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THE DEVELOPMENT OF FEMALE- AND MALE-ORIENTED PARTNER PREFERENCE IN THE MALE LABORATORY RAT: INDICATION FOR THE ORGANIZATIONAL ROLE OF GONADAL HORMONES

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Casey Leigh Henley

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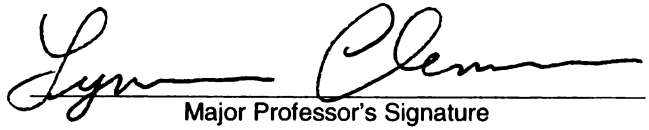

Major Professor's Signature

December 2, 2008

Date

THE DEVELOPMENT OF FEMALE- AND MALE-ORIENTED PARTNER PREFERENCE IN THE MALE LABORATORY RAT: INDICATION FOR THE ORGANIZATIONAL ROLE OF GONADAL HORMONES

By

Casey Leigh Henley

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ABSTRACT

THE DEVELOPMENT OF FEMALE- AND MALE-ORIENTED PARTNER PREFERENCE IN THE MALE LABORATORY RAT: INDICATION FOR THE ORGANIZATIONAL ROLE OF GONADAL HORMONES

By

Casey Leigh Henley

Sexual differentiation of brain and behavior takes place during a critical period in development. In males, this is a time of masculinization, the enhancement of brain systems that mediate male-typical responses, and defeminization, the suppression of brain systems that mediate female-typical responses. In the rat, adult male partner preference is organized early in development by testicular steroids.

There is broad acceptance for the idea that the estradiol metabolite of testosterone is responsible for the organization of the neural systems that mediate reproductive behavior including partner preference in the rat. The notion that estradiol plays an organizational role in development of partner preference is drawn from studies where estrogen action was in some way blocked, either through aromatase or estrogen receptor inhibition during development in male rats. Lack of estrogens neonatally results in a decrease in the male's preference for a female. Therefore, it is likely that estrogens are responsible for the development of female-oriented partner preference in the male.

Super-physiological doses of testosterone (hyper-androgen) during development may also affect partner preference. In the rat, an animal that goes through a male-typical development will show male sexual behavior in adulthood. Administration of

testosterone after neonatal castration also causes masculinization of behavior. However, if an intact male is treated neonatally with testosterone, male sexual behavior in adulthood is disrupted. There is a possibility that the hyper-androgen treatment is altering the male's partner preference, decreasing the male's interest in a female partner, and thus resulting in a suppression of male sexual behavior. Therefore, exposure to super-physiological levels of androgen during development may play a role in the development of male-oriented partner preference in the male.

In this dissertation, I examine the role of early postnatal treatment with steroid hormones in the development of adult partner preference in the laboratory rat. The role of estrogen in the development of female-oriented adult partner preference and sexual behavior in the male rat will be evaluated initially by treating females during the early postnatal period with estradiol and determining if their behavior was masculinized. Then the role of hyper-androgen exposure during development on the expression of male-oriented partner preference and sexual behavior in the male will be examined, followed by a study determining if the estradiol metabolite of testosterone is responsible for the effects seen after hyper-androgen exposure in the male. Finally, to rule out any possibility that the hormonal effects seen were due to changes in maternal behavior, the role of maternal licking and grooming on adult partner preference and sexual behavior in males and females will be assessed.

I would like to dedicate this dissertation to my parents, John and Gail McGovern, and to my husband, Kevin Henley. Without their unwavering support, encouragement, belief, and patience, I would not be the scientist, scholar, or person I am today.

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CHAPTER ONE:

GENERAL INTRODUCTION

Sexual differentiation of brain and behavior takes place during a critical period in development (Baum, 1979; Baum, 2006). This is a time of masculinization, the enhancement of brain systems that mediate male-typical responses, and defeminization, the suppression of brain systems that mediate female-typical responses (Adkins-Regan, 1988; Bakker, 2003). Research on this topic was initiated by studying the organizing effects of testosterone (Phoenix et al., 1959), and since then many studies have been conducted examining the effects of steroid hormones secreted perinatally on sexual differentiation in a number of animal models. In rats, castration at birth eliminates or greatly reduces the expression of male sexual behavior in adulthood (Beach and Holz, 1946) and increases the expression of female receptivity (Beach et al., 1969). Normal masculine behavior is reinstated if testosterone is replaced soon after castration (Beach et al., 1969). In addition, female rats treated neonatally with testosterone show a decrease in receptivity (Barraclough and Gorski, 1962) and, in some cases, an increase in male typical responses (Mullins and Levine, 1968; Whalen and Edwards, 1967). These findings suggest that perinatal endogenous testosterone mediates behavioral sexual differentiation in male rats.

Partner Preference in Animal Models

In addition to male sexual behavior, adult male partner preference is also organized early in development by gonadal hormones. Partner preference can be assessed by giving an

experimental animal a choice between a sexually active male and a sexually receptive female and measuring the amount of time spent in the vicinity of each stimulus animal. There is a broadening literature studying numerous animal models to determine the role steroid hormones play in the development of adult sex-typical partner preference.

Rat

In rats the normal adult preference usually seen is that for an opposite sex partner (Hetta and Meyerson, 1978; Meyerson and Lindstrom, 1973). When given the choice between a stimulus female or a stimulus male, males prefer to spend time with the female, and females prefer the male. This choice has been shown to be influenced by testicular steroids early in development. If a male is castrated at birth and given ovarian hormones in adulthood, he will approach a sexually active male over an estrous female (Hetta and Meyerson, 1978; Matuszczyk et al., 1988). Neonatal testosterone replacement after castration, however, will result in the male-typical preference for an estrous female (Matuszczyk et al., 1988; Meyerson et al., 1980). In addition, if a female is treated early in life with testosterone, and also again in adulthood, she will prefer an estrous female over a stimulus male (Dejonge et al., 1988; Meyerson et al., 1980; Meyerson and Lindstrom, 1973).

Testosterone is a steroid hormone that can have its effects by acting directly on the androgen receptor itself, or by being metabolized into dihydrotestosterone by 5α-reductase (Jaffe, 1969) and into estradiol by aromatase (Ryan et al., 1972). The two metabolites have different effects on the expression of male behavior because they act

upon different steroid receptors. Studying the effects of the metabolites on adult behavior can give insights into the mechanisms that underlie sexual differentiation by testosterone during development. Many accept the idea that estradiol can play an important role in the organization and activation of male-typical sexual behaviors. Male and female rats have a greater ability to aromatize testosterone to estradiol in the hypothalamus and amygdala during perinatal development than at any other time in life (Tobet et al., 1985). Neonatal treatment with estradiol benzoate defeminizes both female and neonatally castrated male rats (Baum, 1979). It also appears that estrogens have some masculinizing effects since neonatal treatment can increase the expression of male-typical sex responses in both females and neonatally castrated males (Baum, 1979).

The effect of perinatal exposure to testosterone on the development of male-typical partner preference is thought to be a result of the aromatization of testosterone to estrogen. Females treated neonatally with dihydrotestosterone (DHT), a nonaromatizable androgen, continue to show a female-typical preference for a stimulus male (Brand and Slob, 1991b). It has also been shown that males castrated at birth and treated neonatally with DHT do not show a preference for an estrous female, an effect similar to non-treated, neonatally castrated males (Brand and Slob, 1991a). The aromatization hypothesis for partner preference development is also supported by studies examining the effect of neonatal exposure to the aromatase inhibitor 1,4,6 androstatriene-3,17-dione (ATD) on adult partner preference. Neonatal treatment of males with ATD has been shown to have effects similar to those of neonatal castration. Whether tested intact or after adult castration and estrogen treatment, males neonatally treated with ATD either do

not show a preference for either stimulus animal or show a preference for the male stimulus (Bakker et al., 1993a; Bakker et al., 1993b; Bakker et al., 1996; Brand and Slob, 1991a). Control males, however, after adult castration and estrogen treatment continue to prefer estrous females (Bakker et al., 1996).

Studies by Matuszczyk and Larrson (1995) give further indication that perinatal exposure to estrogen plays a substantial role in the development of a male-typical partner preference. Males treated with an anti-estrogen during the prenatal period show a reduced preference for females in adulthood. However, males treated with an anti-androgen prenatally continue to prefer an estrous female over a stimulus male.

Finally, previous work from this laboratory shows that females that receive postnatal exposure to polychlorinated biphenyl (PCB) 77 on postnatal days (PND) 1-21 exhibit a lower preference for a sexually active stimulus male than do control females (Cummings, personal communication). PCB 77 has been reported to have estrogenic effects under other circumstances (Jansen et al., 1993; Nesaretnam et al., 1996; Seegal et al., 2005). Similarly, males with the testicular feminizing mutation (Tfm), which is characterized by a dysfunctional androgen receptor (Zuloaga et al., 2008), also indicate an estrogenic role in the development of adult partner preference. Tfm males exhibit female phenotypes due to a lack of androgenization during development. However, these males do have high levels of circulating estrogen and normal estrogen binding in the brain (Roselli et al., 1987). It has been shown that in adulthood, Tfm males show male-like partner preference, approaching an estrous female more than a stimulus male (Hamson, personal

communication), supporting the idea that organizational estrogen is responsible for male-typical preference in adulthood.

The prenatal environment also seems to be important in adult preference behavior. Male rats castrated neonatally, but treated with testosterone in adulthood, show a normal preference for the female (Brand and Slob, 1991a; Meyerson et al., 1980). Likewise, males treated neonatally with ATD and given adult testosterone replacement also show a preference for the female (Bakker et al., 1993b). These effects do indicate that some masculinization of adult partner preference occurs prior to birth, but testosterone is necessary as the activational hormone for the expression of this prenatally organized male-typical behavior.

Ferret

Stockman et al (1985) were the first to study the development of partner preference in the ferret. Males castrated shortly after birth and treated with estradiol in adulthood show a higher preference for a stimulus male than do control males (Stockman et al., 1985). Females ovariectomized in early life and immediately treated with testosterone show a lower preference for the stimulus male than do control females (Martin and Baum, 1986; Stockman et al., 1985). Early neonatal manipulations were unable to completely reverse the partner preference of ferrets, which could mean prenatal hormones are necessary for the display of sex-typical partner preference. When female ferrets are treated with testosterone during the prenatal period, immediately at birth, and during the early postnatal period, they show a preference similar to control males (Baum et al., 1990).

Treatment during only one of these periods leads to a preference that is intermediate to that of control males and control females (Baum et al., 1990).

Perinatal estrogen appears to have a significant role in the development of adult partner preference in the ferret. As mentioned above, female ferrets that receive neonatal testosterone treatment show lower preference for stimulus males than controls. However, neonatal treatment with DHT does not appear to disrupt female-typical partner preference (Martin and Baum, 1986), indicating that testosterone is most likely working through its estrogenic metabolite. Also, males treated with ATD prenatally show decreased latencies to approach a stud male when tested after adult treatment with estradiol (Baum and Tobet, 1986).

Mouse

Mice have been an important model for studying the effects of estrogen during development on adult partner preference due to gene knock out animal availability. Males with the estrogen receptor α gene knocked out (ERαKO), castrated in adulthood and given testosterone replacement show no preference between an estrous female and a stimulus male, whereas wild-type males show a preference for the female (Wersinger et al., 1997). ERαKO males also do not prefer an estrous female over an unreceptive female (Wersinger et al., 1997). Finally, wild-type males prefer to approach and sniff odors from an estrous female, but ERαKO males show no preference (Wersinger and Rissman, 2000).

