were housed singly for one or two days prior to the actual experiment. Groups of ATD and Chol males were allowed to achieve 8 intromissions with an estrous female. Then, these males were then put singly in their home cages for 1 hr, after which they were perfused and their brains processed for Fos-IR. Pairs of ATD and Chol males were matched for number of mounts & intromissions, and testing time. Additional male rats (ATD and Chol; housed singly for one or two days) taken directly from their homecages served as unmated controls. In Exp 1, 10 mated ATD, 10 mated Chol, 5 unmated ATD and 5 unmated Chol males were used.

Experiment 2a: neural c-Fos responses to soiled bedding (containing urine and feces) from estrous females

At the age of approximately 6 months, 5 neonatally ATD treated and 10 Chol treated males were castrated under ether anesthesia using a midline abdominal incision and received estradiol sc through a silastic capsule (inner diameter 0.5 mm; outer diameter 1.0 mm; length 2.5 cm). Two groups of ovariectomized females (6 per group) were injected subcutaneously with estradiol benzoate (20 g) 24-48h prior to a progesterone (1.0 mg) injection. Three hours after the progesterone injection, all females were placed in clean cages which contained fresh sawdust. Bedding was collected 12h later and immediately used in the experiment. The heterosexually experienced ATD and Chol males were housed singly for one to two days prior to use in the experiment in a room containing no other rats. Pairs of ATD and Chol males were perfused after spending 1.5 hr in the cage containing soiled bedding from estrous

females and their brains were processed for Fos-IR. Additional male rats, housed singly in a separate room containing no other rats, were taken directly from their homecages to the perfusion room and served as controls not exposed to pheromones (homecage Subjects (Ss)). Because no differences in *c-Fos* responses were found between the unmated ATD and Chol males in Exp 1, we used only neonatally Chol treated males as homecage Ss. All experiments took place in the early part of the dark phase of the light/dark cycle (between 8:30 and 12:00 hr).

Experiment 2b: neural c-Fos responses to soiled bedding (containing urine and feces) from sexually active males

At the age of approximately 9 months, 7 neonatally ATD treated and 7 Chol treated males were castrated under ether anesthesia using a midline abdominal incision and received estradiol sc through a silastic capsule (inner diameter 0.5 mm; outer diameter 1.0 mm; length 2.5 cm). The female rats used in this experiment were ovariectomized and treated with estradiol benzoate 24-48 hr prior to the experiment. The ATD and Chol males were pretested 2 times with an estrous female (15 min/test) and 2 times with an active male (15 min/test) in order to provide them with sexual experience. The females had been used as stimulus animals during partner preference testing earlier in our laboratory.

Three groups of males (6 per group) were placed in clean cages which contained fresh sawdust. Bedding was collected 72h later and immediately used in the experiment. All experimental animals (ATD, Chol, and females) were housed singly one or two days prior to use in the experiment in a room containing no other rats (females separate from males). The experimental animals were perfused after spending 1.5 hr in the cage containing soiled bedding from active males and their brains were processed for Fos-IR. A control group, comprised of 2 ATD males, 2 Chol males and 2 females, was killed after spending 1.5 hr in clean bedding (clean bedding Ss). We choose this particular composition of the control group, because 1) no differences in Fos-IR were found between unmated ATD and Chol males in Exp 1; 2) neural *c-Fos* responses in homecage Ss in Exp 2a were very low; 3) females have been added to the experimental groups. All experiments took place in the early part of the dark phase of the light/dark cycle (between 8:00 and 11:00 hr).

Immunocytochemistry

Rats were anesthetized with sodium pentobarbital (100 mg/rat, i.p.), given an intracardiac injection of heparin (1000 U/rat), and perfused via the aorta with 0.1 M phosphate-buffer-saline (PBS; pH=7.3) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and post fixed in 4% paraformaldehyde for 2 hours before being placed

in 20%-30% sucrose-PBS for cryoprotection overnight on a shaker at 4°C. Coronal sections of 52 m were cut using a sledge microtome with a freezing stage. In Exp 1 and 2a, consecutive sections were saved at each of four levels, including the Nucleus Accumbens (Core and Shell), Medial POA/bed nucleus of the stria terminalis (BNST), posteroventral and -dorsal portion of the medial amygdala (MePV and MePD) and midbrain Central Tegmental Field (CTF; only in Exp 1). In Exp 2b, consecutive sections were saved at each of three levels, including the olfactory bulb, the nucleus accumbens, and from the medial preoptic anterior hypothalamus till the posterodorsal portion of the medial amygdala. Freefloating sections were rinsed twice in 0.1 M PBS and then incubated overnight on a shaker at room temperature with an anti-Fos antiserum raised in rabbit against the N-terminal sequence of rat Fos amino acids (DCH-1, gift from Dr. David Hancock, London, UK). After incubation in primary antiserum, the brain sections were rinsed 4 times in 0.1 M PBS containing 0.02% triton X-100 and incubated for 2 hr on a shaker at room temperature with biotinylated goat anti-rabbit IgG. After incubation with the secondary antiserum, the sections were rinsed 4 times and incubated for 1,5 hr on a shaker at room temperature with ABC solution (Vector Labs, Elite Kit). The brain sections were again rinsed 4 times in 0.1 M PBS (no Triton X-100) and then reacted with nickel-chloride 3,3'-diaminobenzidine (DAB) and 0.0003% Hydrogenperoxide for 5-10 min (Vector Labs). The sections were rinsed 3 times, mounted onto gelatin-coated slides and coverslipped using Permount. In Exp 1, after the immunocytochemistry for Fos protein was completed, alternate sections were counterstained with cresyl-violet in order to determine the boundaries of the SDN.

Analysis

To quantify the numbers of Fos-IR nuclei, all slides were given a code. In Exp 1, two brain sections at the level of the n. accumbens, medial POA, BNST, MePD and CTF were selected for quantitative analysis for each animal. In Exp 2a, brain sections at the level of the n. accumbens, medial POA, BNST, MePV and MePD were selected for quantitative analysis for each animal. We did not collect the olfactory bulbs in Exp 2a, because Bressler and Baum (submitted for publication) reported similar increments in the number of Fos-IR neurons in the olfactory bulb for males and females. Thus, we had no expectation of finding a difference in the present study. In Exp 2b, brain sections at the level of the accessory olfactory bulb (AOB; mitral and granule cell layer), n. accumbens, medial POA, BNST, MePV, and MePD were selected for quantative analysis for each animal. All of the Fos-IR nuclei in a field of view under the 25x objective (0.27 mm^2) were drawn using a camera lucida and

counted later.

To analyze the distribution of Fos-IR nuclei in the SDN-POA (Exp 1), the number of Fos-IR nuclei inside the SDN was counted for each animal in the counterstained sections. Because the SDN was present in 1-3 sections only, the mean number of Fos-IR nuclei inside the SDN was calculated for each animal. Area measurements of the SDN (coronal sections) were performed unilaterally using the method described by Houtsmuller et al.[21] and a mean SDN area was calculated for each animal. The Fos-IR data of the SDN are expressed as mean number of Fos-IR nuclei inside the SDN / mean SDN area. In some animals (2 Chol unmated, 1 ATD and 1 Chol mated), the sections taken for the mPOA/ BNST did not include the SDN. These animals were excluded from statistical analysis. In addition, camera lucida drawings of Fos-IR nuclei and the SDN were made of consecutive coronal sections through the medial POA of a representative ATD male and a Chol male after mating.

Statistics

All Fos-IR data were first analyzed for normal distribution using the Kolmogorov-Smirnov Test and were subsequently subjected to two-way analysis of variance (ANOVA; Exp 1: neonatal treatment x mating) or one-way analysis of variance (ANOVA; Exp 2: groups), followed by the Student-Newman-Keuls method for pairwise multiple comparison procedures (Student-Newman-Keuls method). If the normality test failed ($p<0.05$), the data were subjected to Kruskal-Wallis one way analysis of variance (ANOVA) on ranks, followed by the Student-Newman-Keuls method for pairwise multiple comparison procedures. In Exp 1, the sections containing the nucleus accumbens were taken too caudal for some animals (2 ATD and 1 Chol unmated, 1 ATD and 1 Chol mated). These animals were excluded from statistical analysis.

RESULTS

Experiment 1: neural c-Fos responses to mating stimulation

Mating significantly augmented the number of Fos-IR neurons in several brain regions of both ATD and Chol males. The magnitude of this *c-Fos* response was very similar in ATD and control males, with exception of the medial preoptic area (POA), which was greater in ATD males than in Chol males. Examples of the effect of mating on Fos-IR in the medial POA and posterodorsal portion of the medial amygdala (MePD) are shown in Figures 1 and 2. Two-way analysis of variance (variables: neonatal treatment x mating) showed that there was a signifi-

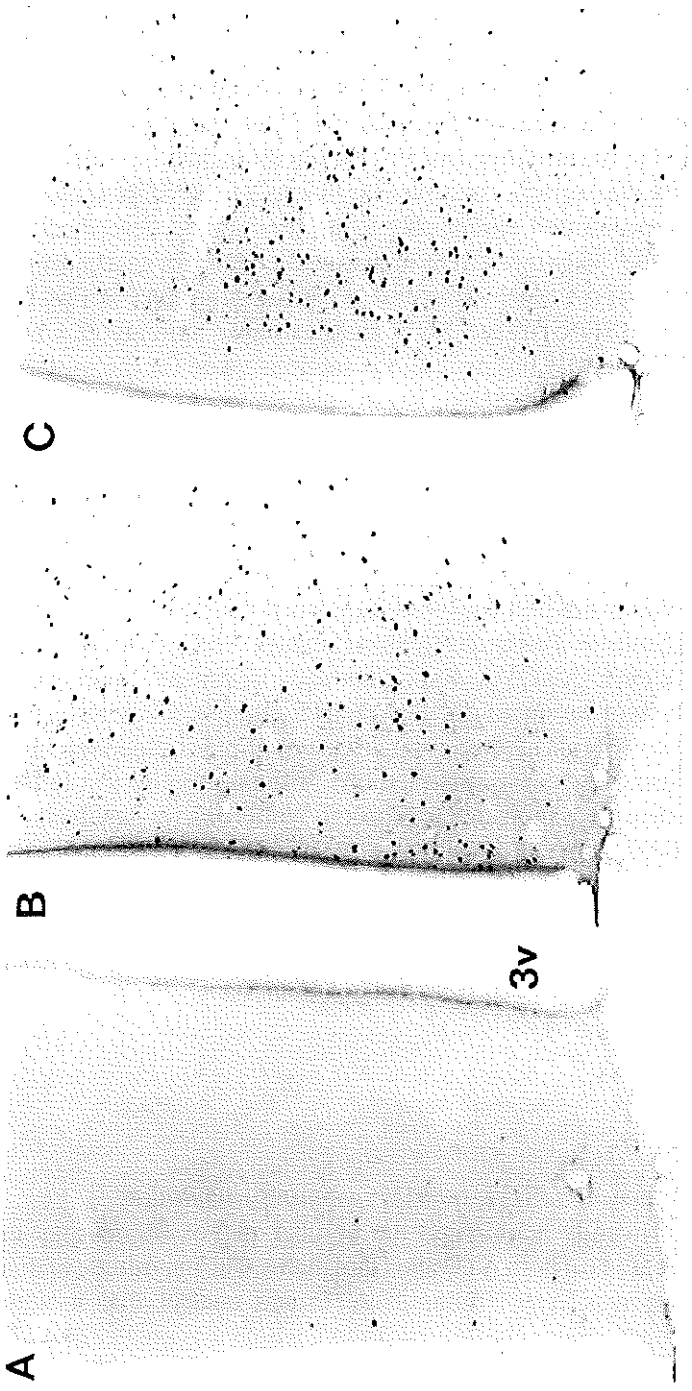

Figure 1. Photomicrographs showing Fos protein-immunoreactivity in the medial preoptic area from an unmated cholesterol-treated male (A), a cholesterol-treated male after 8 intromissions with an estrous female (B) and an ATD-treated male after 8 intromissions with an estrous female (C). All males were castrated in adulthood and subsequently treated with testosterone-propionate.

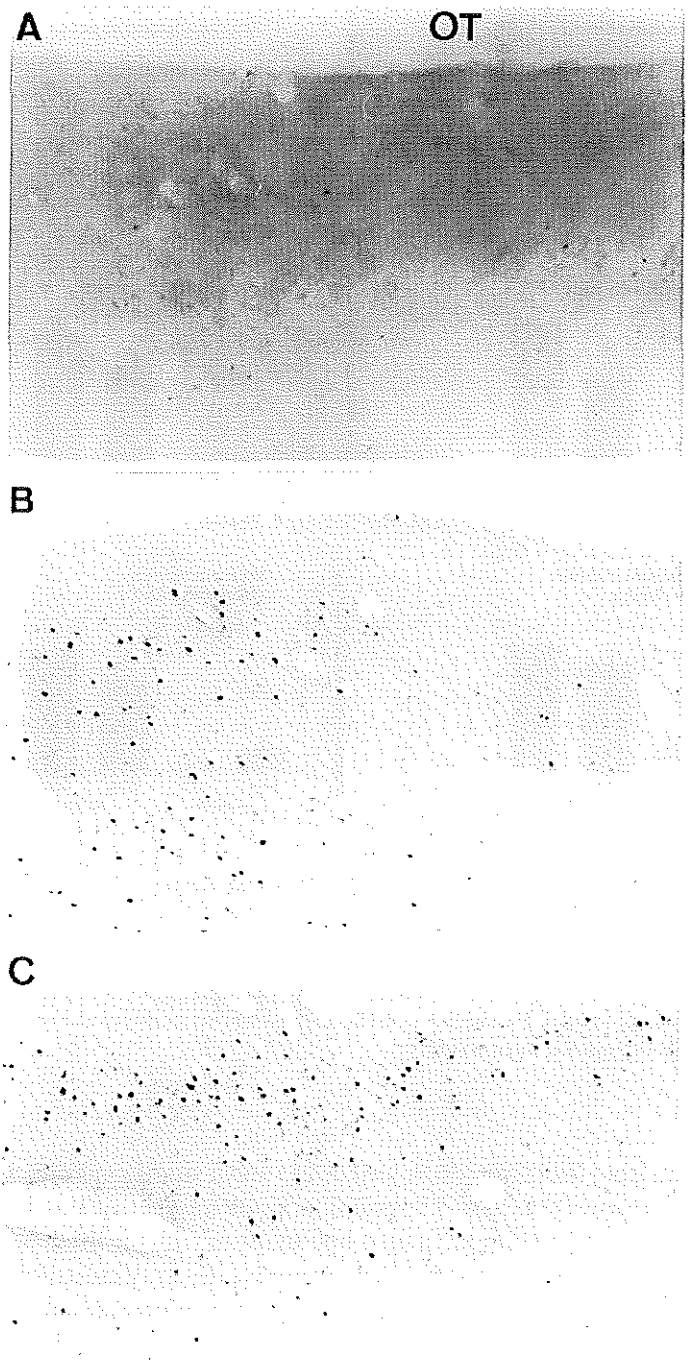

Figure 2. Photomicrographs showing Fos protein-immunoreactivity in the posterodorsal portion of the medial amygdala of an unmated cholesterol-treated male (A), a cholesterol-treated male after 8 intromissions (B) and an ATD-treated male after 8 intromissions (C). All males were castrated in adulthood and subsequently treated with testosterone-propionate.

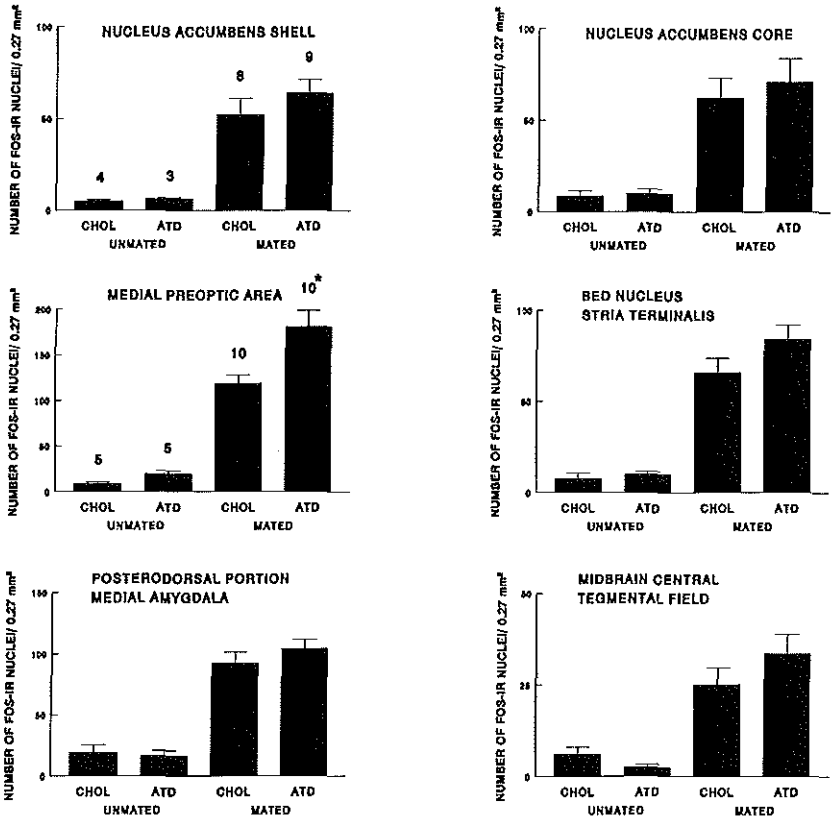

Figure 3. Effect of mating stimulation with an estrous female on the number of Fos protein-immunoreactive nuclei in the nucleus accumbens (shell and core), the medial preoptic area, the bed nucleus of the stria terminalis, the posterodorsal portion of the medial amygdala and the midbrain central tegmental field of neonatally estrogen-deprived males (ATD-treated) and normal males (cholesterol treated). * $p < 0.05$ post-hoc comparisons with the mated cholesterol-treated group (Student-Newman-Keuls method). The numbers in the figures represent the number of animals in each group. Data are expressed as mean ± SEM.

cant effect of mating ($F(1/26)= 80.9$, $p<0.0001$) and a significant effect of neonatal treatment ($F(1/26)= 5.9$, $p=0.02$) on the number of Fos-IR nuclei in the medial POA (Figure 3). Post-hoc analyses with the Student-Newman-Keuls method revealed that there were significantly more Fos-IR nuclei in the medial POA of mated males than of unmated males. Furthermore, mated ATD males had significantly more Fos-IR nuclei in the medial POA than mated Chol males. Mating augmented also the number of Fos-IR nuclei in the BNST ($F(1/26)= 57.2$, $p<0.0001$), MePD ($F(1/26)= 72.8$, $p<0.0001$) and CTF ($F(1/26)= 21.9$, $p<0.0001$; Figure 3) to equivalent numbers in ATD and Chol males. Finally, the n. accumbens

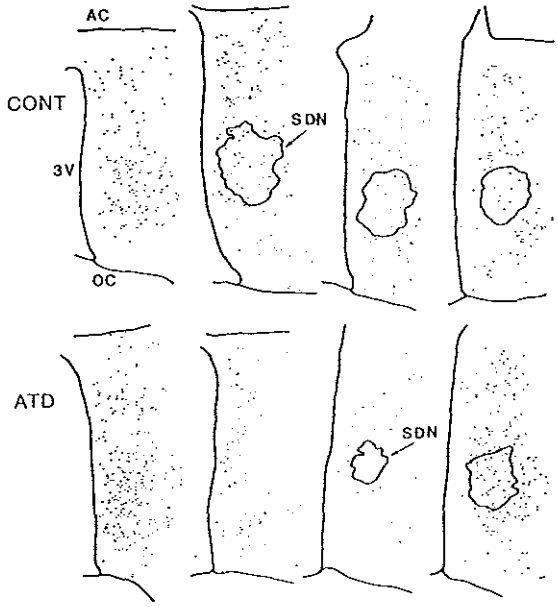

Figure 4. Camera lucida drawings of fos-immunoreactive nuclei (black dots) and the sexually dimorphic nucleus (SDN) in consecutive coronal sections through the preoptic area of a representative cholesterol-treated male (CONT) and an ATD-treated male after 8 intromissions with an estrous female.

shell ($F(1/20)= 28.2$, $p<0.0001$) and core ($F(1/20)= 15.0$, $p=0.001$) also showed increased Fos-IR after mating in both ATD and Chol males (Figure 3).