However, one problem arises with ERα knock out studies. It cannot be determined if the effects are due to the absence of either the organizational or activational effects of estrogen. The lowered preference for the stimulus female shown by the male mice may be due to a lack of estrogen effects during the perinatal critical period but it could also be due to the fact that the adult testosterone treatment cannot have an estrogenic activational effect in adulthood. Other studies have attempted to resolve this issue by studying mice in which the aromatase gene, Cyp-19, has been knocked out (ArKO). Wild-type males prefer estrous female odors over male odors, but ArKO males show no preference for female odors over male odors and do not show a preference for a stimulus female over an empty room (Bakker et al., 2004). The preference for females odors is not increased after adult estradiol treatment, indicating the effects seen can be attributed to the organizational effects of estrogen and not activational effects.

The androgen receptor (AR) may also play a role in the development of partner preference in the mouse, unlike in the rat. Female mice treated with DHT neonatally display a male-like partner preference for both a stimulus male animal and male bedding over an estrous female or female bedding (Bodo and Rissman, 2008), whereas neonatal DHT does not have a masculinizing effect on the partner preference of female rats (Brand and Slob, 1991b). Also, Tfm male mice, unlike Tfm male rats, show a female-typical partner preference (Bodo and Rissman, 2007), indicating that perinatal activation of the AR is necessary for the development of masculine partner preference behavior in the mouse. More research is needed, however, to explain why the ERαKO and ArKO models

described above show feminine partner preference behaviors despite having normal male levels of circulating DHT.

Pig

Pigs are another animal model that have been used to evaluate the role of perinatal exposure to gonadal hormones on adult partner preference. In intact adult pigs, males usually show no preference for either stimulus animal, whereas estrous females show a preference for a male over a female stimulus animal (Signoret, 1970). Male pigs castrated at birth and treated with estradiol in adulthood show a female-typical preference for a boar (Adkins-Regan et al., 1989; Ford, 1983). Males castrated later in life, show no preference, similar to the behavior of intact males (Adkins-Regan et al., 1989; Ford, 1983). Both males and females gonadectomized early in life and then treated with estradiol during the prepubertal period show an adult preference for a stimulus female after adult estradiol treatment (Adkins-Regan et al., 1989). Prepubertal hormone treatment has an effect because the pig appears to have a much longer postnatal critical period than the majority of other animal models studied (Adkins-Regan, 1988).

Hamster

Little work has studied the organizational effects of hormones on the adult expression of partner preference in the hamster. Johnson and Tiefer (1972) studied partner preference in the hamster and discovered that neonatal hormones are important for the display of sex-typical partner preference. A male hamster castrated neonatally and given ovarian hormones in adulthood prefers a stimulus male over a female (Johnson and Tiefer, 1972).

The role of estrogen in the development of partner preference has not been studied in the hamster.

Dog

Similar to the hamster, few studies have researched the role of hormones during development on sexual partner preference in the dog. Beach et al (1977) tested female dogs after pre- and/or postnatal exposure to testosterone. Adult females were first tested after estradiol treatment with a stimulus male and then after testosterone treatment with an estrous female. Even though the paradigm is not a choice test, the females treated with perinatal testosterone spend more time with the stimulus female than they do with the stimulus male. Control females, however, spend more time near the stimulus male than the stimulus female (Beach et al., 1977). The role of estrogen in the development of partner preference has not been studied in the dog.

Partner Preference in Humans

Although numerous studies have examined the role of steroid hormones on the development of brain and behavior, many questions remain. Among these is the role steroid hormones play in the development of human orientation. Understanding hormonal control of the development of adult partner preference and sexual behavior in an animal model such as the laboratory rat, can have a wide range of implications. Examining the endocrine components and neural factors that play a role in sexual motivation and partner preference in laboratory animals, can give insight into understanding the basis of human orientation.

The prenatal environment appears to play a role in the development of male sexual orientation in humans. It has been shown that for each older brother a boy has, his chances of growing up to be homosexual increases by one-third (Blanchard and Bogaert, 1996), and approximately one homosexual male in seven can attribute his orientation to this fraternal birth order effect (Cantor et al., 2002). One explanation could be due to possible hyper-androgen exposure during development. There is the notion that male preference for another male may not always be due to feminizing effects since it has been shown that homosexual men have larger genitalia (Bogaert and Hershberger, 1999) and more masculine auditory evoked potentials (McFadden and Champlin, 2000) than heterosexual men, indicating a higher level of androgen during development. Also, studies indicate that having more older brothers can lead to hyper-masculinization on some measures such as digit length ratios. The index finger (2D) to ring finger (4D) ratio is sexually dimorphic in humans and believed to be affected by prenatal androgen levels (Brown et al., 2002; McFadden et al., 2005; Williams et al., 2000). In females the 2D is similar in length to 4D, whereas males tend to have shorter 2D (Williams et al., 2000). The 2D:4D ratio becomes more masculine in men who have more than two older brothers compared to men with no older brothers, and there is a correlation between number of older brothers and 2D:4D ratio (Williams et al., 2000). Studies also show that homosexual men tend to have a lower, or more masculine 2D:4D ratio than heterosexual men (Rahman, 2005; Rahman and Wilson, 2003; Robinson and Manning, 2000). These findings suggest that men with more older brothers are more likely to be homosexual and are exposed to higher androgen levels prenatally than men with no older brothers.

Further indication for a role of steroid hormones in the development of human sexual orientation can be found in studies of human endocrine disorders. Women with congenital adrenal hyperplasia are exposed to higher than normal levels of androgen throughout development. These women show an increased incidence of bisexual or homosexual orientation (Meyer-Bahlburg et al., 1996; Zucker et al., 1996). In addition, genetic males with complete androgen insensitivity syndrome (CAIS; the human equivalent of Tfm), look like females at birth, are usually raised as female throughout life, and show a preference for males in adulthood. Women that were exposed to the synthetic estrogen diethylstilbestrol (DES) prenatally also have an increased incidence of bisexual orientation compared to women not exposed to DES (Ehrhardt et al., 1985; Meyer-Bahlburg et al., 1995). DES is a nonsteroidal synthetic estrogen used through 1970 to decrease the occurrence of miscarriages. It is interesting to note, however, that although estrogen exposure appears to affect the orientation of women, lack of estrogen plays little role in the development of female-oriented male sexual orientation. Genetic males with CAIS are still exposed to male-typical levels of estrogen during development yet show a preference for men in adulthood. Also, although subjects are limited, individuals who carry a mutant ERα or aromatase gene self report as being heterosexual males (for review, Baum, 2006).

Finally, the role of steroid hormones on human orientation can be assessed in gender reassignment cases. In instances of partial AIS or cloacal exstrophy, genetic males are born with ambiguous genitalia (Gooren, 2006). In the past, the most common solution posed by doctors was to assign the newborn a female identity and surgically reconstruct

female genitalia. In a long term study conducted by Reiner and Kropp (2004), eighteen patients with sexual differentiation disorders were evaluated throughout adolescent life. Of the patients, three were raised as boys and continued to identify with the male gender over the evaluation period. Fifteen were assigned as females at birth, and seven of them reassigned to the male gender before twelve years of age. Of the remaining females, one refused to declare a sexual identity, one died of unknown causes, and five continue to live as females. However, the five patients that remained female had mood scores and social competence evaluations lower than those of the individuals that reassigned to the male gender. Of the individuals (male or female) that were old enough to declare a sexual orientation, all expressed a preference for a female partner. These studies all indicate that prenatal hormones have a profound influence on sexual orientation and gender identity of humans.

Overview of Studies

In this dissertation, the role of early postnatal treatment with steroid hormones in the development of adult partner preference in the laboratory rat will be examined. The role of estrogen in the development of female-oriented adult partner preference and sexual behavior in the male rat will be evaluated initially by treating females during the early postnatal period with estradiol and determining if their behavior was masculinized. Then the role of hyper-androgen exposure during development on the expression of male-oriented partner preference and sexual behavior in the male will be examined, followed by a study determining if the estradiol metabolite of testosterone is responsible for the effects seen after hyper-androgen exposure in the male. Finally, to rule out any possibility

that the hormonal effects seen were due to changes in maternal behavior, the role of maternal licking and grooming on adult partner preference and sexual behavior in males and females will be assessed.

CHAPTER TWO:

ESTROGEN IS RESPONSIBLE FOR THE DEVELOPMENT OF FEMALE-ORIENTED PARTNER PREFERENCE IN THE MALE LABORATORY RAT AS INDICATED BY ITS MASCULINIZING EFFECTS ON ADULT BEHAVIOR IN FEMALES

INTRODUCTION

As reviewed in Chapter One, adult male partner preference is organized early in development, and it appears that the estradiol metabolite is responsible for the ontogeny of partner preference behavior. A normal adult male rat will show a preference for an estrous female over a sexually active male (Hetta and Meyerson, 1978), neonatal castration significantly reduces this preference (Brand and Slob, 1991a), and this reduction in preference is prevented when testosterone is replaced following castration (Brand and Slob, 1991a). However, a male treated with DHT after neonatal castration continues to display a reduced preference for the estrous female (Brand and Slob, 1991a). The success of testosterone administration in overcoming the loss of normal partner preference after castration, but not DHT, is consistent with the idea that estradiol is required for the development of this behavior.

Other studies also support the notion that estrogens plays a role in the organization of adult partner preference behavior. Blocking estrogen action during development, either through aromatase inhibitors (Bakker et al., 1993a; Bakker et al., 1993b), antiestrogens (Matuszczyk and Larsson, 1995), estrogen receptor knock outs (Rissman et al., 1997;

Wersinger and Rissman, 2000; Wersinger et al., 1997) or aromatase gene knock outs (Bakker et al., 2002) results in a decrease in the male's preference for a female.

From these studies, it appears that estradiol plays a significant role in the organization of partner preference behavior in the rat during postnatal development. However, this idea is drawn from studies where estrogen action was in some way blocked in male rats. In the present study, the possible masculinizing effect of estrogens was tested directly by treating female rats during the early postnatal period with estradiol benzoate and examining their adult partner preference and sexual behavior. In addition, the effects of exposure to estradiol on the morphology of the ovaries and cell number in the spinal nucleus of the bulbocavernosus and pelvic ganglia were also measured.

METHODS

Animals

Experimental Females: Time-mated pregnant Long-Evans rats (Charles River, Raleigh, NC) were housed individually with *ad lib* food and water in plastic cages (45.5 x 24 x 21 cm) in a 14:10-hr light dark cycle with lights on at 01:00. For nest building material thirty, one-inch paper towel strips were given to the dams on gestation day (GD) 20. A subset of the female offspring of these dams became the experimental females of this study (see below). On the day of birth, PND 0, the litter was reduced to four male and four female pups. For litter reductions, the anogenital distance (AGD) for each pup was measured, and since the AGD is shorter in females than in males, the four shortest and the four longest were retained (Figure 2.1A).

Stimulus Animals: Sexually experienced, gonadally intact, adult Long Evans rats (Charles River, Raleigh, NC) were used as stimulus animals for the behavioral tests (females at least 60 days old; males at least 90 days old).

Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experimental procedures were approved by the Michigan State University Animal Care and Use Committee.

Hormone Treatments

Silastic capsules (Dow Corning; inner diameter 1.47 mm; outer diameter 1.96 mm; length 5 mm) were used to administer estradiol treatments on the day of birth (Figure 2.1A). Estradiol benzoate (Sigma; EB) was mixed with cholesterol (Sigma; C) to achieve 5% and 10% EB to C mixtures. On PND 0, pups received Silastic capsules containing either cholesterol or 5% or 10% EB-C mixtures. These animals are referred to as C, 5%, and 10% females, respectively. While these estradiol treatments are most likely outside of the physiological range of estrogen exposure, this study was also designed to provide a positive estradiol control to evaluate the claim from studies in this laboratory that environmental contaminants, such as polychlorinated biphenyls, that have estrogenic effects (Jansen et al., 1993; Nesaretnam et al., 1996; Seegal et al., 2005) affect partner preference in female rats (Cummings et al, in press).

Capsules were implanted subcutaneously (s.c.; one treatment per litter) through a small incision on the back of the animal, while the pups were under ice anesthesia. The incision

was closed using superglue (Loctite). Following surgery, pups were warmed under a heat lamp until the incision was dry (approximately one hour) and then returned to their mothers. Capsules were left in place for 3 weeks. On PND 21, at weaning, the pups were anesthetized with isoflurane (Isoflo, Abbot Laboratories), and the implants were removed through an incision made near one end of the capsule (Figure 2.1C). The incision was closed with an Auto Clip (Clay Adams) and covered with First Aid Cream (Johnson and Johnson). Pups were then housed with same-sex littermates. Only female pups were used in this Experiment.

After animals reached 60 days of age, two females (C, n=16; 5%, n=14; 10%, n=16) from each litter were ovariectomized and implanted with a Silastic capsule containing either 25% or 12.5% EB-C mixtures (Figure 2.1D). The capsules were implanted s.c. while the animals were anesthetized with isoflurane; the incision was closed with an Auto Clip and covered with First Aid Cream. After 4 weeks, the capsules begin to lose efficacy because of connective tissue growth around the capsule (personal observation). The capsules were removed and reimplanted via a new incision in the neck. For some tests (see below), the females were injected s.c. with 0.5 mg progesterone (Sigma; P, in sesame oil) four hours prior to data collection. Adult behavioral tests were run after treatment with one of two doses of EB treatment in adulthood and with or without progesterone treatment. This hormonal regime was used to test the females under two naturally occurring hormonal conditions: estrogen alone, typical of early proestrus, and estrogen plus progesterone, typical of late proestrus. Female rats mate during both early proestrus, when estradiol is

available but before the surge in progesterone, and during late proestrus, when both ovarian hormones are present (Blaustein, 2008).

Stimulus females were implanted with a Silastic capsule containing 25% EB-C mixture prior to testing but were not ovariectomized. It is the practice of this laboratory to not ovariectomize stimulus females for testing procedures. The hormone treatments used reliably induce sexual receptivity while avoiding the trauma of an unnecessary surgery. Stimulus females were injected s.c. with 0.5 mg P four hours prior to partner preference and sexual behavior testing.

Maternal Licking and Grooming

Studies have shown that neonatal hormone treatments can alter the display of maternal behavior (Cummings et al., 2005; Moore, 1982), and changes in maternal care can affect the behavior of the offspring (Cameron et al., 2008; Champagne et al., 2001; Champagne and Meaney, 2006; Francis et al., 1999). To determine if the early postnatal treatments caused alterations in maternal care, the maternal licking and grooming behavior of the mothers of the experimental females was analyzed (Figure 2.1B). Maternal behavior was videotaped during the last hour of the light phase and the first hour of the dark phase of the light-dark cycle on PND 1, 2, 4, and 6. These recordings, as well as those for all other behavioral tests, were analyzed using The Observer 5.0 (Noldus), a behavioral data acquisition computer program. The amount of time the dam spent licking and grooming the litter was determined.

Behavioral Testing

Partner Preference: Tests for partner preference were conducted in a three-chamber apparatus (91 x 61 x 41 cm) made of Plexiglas with a transparent front and opaque sides and inner walls. Each chamber (30 x 61 x 41 cm) had openings in the two inner walls at the back of the apparatus which allowed the experimental animal to move freely among the three chambers. One intact male and one sexually receptive female stimulus animal were tethered to a bar at the front end of the two outer chambers using a 25 cm wire fitted with a swivel. The stimulus animals wore a harness that was attached to the bar allowing the experimental animal to make physical contact, but limiting the movement of stimulus animal to its chamber. The middle chamber had no stimulus animal. Both experimental and stimulus animals were adapted to the apparatus, and stimulus animals were adapted to the harness prior to testing. All adult behavioral testing took place under dim red light illumination in the middle part of the dark phase of the light-dark cycle.

During the 20-minute partner preference test, the experimental animal could freely move among the three chambers and interact with the stimulus animals. The test was videotaped, and the following behaviors were quantified: time spent in each chamber, latency to first enter each chamber, and the occurrence of mounts, intromissions, and ejaculations between the experimental and stimulus animals. Preference scores were calculated by subtracting the duration of time spent in the stimulus male chamber from the duration of time spent in the stimulus female chamber. Therefore, a positive preference score indicates more time spent with the stimulus female, whereas a negative

preference score indicates more time spent with the stimulus male. The experimental females were sexually naïve prior to the initial partner preference test.

Female sexual behavior: When tested for female sexual behavior, a barrier with four holes (4.5 x 4.5 cm) was placed in a Plexiglas observation chamber (46 x 58 x 51 cm), which divided it into a female escape area (46 x 22 x 51 cm) and a male area (46 x 36 x 51 cm). The holes in the barrier were too small for the male to get through but gave the female free access to both chambers. The tests lasted 30 minutes and were videotaped. The frequency of male mounts, intromissions and ejaculations were scored, as was the latency to show these behaviors. The latency of the experimental female to approach the male, the amount of time the female spent in the male area of the observation chamber, and sexual receptivity were also recorded. Receptivity was measured using lordosis quotient (LQ), which was calculated for all females who received at least six mounts. For the first 10 mounts (including intromissions or ejaculations) female responses were scored as a 0 (no lordosis) or 1 (lordosis), from which a percentage of number of lordoses per test was calculated. The frequency of exits and latency to return to the male were not measured because the females treated with estradiol during the early postnatal period did not receive intromissions or ejaculations from the stimulus males (see Results). Finally, proceptive behaviors, which include hopping and darting, ear wiggling, and approaching the male, were scored during the test following progesterone treatment.

Male-like sexual behavior: Tests for male-like sexual behavior displayed by the experimental females were conducted in a Plexiglas observation chamber (46 x 58 x 51

cm). During the test, the experimental female had unrestricted access to the female stimulus animal. The tests lasted 30 minutes, and video recordings were analyzed to determine frequency of mounts, intromissions, and ejaculatory patterns shown by the experimental females and the latency to show these behaviors.

Testing Schedule: Experimental animals were tested twice a week for 6 weeks (Figure 2.2). During the first test each week, the females were tested with only the adult estradiol treatment (EB alone). For the second test, each female was injected with 0.5 mg progesterone four hours prior to testing (EB + P). The initial partner preference of the female was tested in week 1 (Figure 2.2A). Each experimental female received sexual and social experience with both male and female stimulus animals during weeks 2 and 3, but data were not collected. During experience weeks, experimental females were partnered with stimulus animals for 30 minutes during which time sexual behavior could occur. During week 4, half of the experimental females were tested for sexual behavior with a male and the other half with a female (Figure 2.2B). The sex of the stimulus animals was switched for week 5 (Figure 2.2C). Sexual behavior during weeks 4 and 5 was recorded and scored. In week 6, the female's final partner preference was evaluated (Figure 2.2D).

Histology

Ovaries: To determine if the early estradiol treatment had an effect on the estrous cycle of the females, the ovaries were examined for the presence of corpora lutea (Figure 2.1D). The ovaries were removed as described above during the ovariectomy surgery and

placed in 0.1 M phosphate-buffered 10% formalin fixative at room temperature for at least 30 days. One ovary from each female was then washed in phosphate-buffered saline (PBS) solution (pH 7.4) and transferred to a 50% ethanol solution. The tissue was embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Stained sections were viewed on a light microscope by an experimenter blind to the treatment groups, and the presence of corpora lutea was determined. Tissue embedding, sectioning, and staining were completed by the Investigative HistoPathology Laboratory at Michigan State University.

Pelvic Ganglia (PG): After behavioral testing was concluded, the experimental females were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (Nembutal, 75 mg/kg). The major pelvic ganglion was fresh dissected from both sides of each animal (Figure 2.1D). The ganglia were placed in a Cryomold (Tissue-Tek; 15 mm x 15 mm x 5 mm), covered with O.C.T. Compound (Tissue-Tek), and immediately frozen in dry ice-cooled isopentane. Ganglia were stored at -80° C until sectioning. Cryostat sections at a thickness of 14 μm were taken in four series of sequential sections and mounted onto gelatin-subbed slides. Slides were stored at -80° C until staining. One series of every fourth section was processed for Nissl staining using thionin. Slides were then dehydrated with ethanol, cleared with xylene (Baker), and coverslipped with Permount (Fisher Scientific). Stained sections were viewed on a light microscope, and the number of neurons present were counted by an experimenter blind to the treatment groups. Neurons were identified as darkly stained, large nucleated cells (Figure 2.3). Only one

female from each litter (10% EB and C treatment groups only) was used for analysis of the pelvic ganglia.

Spinal Nucleus of the Bulbocavernosus (SNB): Directly after pelvic ganglia removal, females were perfused intracardially with chilled saline and then 10% buffered formalin fixative. The lumbosacral spinal cord was dissected and placed into buffered formalin fixative at room temperature for at least 30 days (Figure 2.1D). The spinal cord was then sectioned at 50 μm in three series of sequential sections. Sections were mounted onto gelatin-subbed slides, and one series of every third section was processed for Nissl staining and counted by an experimenter blind to the treatment groups. Motoneurons were identified as darkly stained, large nucleated cells (see Figure 2.10).

Analysis

Maternal licking and grooming data were analyzed using a 3 x 4 (early postnatal treatment x day) ANOVA with repeated measures on the second factor. The data for the behavioral measures during the partner preference tests were analyzed using a 3 x 2 x 2 x 2 (early postnatal treatment x adult estradiol treatment x progesterone treatment x initial or final test) ANOVA with repeated measures on the third and fourth factors. Preference scores and duration of time spent in the female chamber during the partner preference tests were also analyzed within each early postnatal treatment group using a one-sample t-test. This test was used to determine if the animals showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the female above chance (duration = 400 seconds).

The data for the behavioral measures during the sexual behavior tests were analyzed separately for female or male behaviors using a 3 x 2 x 2 (early postnatal treatment x adult estradiol treatment x progesterone treatment) ANOVA with repeated measures on the third factor. The presence or absence of corpora lutea in the ovaries was analyzed using a Chi-Square test. SNB cell counts were analyzed using a one-way ANOVA by early postnatal treatment. PG cell counts were analyzed using an independent samples t-test.

For some variables, the data did not meet homogeneity of variance assumptions, even after the prescribed transformations (i.e. square root). For these measures, nonparametric statistics were used for analyses. In these situations, data from the 5% and 10% females were collapsed into an early postnatal-estradiol treated group (EB females) because no significant differences between the two early postnatal estradiol treatments were found for any of the variables analyzed using nonparametric statistical tests. Also, no significant differences were found with the same tests between the two adult EB doses within the early postnatal treatment groups, so data were collapsed across adult EB doses as well. The Mann-Whitney U and the Fisher's exact probability tests were used for these analyses.

Abbreviations

C females – female pups treated on PND 0-21 with cholesterol

5% females – female pups treated on PND 0-21 with a 5% EB-C mixture

10% females – female pups treated on PND 0-21 with a 10% EB-C mixture

EB females – 5% and 10% females collapsed into one treatment group used for non-parametric analyses

EB alone – adult hormonal testing condition when females were treated with only EB (either 25% or 12.5% dose)

EB + P – adult hormonal testing condition when females were treated with both EB and P

RESULTS

Maternal Licking and Grooming

No significant differences in maternal licking and grooming were seen among treatment groups (p=.292) or days (p=0.078), with no significant interaction (p=0.147; Figure 2.4).

Partner Preference

Effects of Early Postnatal Treatments: Early postnatal treatment with EB altered the partner preference of the female experimental animals. The 10% and 5% females spent more time in the stimulus female chamber (Figure 2.5A; [$F(2,40) = 20.3$, $p<0.001$]) and less time in the stimulus male chamber (Figure 2.5B; [$F(2,40) = 13.7$, $p<0.001$]) than did the C females. Early postnatal treatment did not affect the time the females spent in the middle chamber (Figure 2.5C). The 10% and 5% females also showed a preference score greater than that of the C females (Figure 2.5D; [$F(2,40) = 18.7$, $p<0.001$]). Thus, early estradiol treatments reduced the preference for the male and increased the preference for the female, and these effects were seen with no significant interactions between them and

those of adult treatments or week of test. Also for all conditions, early postnatal treatments did not affect the latencies to enter the male or female chambers.

In both the initial and final partner preference test, the 10% and 5% females had positive preference scores statistically different than chance, indicating a preference for the female stimulus animal. The C females, however, did not show a preference greater than chance for either stimulus animal (Table 2.1). Also, the duration of time spent in the female chamber in both the initial and final tests was significantly greater than chance for the 10% and 5% females, but not for the C females (Table 2.1).

The proportion of experimental females that displayed male-like sexual behavior directed to the stimulus female during partner preference tests did not differ significantly between EB and C females (Fisher's exact probability tests). However, five out of thirty females (four 10% females and one 5% female) that received early postnatal estradiol showed at least one full ejaculatory reflex pattern during the preference tests, whereas no ejaculatory patterns were shown by the C females. The proportion of experimental females that received mounts, intromissions, or ejaculations from the stimulus male differed significantly between early postnatal treatment groups (see Table 2.2 for the specific comparisons). The stimulus males showed more sexual behavior toward the C females compared to the EB females in both partner preference tests.

Effects of Experience and Adult Treatments: Across all hormone treatments, the average latency to enter the male chamber was significantly shorter for the final test compared to

the initial one ($\bar{X}$ ± SEM: all measures are in seconds: 31.9 ± 3.4 vs 13.6 ± 1.5; [F(1,40) = 49.6, p<0.001]). Also, for time spent in the middle chamber, the experimental females spent significantly more time in the middle chamber during the final tests than during the initial tests ($\bar{X}$ ± SEM: 374.9 ± 15.8 vs 330.1 ± 13.4; [F(1,40) = 4.8, p=0.035]).

Two measures were affected by progesterone treatment. Across all tests and conditions, the experimental females spent less time with the stimulus female after EB + P treatment compared to after treatment with adult EB alone ($\bar{X}$ ± SEM: 517.2 ± 15.9 vs 479.1 ± 17.9; [F(1,40) = 6.0, p=0.019]). In addition, the experimental females spent significantly more time in the middle chamber when treated with EB + P than when treated with adult EB alone ($\bar{X}$ ± SEM: 378.3 ± 13.4 vs 326.7 ± 13.1; [F(1,40) = 10.0, p=0.003]).

Both the amount of time spent with the male [F(1,40) = 5.5, p=0.024] and latency to enter the female chamber [F(1,40) = 49.6, p<0.001] were affected by a progesterone by test interaction. During the initial partner preference test, females treated with adult EB alone spent more time in the stimulus male chamber than did females treated with EB + P ($\bar{X}$ ± SEM: 404.1 ± 17.4 vs 361.0 ± 18.6). There was also a decrease in time spent with the male in the adult EB alone treatment group from the initial to final preference test ($\bar{X}$ ± SEM: 404.1 ± 17.4 vs 306.4 ± 23.1) but no change in the EB + P treatment group. The latency to enter the female chamber was significantly reduced from the initial to the final test when the females received adult EB alone ($\bar{X}$ ± SEM: 35.0 ± 4.6 vs 11.6 ± 1.0) but not when progesterone was also given ($\bar{X}$ ± SEM: 15.8 ± 1.8 vs 12.4 ± 2.0). Adding

progesterone significantly reduced the latency to enter the female chamber, but only for the initial test.

Finally, there were significant adult EB by test interactions for time spent with the female [$F(1,40) = 10.8$, $p=0.002$], time spent with the male [$F(1,40) = 5.1$, $p=0.030$], and preference score [$F(1,40) = 10.0$, $p=0.003$]. Individual comparisons showed that females treated with the high adult EB dose increased the time they spent with the female from initial to final test ($\bar{X}$ ± SEM: 478.6 ± 20.8 vs6570.4 ± 30.4), but no significant effect of test was found for the females treated with the low adult EB dose ($\bar{X}$ ± SEM: 495.9 ± 20.8 vs 447.8 ± 30.4). During the final test, the high adult EB females spent more time in the female chamber than their low dose counterparts. Females treated with the high adult EB dose spent significantly less time with the male from initial to final test ($\bar{X}$ ± SEM: 392.6 ± 22.6 vs 278.2 ± 31.2), but females receiving the low adult EB dose remained the same ($\bar{X}$ ± SEM: 372.5 ± 22.6 vs 352.2 ± 31.2). This lead to an increase in preference score for the females treated with the high adult EB dose ($\bar{X}$ ± SEM: 86.0 ± 39.1 vs 292.2 ± 57.4) but no change for the low EB dose females ($\bar{X}$ ± SEM: 123.4 ± 39.1 vs 95.6 ± 57.4).

Female Sexual Behavior

The 10% females spent significantly less time in the male area of the testing arena than did the C females (Figure 2.5; [$F(2,39) = 4.4$, $p = 0.020$]). Also, regardless of other hormone treatments, all females spent less time with the male after EB + P treatment ($\bar{X}$ ± SEM: 560.0 ± 48.5 vs 486.3 ± 49.3; [$F(1,39) = 4.1$, $p = 0.049$]).

Non-parametric statistics were used to analyze the remaining measures for the female sexual behavior tests. A Mann-Whitney U test was used to compare the behaviors displayed by the stimulus males on tests with EB females (combining the 5% and 10% groups as explained in Methods) and with C females treated as adults with EB alone (combining both adult doses) or with EB + P.

Male mount, intromission, and ejaculation frequencies were significantly affected by the early postnatal treatment of the experimental females. The stimulus males displayed fewer mounts, intromissions, and ejaculations in tests with EB females as compared to C females (Figure 2.7). The proportion of experimental females that received mounts, intromissions, and ejaculations by the stimulus male was analyzed using Fisher's exact probability test. In both tests with and without progesterone, significantly fewer EB females received mounts, intromissions, and ejaculations compared to C females (Table 2.3).

Too few EB females (n = 2) received at least six mounts to allow meaningful statistical comparisons of the LQ scores of EB vs. C females. During tests with adult EB alone treatment, one 10% female received six mounts, but showed no lordosis responses. One 5% female received six mounts during the EB + P test, but showed no lordosis responses. In contrast, all the C females that received at least 6 mounts showed lordosis responses with average LQs of 87% (n=12) for the EB alone (combining both adult doses) tests and 97% (n=14) for the EB + P tests.

The data for the proceptive behaviors were analyzed using the Mann-Whitney U test, which revealed that EB females showed significantly fewer proceptive behaviors than C females did (Figure 2.8). Separate statistical tests were used to compare the EB and C females for each specific proceptive behavior.

Male-like Sexual Behavior

Nonparametric tests were used to analyze the data from the male sexual behavior tests. No significant differences were seen between EB and C females for any behavioral measure (Fisher's exact probability tests). However, five out of thirty females (four 10% females and one 5% females) that received early postnatal EB showed at least one full ejaculatory pattern during the sexual behavior tests, whereas no ejaculatory patterns were shown by the C females. Four of these five EB females were the same females that showed ejaculatory patterns during the partner preference tests.

Histology

Estradiol treatment during development significantly affected the presence of corpora lutea in the ovaries of the experimental females (Chi-Square, $p<0.001$; Figure 2.9). Corpora lutea were seen in 13 of 16 C females but in none of the females treated with early postnatal estradiol.

Early postnatal estradiol treatment also altered the number of motoneuron cells seen in the SNB. C females showed fewer cells than did the 5% and 10% female groups (Figure

2.10; [$F(2,37) = 14.3$, $p<0.001$]). No effect was seen on the number of neurons present in the PG of the females ($\bar{X}$ ± SEM: 10% EB:3011.6 ± 340.5 vs C: 2916.0 ± 264.1).

Table 2.1. One-sample t-test analyses of preference scores and duration of time spent in the female chamber during the partner preference tests. This analysis was used to determine if the experimental females showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the stimulus female above chance (duration = 400 sec). The 10% and 5% females showed a preference for the stimulus female and spent more time in the female chamber than expected by chance. Data were collapsed across adult EB doses and P treatment. *Significantly different from chance, $p<0.02$. **Significantly different from chance, $p<0.001$.

	Preference Score (Chance = 0)				**Duration in Female Chamber (Chance = 400)**			
	Initial		**Final**		**Initial**		**Final**	
	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value
10% EB	203.3 (32)	6.3**	389.4 (78.3)	5.0**	546.9 (18.9)	7.8**	640.4 (42.8)	5.6**
5% EB	206.6 (46.3)	4.5*	283.0 (96.5)	2.9*	559.0 (27.9)	5.7**	551.6 (52.2)	2.9*
C	-112.2 (66.2)	-1.8	11.6 (57.4)	0.2	394.2 (31.1)	-0.2	411.2 (40.2)	0.3

Table 2.2. Proportion of experimental females that received sexual behavior from the stimulus male during the partner preference tests. C females were more likely than EB females to receive sexual behavior from the stimulus male. Data from tests after adult treatment with both EB alone and EB + P are shown. Data are collapsed across the two adult EB doses. *Significantly different from cholesterol, $p<0.05$. **Significantly different from cholesterol, $p<0.01$.