The mating increments in Fos-IR occurred in portions of the medial POA which included, but were not restricted to the SDN. Camera lucida drawings of Fos-IR neurons and the SDN in consecutive coronal sections through the medial POA of mated males are shown in Figure 4. Two-way analysis of variance (neonatal treatment x mating) on the mean number of Fos-IR neurons inside the SDN (see Figure 5), showed a significant effect of mating ($F(1/22)= 29.4$, $p<0.0001$) and of neonatal treatment ($F(1/22)= 4.2$, $p=0.05$) on the number of Fos-IR nuclei in the SDN. Posthoc-analyses with the Student-Newman-Keuls method revealed that the number of Fos-IR nuclei was significantly higher in mated males than in unmated males. Furthermore, the SDN of mated ATD males contained more Fos-IR neurons than the SDN of mated Chol males. There were no significant differences between ATD and Chol males in mean SDN area (ATD: 0.066 ± 0.005 mm^2; Chol: 0.078 ± 0.007 mm^2).

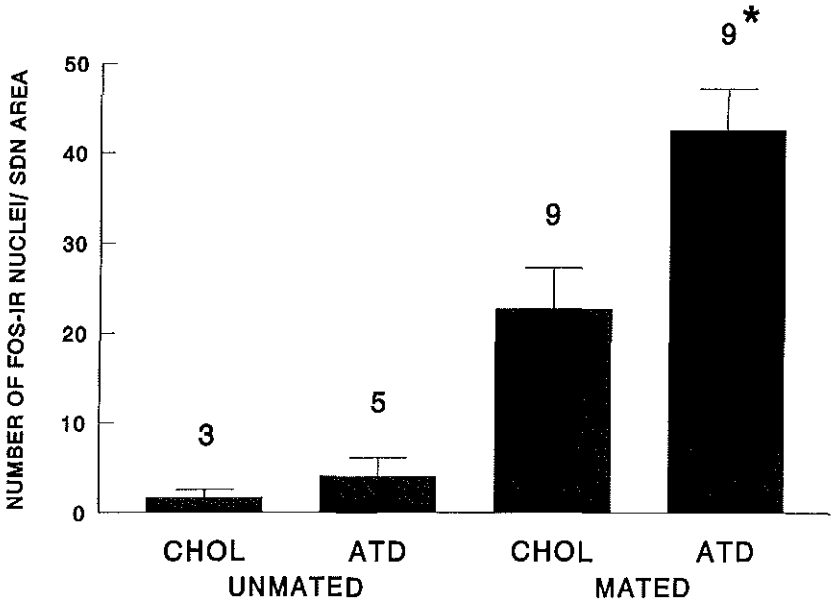

Figure 5. Effect of mating stimulation on the number of Fos protein-immunoreactive nuclei in the sexually dimorphic nucleus of the preoptic area of neonatally estrogen-deprived males (ATD-treated) and normal males (cholesterol treated). The numbers in the figures represent the number of animals in each group. Data are expressed as mean number of Fos protein-immunoreactive neurons inside the SDN per mean SDN area.

Experiment 2: neural c-Fos responses to chemosensory stimulation

Experiment 2a: neural c-Fos responses to urine and feces of estrous females

Exposure to urine and feces of estrous female significantly augmented the number of Fos-IR nuclei at each level of the vomeronasal projection circuit of both ATD and Chol males, compared to control males not exposed to soiled bedding from females (homecage Ss). The magnitude of this *c-Fos* response was very similar in ATD and Chol males. Examples of the effect of chemosensory stimulation on neuronal Fos-IR in the medial POA are shown in Figure 6. One-way analysis of variance revealed that exposure to chemosensory cues from estrous females augmented the number of Fos-IR neurons in the posteroventral portion of the medial amygdala (MePV; $F(2/12)= 8.1$, $p=0.006$) and the MePD ($F(2/12)= 18.6$, $p=0.0002$) to equivalent numbers in ATD and Chol males (Table 1). Chemosensory stimulation also increased Fos-IR in the BNST ($F(2/12)= 8.1$, $p=0.006$) and medial POA ($F(2/12)= 15.1$, $p=0.0005$) similarly in ATD and Chol males (Table 1). Furthermore, there was a

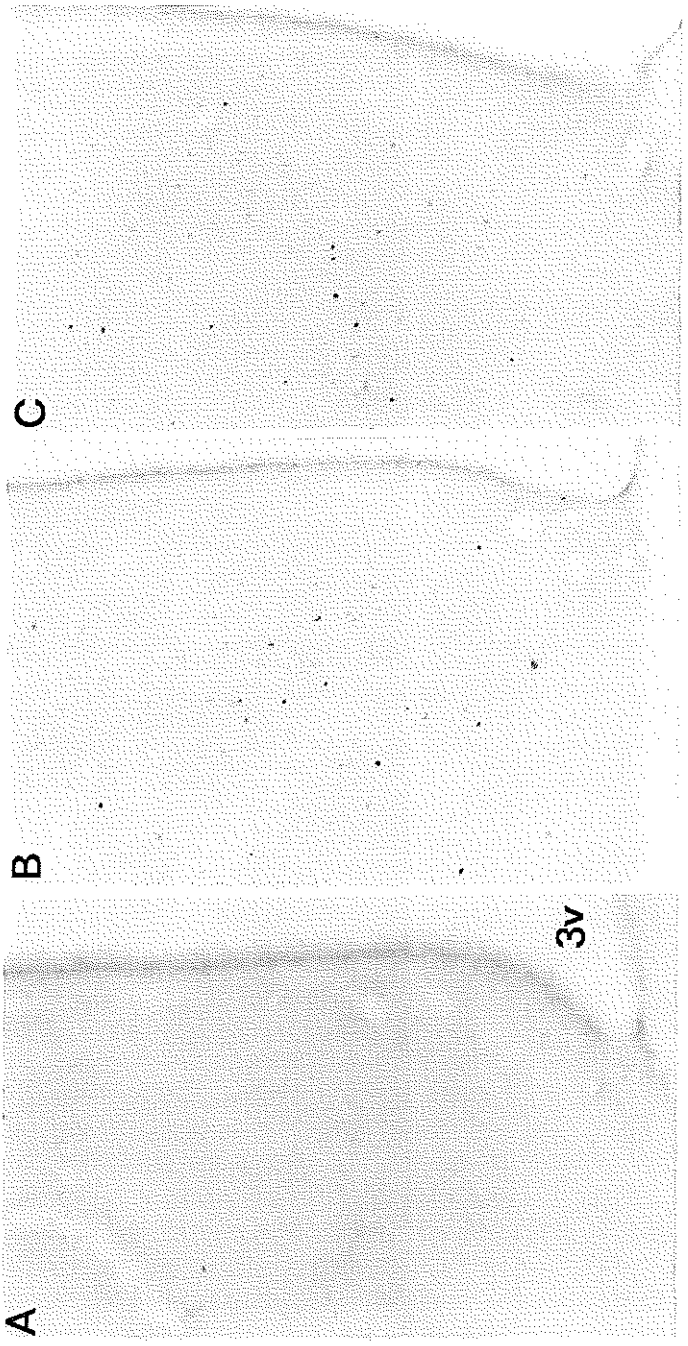

Figure 6. Photomicrographs showing Fos protein-immunoreactivity in the medial preoptic area of a male taken directly from his homecage to the perfusion room (A), a cholesterol-treated male after spending 1,5 hr in soiled bedding from estrous females, and an ATD-treated male after spending 1,5 hr in soiled bedding from estrous females (C). All animals were gonadectomized in adulthood and subsequently treated with estradiol.

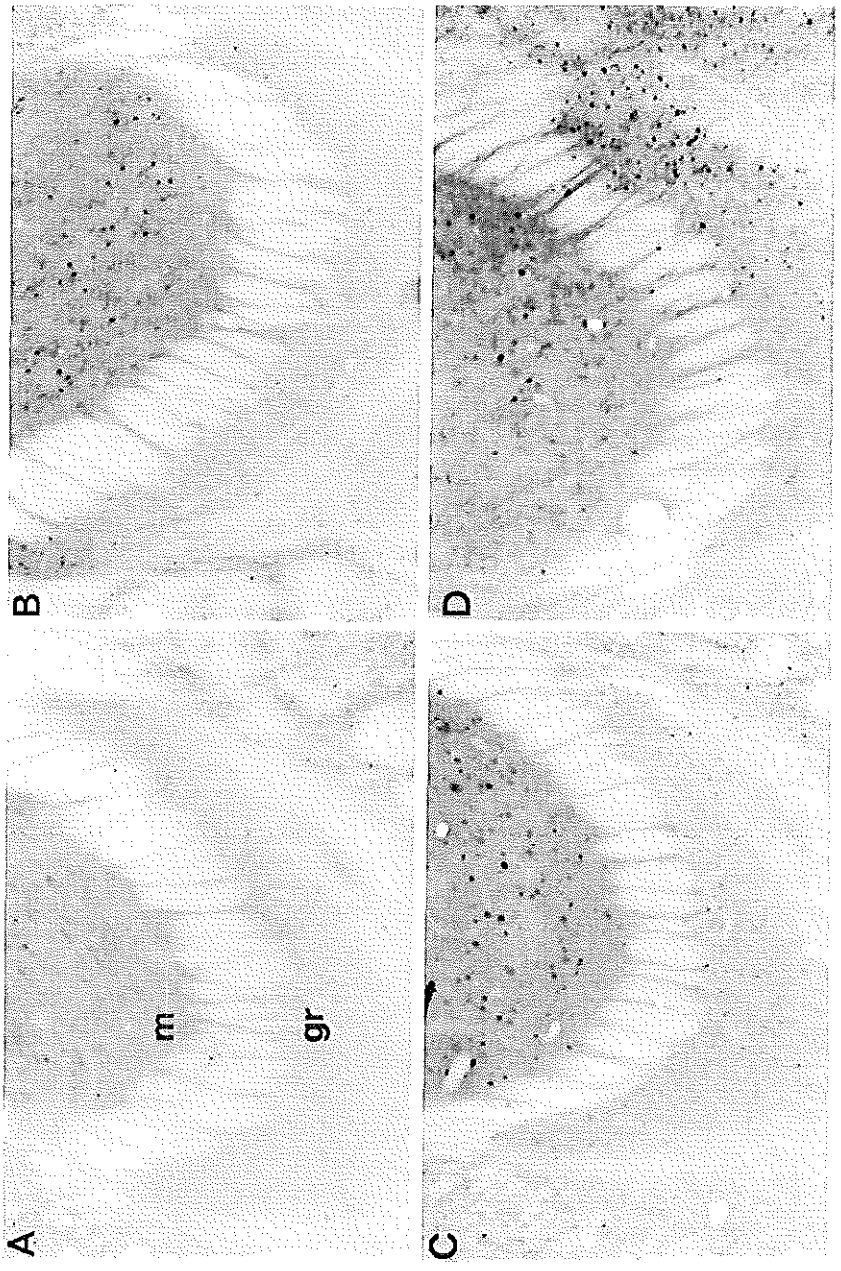

Figure 7. Photomicrographs of the accessory olfactory bulb showing Fos protein-immunoreactivity in the mitral (m) and granular (gr) layers from a male after spending 1,5 hr in clean bedding (A), a cholesterol-treated male after spending 1,5 hr in soiled bedding from sexually active males (B), an ATD-treated male after spending 1,5 hr in soiled bedding from sexually active males (C), a female after spending 1,5 hr in soiled bedding from sexually active males (D). All animals were gonadectomized in adulthood and subsequently treated with estradiol.

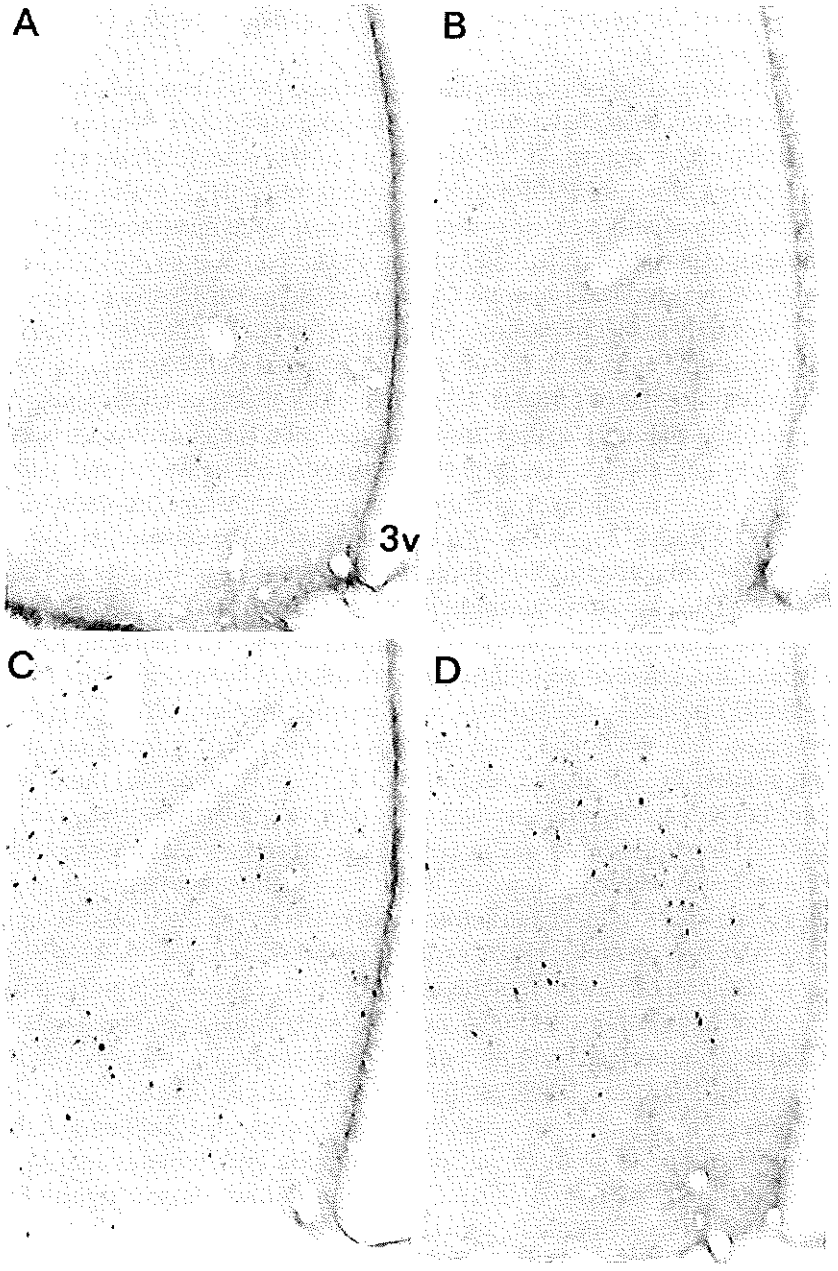

Figure 8. Photomicrographs of Fos protein-immunoreactivity in the medial preoptic area of a male after spending 1,5 hr in clean bedding (A), a cholesterol-treated male after spending 1,5 hr in soiled bedding from sexually active males (B), an ATD-treated male after spending 1,5 hr in soiled bedding from sexually active males, and a female after spending 1,5 hr in soiled bedding from sexually active males (D). All animals were gonadectomized in adulthood and subsequently treated with estradiol.

significant chemosensory-induced increment in Fos-IR in the n. accumbens core ($F(2/12)= 5.9$, $p=0.02$) and shell ($F(2/12= 4.2$, $p=0.04$) to equivalent numbers in ATD and Chol males (Table 1).

Experiment 2b: neural c-Fos responses to urine and feces of sexually active males

There were clear sex differences in neuronal *c-Fos* responses following exposure to soiled bedding (containing urine and faeces) from sexually active males. At the first levels of the vomeronasal pathway (accessory olfactory bulb-posterior medial amygdala), males and females showed similar *c-Fos* responses, but at more distal levels of the vomeronasal pathway (BNST, medial POA) female rats still showed clear *c-Fos* responses, whereas Chol males did not differ from subjects exposed only to clean bedding. ATD males, however, showed a clear *c-Fos* response at each level of the vomeronasal pathway which was equivalent to that of females. Examples of the effect of chemosensory stimulation on Fos-IR in the mitral and granule cell layer of the accessory olfactory bulb (AOB; first level of the vomeronasal pathway) and in the medial POA (distal level of the vomeronasal pathway) are shown in Figures 7 and 8. One-way ANOVA showed that exposure to soiled bedding from active males significantly augmented the number of Fos-IR nuclei in the AOB-mitral cell layer ($F(3/13)= 10.1$, $p=0.001$) to equivalent numbers in males and females (Table 1). The number of Fos-IR was also augmented in the AOB-granule cell layer ($F(3/13)= 15.1$, $p<0.001$) in males and females, with females showing a higher *c-Fos* response than ATD and Chol males. Chemosensory stimulation also augmented Fos-IR in the MePV ($F(3/17)= 26.0$, $p<0.0001$) and the MePD (Kruskal-Wallis one-way ANOVA on ranks: $H= 8.5$, ($df=3$), $p=0.04$) to similar numbers in both sexes. At more distal levels in the vomeronasal pathway, however, there were clear sex differences in *c-Fos* responses to odors from active males in BNST (Kruskal-Wallis one-way ANOVA on ranks; $H=14.9$ ($df=3$), $p=0.002$) and medial POA ($F(3/17)= 8.8$, $p=0.001$) with females and ATD males showing significantly larger numbers of Fos-IR nuclei in BNST and medial POA compared to Chol males and clean bedding Ss. Chol males showed no responsiveness in medial POA and BNST equivalent to clean bedding Ss (Table 1). Exposure to odors from sexually active males also augmented the number of Fos-IR in the n. accumbens core in both females and ATD males ($F(3/16)= 5.9$, $p=0.006$). Chol males did not show a significant increase in Fos-IR in the n. accumbens core.

DISCUSSION

Remarkably, heterosexual mating (mounts, intromissions, no ejaculations) led to a greater neuronal *c-Fos* response in the medial POA of male rats, in which brain estrogen synthesis was inhibited neonatally by treatment with the aromatase inhibitor ATD, compared to the medial POA of Chol males. Thus, neurons in the medial POA of ATD males, in which the normal processes of estrogen-dependent masculinization and defeminization of sexual behavior are attenuated, seem to be more responsive to heterosexual mating stimulation than those in the mPOA of control males. The larger number of Fos-IR neurons seen in the medial POA of mated ATD males is reminiscent of the observation of Wersinger et al.[39] that intromissive stimulation (received) induced significantly more Fos-IR in mPOA neurons of the female partner than in the mPOA of the male partner of a heterosexual mating pair of rats.

In both ATD and Chol males, mating-induced increments in Fos-IR occurred in portions of the medial POA which included, but were not restricted to the SDN. The number of Fos-IR neurons was greater in the SDN of ATD males than in the SDN of Chol males. Thus, the SDN of ATD males seems to be more responsive to heterosexual mating stimulation than the SDN of Chol males. To date, the volume of the SDN has only been measured in prenatally and pre- and neonatally ATD treated males with the latter males showing the largest reduction in SDN volume[21]. These findings indicate that neonatal estrogens are also required for the sexual differentiation of the male rats' SDN. Presumably, the volume of the male SDN is also reduced by neonatal ATD treatment alone, although in the present study no differences were found in mean SDN area between ATD and Chol males. An explanation for this latter finding could be that we used 50 m sections while the SDN measurements were done in 6 m sections[21]. Additionally, we did not have all the SDN sections in the present study. We are currently studying the SDN volume in neonatally ATD treated males.

In the study by Houtsmuller et al.[21], it was found that the size of the SDN was positively correlated with the frequency of masculine sexual behaviors: SDN volumes smaller than 10×10^{-3} mm^{-3} characterized nonejaculators, whereas volumes larger than 16×10^{-3} mm^{-3} were typical of ejaculators. However, female rats ovariectomized and treated chronically with high doses of either TP or EB can show all the elements of masculine sexual behavior, including mounts as well as intromissive and ejaculation behavior[5,8,14]. Apparently, the typical sex dimorphism in size of the SDN is not predictive of rats' capacity to display these masculine sexual behaviors.

Table 1: Effect of chemosensory stimulation on the mean (± SE) number of Fos-IR neurons (counted per standard area (0.27 mm²)). All subjects were gonadectomized and treated with estradiol or estradiol benzoate at the time of the experiment. AOB, accessory olfactory bulb; MePV, posteroventral portion medial amygdala; MePD, posterodorsal portion medial amygdala; BNST, bed nucleus of the stria terminalis; mPOA, medial preoptic area; Ss, subjects.

Group	N	AOB		MePV	MePD	BNST	mPOA	n.accumbens	
		mitrall	granule					core	shell
Exp 2a: odors from estrous									
homecage Ss	5	not determined		14 ± 4	7 ± 3	2 ± 1	7 ± 2	10 ± 3	4 ± 2
Chol males	5	not determined		101 ± 21*	79 ± 12*	75 ± 17*	65 ± 11*	45 ± 9*	14 ± 3*
ATD males	5	not determined		100 ± 16*	66 ± 6*	74 ± 15*	65 ± 8*	36 ± 7*	10 ± 3*
Exp 2b: odors from active									
clean bedding Ss	6	5 ± 2	2 ± 1	28 ± 8	20 ± 4	2 ± 1	15 ± 4	5 ± 2	2 ± 1
Chol males	5	82 ± 10*	86 ± 17*	135 ± 10*	70 ± 15*	15 ± 4	28 ± 7	14 ± 4	6 ± 2
ATD males	5	60 ± 15*	64 ± 13*	127 ± 11*	60 ± 13*	31 ± 4*	86 ± 12*	25 ± 6*	12 ± 3
females	5	76 ± 11*	123 ± 13*#	146 ± 12*	81 ± 18*	50 ± 11*	102 ± 23*	31 ± 6*	7 ± 3

* significantly ($p<0.05$) higher compared to homecage Ss (Exp 2a) or clean bedding Ss (Exp 2b)
# significantly ($p<0.05$) higher in females compared to males (ATD and Chol; Exp 2b).

The male's SDN may be involved in the suppression of feminine sexual behaviors, as was suggested by Hennesey et al.[19]. Some support for a possible functional role of the SDN in the inhibition of feminine sexual behaviors can be found in the study by Houtsmuller et al.[21]. They reported that the largest reduction in SDN volume was found in pre- and neonatally ATD treated male rats, which showed, while gonadally intact, the highest lordosis quotients in a pair test with an active male[9], compared to prenatally ATD treated males and control males. So, there seems to be a negative correlation between the size of the SDN and the display of feminine sexual behaviors. However, bilateral lesions of the SDN in testosterone-treated female rats did not affect their proceptive and receptive behaviors, but did affect their masculine sexual behaviors[37].

Less controversial is the inhibitory role of medial POA neurons in the display of receptive behaviors in female rats. Bilateral lesions of the mPOA/AH enhanced females' receptivity indicating an inhibitory effect of POA neurons in the lordosis reflex[20,31,41].

The distribution and intensity of increments in neuronal Fos-IR following exposure to soiled bedding from estrous females were very similar in both ATD and Chol males. *c-Fos* Responses were evident at each level of the vomeronasal pathway. Thus, there was no effect of neonatal estrogen deprivation on male rats' later neuronal responsiveness to odors from estrous females. This was rather surprising, because in a previous behavioral study[2] it was found that ATD males, castrated and treated with estradiol in adulthood (the same hormone conditions we used in the present study), spent significantly more time with a sexually active male to whom they show high levels of feminine sexual behaviors, when they were given free access to an estrous female and a sexually active male. Apparently, the behavioral findings of this previous study do not predict neuronal *c-Fos* responses in the chemosensory pathway when exposed to odors from estrous females. A similar discrepancy between behavior and neuronal *c-Fos* responses was recently found by Bressler and Baum (submitted for publication). In that study, these authors investigated odor preference (urine-soaked bedding from estrous vs anestrous females) and neuronal *c-Fos* responses to soiled bedding from estrous and anestrous females in gonadectomized, testosterone propionate-treated male and female rats. Both males and females preferred to investigate the estrous bedding for more time than the anestrous bedding, although they had similar increments in Fos-IR after exposure to bedding from either estrous or anestrous females at each level of the vomeronasal projection system. This result was not predicted by the behavioral findings. Thus, there is no clear sexual dimorphism in the functional activity of the

vomeronasal system after exposure to odors from estrous females, which is surprising since large morphological differences at all levels of the system have been reported between the sexes[35]. Additionally, the functional activity of the vomeronasal system to odors from females seems to be independent of neonatal estrogens, i.e. it is not sexually differentiated.

There was by contrast, a clear sexual dimorphism in functional activity, as revealed by Fos-IR, of the vomeronasal system after exposure to soiled bedding from sexually active males. At the beginning of the vomeronasal pathway, i.e. the AOB-mitrall cell layer and the posterior medial amygdala, there were no differences in the number of Fos-IR neurons between the sexes. Only the number of Fos-IR neurons in the granule cell layer differed between the sexes, with females showing a higher response than males. At more distal levels of the vomeronasal pathway, i.e. the BNST and mPOA, sex differences became evident. In female rats, the BNST and medial POA were clearly responsive to odors from active males, whereas in Chol males these brain areas were not responsive. Interestingly, ATD males showed a female-like responsiveness, i.e. *c-Fos* responses were evident at each level of the vomeronasal system. In this case, the behavioral findings found in earlier studies[2,23,24] do predict the neuronal *c-Fos* responses in the chemosensory pathway when exposed to odors from active males. When tested for sexual partner preference (choice: estrous female vs sexually active male), both females[23,24], ovariectomized and treated with estradiol benzoate, and neonatally ATD treated males[2], castrated and treated with estradiol in adulthood, spent significantly more time approaching and interacting sexually with a sexually active male than with an estrous female, whereas Chol males[2] spent significantly more time approaching and interacting sexually with an estrous female than with a sexually active male. Thus, there is a sexual dimorphism in the functional activity as revealed by Fos-IR of the vomeronasal pathway following exposure to odors of sexually active males, which is clearly dependent on the action of neonatal estrogens in males.

As mentioned in the Introduction, the vomeronasal system is a sexually dimorphic network with males having larger number of neurons at each level of the vomeronasal pathway. The differentiation of the vomeronasal system seems to be dependent on the presence of perinatal androgens[35]. Early postnatal castration decreases the number of cells and/or the volumetric measures in the vomeronasal organ, AOB, the medial posterior division of the BNST, and the bed nucleus of the olfactory tract. Early postnatal androgenization of females increases the number of neurons and/or the volumetric measures in vomeronasal organ, AOB, medial

posterior division of the BNST, and the bed nucleus of the olfactory tract[35]. These androgen effects on the developing vomeronasal projection circuit are most likely mediated by its estrogenic metabolite as has already be shown for the medial amygdala, AOB and bed nucleus of the olfactory tract[35]. Presumably, the vomeronasal system of ATD males functionally resembles that of females; the morphology of this system in ATD males has not yet been studied, however except for the medial POA. The supposition that ATD males show a female-like vomeronasal system is supported by the present study, in which ATD males showed a female-like responsiveness in the chemosensory pathway to odors of sexually active males.

Numerous experiments[15] have shown that the mesolimbic dopaminergic system is involved in motivational aspects of sexual behavior. Mitchell and Gratton[28] reported that male rats exposed to soiled bedding from estrous females had an increase in dopamine release in the nucleus accumbens. This response became larger and peaked earlier in time with repeated presentations. When male rats were exposed to other males' bedding or anestrous females' bedding, however, dopamine did not increase. These findings show that reproductively relevant stimuli have access to the mesolimbic dopamine system, activating dopamine release. This correlation between reproductively relevant chemosensory stimuli and the nucleus accumbens, is supported by the present study, in which we found that exposure to bedding from estrous females significantly increased the number of Fos-IR neurons in the nucleus accumbens (core and shell) in both ATD and Chol males. When males (ATD and Chol) and females were exposed to odors of sexually active males, the number of Fos-IR neurons in the nucleus accumbens core was increased in females and ATD males, but not in Chol males.

CONCLUSIONS

Neurons in the medial preoptic area, including the sexually dimorphic nucleus, of ATD males were more responsive to heterosexual mating stimulation than those in the medial preoptic area, including the sexually dimorphic nucleus, of control males.

There was no effect of neonatal estrogen deprivation on male rats' adult responsiveness of the vomeronasal projection pathway to odors from estrous females. By contrast, neonatal estrogens clearly mediates male rats' neuronal responsiveness of the vomeronasal projection pathway to odors from sexually active males.

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Chapter X

QUANTITATIVE ESTIMATION OF ANDROGEN AND ESTROGEN RECEPTOR-IMMUNOREACTIVE CELLS IN THE FOREBRAIN OF NEONATALLY ESTROGEN-DEPRIVED MALE RATS

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ABSTRACT

Previous studies have shown that estrogen and androgen receptor concentrations are sexually dimorphic in several areas of the rat brain. In general, adult female rats have a higher estrogen-binding capacity in specific brain areas than male rats, wheras male rats have a higher androgen-binding capacity in several brain areas than female rats. These sex differences are presumably established perinatally under the influence of endogenous circulating sex hormones in the male rat brain. In the present study, we evaluated the possible contribution of postnatal estradiol in sexual differentiation of brain estrogen and androgen receptors. On postnatal days 0-10, male rats received sc silastic capsules containing 1,4,6-androstatriene-3,17-dione (ATD), which inhibits the aromatization of testosterone to estradiol, or empty silastic capsules (control males). Using quantitative immunocytochemical techniques, we localized androgen and estrogen receptors in a large number of hypothalamic and limbic nuclei of male rats.

Neonatally ATD-treated males showed a significantly higher estrogen receptor-immunoreactivity than control males in the periventricular preoptic area and the ventrolateral portion of the ventromedial nucleus of the hypothalamus, i.e. those brain areas, in which sex differences have been reported. Thus, neonatal estrogens might be responsible for the lower levels of estrogen receptors in the normal male periventricular preoptic area and the ventromedial nucleus of the hypothalamus. No significant differences in the distribu-

tion and density of androgen receptors were found between ATD and control males. Apparently, neonatal estradiol does not play a significant role in sexual differentiation of androgen receptors.

INTRODUCTION

In mammals, testosterone and estradiol derived from neural aromatization of testosterone, act synergistically in the developing male brain to organize its structures and functions, i.e. to masculinize and defeminize the neural substrates that later control sexual behavior and neuroendocrine function. In the rat brain, masculinization results in the display of male-typical sexual behavior (mounts, intromissions, and ejaculations) in adulthood. Defeminization results in a loss of cyclic release of gonadotropins necessary for ovulation and a loss of female-typical sexual behavior (lordosis, presenting, earwiggling, hop and dart) in adulthood (e.g. MacLusky and Naftolin, 1981). Thus, gonadally intact male rats display the complete pattern of male coital behavior when pair tested with an estrous female and no female-typical sexual behavior when pair tested with a sexually active male. However, lordosis behavior can be induced in castrated male rats by administering estradiol and progesterone, although the behavior displayed is usually less intense than that of females, and that considerably more estradiol is required for its excitation (Davidson, 1969; Whalen, Luttge, and Gorzalka, 1971; Olster and Blaustein, 1988).

The development of the female rat brain is generally believed to proceed in the absence of testosterone and estradiol. Gonadally intact female rats display periods of behavioral proceptivity, i.e. seeking out the male partner, and receptivity, i.e. the display of lordosis in response to mounts, around the time of ovulation (Beach, 1976). However, male-typical sexual behavior, such as intromission-like and ejaculation-like behavior can be induced in ovariectomized female rats by administering testosterone or estradiol, although higher doses are required for its activation than in castrated male rats (Beach, 1942; Emery and Sachs, 1975; Baum, Södersten, and Vreeburg, 1974; Oboh, Paredes, and Baum, 1995).

These sex differences in responsiveness to testosterone and estradiol might be influenced by sex differences in the concentration and distribution of androgen and estrogen receptors in the brain. To date, there is evidence indicating that estrogen and androgen receptor concentrations are sexually dimorphic in several areas of the rat brain. Adult female rats

have a higher estrogen-binding capacity in the periventricular preoptic area (PVP), the medial preoptic area (mPOA), the arcuate (Arc) and in the ventromedial (VMH) nuclei of the hypothalamus than adult male rats (Brown, Hochberg, Zielinski, and MacLusky, 1988; Brown, MacLusky, Shanabrough, and Naftolin, 1990). Sex differences have also been found in basal estrogen receptor mRNA levels in the ventrolateral portion of the VMH and in the Arc with female rats showing higher concentrations than male rats (Lauber, Mobbs, Muramatsu, and Pfaff, 1991). The sex differences in estrogen receptor biosynthesis are already present early in development (DonCarlos and Handa, 1994; Kühnemann, Brown, Hochberg, and MacLusky, 1994). Using immunocytochemical procedures, sex differences have been found in both newborn and adult rats, with females showing a higher density of estrogen receptor-immunoreactivity (ER-IR) than males (Yokosuka and Hayashi, 1995). Sex differences with respect to the androgen receptor have also been reported: nuclear androgen receptor concentrations were found to be higher neonatally in male rats than in female rats; this was most clearly seen in the amygdala (Meaney, Aitken, Jensen, McGinnis, and McEwen, 1985). Adult male rats, castrated and treated with physiological doses of testosterone, exhibit significantly higher levels of androgen receptor binding compared to like-wise treated females in the bed nucleus of the stria terminalis (BNST), PVP, and VMH (Roselli, 1991). Presumably, perinatal androgens are involved in the sexual differentiation of brain estrogen and androgen receptors.