Initial Tests	**Adult Estradiol Alone**		**Adult Estradiol and Progesterone**		
Behavior	Mount	Intromission	Mount	Intromission	Ejaculation
Cholesterol	9/16	7/16	8/16	8/16	4/16
Estradiol *	6/30	1/30	4/30	3/30	0/30
Final Tests	**Adult Estradiol Alone**		**Adult Estradiol and Progesterone**		
Behavior	Mount	Intromission	Mount	Intromission	Ejaculation
Cholesterol	7/16	6/16	12/16	9/16	6/16
Estradiol **	2/30	1/30	1/30	1/30	0/30

Table 2.3. Proportion of experimental females that received sexual behavior from the stimulus male during the female sexual behavior tests. C females received more mounts, intromissions and ejaculations from the stimulus male in both female sexual behavior tests than did EB females. Data from tests after adult treatment with both EB alone and EB + P are shown. Data are collapsed across the two adult EB doses. *Significantly different from cholesterol, $p<0.001$

	Adult Estradiol Alone			**Adult Estradiol and Progesterone**		
Behavior	Mount	Intromission	Ejaculation	Mount	Intromission	Ejaculation
Cholesterol	14/16	12/16	10/16	14/16	14/16	12/16
Estradiol *	6/29	3/29	0/29	6/30	1/30	0/30

A PND 0	B PND 1,2,4,6	C PND 21	D After PND 60		
Litter Reduction Silastic Capsules -Cholesterol -5% EB-C -10% EB-C	Maternal Licking and Grooming	Capsule Removal Weaning	Ovariectomy -Ovaries Collected Silastic Capsules -12.5% EB-C -25% EB-C	Partner Preference Female Sexual Behavior Male-like Sexual Behavior	Histology -PG -SNB

Figure 2.1. Treatment and testing schedule for the experimental females. (A) On the day of birth, PND 0, litters were reduced to four males and four females and received Silastic capsules of cholesterol (C, n=16) or estradiol benzoate [EB either 5% (n=14) or 10% (n=16)]. (B) Maternal licking and grooming behavior was observed on PND 1, 2, 4, and 6. (C) At weaning on PND 21, the Silastic capsules were removed. (D) After the females reached 60 days of age, they were ovariectomized and given Silastic capsules of either 12.5% or 25% EB. The ovaries were collected at this time. Females were tested for partner preference, female sexual behavior, and male-like sexual behavior. After testing was concluded, the pelvic ganglia (PG) and spinal nucleus of the bulbocavernosus (SNB) were removed.

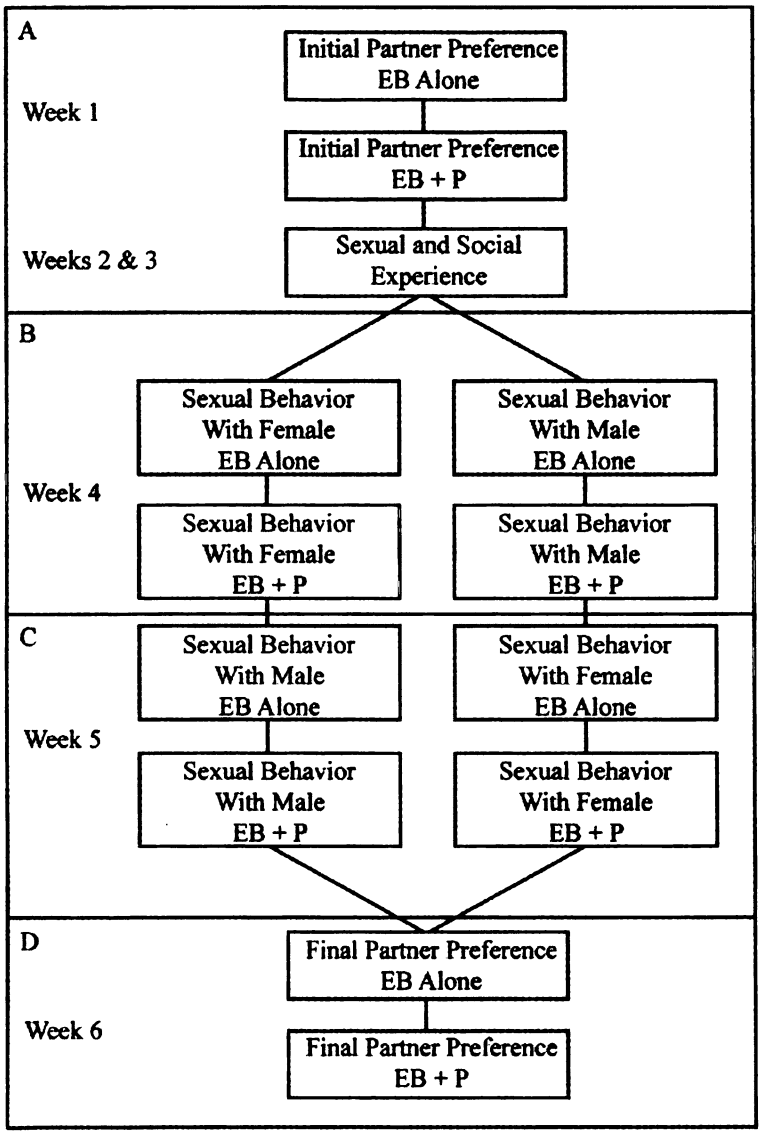

Figure 2.2. Behavioral testing schedule for the experimental females receiving early postnatal treatments of cholesterol (C, n=16) or estradiol benzoate [EB either 5% (n=14) or 10% (n=16)]. Data were not collected during weeks 2 and 3 during which the animals received sexual and social experience. EB and P represent adult hormone treatments prior to each behavioral test. Note: Although not shown here, there were two doses (12.5% and 25%) of adult EB treatment (see text for more details).

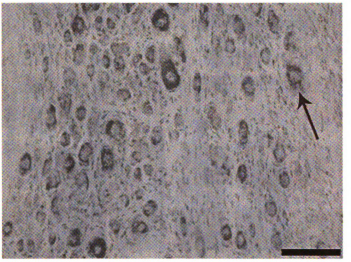

Figure 2.3. Photomicrograph of pelvic ganglion tissue processed for Nissl staining. Neurons were identified as darkly staining, nucleated cells (arrow). Scale bar: 100 μm.

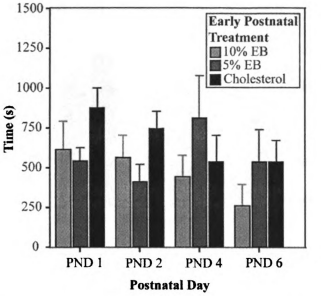

Figure 2.4. Time (in seconds) mothers of experimental females spent licking and grooming during the first postnatal week. No significant differences were seen among treatment groups or days, with no significant interaction.

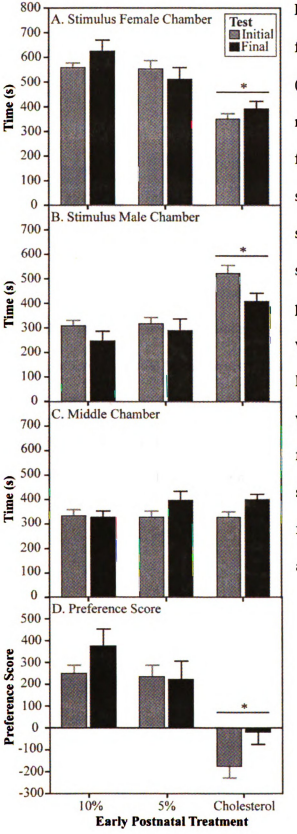

Figure 2.5. Duration of time experimental females spent (A) with the stimulus female, (B) with the stimulus male, and (C) in the middle chamber during both the initial and final partner preference tests. Preference score (D) is calculated as time spent with stimulus female minus the time spent with stimulus male. Therefore, a positive preference score indicates more time spent with the stimulus female. C females spend less time with the female and more time with the male compared with 10% and 5% females. Also, the 10% and 5% females show a clear preference for the stimulus female. *Significantly different from 10% and 5% females, $p<0.001$.

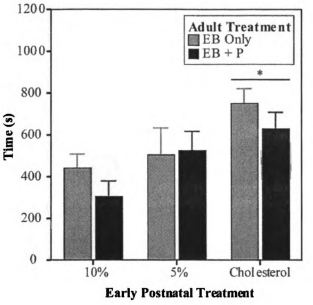

Figure 2.6. Time experimental females spent in the male area of the observation chamber during the female sexual behavior tests. Data for tests after adult treatment with EB alone and EB + P are shown. Regardless of progesterone treatment, females treated with 10% EB spent significantly less time with the male than did females treated with cholesterol. *Significantly different from 10% females, p=0.02.

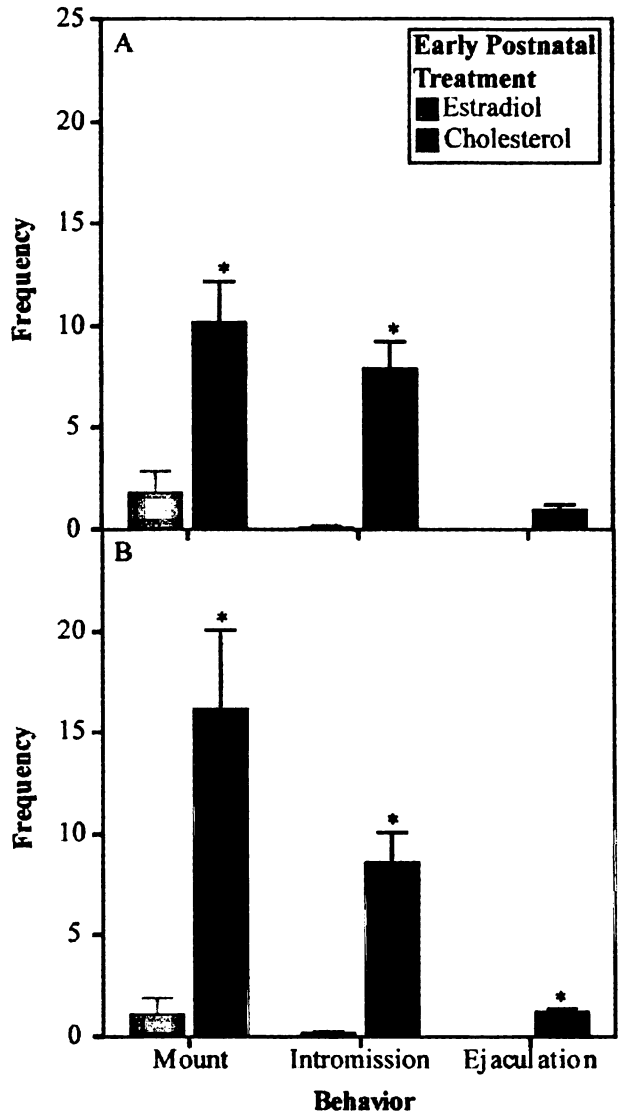

Figure 2.7. Frequencies of mounts, intromissions, and ejaculations displayed by the stimulus male during the female sexual behavior tests. Males showed fewer sexual behaviors when paired with the EB females compared to the C females. (A) Test after adult treatment with EB alone. (B) Test after treatment with EB + P. * Significantly different from EB females, $p<0.001$.