We have previously shown that male rats in which brain estrogen formation was inhibited neonatally by administration of the steroidal aromatization blocker 1,4,6-androstatriene-3,17-dione (ATD) can readily show male-typical as well as female-typical sexual behavior (e.g. Bakker, van Ophemert, and Slob, 1993). In addition, partner preference of such ATD males is also incompletely differentiated: when given free access to an estrous female and a sexually active male, ATD males approached both the estrous female to whom they showed male-typical sexual behavior (mounts, intromissions, but rarely ejaculations) and the sexually active male to whom they showed feminine sexual behavior (lordosis, presenting, earwiggling, hop and dart) (Bakker, van Ophemert, and Slob, 1993; Bakker, van Ophemert, Timmerman, de Jong, and Slob, 1995). Furthermore, ATD males showed a response to estrogens which differs from that of control males. When castrated in adulthood and subsequently treated with estradiol, ATD males displayed high levels of feminine sexual behavior and showed a clearcut preference for a sexually active male over an estrous female. Like-wise treated control males display high levels of masculine sexual behavior and showed a clear preference for an estrous female over a sexually active male (Bakker,

Brand, van Ophemert, and Slob, 1993; Bakker, van Ophemert, and Slob, 1995).

In the present study, we evaluated the possible contribution of neonatal estradiol in the sexual differentiation of brain estrogen and androgen receptors using quantitative immunocytochemical techniques.

EXPERIMENTAL PROCEDURES

Animals and treatments

Male and female Wistar rats (Wu-strain, outbred) commercially obtained (Harlan, Zeist) were housed in single-sex groups of 2 to 3. Food and water was available ad libitum. Female rats were time-mated and parturition occurred 22 days later. Within 2-4 hours after birth, the newborn males received under ice anesthesia subcutaneously in the neck a silastic capsule (SR3: inner diameter 1.5 mm, outer diameter 2.1 mm, length 5 mm) containing crystalline ATD or empty silastic capsules (control males). The implants were removed at 21 days of age and the animals were housed 2-3 to a cage of the same treatment. The males were left undisturbed until the age of approximately 6 months. One week before estrogen receptor-immunocytochemistry, 9 ATD and 9 control males were castrated under ether anesthesia through a midline abdominal incision.

Immunocytochemistry

Males were anesthetized with sodium pentobarbital (100 mg/rat, i.p.), given an intracardiac injection of heparin (1000 U/rat), and perfused via the aorta with 0.1 M phospate-buffer saline (PBS; pH= 7.3) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (300 ml per animal). Brains were removed and postfixed in 4% paraformaldehyde for 2 h and placed in 0.1 M PBS. Sections of 50 m were cut on a Vibratome using ice-cold Tris-buffered saline (TBS; 0.05 M, 0.9% NaCL, pH= 7.6). TBS was used to rinse the sections for at least 1 h (three times with TBS) between all steps of the immunocytochemical procedure. All antibodies were diluted in 0.05 M TBS-containing 0.5 M NaCl and 0.5% Triton-X100 (pH= 7.6). For estrogen receptor immunoreactivity, the antibody ER 21 (gift of G. Greene, University of Chicago), which is a rabbit polyclonal antibody directed against the N-terminus (amino acids 1-21), i.e. the estrogen-binding domain of the estrogen receptor, has been used (1 g/ml). ER 21 has been validated previously (Blaustein, 1992; 1993; Meredith, Auger, and Blaustein, 1994). For androgen receptor immunoreactivity, the antibody PG 21 was used (gift of G. Greene,

University of Chicago), which is a rabbit polyclonal antibody directed against a synthetic peptide corresponding to the first 21 amino acids of the rat androgen receptor (2 g/ml) (e.g. Prins, Birch, and Greene, 1991). PG 21 has been validated extensively (e.g. Wood and Newman, 1993a; Wood and Newman, 1993b; Huang and Harlan, 1993; Clancy, Whitman, Michael, and Albers, 1994).

Free floating sections were incubated with primary antiserum (ER 21 or PG 21) and left for 1 h at room temperature and subsequently at 4°C overnight. Goat-anti-rabbit (Betsy, 1:100; Netherlands Institute for Brain Research) was applied for 1 h at room temperature followed (after rinsing with TBS) by peroxidase-anti-peroxidase (PAP; 1:1000; Netherlands Institute for Brain Research) for 1 h. All incubations were done on a rocking table. As chromogen, 3,3'diaminobenzidine tetrahydrochloride (DAB; 0.5 mg/ml) with 0.01% H_2O_2 and 0.2% nickel-ammonium sulphate in 0.05 M TBS (pH= 7.6) was used. Sections were then mounted on chrome-aluin coated slides, dehydrated and coverslipped using ethallan.

Quantitative analysis

The densitometrical analyses were performed on an IBAS-KAT image analysis system (Kontron). The image analyser was connected to a Bosch TYK9B TV camera equipped with a chalnycon tube mounted on a Zeiss microscope. The microscope was equipped with planapo objective, a light source connected to a stabilized power supply and a scanning stage under control of both a joystick and the image analyser. All measurements were done using a 560 nm small band filter (Schott), which coincides with the absorption maximum of the DAB-Nickel precipitate in the sections. For each section, the analysis consisted of the following steps: 1) loading of a shading image at a 10x magnification for measurement of the background; 2) loading of a 2.5x magnification image covering that part of the section which contained the area(s) of interest; 3) loading of 12 (4 x 3) 768 x 512 images with a 10x magnification; 4) manual outlining of the measuring areas (anatomical structures) in the reconstituted 2.5x magnification image; 5) feeding in the computer the names and the left/right side dimensions of the outlined structures; 6) calculation of a mask for the immunoreactive cells in each outlined structure and measurement of the total area of that mask in each outlined structure (areamask); 7) measurement of the area of the outlined structure (areafield); 8) calculation of the optical density of the areamask (ODmask) which measures the concentration of DAB in the outlined area (mg/m^3; 9) calculation of the integrated optical density (IOD) (IODmask = ODmask x areamask). In this way, the total amount of DAB, which is an estimate for the total number of estrogen or androgen receptors, was measured in each outlined area. The obtained data were first analyzed for

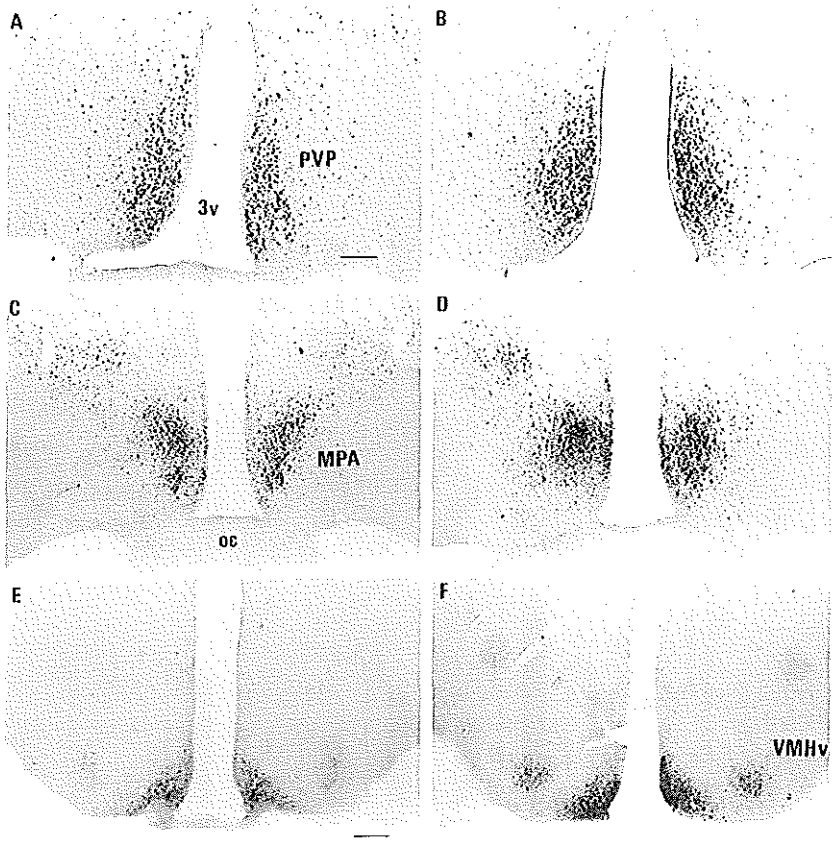

Figure 1. Photomicrographs showing estrogen receptor-immunoreactivity in neonatally ATD-treated male and control male rats. A and B the periventricular preoptic area of a control male (A) and of an ATD male (B), C and D the medial preoptic area of a control male (C) and of an ATD male (D), E and F the ventromedial nucleus of the hypothalamus of a control male (E) and of an ATD male (F). In A and B, 1 cm is 195 μm; in C-F, 1cm is 310 μm.

normal distribution using the Kolmogorov Smirnov Test and the group means for each area of interest were subsequently subjected to the Student's t-test. The $p = 0.05$ level is used as the upper limit for statistical significance.

RESULTS

Estrogen receptor-immunoreactivity

In both ATD and control males, dense populations of ER-IR cell nuclei were observed in the PVP, in the anterior portion of the mPOA, in the

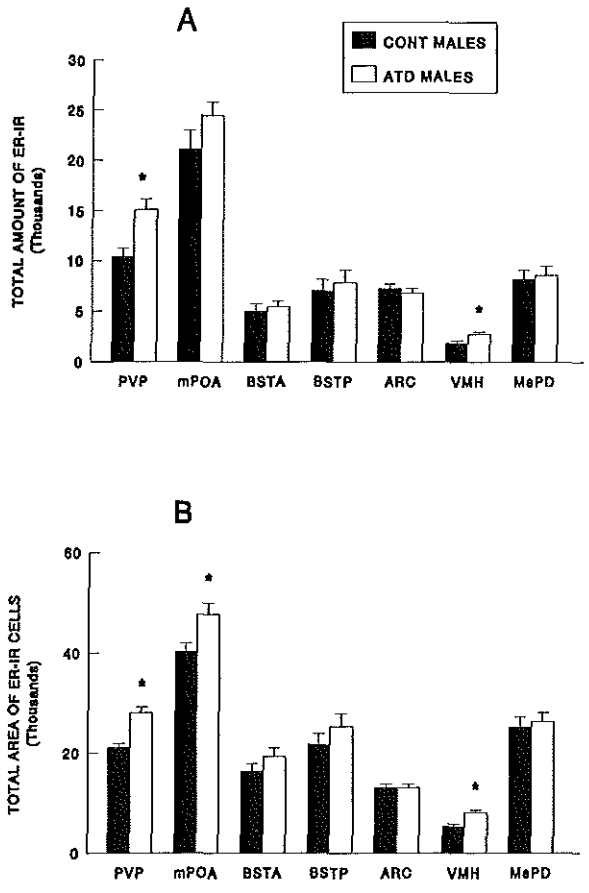

Figure 2. Estrogen receptor-immunoreactivity (ER-IR) of neonatally ATD-treated male rats and control male rats in several hypothalamic and limbic nuclei. A the total amount of ER-IR in each selected brain area, B the total area of ER-IR cells. * $p < 0.05$ student's t-test. Data are expressed as mean + SEM.

BNST, specifically in the anterior and posterior medial division, in the Arc, in the ventrolateral portion of the VMH, and in the medial amygdaloid nucleus posterodorsal (MePD). Furthermore, the PVP and the mPOA contained the greatest numbers of ER-IR nuclei. Examples of ER-IR in the PVP, mPOA and the VMH are shown in Figure 1. Analysis with Student's t-test showed that densities of ER-IR, i.e. the IOD, of ATD males were significantly higher compared to control males in the PVP ($t= 3.39$ (df= 16), $p=0.01$) and in the VMH ($t= 2.42$ (df= 15), $p=0.05$) (Fig 4A). ATD males had similar ER-IR densities as control males in the mPOA, BNST, Arc, and MePD (Fig 2A). Analyses with Student's t-test revealed that the total area of ER-IR cells, i.e. the areamask, was

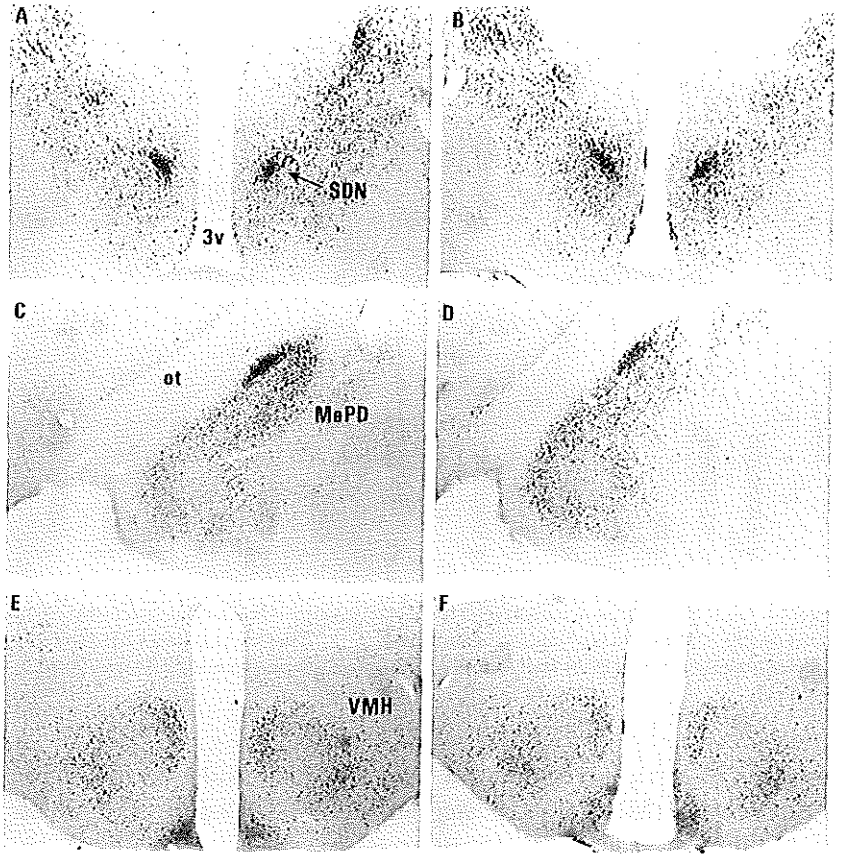

Figure 3. Photomicrographs (1 cm is 310 μm) showing androgen receptor-immunoreactivity of neonatally ATD-treated male and control male rats. A and B the medial preoptic area including the sexually dimorphic nucleus of a control male (A) and of an ATD male (B), C and D the posterodorsal medial amygdaloid nucleus of a control male (C) and of an ATD male (D), E and F the ventromedial nucleus of the hypothalamus of a control male (E) and of an ATD male (F).

significantly higher in the PVP ($t = 4.8$ ($df = 16$), $p < 0.001$), the mPOA ($t = 2.6$ ($df = 16$), $p = 0.02$), and the VMH ($t = 3.6$ ($df = 15$), $p = 0.01$) of ATD males compared to control males (Fig 2B).

Androgen receptor-immunoreactivity

In both ATD and control males, androgen receptor-immunoreactive (AR-IR) cell nuclei were found in the septohypothalamic nuclei (SHy), the mPOA containing the sexually dimorphic nucleus (SDN-POA), the anterior and posterior division of the BNST, Arc, the VMH, and through the rostrocaudal extent of the medial amygdaloid nucleus (Me), i.e.

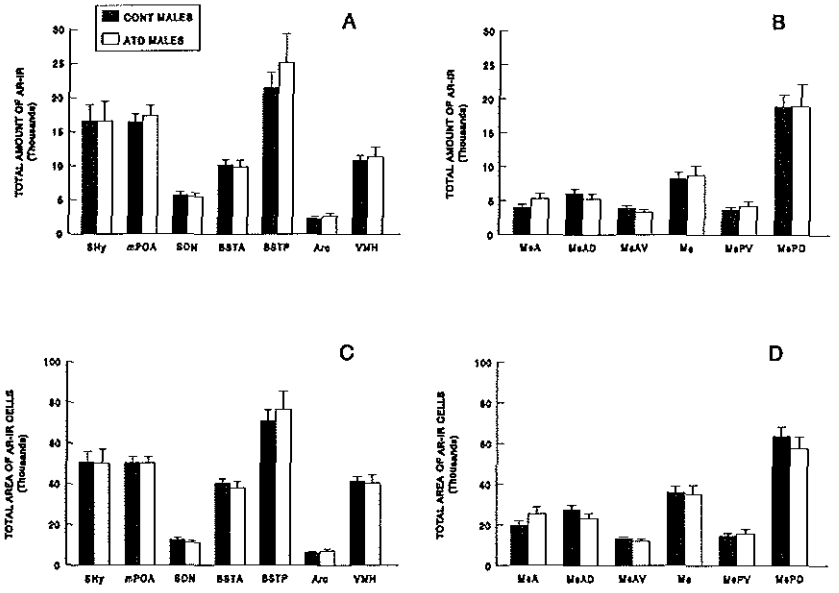

Figure 4: Androgen receptor-immunoreactivity (AR-IR) in several hypothalamic and limbic nuclei of neonatally ATD-treated male rats and control males. A and B the total amount of AR-IR in each selected brain area, C and D the total area of AR-IR cells. Data are expressed as mean + SEM.

anterior (MeA), anteroventral (MeAV), anterodorsal (MeAD), posteroventral (MePV), and posterodorsal (MePD) (see Fig 4). Furthermore, the greatest density in AR-IR was observed in the SHy, the mPOA, the posterior division of the BNST, and the MePD. Examples of AR-IR in the mPOA containing the SDN, the posterior division of the BNST, and medial amygdaloid nucleus posterior, are presented in Figure 3. Analysis with Student's t-test revealed that ATD and control males had similar densities of AR-IR, i.e. the IOD, in each area measured (Fig 4, panels A and B). Also, no differences between ATD and control males were found in the total area of AR-IR cells, i.e. the areamask (Fig 4, panels C and D).

DISCUSSION

Estrogen receptor-immunoreactivity

High densities of ER-IR were observed in the PVP, an area known to be involved in gonadotropin release, and the mPOA. The total amount of estrogen receptors was significantly higher in the PVP and the ventrolateral portion of the VMH of male rats, in which brain estrogen formation was inhibited neonatally by administration of the aromatase inhibitor ATD,

compared to control males. As described in the introduction, sex differences have been reported in those areas with female rats showing a higher estrogen-binding capacity (Brown, MacLusky, Shanabrough, and Naftolin, 1990; Kühnemann, Brown, Hochberg, and MacLusky, 1994) or a higher ER-IR (Yokosuka and Hayashi, 1995) than male rats. These male-female differences are presumably established within 24 hours after birth for the PVP and within 5-10 days for the VMH (Kühnemann, Brown, Hochberg, and MacLusky, 1994). Castration of males and treatment with testosterone propionate of females within 24 hours after birth can reverse brain ER levels with castrated males showing increased ER levels and androgenized females showing reduced ER levels (Kühnemann, Brown, Hochberg, and MacLusky, 1995). The present findings in neonatally estrogen-deprived males suggest that estrogen, derived from neural aromatization of testosterone, is responsible for the lower levels of ER in the PVP and the VMH of the adult male rat. However, the total amount of ER-IR in the mPOA and the Arc of neonatally ATD-treated males were similar to that of control males, although the total area occupied by ER-IR cells in the mPOA of ATD males was higher than in the mPOA of control males (see Fig 1). Kühnemann et al (1995) reported that castration immediately after birth increased ER levels in those areas, whereas castration 1 day after birth failed to do so. Presumably, the sex differences in brain estrogen levels in the mPOA and the Arc might represent an extremely rapid response to the hypothalamic surge in estradiol occurring in males between 0 h *in utero* and 1 h after delivery (Rhoda, Corbier, and Roffi, 1984). Since ATD treatment was initiated not until 2-4 hours after birth, ATD males were exposed to this hypothalamic surge in estradiol. Therefore, ATD males' brains could have been already partly organized in a male direction, i.e. ER levels were already lowered partly in the mPOA and almost completely in the Arc. In support of the role of postnatal estrogen in the sexual differentiation of ER in the preoptic area are the findings by DonCarlos et al (1995). Female rats neonatally treated with the synthetic estrogen, diethylstilbestrol, showed at the age of 28 days reduced ER mRNA levels in the preoptic area, comparable to the levels seen in intact males. The non-aromatizable androgen, dihydrotestosterone, had no effect on ER mRNA in the preoptic area of females.

The sex differences in ER levels in different brain areas seemed to be expressed asynchronously, providing a possible mechanism for variation in the duration of 'critical periods' for androgen or estrogen-mediated sexual differentiation of specific neural functions. Masculinization and defeminization of sexual behavior are probably two separable processes, which occur at slightly different times during development (e.g. Baum, 1979; Ward and Ward, 1985; Beatty, 1992). Our behavioral data suggest

that defeminization of the male rat brain takes place primarily neonatally under the action of estradiol and that masculinization of the brain occurs mainly both prenatally and immediately after birth under the synergistic action of testosterone and estradiol.

The sex differences in densities of ER-IR cells in the PVP, the mPOA, the Arc and the VMH may contribute to the regulation of sex-specific estrogen-dependent functions. For instance, female rats are capable of showing a preovulatory LH surge after adult gonadectomy and treatment with a large dose of estradiol, whereas male rats are not (Neill, 1972). Neonatal castration makes it possible for males to show a preovulatory LH surge in response to estrogen, whereas neonatal treatment of female rats with testosterone eliminates this capacity to exhibit estrogen-induced surges (for overview see Dyer, 1984). Whether functional responses, such as an estrogen-induced LH surge, are directly proportional to the number of ER containing neurons in a particular brain area remains to be investigated. Interestingly, a coexistence of ER and neurotensin have been found in the PVP (Axelson, Shannon, and Van Leeuwen, 1992). Neurotensin appears to be involved as an interneuron in the regulation of the release of luteinizing-releasing hormone (e.g. Alexander, Mahoney, Ferris, Carraway, and Leeman, 1989).

For activation of feminine sexual behavior in the male rat a much higher dose of estradiol is required than in the female rat (Goy and McEwen, 1980; Davidson, 1969; Whalen, Luttge, and Gorzalka, 1971; Olster and Blaustein, 1988). This is confirmed by previous behavioral findings in our laboratory (Brand, Brand, van Ophemert, and Slob, 1993; Bakker, van Ophemert, and Slob, 1995). Neonatally ATD-treated male rats, castrated in adulthood and subsequently treated with estradiol, show high levels of feminine sexual behaviors and a partner preference for a sexually active male, whereas like-wise treated control males show high levels of masculine sexual behaviors and a partner preference for an estrous female. Only when treated with extremely high doses of estradiol (blood serum levels > 1000 pmol/l; normal blood serum levels 7 pmol/l), normal males show feminine sexual behaviors (unpublished findings in our laboratory). These findings suggest that the reduced capacity to respond to estrogen in adulthood is associated with lower levels of ER in the male rat brain.

Meredith et al (1994) reported that the ER 21 antiserum is able to detect both occupied and unoccupied ER in the guinea pig, since ER 21 immunostaining was not affected by a high dose of 17ß-estradiol (50 g). In a pilot study, we found a more intense staining with the ER 21 antiserum after the male rats were castrated. It has been reported that castration leads to an up-regulation of ER mRNA (Shughrue, Bushnell, and Dorsa, 1992), whereas acute estradiol treatment down-regulates ER

mRNA (Lauber, Mobbs, Muramatsu, and Pfaff, 1991). In the present study, castration presumably has led to an up-regulation of ER in both ATD and control males. Thus, one could say that we have measured the maximal capacity of ER rather than physiological levels of ER. It should be noted that the period of time between castration and estrogen receptor-immunocytochemistry was similar in both ATD and control males. Therefore, we believe that castration up-regulated ER to the same extent in ATD as in control males.

Androgen receptor-immunoreactivity

The highest densities of AR-IR were observed in the Shy, the mPOA, the posterior division of the BNST and the posterodorsal division of the medial amygdaloid nucleus. No significant differences in distribution or density of AR-IR were found between neonatally estrogen-deprived males and control males. Apparently, postnatal estradiol does not play a significant role in the sexual differentiation of androgen receptor levels in adulthood. To what extent androgen receptors are sexually differentiated, i.e. sexually dimorphic in adulthood, is not clear. Reports on sex differences in androgen binding capacity have been conflicting. Biochemical assays of androgen binding in whole preoptic area and/or hypothalamus dissections have not revealed sex differences (Handa, Reid, and Resko, 1986; Roselli, Handa, and Resko, 1989). However, measuring androgen receptor binding in more discrete nuclei within the preoptic area and hypothalamus have demonstrated sex differences (Roselli, 1991). Especially the caudal part of the mPOA showed sex differences with males having more testosterone target neurons that project to the midbrain than females (Lisciotto and Morrell, 1994). This might not be proof for sex differences in androgen receptors, since testosterone can be aromatized to estradiol within the central nervous system. Therefore, a labelled cell following an injection of radioactively labelled testosterone may represent estrogen binding to estrogen receptors. Sex differences in androgen receptor levels have been found during the first 10 days of life, especially in the amygdala (Meaney, Aitken, Jensen, McGinnis, and McEwen, 1985). In this latter study, however, males and females were left gonadally intact, which makes it very likely that the sex differences in androgen receptor levels reflect sex differences in endogenous testosterone or dihydrotestosterone levels. Presumably, there are no large sex differences in the distributions and densities of androgen receptor containing cells, although small subtle quantitative differences in certain regions of the male and female rat brain cannot be ruled out. It seems unlikely that possible sex differences are caused by the actions of postnatal estradiol during early development, since ATD males showed similar distributions and densities of AR-IR as control males. To date, we

cannot determine whether or not sexual differentiation of androgen receptor levels occurs and if it happens, whether it takes place prenatally under the influence of androgens/estrogens and/or postnatally under the influence of androgens (Resko, Feder and Goy, 1968; Pang, Caggiula, Gay, Goodman, and Pang, 1979; Weisz and Ward, 1980; Slob, Ooms, and Vreeburg, 1980; Baum, Brand, Ooms, Vreeburg, and Slob, 1988; Baum, Woutersen, and Slob, 1991). Future studies should address this question.

Interestingly, a dense cluster of AR-IR was found in the SDN-POA (see Fig 3). This was not seen with ER-IR. In the rat, the adult volume of the SDN-POA is determined by the perinatal action of androgens (Döhler, Coquilin, Davis, Hines, Shryne, and Gorski, 1982; Döhler, Coquilin, Davis, Hines, Shryne, and Gorski, 1984; Döhler, Srivastava, Shryne, Jarzab, Sipos and Gorski, 1984; Jacobson, Csernus, Shryne, and Gorski, 1981; Houtsmuller, Brand, de Jonge, Joosten, Van de Poll, and Slob, 1994). Jacobson et al (1987) have found that gonadectomized and adrenalectomized male rats showed a higher percentage of radioactively labelled cells in the SDN-POA following exposure to [^{3}H]testosterone than gonadectomized and adrenalectomized females (Jacobson, Arnold, and Gorski, 1987). This sex difference might be due to a difference in androgen receptor levels in the mPOA (Handa, Reid, and Resko, 1986). In the present study, no differences were found in androgen receptor levels in the SDN-POA between neonatally ATD-treated males and control males. At present, the volume of the SDN-POA of neonatally ATD-treated males has not yet been measured, however, we expect it to be smaller than that of control males, since neonatal castration of male rats reduces SDN-POA volume in adulthood (Jacobson, Csernus, Shryne, and Gorski, 1981). We are currently studying the SDN-POA volume in neonatally ATD-treated male rats.

CONCLUDING REMARKS

Using quantitative immunocytochemical procedures, the total amount of estrogen and androgen receptors were estimated in a large number of hypothalamic and limbic nuclei of male rats, in which brain estrogen formation was inhibited neonatally by treatment with the aromatase inhibitor ATD. The highest densities of ER-IR was observed in the periventriculair preoptic area (PVP) and the mPOA. ATD males showed a higher ER-IR in those brain areas, i.e. the PVP and the ventrolateral portion of the VMH, in which sex differences have been reported with female rats showing a greater estrogen binding capacity than male rats. These findings suggest that estrogen, derived from neural aromatization of testosterone, is responsible for the lower levels of ER in the normal male PVP and the VMH.

The highest density of AR-IR was found in Shy, the mPOA, the posterior division of the BNST and the posterodorsal division of the medial amygdaloid nucleus. No significant differences in distribution or density of AR-IR were found between neonatally estrogen-deprived males and control males. Apparently, postnatal estradiol does not play a significant role in the sexual differentiation of androgen receptor levels.

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ABBREVIATIONS

Arc	arcuate nucleus
AR-IR	androgen receptor-immunoreactivity
ATD	1,4,6-androstatriene-3,17-dione
BNST	bed nucleus of the stria terminalis
DAB	3,3' diaminobenzidine tetrahydrochloride
ER-IR	estrogen receptor-immunoreactivity
IOD	integrated optical density
Me	medial amygdaloid nucleus
MeA	anterior medial amygdaloid nucleus
MeAV	aneroventral medial amygdaloid nucleus
MeAD	anterodorsal medial amygdaloid nucleus
MePD	posterodorsal medial amygdaloid nucleus
MePV	posteroventral medial amygdaloid nucleus
mPOA	medial preoptic area
PBS	phosphate-buffer saline
PVP	periventricular preoptic area
SDN-POA	sexually dimorphic nucleus of the preoptic area
Shy	septohypothalamic nuclei
TBS	tris-buffer saline
VMH	ventromedial nucleus of the hypothalamus

Chapter XI

GENERAL DISCUSSION

In this chapter, the main results and some future lines of research to further study the role of perinatal estradiol in the sexual differentiation of the male rat brain will be discussed.

Sexual differentiation of the male rat brain

The studies presented in this thesis show that neonatal aromatization of testosterone to estradiol plays a pivotal role in the sexual differentiation of the male rat brain. Adult, gonadally intact male rats, in which brain estrogen formation was inhibited neonatally by administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD), can readily display masculine as well as feminine sexual behaviors. Such ATD males apparently developed the neural mechanisms needed for the adult expression of male-typical sexual behaviors without losing those needed for the adult expression of female-typical sexual behaviors. Remarkably, ATD males, studied in adulthood when gonadally intact, can exhibit both types of sexual behavior intermittently, depending on the partner they are with and the phase of the dark period of the light/dark cycle they are tested. When given free access to a tethered estrous female and a tethered sexually active male, ATD males run back and forth between the sexually active male to whom they show female-typical sexual behaviors and the estrous female to whom they display male-typical sexual behaviors. This is most outspoken when tested during the early part of the dark period of the light/dark cycle.

In all studies presented in this thesis, ATD treatment was initiated neonatally. This was done because in a previous study (13), it was found that the critical period for the sexual differentiation of partner preference and coital behavior appeared to be primarily early neonatal. Prenatal ATD treatment only slightly decreased the preference for an estrous female over a sexually active male, whereas neonatal ATD treatment made the preference for an estrous female virtually disappear (13). Likewise, prenatal ATD treatment had no major effect on ejaculatory behavior, whereas neonatal ATD treatment impaired the ability of most male rats to ejaculate (13). However, combined pre- and neonatal ATD treatment decreased the preference for an estrous female over a sexually active male more dramatically than neonatal ATD treatment alone (13). These findings suggest that the process of sexual differentiation of partner preference and coital behavior begins prenatally. This differentiation process of partner preference and coital behavior presumably consists of multiple steps, as was earlier suggested for the sexual differentiation of the sexually dimorphic nucleus of the preoptic area

(e.g. 54). Our behavioral data presented in chapter 5 (page 101) demonstrate the importance of prenatal estradiol in the sexual differentiation of partner preference. We found that partner preference of neonatally ATD-treated male rats, gonadectomized in adulthood and treated with estradiol was clearly intermediate between the extremes of the male and female control group, both groups also gonadectomized in adulthood and treated with estradiol. Presumably, pre- and neonatally ATD-treated male rats would be indistinguishable from control females in their partner preference. However, to date, we have not studied this. It would be very interesting to test this hypothesis in the future.

With respect to coital behavior, neonatally ATD-treated males are masculinized but not defeminized. Gonadally intact ATD males can readily display masculine sexual behavior, that is, mounts and intromissions although ejaculatory behavior seems to be impaired when tested early in the dark phase of the light/dark cycle. However, when tested late in the dark phase, ATD males can readily ejaculate (chapters 6 and 7, page 119, 139). Furthermore, neonatally ATD-treated male rats, studied in adulthood when castrated and estradiol-treated, were indistinguishable from control females given the same adult hormone treatment in the display of feminine sexual behavior, that is, proceptive and receptive behavior (chapter 5, page 101). Our data suggest that behavioral masculinization takes place pre- and neonatally, possibly through a synergistic action of testosterone and estradiol, and that behavioral defeminization occurs primarily neonatally under the action of estradiol. Indeed, it has been postulated that masculinization and defeminization of sexual behavior are two separable processes that occur at slightly different times in development and are differentially affected by the organizational actions of testosterone and estradiol (e.g. 8,12,18,25,58).

In all studies presented in this thesis, the neural conversion of endogenous testosterone to estradiol was blocked with the steroidal aromatization blocker ATD. It was previously found that ATD administered neonatally by silastic capsules affected the sexual differentiation of the brain more profoundly than ATD administered via subcutaneous injections (58). In addition, it was found that two days after implantation of silactic capsules containing crystalline ATD (postnatal Day 3), blood plasma concentrations of testosterone were normal, while the concentration of ATD in the plasma was high (520 ng/ml plasma) (58). Therefore, in all our studies we used the same method of neonatal treatment as Vreeburg and co-workers (58). However, there is some evidence that ATD also acts as an androgen receptor blocker, in addition to blocking aromatization (37). It was found that ATD reduced nuclear androgen receptor binding *in vivo*, whereas *in vitro* ATD competed for cytosolic androgen receptors (37). Tobet and co-workers (55) reported that ATD given to mothers com-

pletely blocked aromatase activity in the fetal hypothalamus and that the effects of ATD on the development of the male nucleus in the preoptic area could not be duplicated by treatment of the mothers with the anti-androgen flutamide (55). In our studies, we have not measured brain aromatase activity during neonatal ATD treatment of male rats. Therefore, we have only behavioral evidence that ATD acted via an inhibition of brain aromatase activity. On the other hand, we also found that gonadally intact male rats treated on postnatal days 0-10 with ATD plus estradiol benzoate given subcutaneously were indistinguishable from control males in adult partner preference (unpublished data). Thus, neonatal treatment with estradiol neutralized the effects of ATD treatment, indicating that ATD treatment blocked brain estrogen formation. Apparently, estradiol benzoate could enter the brain regardless of the presence of high concentrations of alpha-fetoproteins in the plasma. Montano and co-workers (43) found that when 2-day old female pups were treated with silastic capsules, which were implanted subcutaneously in the neck, containing [^{3}H]estradiol, [^{3}H]estradiol was detected specifically bound to brain cell nuclei (43). This means that alpha-fetoproteins cannot entirely protect the rat brain from circulating estradiol.

Recently, the nonsteroidal aromatase inhibitor Fadrozole, which has no affinity for androgen receptors, has been used for investigating the role of estradiol in the activation of sexual behavior in the male rat (e.g. 17). It was found that mounting and ejaculation were significantly decreased in rats given Fadrozole in the medial preoptic area compared with the behavior of control male rats (17). Clancy and co-workers (17) obtained the same results as Christensen and Clemens (16) who administered ATD intracranially. In future studies, it would be interesting to compare adult partner preference of male rats treated neonatally with ATD with that of male rats treated neonatally with Fadrozole.

Sexual differentiation of partner preference in the male rat

Most studies presented in this thesis were conducted to determine the influence of neonatal estradiol action on the neural mechanisms controlling sexual differentiation of partner preference in the male rat. The vast majority of studies concerning the influence of perinatal sex steroids on sexual differentiation have concentrated on the animal's coital performance. Relatively few studies have been dedicated to the influence of perinatal sex steroids on sexual differentiation of motivational aspects of sexual behavior. Motivational aspects of sexual behavior can be studied by assessment of an animal's partner preference (e.g. 13,39,41,44,57). Our data clearly demonstrated that the sexual differentiation of partner

preference in the male rat requires neonatal actions of estradiol. Neonatally ATD-treated male rats show in adulthood an altered partner preference. Such gonadally intact ATD males approach both the estrous female to whom they display masculine sexual behaviors and the sexually active male to whom they show feminine sexual behaviors. In contrast with normal males, ATD males' partner preference is differentially activated by gonadal hormones in adulthood. If gonadally intact or castrated and testosterone-treated, ATD males' partner preference fluctuates between a low preference for a sexually active male, no preference at all, and a low preference for an estrous female (chapters 4, 6, and 7). Control males given the same adult hormone treatments show a clear preference for an estrous female. If castrated in adulthood and treated with estradiol, ATD males show a clearcut preference for a sexually active male over an estrous female, whereas control males prefer an estrous female over a sexually active male (chapters 3 and 5).