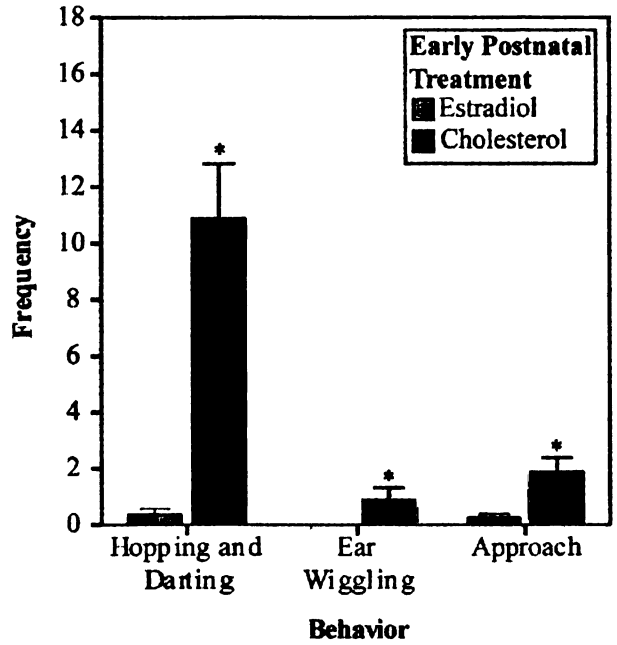

Figure 2.8. Frequency of proceptive behaviors shown by experimental females during the female sexual behavior test after adult treatment with EB + P. EB females exhibited fewer proceptive behaviors than did C females. *Significantly different from EB females, $p<0.01$.

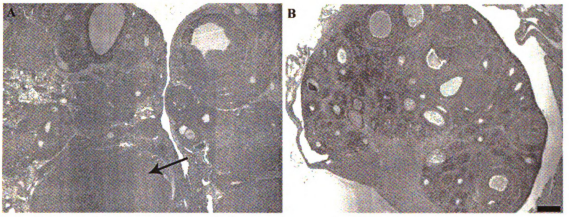

Figure 2.9 Photomicrograph of cross-sections through ovarian tissue processed for hematoxylin and eosin staining. (A) Corpora lutea were present (arrow) in the ovaries of C females, indicating a normal estrous cycle. (B) Ovaries of EB females showed no corpora lutea. Scale bar: 200 μm.

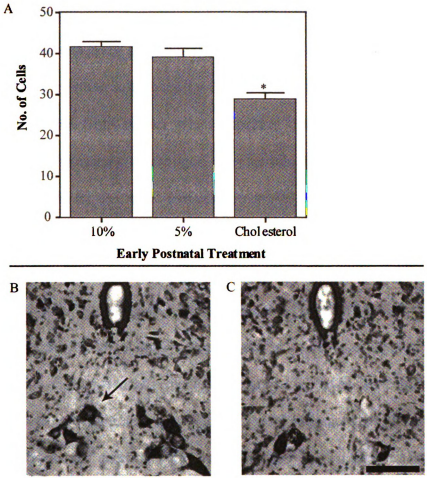

Figure 2.10. Number of motoneurons present in the SNB of experimental females. (A) 5% and 10% females showed more neurons than did C females. (B,C) Photomicrographs of spinal cord section processed for Nissl staining at the level of the SNB. EB females (B) had more motoneurons (arrow) than did C females (C). Scale bar: 100 μm. *Significantly different from 5% and 10% females, $p<0.001$.

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DISCUSSION

The overreaching hypothesis of this study was that adult partner preference in the laboratory rat is influenced by the presence of estradiol during early development. This idea stems from studies where blocking estrogen action during development, either through aromatase or estrogen receptor inhibition, results in a decrease in a male rat's preference for an estrous female compared to control males.

Effects of Early Postnatal Treatments

Behavior: The principal finding is that increased postnatal EB exposure during development masculinizes female partner preference. As indicated by the preference scores, female rats that receive exogenous EB during development spent more time with an estrous female and less time with a sexually active male than did C females. These differences in duration were not a result of treatment differences in time spent alone, but were due to differences in the time spent with specific stimulus animals. Also, the 5% and 10% females showed positive preference scores significantly different from chance indicating a preference for the female stimulus animals. This preference was a result of spending more time in the female chamber than would be expected by chance. The early postnatal treatments did not alter the amount of maternal licking and grooming received by the litters; therefore, it is unlikely that the early postnatal treatment group differences were mediated by changes in maternal care. The effect of early EB exposure on partner preference was very robust and evident across different adult hormonal conditions and amounts of sexual experience.

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These findings are consistent with the results of other studies that indirectly tested the effect of estrogens on partner preference during development. Male rats treated neonatally with the aromatase inhibitor ATD either do not show a preference for an estrous female over a sexually active male or show a preference for the male stimulus (Bakker et al., 1993a; Bakker et al., 1993b). Males neonatally castrated and treated with 5α-dihydrotestosterone propionate (DHTP), a non-aromatizable androgen, exhibit a lower preference for a stimulus female than do males that are treated with testosterone propionate (TP) after neonatal castration (Brand and Slob, 1991a). Also, compared to controls, male rats treated prenatally with an antiestrogen, nitromifene citrate (CI628), show decreased preference for a female (Matuszczyk and Larsson, 1995). In addition, when male mice lacking a functional estrogen receptor α (ERα-KO) are castrated and treated with testosterone in adulthood, they spend less time with an estrous female than do wild-type males (Rissman et al., 1997). Further, ERα-KO males do not prefer an estrous female over an unreceptive female (Wersinger et al., 1997), and they approach the odors of an estrous female less than do wild type males (Wersinger and Rissman, 2000). Studies have also been conducted with mice in which the aromatase gene, Cyp-19, has been knocked out (ArKO). In these studies, ArKO males show no preference for female odors over male odors and do not show a preference for a female over an empty chamber (Bakker et al., 2002). Further, recent work from our laboratory also shows that female rats that receive postnatal exposure to the polychlorinated biphenyl congener 3, 4, 3', 4'-tetrachlorobiphenyl (PCB 77), which has been shown to have estrogenic effects (Jansen et al., 1993; Nesaretnam et al., 1996; Seegal et al., 2005), exhibit a lower preference for a sexually active stimulus male than do controls.

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Additional support for the idea that early estrogen exposure mediates a preference for females can be found in the human literature. Diethylstilbestrol (DES) is a nonsteroidal synthetic estrogen used through 1970 to decrease the occurrence of miscarriages. Because it was found to increase the risk of cervical cancer in female offspring (Herbst et al., 1971), its clinical use was discontinued. Follow-up studies suggest that DES may have affected sexual orientation of female offspring (but see Titus-Ernstoff et al. (2003) for a report of negative results). When interviewed regarding their sexual orientation, DES-exposed women are found to have a higher incidence of bisexuality or homosexuality than non-exposed women (Ehrhardt et al., 1985; Meyer-Bahlburg et al., 1995).

In the present study, early postnatal EB treatment also affected the sexual behavior of the female rats. The 10% EB dose reduced the time the female spent with the male during the sexual behavior test, and none of the females that received postnatal EB showed lordosis in response to male mounts. This effect on female receptivity is in agreement with other studies showing that neonatal EB treatment decreases the frequency to show lordosis in females and neonatally castrated males (Levine and Mullins, 1964; Whalen and Edwards, 1967; Whalen and Nadler, 1963). Also, perinatal treatment with ATD enhances the tendency of male rats to show lordosis after castration and treatment with ovarian hormones in adulthood (Clemens and Gladue, 1978; McEwen et al., 1977; Whalen et al., 1986), and perinatal treatment with antiestrogen, but not antiandrogen, increases the frequency of lordosis in male rats (Matuszczyk and Larsson, 1995).

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In both the partner preference tests and the female sexual behavior tests, the EB females were less likely than C females to receive mounts, intromissions, or ejaculations from the stimulus male. The male's reduced interest for the EB females could reflect multiple factors, which are not mutually exclusive. The EB females could be less proceptive, they could be less attractive to the males, or they could be actively avoiding the males, any of which would lead to decreased copulatory behavior by the stimulus male. Proceptive behaviors were measured during the female sexual behavior test after the experimental females had received adult EB + P treatment. EB females show significantly fewer instances of ear wiggling, hopping and darting, and approaches to the stimulus male compared to C females. Attractivity was not measured in this study but could also be an explanation for the low frequency of male behaviors directed toward the EB females. Stimulus males may find the EB females less attractive than C females resulting in the male showing less sexual behavior. Further support for this idea comes from the fact that the differential behavior of the male was also seen during tests when only adult EB was given, which is a hormonal condition associated with very little proceptivity even for control females (Frye et al., 1998). Finally, avoidance behaviors were not measured, but the lack of sexual stimulation by the male may be due to the EB females actively avoiding contact with the males.

The argument could be made that since the EB females are not receiving sexual behavior in the partner preference tests, the validity of the preference test is questionable. However, Adkins-Regan (1988) made the distinction that partner preference and sexual behavior are not necessarily linked. This outcome was also observed in other work from

this laboratory where perinatal exposure to PCBs was found to alter partner preference without affecting sexual receptivity (Cummings, personal communication). Therefore, we abide by the common convention (Baum, 2006) that partner preference may be studied independently of sexual receptivity and behavior.

There were no statistically significant differences between EB females and C females in the display of male sexual behavior. The two treatments did not differ in the frequency of mounts or intromission patterns shown when placed with an estrous stimulus female. Although the expression of ejaculation patterns did not reach statistical significance, it is interesting that a total of six females treated during the early postnatal period with EB showed at least one ejaculation pattern during either the partner preference or male-like sexual behavior test. Four 10% females showed ejaculatory patterns in both tests, while two 5% females showed ejaculation patterns in one of the tests. None of the C females showed ejaculation patterns in any of the tests. Despite not reaching statistical significance, this effect agrees with previous findings that show estrogen may play a role in the expression of ejaculatory patterns. Brand et al. (1991) found that compared with control males, fewer neonatally ATD-treated males ejaculate when paired with an estrous stimulus female. A similar decrease in male sexual behavior is also seen after neonatal treatment with an antiestrogen (Matuszczyk and Larsson, 1995).

Histology: The experimental females treated with early postnatal EB were shown to be anovulatory prior to ovariectomy and treatment with adult hormones. The early postnatally treated females showed no corpora lutea present in the ovaries compared with

the majority of C females showing corpora lutea, and therefore, regular estrus cycles. This effect of estradiol on ovarian function is in agreement with other studies that demonstrate that neonatal EB treatment is sufficient to completely prevent the formation of corpora lutea in the adult ovaries (Harris and Levine, 1965; Levine and Mullins, 1964).

The SNB is a sexually dimorphic cluster of motoneurons located in the dorsomedial aspect of the ventral horn of the fifth and sixth segments of the lower lumbar spinal cord in the rat. The SNB motoneurons innervate four striated perineal muscles: the sexually dimorphic levator ani (LA) and paired bulbocavernosus (BC) muscles (Breedlove and Arnold, 1980) and the sexually monomorphic external anal sphincter (EAS) (Sengelaub and Arnold, 1986). The BC/LA complex is present in adult males and functions in penile erections and ejaculations. The BC/LA muscles, however, are completely absent or vestigial in adult females (Breedlove and Arnold, 1980; Cihak et al., 1970).