C-fos expression as a marker of neuronal activity

We used *c-Fos* expression as a marker of neuronal activity to reveal possible differences between neonatally ATD-treated male and control male rats' responses to genital and chemosensory stimulation associated with sexual behavior (chapter 9, page 177). The advantage of monitoring the expression of immediate-early genes, such as *c-Fos*, as a method of identifying the neural systems involved in sexual behavior, is that it sheds new light on the functional relationships between the neural structures that control sexual behavior. However, one should discriminate between circuits that are activated as a result of an animal's sexual behavior and the neural mechanisms that control the behavior in the first place. There is probably an overlap between the two systems, but it should be realized that with *c-Fos* expression, actually an effect of intense sensory (olfactory or somatosensory) stimulation on certain neurons is studied. These activated neurons may or may not have anything to do with the ongoing expression of the studied behavior that has led to the sensory stimulation causing the induction of neuronal *c-Fos*. For instance, intromissive stimulation received induced significantly more Fos protein-immunoreactivity in neurons in the medial preoptic area of the female rat than in the medial preoptic area of the male rat of a heterosexual mating pair (59). Bilateral lesions of the medial preoptic area/anterior hypothalamus enhanced female receptivity, indicating an inhibitory effect of POA neurons in the lordosis reflex (33,46,60). Therefore, it seems likely that the Fos protein-immunoreactive neurons in the medial preoptic area are not involved in the ongoing expression of feminine

sexual behavior. Our data revealed that heterosexual mating (mounts, intromissions, no ejaculations) led to a greater neuronal *c-Fos* expression in the medial preoptic area of neonatally ATD-treated male rats than in the medial preoptic area of control males. In addition, the number of Fos protein-immunoreactive neurons was greater in the SDN of ATD males than in the SDN of control males. These findings were unexpected. We had hypothesized that *c-Fos* expression would be lower in the medial preoptic area of ATD males than in the medial preoptic area of control males, in a manner corresponding to the possibly reduced volume of the SDN in such ATD males versus control males. At present, the volume of the SDN has only been measured in male rats which were treated with ATD prenatally and in male rats treated with ATD both pre- and neonatally (34). It was found that the volume of the SDN was reduced in both ATD-treated groups compared with control males. At present, the volume of the SDN of neonatally ATD-treated males has not yet been measured; however, we expect it to be smaller than that of control males, since neonatal castration of male rats reduces SDN volume in adulthood (35). It is not clear at present how the higher *c-Fos* expression in the medial preoptic area, including the SDN, of neonatally ATD-treated male rats has to be explained. One could hypothesize that heterosexual mating activated both stimulatory and inhibitory neurons involved in the regulation of sexual behavior in the medial preoptic area of ATD males, whereas in control males only stimulatory neurons were activated. In a future study, it would be interesting to compare the distribution and density of Fos-immunoreactive neurons in the medial preoptic area of ATD males after either heterosexual mating (masculine sexual behavior) or homosexual mating (feminine sexual behavior).

In accordance with their partner preference, i.e. approaching and mating alternately with both the estrous female and the sexually active male, neonatally ATD-treated male rats showed a neuronal responsiveness, as measured by *c-Fos* expression, of the vomeronasal system to odors of estrous females as well as to odors of sexually active males (chapter 9, page 177). Control males only showed a neuronal responsiveness of the vomeronasal system to odors of estrous females (chapter 9), whereas control females, like ATD males, showed a neuronal responsiveness of the vomeronasal system to both odors of estrous females (14) and of sexually active males (chapter 9, page 177). As mentioned in the Introduction, the vomeronasal system is a sexually dimorphic network with males having larger number of neurons at each level of the vomeronasal pathway. The differentiation of the vomeronasal system seems to be dependent on the presence of perinatal testosterone, presumably mediated by estradiol (47). Therefore, the vomeronasal system of ATD males functionally resembles that of females. The morphology of

this system in ATD males has not yet been studied, however except for the medial preoptic area. It would be interesting to investigate in future studies the morphology of the vomeronasal system, i.e. the accessory olfactory bulbs, the medial amygdala, and the bed nucleus of the stria terminalis.

Nocturnal fluctuations in sexual behavior

A new finding is that coital performance and partner preference of neonatally ATD-treated male rats are affected by the phase of the dark period of the light (L)/dark (D) cycle (chapters 6 and 7). When tested early in the dark period, gonadally intact ATD males readily show feminine sexual behavior and prefer the sexually active male over an estrous female. By contrast, when tested late in the dark period, ATD males easily show masculine sexual behavior and prefer the estrous female over the sexually active male (chapters 6 and 7). These nocturnal fluctuations in sexual behavior are absent in control males. It was earlier reported by Södersten, Hansen, and co-workers (31,48) that a sex difference existed in the diurnal rhythm of sexual behavior in rats. Unlike male rats, female rats, ovariectomized and treated with estradiol-filled silastic capsules display low levels of lordosis during the light period of the LD cycle and high levels during the dark period of the LD cycle (30,31). Furthermore, neonatal castration induced this rhythmic pattern of lordosis behavior in male rats, whereas neonatal testosterone treatment abolished this rhythmic pattern of lordosis behavior in female rats (31). Södersten, Hansen, and co-workers (49) postulated that perinatal androgens uncouple the central rhythm generator, i.e. the suprachiasmatic nucleus (SCN), from the neural substrates that control sexual behavior in rat, since SCN lesions disrupted the daily rhythmicity in sexual behavior. It should be noted, however, that other investigators have failed to find a diurnal rhythm in female rats' lordosis behavior (23,32). At present, we do not know whether female rats show nocturnal fluctuations in partner preference and coital behavior. We are currently studying this question.

A strong indication was obtained that the SCN is a crucial structure in controlling the nocturnal fluctuations in partner preference and coital behavior of ATD males (chapter 8, page 159). Lesions of the SCN disrupted the nocturnal fluctuations in partner preference and coital behavior. Interestingly, partner preference of ATD males, but not that of control males, is clearly affected by SCN-lesions (chapter 8, page 159). SCN-lesioned ATD males, regardless whether or not the lesions were complete, showed a much greater preference for the estrous female

following the lesions than before the lesions were made. In fact, SCN-lesioned ATD males did no longer differ from control males in partner preference, suggesting that the SCN plays a role in the display of partner preference in neonatally ATD-treated male rats, but not in control male rats. It was recently reported that the SCN of pre- and neonatally ATD-treated male rats contained more vasopressin-expressing neurons than the SCN of prenatally ATD-treated male and control male rats (53). This suggests that perinatal estradiol normally is involved in the development of the vasopressing-expressing neurons in the SCN. It could be hypothesized that in the normal male rat perinatal estradiol suppresses the survival of SCN neurons which eventually express vasopressin or that perinatal estradiol reduces the expression of the vasopressin gene. At present, there is no evidence that the vasopressin-expressing neurons in the SCN and the vasopressinergic projections of the SCN are hormonally dependent in the adult rat, i.e. castration or subsequent hormone treatment had no effect (20). Furthermore, the adult rat SCN contains hardly any steroid-binding cells (45,62). However, the possibility of transient estrogen receptor expression during certain periods of development or receptors in neurons outside the SCN that project to this nucleus cannot be ruled out. Future workers should investigate the presence of estrogen receptors in the perinatal rat SCN. From our data, we cannot determine whether there is a functional link between increased numbers of vasopressin-expressing neurons in the SCN and the nocturnal fluctuations in partner preference of ATD-treated male rats. It would be very interesting to investigate the role of the vasopressin-expressing neurons in the SCN in sexual partner preference of perinatally ATD-treated male rats. Future workers should also investigate ATD males' partner preference after blocking vasopressin synthesis in the SCN by antisense oligonucleotides or after infusion of a vasopressin-antagonist into the SCN.

The relevance of animal studies for human sexuality

We have found that experimentally-induced variations in the availability of sex hormones during neonatal life affect male rats' adult choice of male versus female sexual partners. Similar results have been obtained in other mammalian species, such as ferrets (10), hamsters (36), dogs (11), and pigs (24). Likewise, in zebra finches immediate post-hatching treatment of females with estradiol benzoate masculinizes their preference, i.e. such females form pair-bonds with other females in adulthood, in a manner similar to males (2). We found that social developmental rearing conditions did not affect later partner preference in neonatally

ATD-treated male and control male rats (chapter 4 page 85). In adulthood, however, social interactions can affect partner preference. The data presented in this thesis demonstrated that the absence or presence of physical interaction with the stimulus animals during partner preference testing plays a crucial role in the expression of partner preference. When tested in the three-compartment box with stimulus animals behind wire mesh (no physical interaction possible), ATD and control males did not differ in partner preference (chapters 4-6). However, in the study by Brand, Kroonen, Mos, and Slob (13), ATD males showed a lower preference for an estrous female than control males when tested with stimulus animals behind wire mesh. We have no explanation for this discrepancy. In chapter 5 (page 101), both ATD and control males preferred the sexually active male over an estrous female. Presumably, control males' preference for the sexually active male has been caused by nonsexual factors, such as social affiliation with the stimulus male. Physical interaction with the sexually active male and the estrous female appeared to be a prerequisite for revealing differences in partner preference between ATD and control males (chapters 4-6). Adkins-Regan (1) made the point that prevention of physical interaction with the stimulus animals more accurately reflects appetitive/motivational aspects of mating preference. However, the disadvantage of preventing physical contact between the experimental animal and the stimulus animals is that it is unclear whether the choice was made on the basis of sexual preference or social affiliation. We believe that allowance of physical interaction with the stimulus animals more accurately reflects sexual partner preference.

In general, as one ascends the phylogenetic scale, the importance of social influences for the display of sexual behavior seems to increase. In contrast to rats, male macaques frequently signal subordinance to a more dominant animal by displaying the posture normally associated with feminine receptivity (9,29). It should be noted that perinatal exposure to androgens completely defeminizes sexual behavior in rats, but not in rhesus monkeys (26). Administration of testosterone propionate to pregnant rhesus monkeys between Days 40 and 100 of gestation did not defeminize the female offsprings' behavior: these androgenized females present to other animals as frequently as control females (27). Nevertheless, the interaction between social influences and perinatal sex hormones in the expression of sexual behavior seems to be stronger in nonhuman primate species than in rats. It is generally thought that the interactive role of social rearing conditions and adult sexuality is even more striking in humans than in animals. However, there is no empirical support for this view. By contrast, Green (28) reported that children being raised by transsexual or homosexual parents do not differ appreciably on

macroscopic measures of sexual identity from children raised in more conventional family settings. In the literature, there are some examples of possible effects of prenatal exposure to sex hormones on adult sexual orientation. As mentioned in the *Introduction*, women exposed to elevated androgen levels during gestation due to congenital adrenal hyperplasia reported increased levels of homosexual experiences compared to control women (21,42). Another example relevant to the question whether perinatal sex hormones influence the differentiation of sexual orientation in humans, is that women with a history of prenatal exposure to diethylstilbestrol (DES), a nonsteroidal synthetic estrogen, show a higher incidence of bisexuality or homosexuality than control women (22,40). These data suggest that prenatal sex hormones do play a role in the development of human sexual orientation. The human data are interesting since they bear some resemblance to those obtained in animal studies. However, whether the findings of altered sexual behavior in women prenatally exposed to DES or in women with congenital adrenal hyperplasia can be explained in other terms than specific hormonal effects on the developing brain has been the subject of heated debate. The opponents of a possible involvement of perinatal sex hormones in determining sexual orientation in women with congenital adrenal hyperplasia have argued for a social instead of a hormonal explanation for variations in sexual orientation, based on the assumption of subtle effects of genital ambiguity and surgery on parental child rearing and the child's own behavior and feelings (40). At present, there is no empirical support for this view. The opponents of either a possible neuroendocrine or a genetic basis of sexual orientation in humans, generally believe that homosexuality is a 'freely-chosen' behavior. In contrast to the social explanations for individual variations in sexual orientation, neuroanatomical data of the human hypothalamus have indicated that perinatal sex hormones mediate the differentiation of the neural substrates presumed to be involved in the regulation of sexual behavior. As already mentioned in the *Introduction*, morphological sex differences exist between the brains of men and women (3,4,6,38,50,63). The controversy about the sexual dimorphism of the human SDN (6) can be explained by the strong age-dependence of the sex difference (52). In addition, differences have been found between the brains of heterosexual and homosexual men (5,38,51). Interestingly, the results obtained in the human hypothalamus bear some resemblance to those obtained in rats. For instance, the human SDN is probably homologous to the SDN-POA in the rat, as jugded by its sex difference in size and cell number, localization and cytoarchitecture and the presence of galanin- and thyrotropin-releasing hormone (TRH)-containing neurons (52). In addition, the size of the vasopressin-containing subregion of the SCN, which

is twice as large in homosexual men as in heterosexual men (51) corresponds with the increased size of the vasopressin-containing subregion of the SCN in pre- and neonatally ATD-treated male rats (53). These analogies between rats (of which it is generally agreed that adult sexual behavior is dependent on the action of perinatal sex hormones) and humans suggest that perinatal sex hormones are involved in the development of the SDN and the vasopressin-containing neurons in the SCN in humans. Thus, the empirical support for a hormonal basis of sex differences in the human hypothalamus is largely indirect, that is, obtained from animal studies.

At present, there is no evidence that the reported differences in SDN volume between heterosexual men and women and the differences in vasopressin-content of the SCN between heterosexual men and homosexual men contribute in any way to the differences between these groups in their sexual identity. Animal studies, which allow us to investigate the contribution of the SDN-POA in the expression of sexual behavior by lesioning this nucleus, have shown that there is no clear correlation between the SDN-POA and the display of masculine coital behavior in rats (7,19) and ferrets (15). By contrast, lesions of the sexually dimorphic area in male gerbils disrupt mating (61). This inter-species difference in the functional role of the SDN in sexual behavior is just one of the many reasons to be wary of easy extrapolation from animal studies to human sexual behavior. At present, the contribution of the SDN to partner preference has not yet been investigated. Future studies should address this question.

Paredes and Baum (44) reported that male ferrets with bilateral lesions of the medial preoptic area/anterior hypothalamus approached a stimulus male significantly more often than control males. The limitation of the ferret model for human sexuality is that the increased preference for the male partner in male ferrets with bilateral lesions of the medial preoptic area/anterior hypothalamus was associated with significant reductions in masculine coital performance (44). Obviously, coital function does not differ between homosexual and heterosexual men. Even so, homosexual men are sexually more active, i.e. they have more sexual partners during lifetime, than heterosexual men (56). Our rat data strongly suggest that the SCN is involved in the display of partner preference of neonatally ATD-treated males (chapter 8, page 159). Bilateral lesions of the SCN dramatically increased the preference for an estrous female over a sexually active male in neonatally ATD-treated male rats. Obviously, one clear limitation of the ATD male rat model for homosexual orientation in humans is that ATD males prefer to mate with both the estrous female and sexually active male. As such the ATD male rat could be at the most a model for bisexual orientation in the human male.

In my opinion, animal studies can provide guidelines for the neural pathways possibly involved in human sexuality and the possible hormone-dependence of these neural pathways.

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SUMMARY

Chapter 1

General introduction

This first chapter provides an overview of the effects of gonadal hormones on the sexual differentiation of the brain, with an emphasis on functional sex differences in behavior and neuroendocrine function in possible relation to morphological sex differences in the rat brain. The first step in the process of sexual differentiation is the establishment of the genetic sex at the time of conception. In mammalian species, females are the homogametic sex (XX), males the heterogametic sex (XY). If a Y-chromosome is present, the SRY gene will be expressed and consequently the undifferentiated gonads develop into testes and consequently male-typical internal genitalia develop under the influence of androgens and Anti-Müllerian Hormone, both secreted by the fetal testes. Masculinization of the external genitalia requires the presence of 5αdihydrotestosterone (DHT) converted from testosterone by the enzyme 5αreductase. In the absence of a Y-chromosome, the undifferentiated gonads will develop into ovaries and female-typical internal and external genitalia are formed. In the presence of testes, the large quantities of testosterone released into the circulation act in the brain to masculinize and defeminize the neural substrates that control gonadotropin secretion and sexual behavior in adulthood. Masculinization results in the display of male-typical sexual behavior in adulthood. Defeminization results in a loss of cyclic release of gonadotropins necessary for ovulation and the loss of the display of female-typical sexual behavior in adulthood. Thus, in the rat, gonadally intact males show the complete pattern of masculine sexual behavior (mounts, intromissions, ejaculations) when paired with an estrous female and no feminine sexual behavior (proceptive and receptive behavior) when paired with a sexually active male. The development of the female brain is generally believed to proceed in the absence of testosterone. Gonadally intact female rats display periods of behavioral proceptivity, i.e. seeking out the male partner, and receptivity, i.e. the display of lordosis in response to mounts, around the time of ovulation.

Numerous studies on mammalian species have shown that local conversion of testosterone to estradiol is a crucial step in the sexual differentiation of the brain. Thus, estradiol mediates many of the effects of testosterone on brain function. This represents the *aromatization hypothesis*.

Not only coital behavior, but also motivational aspects of sexual behavior, i.e. partner preference, are masculinized and defeminized by

testosterone and/or estradiol during early development. In adulthood, gonadally intact male rats prefer estrous females over males, and gonadally intact female rats at proestrus prefer males over females as mating partners.

Brain lesioning techniques have been used to identify brain circuits which control sexual behavior. In addition to lesion studies, researchers have also performed electrical stimulation and recording studies to locate the neural pathways of sexual behavior. Brain implant studies have been used to discover the sites of hormone action in the central nervous system. It has been found that the medial preoptic area plays a pivotal role in the regulation of male sexual behavior in all vertebrate species studied to date. Bilateral lesions of this area completely disrupt male coital performance. Additionally, gonadal hormones act in the medial preoptic area mediating sexual behavior. In the female rat, however, neurons in the medial preoptic area have an inhibitory effect on female-typical sexual behavior. Bilateral lesions of this brain area in female rats facilitate the display of lordosis, whereas electrical stimulation of preoptic neurons has the opposite effect: it suppresses the lordosis reflex in female rats. The ventromedial nucleus of the hypothalamus plays an important role in the hormonal control of lordosis behavior in the female rat.

The functional sex differences in behavior and neuroendocrine function might be related to morphological differences between the brains of males and females. Such evidence became available in 1976 when two investigators working with zebra finches and canaries reported that the volume of the forebrain nuclei which control singing was significantly greater in males than in females. This sex difference correlated very nicely with the dimorphic control of singing in these species, i.e. only males sing. In several mammalian species, clear sex differences have been found in the preoptic area. Within the preoptic area lies a nucleus which is severalfold larger in males than in females. This nucleus was first described in rats and named the sexually dimorphic nucleus of the preoptic area (SDN-POA). Furthermore, the vomeronasal system is a sexually dimorphic network with male rats having more vomeronasal receptors and a larger number of neurons than female rats throughout this chemosensitive pathway. Another type of morphological sex difference has become obvious with the application of immunocytochemical techniques for localizing neurotransmitter (e.g. vasopressin) or neuropeptide (e.g. gonadotropin-releasing hormone (GnRH)) systems. Though the sex differences reported in the distribution of GnRH neurons are very subtle compared to the sex differences in the distrubion of vasopressinergic neurons.

Sex differences have also been found in various non-sexual behaviors, such as locomotor activity, i.e. females are more active than males, and in aggression, i.e. males are more aggressive than females.

The studies reported in this thesis have been conducted to investigate the role of early postnatal androgen aromatization in the sexual differentiation of the male rat brain. It has been shown that estradiol plays a pivotal role in the masculinization and defeminization of coital behavior. Relatively little attention has been paid to the sexual differentiation of partner preference. In all studies presented in this thesis, brain estrogen formation has been inhibited by implanting silastic capsules containing the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) sc in the back of the newborn male. Control males were implanted with silastic capsules which were either left empty or contained cholesterol (a hormonally-inert precursor of gonadal steroids). All silactic capsules were removed either at the end of the critical period of steroid action in the brain (at the age of 10 days) or at weaning (at the age of 21 days).

Chapter 2

A semiautomated test apparatus for studying partner preference behavior in the rat

The semiautomated three compartment box automatically records the rat's time spent in each compartment, as well as the locomotor activity, i.e. the number of visits an animal pays to each compartment. Software was developed to calculate preference scores. The use of the apparatus was validated by studying adult partner preference of neonatally ATD-treated male rats. The collected data fully corroborated previous results. A new finding was the much higher locomotor activity of ATD males compared to control males.

Chapter 3

Hormonal regulation of adult partner preference behavior in neonatally ATD-treated male rats

When gonadally intact, ATD males showed a much lower preference for an estrous female than control males. After castration (no hormone treatment), the preference for the estrous female declined in all males with ATD males still showing a lower preference for the estrous female than control males. Postcastration treatment of control males with estradiol was sufficient to produce a partner preference for an estrous female, although estradiol treatment together with DHT restored the preference for an estrous female to precastration levels. Postcastration treatment with DHT alone had no effect on partner preference in either

ATD or control males. ATD males showed a different behavioral response to estradiol when compared with control males. Castrated estradiol-treated ATD males clearly preferred the sexually active male. Estradiol treatment together with DHT produced a low preference in ATD males for the estrous female.

Chapter 4

Postweaning housing conditions and partner preference and sexual behavior of neonatally ATD-treated male rats

At weaning, ATD and control males were either housed alone or in small groups (2-3 animals). In adulthood, they were tested while gonadally intact for partner preference, i.e. they were given free access to a sexually active male and an estrous female. Social isolation did not have major effects on adult partner preference except when physical interaction with the stimulus animals was prevented. In this case, isolates (ATD and control) showed a greater preference for the estrous female. When physical interaction with the stimulus animals was allowed, ATD males showed a much lower preference for an estrous female than control males. When they were given free access to an ATD-male (instead of a normal male) and an estrous female, ATD males spent more time with the stimulus ATD-male than they had done in previous tests with the normal stimulus male. In contrast to partner preference, coital behavior was clearly affected by social isolation. Isolates (ATD and control) displayed lower frequencies of mounts and intromissions. Ejaculation behavior of these isolates was not affected. These effects persisted over tests.

Chapter 5

Sexual differentiation of odor and partner preference in the rat

In this chapter, adult male (ATD and control) and female rats, all gonadectomized and treated with estradiol, were tested for odor and partner preference. Sex differences in preference were evident when subjects were given a choice between soiled bedding (containing urine and feces) from estrous females and from sexually active males. ATD and control males preferred to sit significantly more on soiled bedding from estrous females than on soiled bedding from sexually active males. Control females preferred to sit significantly more on soiled bedding from sexually active males than on soiled bedding from estrous females. More distal cues like actually seeing and hearing the stimulus animals (stimulus animals were placed behind wire mesh), revealed differences in preference between control males and females, but not between ATD and control males. When physical interaction with the stimulus animals

was allowed, sex differences in partner preference became the most explicit. Control males preferred to mate with the estrous female, whereas control females showed a clearcut preference for the sexually active male. ATD males showed a preference for the sexually active male, albeit significantly lower compared to females.

Chapter 6

Organization of partner preference and sexual behavior and its nocturnal rhythmicity in male rats

Male rats were treated with ATD, beginning within 2-4 h after birth (Day 0) or on Day 2 or Day 5 after birth. Each ATD group had a corresponding control group that received a silastic capsule with cholesterol (Day 0, Day 2, and Day 5). In adulthood, ATD and control males were tested for partner preference, i.e. they were given free access to a tethered sexually active male and a tethered estrous female. Early in the dark phase of the light/dark cycle, male rats that received ATD-treatment shortly after birth (ATD Day 0 males), preferred to approach the sexually active male to whom they showed female-typical sexual behavior, whereas control males preferred to approach and mate with the estrous female. Male rats that had received ATD-treatment beginning on Day 2 or 5 showed preference scores intermediate between those of ATD Day 0 males and control males. A new finding was that sexual behavior of ATD males, but not that of control males, fluctuated over the dark phase. When tested late in the dark phase, both ATD and control males preferred to approach and mate with the estrous female. ATD Days 2 and 5 males did no longer differ from control males in partner preference, whereas ATD Day 0 males still showed a significantly lower preference for the estrous female than control males.

Chapter 7

Endogenous reproductive hormones and nocturnal rhythms in partner preference and sexual behavior of ATD-treated male rats

Blood was collected early and late in the dark phase of adult ATD males and control males which were not exposed to behavioral tests. It was found that both testosterone and the gonadotropins LH and FSH fluctuated nocturnally with highest levels late in the dark phase. ATD males had significantly higher FSH levels than control males. Nocturnal levels of inhibin, a selective suppressor of pituitary FSH secretion, also fluctuated in both ATD and control males, with lowest levels late in the dark phase. Another group of ATD and control males were tested for partner preference, i.e. they were given free access to a tethered sexually

active male and a tethered estrous female, before and after castration with subsequent testosterone treatment using silastic capsules. The latter treatment ensures a constant blood testosterone level. When gonadally intact, sexual behavior of ATD males fluctuated over the dark phase. When tested early in the dark phase, ATD males preferred the vicinity of and interaction with the stimulus male. When tested late in the dark phase, this preference shifted to a preference for the stimulus female. These nocturnal fluctuations were absent in control males. The latter males showed a constant preference for the stimulus female. After castration and subsequent testosterone treatment, ATD males' sexual behavior still fluctuated nocturnally.

Chapter 8

Lesions of the suprachiasmatic nucleus disrupt the nocturnal fluctuations in partner preference of neonatally estrogen-deprived male rats

Lesions of the suprachiasmatic nucleus (SCN) disrupted the nocturnal fluctuations and at the same time dramatically increased ATD males' preference for an estrous female: the SCN-lesioned ATD males no longer differed from control males in partner preference.

Chapter 9

Neonatal inhibition of brain estrogen synthesis alters adult neural Fos responses to mating and pheromonal stimulation in the male rat

Heterosexual mating, i.e. 8 intromissions with an estrous female, significantly augmented the number of Fos protein-immunoreactive neurons in the bed nucleus of the stria terminalis, the posterodorsal portion of the medial amygdala and the midbrain central tegmental field to equivalent numbers in neonatally ATD-treated and control males. Mating led to a greater neuronal *c-Fos* expression in the medial preoptic area of ATD males compared to the medial preoptic area of control males. The number of Fos protein-immunoreactive neurons was also augmented in the sexually dimorphic nucleus of the preoptic area of both ATD and control males following mating, with ATD males showing a greater *c-Fos* expression.

The distribution and intensity of Fos protein-immunoreactivity following exposure to soiled bedding from estrous females were very similar in both ATD and control males. *c-Fos* Expression was evident throughout the vomeronasal pathway. By contrast, the distribution and intensity of Fos protein-immunoreactivity following exposure to soiled bedding from active males differed between males (ATD and control) and females. At the beginning of the vomeronasal pathway, i.e. the accessory

olfactory bulb and the posterior medial amygdala, there were no differences in the number of Fos protein-immunoreactive neurons between the sexes. At more distal levels of the vomeronasal pathway, i.e. the bed nucleus of the stria terminalis and the medial preoptic area, sex differences became apparent. In female rats, the bed nucleus of the stria terminalis and the medial preoptic area were clearly responsive to odors of sexually active males, whereas in control males these brain areas were not responsive. ATD males showed a female-like responsiveness, i.e. *c-Fos* expression was evident at each level of the vomeronasal system.

Chapter 10

Quantitative estimation of androgen and estrogen receptor-immunoreactive cells in the forebrain of neonatally estrogen-deprived male rats

Neonatally ATD-treated male rats showed a significantly higher estrogen-receptor immunoreactivity in the periventricular preoptic area (PVP) and the ventrolateral portion of the ventromedial nucleus of the hypothalamus (VMH) compared with control males. No significant differences in the distribution and density of androgen receptors were found between the brains of ATD and control males.

CONCLUSIONS

Neonatal estradiol plays a pivotal role in the sexual differentiation of sexual partner preference and coital behavior in the male rat.

In adulthood, estradiol treatment is very effective in activating partner preference and coital behavior in castrated male rats and revealed the most robust differences in partner preference between neonatally ATD-treated and control male rats.

The absence or presence of postweaning sociosexual interactions did not play a significant role in determining adult partner preference in both neonatally ATD-treated and control male rats. It did affect, however, to some extent mounting and intromissive behavior in both neonatally ATD-treated and control male rats.

Physical interaction with the estrous female/sexually active male appeared to be a prerequisite for revealing differences in partner preference between ATD and control males. ATD males' partner preference was clearly intermediate between the extremes of the male and female control group.

Locomotor activity was also affected by neonatal ATD treatment.

ATD males made more excursions to each compartment of the three compartment box than control males.

Partner preference and coital behavior of ATD males fluctuated over the dark phase. These nocturnal fluctuations in sexual behavior were not a reflection of fluctuating testosterone levels. The suprachiasmatic nucleus appears to be a crucial structure in controlling the nocturnal fluctuations in ATD males' sexual behavior and at the same time plays a role in the display of ATD males' partner preference.

Neurons in the medial preoptic area, including the sexually dimorphic nucleus, of ATD males were more responsive to heterosexual mating stimulation than those in the medial preoptic area, including the sexually dimorphic nucleus, of control males.

There was no effect of neonatal estrogen deprivation on male rats' adult responsiveness of the vomeronasal projection pathway to odors from estrous females. By contrast, neonatal estrogens clearly mediates male rats' neuronal responsiveness of the vomeronasal projection pathway to odors from sexually active males.

Neonatal estradiol might be responsible for the lower levels of estrogen receptors in the periventricular preoptic area and the ventrolateral portion of the ventromedial nucleus of the hypothalamus of normal male rats. By contrast, neonatal estradiol does not play a significant role in the sexual differentiation of androgen receptors in the forebrain in the male rat.

SAMENVATTING

Hoofdstuk 1

Algemene Introductie

In dit hoofdstuk wordt een overzicht gegeven van de rol die geslachtshormonen spelen in de seksuele differentiatie van de hersenen. De nadruk van dit hoofdstuk ligt op geslachtsverschillen in gedrag en neuroendocriene functies, die mogelijkerwijs gerelateerd zijn aan geslachtsverschillen in de morfologie en het functioneren van de hersenen. De eerste stap in de seksuele differentiatie is het ontstaan van het genetische geslacht bij de bevruchting. Zoogdieren hebben in de kernen van hun lichaamscellen een voor elk soort specifiek aantal gepaarde autosomen en twee geslachtschromosomen. Vrouwen hebben twee X-chromosomen en mannen één X- en één Y-chromosoom. Als er een Y-chromosoom aanwezig is, dan ontstaan testikels uit de ongedifferentieerde gonaden met als gevolg mannelijke inwendige en uitwendige genitaliën. De mannelijke inwendige genitaliën ontwikkelen zich onder invloed van testosteron en het Anti-Müllerian Hormoon (AMH). Beide hormonen worden geproduceerd door de zich ontwikkelende testikels. Vermannelijking van de uitwendige genitaliën gebeurt onder invloed van 5αdihydrotestosteron (DHT), wat door reductie ontstaan is uit testosteron. In de afwezigheid van een Y-chromosoom zullen de ongedifferentieerde gonaden zich ontwikkelen tot ovariën met als gevolg vrouwelijke inwendige en uitwendige genitaliën. Het door de testikels geproduceerde testosteron vermannelijkt en ontvrouwelijkt in de hersenen de neurale substraten die seksueel gedrag en de afgifte van gonadotrophines reguleren op volwassen leeftijd. In de mannelijke rat resulteert vermannelijking in het kunnen vertonen van mannelijke seksueel gedrag op volwassen leeftijd. Ontvrouwelijken in de rat resulteert in het niet meer kunnen vertonen van een cyclische afgifte van gonadotrophines wat noodzakelijk is voor ovulatie, en het niet meer kunnen vertonen van vrouwelijk seksueel gedrag op volwassen leeftijd. Dus, gonadaal intacte mannelijke ratten vertonen op volwassen leeftijd het complete patroon van mannelijk seksueel gedrag (beklimmingen, intromissies, en ejaculaties) wanneer ze gepaard worden met een bronstige vrouwelijke rat, en vertonen geen vrouwelijk seksueel gedrag (proceptieve en receptieve gedragingen) wanneer ze gepaard worden met een seksueel actieve mannelijke rat. In het algemeen wordt verondersteld dat de hersenen zich in vrouwelijke richting ontwikkelen als er geen androgenen aanwezig zijn. Gonadaal intacte vrouwelijke ratten vertonen rond het tijdstip van ovulatie proceptief gedrag, dat wil zeggen het actief opzoeken van een mannelijke partner, en receptief gedrag, dat wil zeggen het

vertonen van lordose wanneer ze worden beklommen.

Inmiddels hebben vele studies aangetoond dat de aromatisatie van testosterone naar oestradiol in de hersenen zeer belangrijk is voor de seksuele differentiatie van de mannelijke hersenen. Dit betekent dat een groot gedeelte van de effekten van testosteron op hersenfuncties kunnen worden nagebootst met oestradiol. Dit wordt ook wel de *aromatisatie hypothese* genoemd.

Niet alleen coïtaal gedrag, maar ook motivationele aspecten van seksueel gedrag, zoals bijvoorbeeld partner preferentie, wordt vermannelijkt en ontvrouwelijkt door testosteron en/of oestradiol tijdens de vroege ontwikkeling in de mannelijke rat. Als gonadaal intacte ratten op volwassen leeftijd kunnen kiezen tussen een seksueel actieve mannelijke rat en een bronstige vrouwelijke rat, dan hebben mannelijke ratten een duidelijke voorkeur voor de nabijheid van en het paren met een bronstige vrouwelijke rat, terwijl vrouwelijke ratten een voorkeur hebben voor het paren met een seksueel actieve mannelijke rat.

Het uitschakelen van bepaalde gebieden in de hersenen met behulp van electrische stroom via electroden, is een veel gebruikte methode om uit te zoeken waar precies in de hersenen seksueel gedrag wordt gereguleerd. Naast deze lesie-studies hebben onderzoekers ook gebruik gemaakt van het elektrisch stimuleren van bepaalde hersengebieden om zo de neurale circuits die betrokken zijn bij de regulatie van seksueel gedrag, te localiseren. Het implanteren van hormonen in de hersenen is gebruikt om zo de hormoon-gevoelige gebieden te vinden. Met behulp van deze technieken heeft men gevonden dat het mediaal preoptisch gebied een sleutelrol speelt in de regulatie van mannelijk seksueel gedrag in verscheidene zoogdieren. Tweezijdige lesies in dit gebied verstoort compleet het mannelijk coïtaal gedrag. Tevens stimuleren geslachtshormonen geimplanteerd in het mediaal preoptisch gebied seksueel gedrag. Echter in de vrouwelijke rat hebben de neuronen in het mediaal preoptisch gebied een remmend effekt op vrouwelijk seksueel gedrag. Tweezijdige lesies van dit gebied in vrouwelijke ratten vergemakkelijkt het vertonen van lordose, terwijl electrische stimulatie van neuronen in het mediaal preoptisch gebied het tegenovergestelde effekt heeft: het onderdrukt het lordose gedrag in vrouwelijke ratten. In vrouwelijke ratten speelt de ventromediale nucleus in de hypothalamus een belangrijke rol in de hormonale controle van lordose.

Mogelijkerwijs zijn de geslachtsverschillen in gedrag en afgifte van gonadotrophines gerelateerd aan morfologische verschillen tussen de hersenen van mannen en vrouwen. Het eerste overtuigende bewijs hiervoor werd gevonden in 1976, toen twee onderzoekers die met zebravinken en kanaries werkten, vonden dat het volume van bepaalde kernen in het telencephalon (vooraan in de hersenen) die het zingen

reguleren, groter was in mannetjes dan in vrouwtjes. Dit geslachtsverschil correleerde heel mooi met het geslachtsverschil in zingen in deze vogelsoorten, namelijk alleen mannetjes zingen. Later werden in verscheidene zoogdiersoorten duidelijke geslachtsverschillen gevonden in het preoptische gebied. Zo ligt er in het preoptische gebied een kern die een aantal malen groter is in mannen dan in vrouwen. Deze kern is het eerst gevonden in ratten en werd de seksueel dimorfe kern van het preoptische gebied genoemd. Tevens zijn er geslachtsverschillen gevonden op elk niveau in het vomeronasale systeem, een neuronaal netwerk wat zeer belangrijk is voor het detecteren van feromonen. Mannelijke ratten hebben meer vomeronasale receptoren en een groter aantal neuronen in dit vomeronasale systeem dan vrouwelijke ratten. Een ander type van morfologische geslachtsverschillen kwam naar voren door nieuwe immunohistochemische technieken voor het localiseren van neurotransmitters (bijvoorbeeld vasopressine) of neuropeptides (bijvoorbeeld luteïne-hormoon-releasing-hormoon (LHRH)).