In the present study, early postnatal estradiol treatment increased the number of motoneurons seen in the SNB of the experimental females. This finding conflicts with the current literature on SNB development. The SNB system has previously been shown to be sensitive to steroid hormones. For example, the motoneurons accumulate radioactivity after injection with tritiated testosterone and the non-aromatizable androgen, dihydrotestosterone, but not estradiol (Breedlove and Arnold, 1980). Persistence of the neuromuscular system postnatally is androgen dependent. Exogenous perinatal androgen treatment can affect the development of the SNB system in the female. If females are treated with testosterone propionate both pre- and postnatally, they display the same

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developmental patterns as male pups (Nordeen et al., 1985). Perinatal androgen treatment of females results in an increase in the number of SNB motoneurons, the cell soma size, and the number of target muscle fibers (Breedlove and Arnold, 1983). Treatment with estradiol benzoate does not masculinize the SNB in female pups suggesting that androgens, and not estrogens, control the development of the SNB system (Breedlove et al., 1982).

The reason for the discrepancy may lie in differences in the treatment paradigm. Breedlove et al (1982) limited neonatal estradiol treatment to one injection on PND 1. In the present study, EB was received constantly through the Silastic capsule for 21 days. The increase in dose and length of treatment may have different effects on the SNB system. The current hypothesis regarding SNB development is that androgens act at the BC/LA muscles preventing them from involution, thereby rescuing the SNB motoneurons indirectly. Also, it has been shown that neonatally treating either castratred males (McAbee and Doncarlos, 1999) or females (Suzuki and Nishihara, 2002) with estrogens increases the expression of androgen receptor (AR) mRNA in the hypothalamus. Since measurable levels of testosterone are present in the plasma of female rats postnatally (Weisz and Ward, 1980), perhaps the early EB treatment is increasing the number of AR present in the BC/LA muscles, thereby allowing the testosterone present to act on the increased receptors, saving the SNB motoneurons from cell death. The increase in motoneuron number seen, however, may be playing a role in the expression of male-like ejaculatory patterns shown by the females treated with the early postnatal estradiol. The early postnatal treatments did not alter the amount of

maternal licking and grooming received by the litters; therefore, these data suggest a direct effect of EB on motorneuron number rather than an increase seen due to maternal mediation.

The PG is a sexually dimorphic structure that in the male rat contains 2 to 4 times more cells than in the female (Greenwood et al., 1985). Gonadal steroids have been shown to have an effect on the number of cells present in the PG. Preliminary work in our laboratory has shown that neonatal treatment of female pups with one injection of testosterone propionate (50 μg) on PND 3 resulted in a significant increase in the number of adult PG cells (Fang and Clemens, unpublished results). Another study looked at the number of cells in Tfm males. The number of cells in the PG of Tfm males was significantly less than their wild-type brothers and did not differ from normal females (Fang, Jordan, Breedlove, and Clemens, unpublished results). These studies indicate the importance of androgens in the development of the male PG. However, it is not clear what mechanism is responsible for these androgenic effects.

The results from the present study seem to indicate that the development of the PG does not rely on the estrogenic metabolite of testosterone. Females treated with either the 5% or 10% dose of early postnatal EB showed the same number of neurons present in the PG as did the C females.

Effects of Experience and Adult Treatments

Although the alteration of adult female partner preference and sexual behavior by early postnatal EB treatment did not change across adult hormone or testing conditions, some effects of adult treatment and experience were evident. The most salient of these effects was that all experimental females, regardless of hormonal condition, decreased their latency to enter the male chamber and increased the time spent in the middle chamber from the initial to the final partner preference test. This effect may be due to the sexual and social experience the females received during the sexual behavior tests since sexual experience can play a role in the display of partner preference. Some studies show that hormonally primed females display a preference for the male stimulus both before and after sexual experience (Dejonge et al., 1986; Slob et al., 1987). Others find a change in preference between naïve tests and experienced tests, such that the females show no preference initially but after sexual experience they prefer the male (Matuszczyk and Larsson, 1991). These different outcomes may be due to the different methods used by each study. Some experiments use short term treatment (one injection) of EB (Dejonge et al., 1986), while others use long term (Silastic capsules or repeated injections; present study, Matuszczyk and Larsson, 1991; Slob et al., 1987). Also, the partner preference tests differ in terms of whether the experimental female is allowed to interact with the stimulus animals.

Also, compared to treatment with EB alone, adult EB + P treatment decreased the amount of time the experimental females spent within the stimulus animals' chambers during the partner preference tests, while increasing the time spent in the middle chamber. In

addition, EB + P treatment decreased the amount of time the experimental females spent in the male chamber during the female sexual behavior test compared to treatment with EB alone. Although these effects were seen across all the early postnatal treatment groups, the mechanism behind these effects may be different for C versus EB females, since sexual behavior is facilitated by progesterone only in the case of the C females. Thus, in the C females the addition of progesterone treatment increases sexual receptivity and the likelihood of a female-paced copulatory bout (Dejonge et al., 1986; Frye et al., 1998). During a female-paced sexual behavior test, a receptive female would approach the stimulus male, be mounted, and then leave the male chamber and enter the escape chamber leading to a decrease in the overall time spent with the male. Similarly, in a partner preference test, a receptive female would enter the male chamber, be mounted, and then move into the middle chamber, thereby decreasing the time spent in the stimulus chambers and increasing the duration in the middle chamber. Therefore, the differences seen for both tests of sexual behavior and partner preferences between the EB and the EB + P conditions can be explained for the C females as a consequence of the enhanced receptivity induced by the addition of progesterone in this group.

While enhanced receptivity and the pacing of copulation may account for the increase in time the C females spent in the escape chambers when given progesterone in addition to EB, a similar explanation cannot be made for the females that received early postnatal EB, since they did not receive any intromissions or ejaculations, and evidently were not pacing copulatory bouts. Failure of the postnatally estrogenized females to show sexual receptivity following progesterone treatment is consistent with earlier reports showing

that perinatal steroid treatments interfere with progesterone responsiveness. A major effect of perinatal treatment with aromatizable androgens is to eliminate or severely reduce progesterone enhancement of sexual receptivity (Clemens, 1970; Davidson and Levine, 1969). But the reduction in the amount of time the EB females in the current study spent with the stimulus animals following adult EB + P treatment may reflect other actions of progesterone different from the facilitation of female receptivity. For example, progesterone has been shown to have anesthetic or tranquilizing effects (Bixo and Backstrom, 1990; Merryman et al., 1954; Selye, 1941) that use a progesterone receptor (PR)-independent mechanism of action (Reddy and Apanites, 2005). Such effects might easily account for the female's tendency to stay in the middle chamber in the partner preference test and the escape chamber in the sexual behavior test in the absence of progesterone-induced sexual receptivity.

The effect of adult progesterone treatment on the amount of time spent in the male chamber showed an interaction with sexual experience. Sexually naïve experimental females treated in adulthood with EB + P spent less time in the male chamber than did females treated with EB alone. However, this effect of progesterone treatment was absent on the final test, after the females had received sexual experience.

In the control females, the decrease in time spent in the male chamber from initial to final test shown by females treated with adult EB alone may be due to dopamine action on the PR. Adult EB treatment has been shown to increase the number of PR in hypothalamic areas important for the expression of female sexual behavior (Maclusky and McEwen,

1978; Maclusky and McEwen, 1980). It has also been shown that PR can be activated by hormonally-independent mechanisms. Dopamine agonists have been shown to mimic the facilitating effect of progesterone on female sexual behavior, and these effects are blocked by treating with progesterone receptor antagonists or antisense oligonucleotides to PR mRNA (Mani et al., 1994). Sexual experience is known to increase dopamine levels in the brain of female rats (Jenkins and Becker, 2003; Mermelstein and Becker, 1995; Pfaus et al., 1995). It is possible that the progesterone treatment of control females during the initial test increases the female's sexual motivation, causing her to pace the copulatory bout, and by doing so decrease the time spent in the male chamber compared to females that received EB alone. However, when the control females are returned to the preference chamber for the final test after sexual experience, the females treated with adult EB alone may have an increase in dopamine levels because previous interactions with a male have been rewarding for her. This increase in dopamine could act on PR, mimicking the effects of progesterone treatment and leading to pacing behavior similar to that of the EB + P-treated females, thus resulting in a decrease in the time spent in the male chamber.

The explanation provided above for the differential effects of progesterone during the initial and final partner preference tests does not generalize to the females that received early postnatal EB. In the early postnatally treated females, progesterone does not facilitate sexual receptivity, and hence their sexual experience during the sexual behavior tests are unlikely to be rewarding and in fact may have been aversive. Consequently, this

experience may have caused the early postnatally estrogenized females treated with adult EB to avoid the stimulus male chamber during the final partner preference test.

During the initial partner preference test, females treated with adult EB alone took longer to enter the female stimulus chamber than did females treated with EB + P. However, by the last test the two adult hormone groups were similar on this measure. The progesterone treatment may be decreasing anxiety behaviors in the females (Frye and Walf, 2004), but this effect may be salient only during the initial test. In the group treated with adult E alone, the social and sexual experience the experimental females received during the sexual behavior tests may have caused a reduction in fear of the novel stimulus animals in the partner preference chamber. Therefore, during the final test, experienced females showed less fear, and thus shorter latencies to enter the female chamber, even without progesterone treatment.

Overall

The most robust finding of this study was that postnatal EB treatment alters female partner preference and sexual behavior. Estradiol seems to have a masculinizing and defeminizing effect. Females treated with postnatal EB preferred to spend more time with an estrous female and less time with a sexually active male than did C females. Also, EB females showed less female sexual behavior, receptivity, proceptivity, and possibly attractivity to the males, while occasionally showing ejaculatory patterns when placed with an estrous female. These findings lead to the suggestion that estrogens during the

postnatal critical period plays a role in the development of male partner preference and sexual behavior.

CHAPTER THREE:

HYPER-ANDROGEN EXPOSURE DURING DEVELOPMENT ALTERS ADULT PARTNER PREFERENCE AND REPRODUCTIVE BEHAVIOR IN GONADALLY INTACT MALE RATS

INTRODUCTION

As previously described, exposure to testosterone during development organizes both the brain and behavior of male rats (Baum, 1979). However, there are also studies showing that high levels of exogenous testosterone administered to an intact male early in life may alter adult behavior (Pollak and Sachs, 1975). In the rat, neonatal administration of testosterone propionate to a castrated male causes both defeminization and masculinization of behavior (Beach et al., 1969). However, if an intact male is treated neonatally with testosterone, there is a disruption of normal male sexual behavior (Pollak and Sachs, 1975). There is a chance the hyper-androgen treatment is altering the male's partner preference, thereby decreasing the male's interest in a female partner. Therefore, the intact male rats that receive high androgen exposure may be exhibiting a suppression of male sexual behavior as a result of an altered partner preference.

Prenatal hyper-androgen exposure may also play a role in human sexual orientation. Homosexual men have been shown to have larger genitalia (Bogaert and Hershberger, 1999), more masculine auditory evoked potentials (McFadden and Champlin, 2000), and more masculine 2D:4D digit length ratios (Rahman, 2005; Rahman and Wilson, 2003;

Robinson and Manning, 2000; reviewed in Chapter One) than heterosexual men indicating a higher level of androgen during development.