Geslachtsverschillen zijn ook gevonden in verscheidene niet-seksuele gedragingen, zoals bijvoorbeeld in locomotor activiteit, waarbij vrouwelijke ratten gemiddeld meer actief zijn in loopgedrag dan mannelijke ratten, en in agressiviteit, waarbij mannelijke ratten gemiddeld aggressiever zijn dan vrouwelijke ratten.

De onderzoeken die vermeld worden in dit proefschrift zijn uitgevoerd om de betekenis van vroeg postnataal oestradiol op de seksuele differentiatie van de hersenen van de mannelijke rat te onderzoeken. Eerder was aangetoond dat oestradiol een sleutelrol speelt in het vermannelijken en ontvrouwelijken van mannelijk coïtaal gedrag. Tot nu toe was er relatief weinig aandacht besteed aan de seksuele differentiatie van seksuele partner preferentie. In alle onderzoeken die in dit proefschrift gepresenteerd worden, werd in pasgeboren mannelijke ratten de synthese van oestradiol in de hersenen geremd door het onder de huid implanteren van siliconerubberen buisje met daarin een remmer van het aromatase enzym, namelijk 1,4,6-androstatrien-3,17-dion (ATD). Controle dieren kregen onderhuids siliconerubberen buisjes die ofwel leeg waren ofwel cholesterol (een hormonaal-inactieve voorloper van testosteron) bevatte. Alle siliconerubberen buisjes werden verwijderd ofwel aan het einde van de kritische periode voor de seksuele differentiatie (na dag 10 na de geboorte) ofwel bij het spenen (dag 21 na de geboorte).

Hoofdstuk 2

Een semi-geautomatiseerde drie compartimenten kooi voor het bepalen van partner preferentie van de rat

De semi-geautomatiseerde drie compartimenten kooi berekent automatisch de tijd doorgebracht in elk compartiment en registreert de locomotor activiteit, dat wil zeggen het aantal excursies dat een dier brengt aan elk compartiment. Speciaal ontwikkeld software berekent automatisch een preferentie score, dat wil zeggen aan het einde van elke keuzetest wordt de tijd doorgebracht bij de seksueel actieve man afgetrokken van de tijd doorgebracht bij de bronstige vrouw. Dus een positieve preferentiescore betekent een voorkeur voor een bronstige vrouw, een negatieve preferentiescore betekent een voorkeur voor een seksueel actieve man. Het gebruik van het nieuwe apparaat werd gevalideerd door partner preferentie te bestuderen van volwassen neonataal met ATD behandelde mannelijke ratten. De nieuwe data kwamen volledig overeen met wat eerder was gevonden. Nieuw was echter dat ATD mannen een veel hogere locomotor activiteit hadden dan controle mannen.

Hoofstuk 3

Hormonale regulatie van volwassen partner preferentie in neonataal met ATD behandelde mannelijke ratten

Gonadaal intacte ATD mannen vertoonden een veel lagere voorkeur voor een bronstige vrouw, dat wil zeggen zij brachten veel minder tijd door bij de bronstige vrouw en meer tijd bij de seksueel actieve man, dan gonadaal intacte controle mannen. Na castratie (geen hormoonbehandeling) daalde de voorkeur voor de bronstige vrouw in alle mannen doch ATD mannen vertoonden nog steeds een veel lagere voorkeur voor de bronstige vrouw dan controle mannen. Behandeling van gecastreerde controle mannen met oestradiol was voldoende om een preferentie voor een bronstige vrouw te induceren, hoewel oestradiol behandeling tesamen met dihydrotestosteron (DHT) de voorkeur voor de bronstige vrouw geheel herstelde tot de voorkeur die ze hadden toen ze gonadaal intact waren. Behandeling van gecastreerde ATD en controle mannen met alleen DHT had geen effekt op hun partner preferentie. ATD mannen reageerden anders op oestradiol dan controle mannen. Gecastreerde ATD mannen behandeld met oestradiol vertoonden een duidelijke voorkeur voor een seksueel actieve man. Behandeling van gecastreerde ATD mannen met oestradiol en DHT produceerden een lage voorkeur voor een bronstige vrouw. Dus, behandeling met oestradiol alleen is zeer effektief in het activeren van partner preferentie in gecastreerde mannelijke ratten en onder deze hormoonconditie verschilden ATD en controle mannen het meest in hun partner preferentie.

Hoofdstuk 4

Huisvestingscondities en partner preferentie en seksueel gedrag van neonataal met ATD behandelde mannelijke ratten

Bij het spenen, werden ATD en controle mannen ofwel alleen gehuisvest, ofwel in kleine groepjes (2-3 dieren per kooi). Op volwassen leeftijd werden ze getest voor partner preferentie, dat wil zeggen ze kregen vrij toegang tot een seksueel actieve man en een bronstige vrouw in de drie compartimenten kooi. Sociale isolatie had geen grote effekten op partner preferentie. Alleen wanneer seksuele interactie met de stimulus dieren was verhinderd vertoonden de solitair gehuisvestte ATD en controle mannen een grotere voorkeur voor een bronstige vrouw dan de sociaal gehuisvestte ATD en controle mannen. Wanneer seksuele interactie met de vastgebonden stimulus dieren mogelijk was, vertoonden ATD mannen een veel lagere voorkeur voor een bronstige vrouw dan controle mannen. Wanneer ATD en controle mannen konden kiezen tussen een ATD man (in plaats van een normale man) en een bronstige vrouw brachten ATD mannen veel meer tijd door bij de stimulus ATD man dan dat ze hadden gedaan bij de normale stimulus man in de eerdere keuzetesten. In tegenstelling tot partner preferentie was coïtaal gedrag wel beinvloed door sociale isolatie. Solitair gehuisvestte ATD en controle mannen vertoonden minder beklim- en intromissiegedrag dan sociaal gehuisvestte ATD en controle mannen. Dit verschil bleef aanwezig tijdens alle testen. De conclusie is dat de af- of aanwezigheid van socioseksuele interacties na het spenen geen belangrijke rol speelt in het bepalen van partner preferentie. Het heeft echter wel een effekt op beklim- en intromissiegedrag.

Hoofdstuk 5

Seksuele differentiatie van geur en partner preferentie van de rat

Op volwassen leeftijd werden bij mannelijke (ATD en controle) en vrouwelijke ratten de gonaden verwijderd en werden ze vervolgens behandeld met oestradiol. Daarna werden ze getest voor geur (= zaagsel) en partner preferentie. Geslachtsverschillen in preferentie waren al duidelijk aanwezig wanneer ze de keuze kregen tussen zaagsel waarop bronstige vrouwen hadden gezeten en zaagsel waarop seksueel actieve mannen hadden gezeten. Dit zaagsel was bevuild met urine en faeces en bevatte dus feromonen. ATD en controle mannen prefereerden het zaagsel van bronstige vrouwen boven dat van actieve mannen. Vrouwen prefereerden het zaagsel van actieve mannen boven dat van bronstige vrouwen. Wanneer mannen (ATD en controle) en vrouwen vrij toegang kregen tot een seksueel actieve man en een bronstige vrouw waarbij seksuele interactie was verhinderd (beide stimulus dieren waren geplaatst

achter gaas), waren er duidelijke verschillen in preferentie tussen controle mannen en vrouwen, maar niet tussen ATD en controle mannen. Seksuele interactie met de stimulus dieren bleek noodzakelijk om het bekende verschil in partner preferentie te zien tussen ATD en controle mannen. ATD mannen vertoonden dan een duidelijke voorkeur voor een seksueel actieve man, terwijl controle mannen een voorkeur hadden voor een bronstige vrouw. Qua partner preferentie zitten ATD mannen tussen normale mannen en vrouwen in.

Hoofdstuk 6

Organisatie van partner preferentie en seksueel gedrag en een nachtelijk ritme in seksueel gedrag van mannelijke ratten

Mannelijke ratten werden behandeld met ATD, direkt vanaf de geboorte (dag 0), of vanaf dag 2 of dag 5 na de geboorte. Elke ATD groep had een controle groep die siliconerubberen buisjes met cholesterol ontvingen (dag 0, dag 2, dag 5). Op volwassen leeftijd werden de ATD en controle mannen getest voor partner preferentie, dat wil zeggen ze kregen vrij toegang tot een vastgebonden seksueel actieve man en een bronstige vrouw in de drie compartimenten kooi. Vroeg in de donkerperiode van de dag/nacht cyclus vertoonden mannelijke ratten, die met ATD behandeld werden direkt na de geboorte (ATD dag 0) een voorkeur voor de seksueel actieve man met wie ze vrouwelijk seksueel gedrag vertoonden. Controle mannen prefereerden de bronstige vrouw met wie ze mannelijk seksueel gedrag vertoonden. Mannelijke ratten, die met ATD behandeld werden vanaf dag 2 of 5 na de geboorte, hadden preferentie scores die tussen die van de ATD dag 0 en controle mannen inlagen. Dit betekent dat het remmen van de oestradiol synthese in de hersenen vanaf dag 5 na de geboorte nog steeds volwassen partner preferentie beinvloedt. Tevens fluctueerden seksueel gedrag van ATD mannen, maar niet dat controle mannen, over de donkerperiode. Laat in de donkerperiode hebben zowel ATD als controle mannen een voorkeur voor de bronstige vrouw. In de late donkerperiode verschilden ATD dag 2 en 5 mannen niet meer van controle mannen in keuzegedrag, terwijl ATD dag 0 mannen nog steeds een lagere voorkeur vertoonden voor de bronstige vrouw dan controle mannen. Deze resultaten veronderstellen dat neonataal oestradiol het bestaan van nachtelijke fluctuaties in seksueel gedrag onderdrukt.

Hoofdstuk 7

Endogene geslachtshormonen en nachtelijke ritmes in partner preferentie en seksueel gedrag van mannelijke ratten behandeld met ATD

Op twee verschillende tijdstippen in de donkerperiode werd bloed afgenomen van volwassen ATD en controle mannen, die geen gedragstesten ondergingen. Het bleek dat zowel testosteron als LH en FSH over de donkerperiode fluctueerden: de hoogste concentraties laat in de donkerperiode, de laagste concentratie vroeg in de donkerperiode. FSH was hoger in ATD mannen. Ook inhibine, een door de testikels geproduceerd hormoon dat selectief de afgifte van FSH uit de hypophyse onderdrukt, fluctueerde over de donkerperiode met de laagste concentraties laat in de donkerperiode. Een andere groep ATD en controle mannen werd getest voor partner preferentie, dat wil zeggen ze kregen in de drie compartimenten kooi vrij toegang tot een vastgebonden seksueel actieve man en een bronstige vrouw, voor en nadat ze gecastreerd werden en behandeld met testosteron via siliconerubberen buisjes. Hormoonbehandeling via siliconerubberen buisjes zorgt voor een gelijkmatige afgifte van het hormoon. Seksueel gedrag van gonadaal intacte ATD mannen, maar niet dat van controle mannen, fluctueerden duidelijk over de donkerperiode. In de vroege donkerperiode hadden ATD mannen de voorkeur om te copuleren met een seksueel actieve man. In de late donkerperiode verschoof deze voorkeur naar een voorkeur om met een bronstige vrouw te copuleren. Controle mannen vertoonden een duidelijke voorkeur voor een bronstige vrouw ongeacht of ze vroeg of laat in de donkerperiode werden getest. Na castratie en testosteron behandeling vertoonden ATD mannen nog steeds nachtelijke fluctuaties in seksueel gedrag. Dit betekent dat het bestaan van nachtelijke fluctuaties in seksueel gedrag het gevolg is van de afwezigheid van oestradiol na de geboorte en dat het niet veroorzaakt wordt door nachtelijke fluctuaties in geslachtshormonen.

Hoofdstuk 8

Lesies van de nucleus suprachiasmaticus verstoren de nachtelijke fluctuaties in partner preferentie van neonataal oestradiol-gedepriveerde mannelijke ratten

Na het aanbrengen van lesies in de nucleus suprachiasmaticus (SCN), fluctueerde seksueel gedrag van ATD mannen niet meer over de donkerperiode. Tevens vonden we dat de voorkeur voor een bronstige vrouw enorm was toegenomen in ATD mannen nadat er lesies waren aangebracht in de SCN. De SCN-geledeerde ATD mannen verschilden niet meer van controle mannen in partner preferentie. Deze resultaten veronderstellen dat de SCN een zeer belangrijke rol speelt in het ontstaan

van de nachtelijke fluctuaties in seksueel gedrag en partner preferentie van ATD mannen.

Hoofdstuk 9

Neonatale remming van de oestradiol synthese verandert volwassen neuronale Fos responses op een seksuele stimulus in de mannelijke rat

Heteroseksuele copulatie, dat wil zeggen 8 intromissies met een bronstige vrouw, gaf een significante toename in het aantal Fos eiwit-immunoreactieve neuronen in de bed nucleus van de stria terminalis, het posterodorsale gedeelte van de mediale amygdala en het centraal tegmentum in de middenhersenen in zowel ATD als controle mannen. Copulatie leidde echter tot een groter aantal Fos eiwit-immunoreactieve neuronen in het mediaal preoptische gebied van ATD mannen vergeleken met het mediaal preoptische gebied van controle mannen. Fos eiwit-immunoreactieve neuronen waren ook aanwezig in de seksueel dimorfe nucleus van het preoptisch gebied (SDN-POA) in zowel ATD als controle mannen. ATD mannen hadden echter meer Fos eiwit-immunoreactieve neuronen in de SDN dan controle mannen.

De verdeling en de hoeveelheid Fos eiwit-immunoreactieve neuronen na te zijn blootgesteld aan zaagsel van bronstige vrouwen verschilden niet tussen ATD en controle mannen. Activatie van het proto-oncogen *c-Fos* kon worden waargenomen in het gehele vomeronasale systeem. Echter, er waren duidelijke sekseverschillen in neuronale activatie van het vomeronasale systeem door geuren van seksueel actieve mannen. Voor in het vomeronasale systeem, namelijk de accessoire olfactorische bulb en het achterste gedeelte van de medial amygdala, waren geen verschillen in het aantal Fos eiwit-immunoreactieve neuronen tussen mannelijke en vrouwelijke ratten. Meer achter in het vomeronasale systeem, namelijk de bed nucleus van de stria terminalis en het mediaal preoptische gebied, kwamen geslachtsverschillen naar voren. Vrouwelijke ratten vertoonden een duidelijke activatie van het proto-oncogen *c-Fos* in de bed nucleus van de stria terminalis en het mediaal preoptische gebied na te zijn blootgesteld aan geuren van seksueel actieve mannen. Controle mannen vertoonden in deze gebieden geen *c-Fos* expressie. Dit geslachtsverschil was duidelijk afhankelijk van neonataal oestradiol: ATD mannen vertoonden een vrouwelijke respons, dat wil zeggen Fos eiwit-immunoreactieve neuronen waren aanwezig in het gehele vomeronasale systeem.

Hoofdstuk 10

Kwantitatieve analyse van androgeen en oestrogeen-immunoreactieve cellen in de hersenen van neonataal oestrogeen-gedepriveerde mannelijke ratten

Neonataal met ATD behandelde mannen bleken meer oestrogeenreceptoren te hebben dan controle mannen in de periventriculaire preoptisch gebied (PVP) en het ventrolaterale gedeelte van de ventromediale nucleus in de hypothalamus (VMH) dan controle mannen. De verdeling en de hoeveelheid androgeenreceptoren verschilden niet tussen de hersenen van ATD en controle mannen.

CURRICULUM VITAE

Julie Bakker werd geboren op 1 september 1968 in Badhoevedorp. Zij deed in 1986 eindexamen aan het Ignatius Gymnasium te Amsterdam, waarna ze begon aan de studie Biologie aan de Universiteit van Amsterdam. In het kader van een afstudeerstage, deed zij 1 jaar onderzoek bij de vakgroep Experimentele Dierkunde onder leiding van Dr. W. Ghijsen en Prof.Dr. W. Schreurs (CIVO-TNO, Zeist). Het project was een gezamelijk onderzoeksprogramnma tussen de Universiteit van Amsterdam en TNO Voeding te Zeist en omvatte onderzoek naar de effekten van vitamine B6 deficiëntie op GABA metabolisme en GABA afgifte in de hippocampus van de volwassen rat. Hierna liep ze stage bij de Vakgroep Gezondheidswetenschappen aan de Universiteit van Amsterdam. In deze 4 maanden durende stage is onderzoek gedaan naar de blootstelling aan organische oplosmiddelen bij autospuiters. Na het behalen van het dokteraaldiploma in juni 1991, heeft zij korte tijd stage gelopen bij het Centraal Laboratorium voor de Bloedtransfusiedienst te Amsterdam. Hier heeft ze onderzoek gedaan naar allergenen van de hond, waarbij gebruik werd gemaakt van verscheidene immunologische technieken. Aansluitend begon ze te werken als assistente in opleiding bij de afdeling Endocrinologie en Voortplanting van de Erasmus Universiteit in Rotterdam. In dit proefschrift staat het onderzoek beschreven wat zij daar gedurende 4 jaar verrichtte. Tevens heeft ze de basiscursus Seksuologie gevolgd. In het voorjaar van 1994 bracht zij een werkbezoek van 2 maanden aan de afdeling Biologie van de Universiteit van Boston (USA), onder begeleiding van Prof.Dr. M.J. Baum. Vanaf januari 1996, zal zij daar gaan werken als post-doc en gaan samenwerken met Dr. B.S. Rubin, afdeling anatomie en cellulaire biologie, Tufts University, Boston.

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