In the present study, the effect of exogenous testosterone on adult behavior was tested by treating intact male rats during either the early postnatal period or prenatal period with testosterone propionate and examining their adult partner preference. In addition, the effects of early postnatal exposure to testosterone on serum testosterone and estradiol levels and cell number in the spinal nucleus of the bulbocavernosus and pelvic ganglia were also measured.

METHODS

Animals

Experimental Males (Experiments 1-3): Time-mated pregnant Long-Evans rats (Charles River, Raleigh, NC) were housed individually with *ad lib* food and water in plastic cages (45.5 x 24 x 21 cm) in a 14:10-hr light dark cycle with lights on at 01:00. For nest building material thirty, one-inch paper towel strips were given to the dams on GD 20. A subset of the male offspring of these dams became the experimental males of this study (see below). In Experiments 1 and 2, on the day of birth, PND 0, the litter was reduced to four male and four female pups (Figure 3.1A). For litter reductions, the AGD for each pup was measured, and since the AGD is shorter in females than in males, the four shortest and the four longest were retained. In Experiment 3, since prenatal testosterone increases AGD of both sexes, the seven animals with the largest AGD were kept to

maximize the chance of picking a male and the animal with the smallest AGD was kept in an attempt to prevent forming an all-male litter (Figure 3.2B).

Experimental Male Hormone Treatments (Experiments 1,2): Silastic capsules (Dow Corning; inner diameter 1.47 mm; outer diameter 1.96 mm; length 5 mm) were used to administer either testosterone propionate (Sigma; TP) or cholesterol (Sigma; C) treatments on the day of birth (Figure 3.1A). Capsules were implanted subcutaneously (s.c.; one treatment per litter) through a small incision on the back of the animal while the pups were under ice anesthesia on PND 0. These animals are referred to as TP or C males, respectively. The incision was closed using superglue (Loctite). Following surgery, pups were warmed under a heat lamp until the incision was dry (approximately one hour) and then returned to their mothers. Capsules were left in place for 3 weeks. On PND 21, at weaning, the pups were anesthetized with isoflurane (Isoflo, Abbot Laboratories), and the implants were removed through an incision made near one end of the capsule (Figure 3.1B). The incision was closed with an Auto Clip (Clay Adams) and covered with First Aid Cream (Johnson and Johnson). Pups were then housed with same-sex littermates. Only male pups were used in this Experiment. Behavioral tests began after the animals reached 90 days of age. (Experiment 1: TP n=18, C n=14; Experiment 2: TP n=19, C n=20; Figure 3.1C).

Experimental Male Hormone Treatments (Experiment 3): Pregnant dams were treated with either TP in sesame oil (2 mg/0.1 ml/day) or oil vehicle on GD 16-20 (Figure 3.2A). Pups were weaned on PND 21 (Figure 3.2C) and housed with same-sex littermates until

90 days of age when behavioral tests were conducted (Figure 3.2D). These males are referred to as TP (n=9) and Oil (n=13) males, respectively. Only male pups were used in this Experiment. Behavioral tests began after the animals reached 90 days of age.

Stimulus Females (Experiments 1-3): Sexually experienced, gonadally intact, adult Long Evans female rats at least 60 days old (Charles River, Raleigh, NC) were used as stimulus animals for the behavioral tests. Stimulus females were implanted with a Silastic capsule containing 25% EB-C mixture prior to testing but were not ovariectomized. The capsules were implanted s.c. while the animals were anesthetized with isoflurane; the incision was closed with an Auto Clip and covered with First Aid Cream. After 4 weeks, the capsules begin to lose efficacy because of connective tissue growth around the capsule (personal observation). The capsules were removed and reimplanted via a new incision in the neck. Stimulus females were injected s.c. with 0.5 mg progesterone four hours prior to partner preference and sexual behavior testing.

Stimulus Males (Experiments 1,3): Sexually experienced, gonadally intact, adult Long Evans male rats at least 90 days old (Charles River, Raleigh, NC) were used as stimulus animals for the behavioral tests.

Stimulus Males (Experiment 2): The stimulus males in Experiment 2 received ATD (Sigma) postnatally. Male neonates were treated with Silastic capsules containing ATD on PND 0 through 21. Procedures were the same as those used for the early postnatal TP and C treatments. This treatment was used because Experiment 1 attempted to determine

if the experimental males would show sexual behavior with another male. However, during the social and sexual behavior tests the experimental and stimulus males often fought. It was also found that during the final partner preference test of Experiment 1, both the TP and the C males spent more time alone in the middle chamber than would be expected by chance (data not shown). The social and sexual tests with the stimulus male may have been aversive to the experimental males, causing them to remain in the middle chamber. In an attempt to decrease the aggression shown between the experimental and stimulus males, ATD was used. Males treated postnatally with ATD have been shown to display lordosis when paired with another male (Bakker et al., 1996).

Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experimental procedures were approved by the Michigan State University Animal Care and Use Committee.

Behavioral Testing (Experiments 1-3)

Partner Preference: Tests for partner preference were conducted as described in Chapter Two.

Social and sexual behavior: Tests for social and sexual behavior displayed by the experimental males were conducted in a Plexiglas observation chamber (46 x 58 x 51 cm). During the test, the experimental male had unrestricted access to the stimulus animal. The tests lasted 25 minutes or until ejaculation, whichever occurred first. Behavioral testing took place under dim red light illumination in the middle part of the

dark phase of the light-dark cycle. Video recordings of these tests were analyzed to determine frequency of mounts, intromissions, and ejaculatory patterns shown by the experimental males and the latency to show these behaviors.

Testing Schedule: The six-week testing schedule is summarized in Figure 3.3. The initial partner preference of the male was tested in week 1. Each experimental male received social and sexual experience with both male and female stimulus animals during weeks 2 and 3, but data were not collected. During experience weeks, experimental males were partnered with stimulus animals for 30 minutes during which time sexual behavior could occur. During week 4, half of the experimental males were tested for social and sexual behavior with a male and the other half with a female. The sex of the stimulus animals was switched for week 5. Sexual behavior during weeks 4 and 5 was recorded and scored. In week 6, the male's final partner preference was evaluated.

Histology (Experiment 1)

Pelvic Ganglia and Spinal Nucleus of the Bulbocavernosus: Tissue removal and histological processing were conducted as described in Chapter Two. A subset of males from Experiment 1 were analyzed for cell counts (Figure 3.1C).

Hormone Analysis (Experiments 1,2)

Prior to perfusion, blood was collected intracardially into heparinized tubes (Figure 3.1C). The samples were kept on ice for 30 minutes, and then centrifuged at 8° C for 20 minutes at 3000 rcf. The supernatant was removed and stored at -20° C.

A solid-phase radioimmunoassay that uses ^{125}iodine-labeled steroid, which competes with steroid in the sample for antibody sites in a polypropylene tube, was used to determine the amount of hormones in the samples. Testosterone and estradiol were measured using the Coat-A-Count Testosterone (Siemens) and Active Estradiol (Diagnostic Systems Laboratories, Inc.) radioimmunoassay kits, respectively. After incubation for 3 hours (testosterone) or 2 hours (estradiol) at 37° C, the tube was decanted and counted in a gamma counter. The quantity of hormone present in the sample was determined from a calibration curve. Radioimmunoassays were completed by the Diagnostic Center for Population and Animal Health at Michigan State University. A subset of males from both Experiment 1 and Experiment 2 were used for the radioimmunoassays.

Analysis

The data for the behavioral measures during the partner preference tests were analyzed using a 2 x 2 (perinatal treatment x initial or final test) ANOVA with repeated measures on the second factor. Preference scores and duration of time spent in the male chamber during the partner preference tests were also analyzed within each early postnatal treatment group using a one-sample t-test. This test was used to determine if the animals showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the male above chance (duration = 400 seconds). The data for the behavioral measures during the social and sexual behavior tests, histological cell counts, and hormones assays were analyzed using an independent samples t-test.

For some variables, the data did not meet homogeneity of variance assumptions, even after the prescribed transformations (i.e. square root). For these measures, nonparametric statistics were used for analysis. The Mann-Whitney U and the Fisher's exact probability tests were used for these analyses.

RESULTS

Experiment 1 – Early Postnatal TP

Partner Preference: Early postnatal treatment with testosterone altered the partner preference of the male experimental animals. The TP males spent more time in the stimulus male chamber during both partner preference tests than did the C males (Figure 3.4; [$F(1,27) = 18.0$, $p<0.001$]. Also, the C males showed a higher preference score than that of the TP males (Figure 3.5; [$F(1,27) = 10.9$, $p=0.003$]).

In both the initial and final partner preference test, the C males had positive preference scores significantly different from chance, indicating a preference for the female stimulus animal. The TP males, however, did not show a preference greater than chance for either stimulus animal (Table 3.1). Also, the duration of time spent in the male chamber in both the initial and final tests was significantly less than chance for the C males, but not for the TP males (Table 3.1).

The latency to enter the stimulus female chamber [$F(1,27) = 12.7$, $p=0.001$] and time spent with the male [$F(1,27) = 4.2$, $p=0.05$] were affected by a main effect of week of test. Regardless of early postnatal hormone treatment, the males decreased their latency

to enter the female chamber from initial to final test ($\bar{X}$ ± SEM: all measures in seconds: 39.3 ± 4.2 vs. 23.2 ± 2.8). They also decreased the amount of time spent in the stimulus male chamber from initial to final test ($\bar{X}$ ± SEM: 335.2 ± 22.7 vs. 252.0 ± 35.1).

Non-parametric statistics were necessary to analyze the amount of time spent in the middle chamber. TP males spent less time alone in the middle chamber during the initial partner preference test than during the final test ($\bar{X}$ ± SEM: 366.8 ± 41.8 vs. 472.6 ± 47.6).

The proportion of males that showed sexual behavior with the stimulus female during the partner preference test was analyzed using the Fisher's exact probability test. More C males showed mounts and intromissions during the initial test, and more mounts, intromissions, and ejaculations during the final test compared with the TP males (Table 3.2). No differences were seen in behaviors shown with the stimulus male.

Social and Sexual Behavior: Early postnatal testosterone treatment altered the expression of male sexual behavior when paired with a stimulus female. TP males showed fewer mounts, intromissions, and ejaculations during the test than did C males (Figure 3.6; Mann-Whitney). Also, the proportion of males that showed behaviors when paired with a female was higher in the C male group than in the TP male group (Table 3.3). No significant differences were seen in behaviors shown when paired with the stimulus male.

Hormone Assays: The amount of circulating testosterone was affected by the early postnatal testosterone treatment during early life (t = -2.7, p=0.012). The C males had significantly higher circulating testosterone levels than did the TP males (Figure 3.7). To rule out the possibility that changes in behavior were seen due to different levels of circulating testosterone, a correlation was run between serum testosterone levels and preference score. No correlation between the two measures was seen. Circulating levels of estradiol were not affected by the early hormone treatments.

Histology: Early postnatal testosterone did not have an effect on cell number in either the PG ($\bar{X}$ ± SEM: TP: 6261.7 ± 432.2; C: 5983.4 ± 635.5; p>0.05) or the SNB ($\bar{X}$ ± SEM: TP: 75.2 ± 3.3; C: 86.0 ± 4.4; p>0.05).

Experiment 2 – Early Postnatal TP and ATD Stimulus Male

Partner Preference: Treatment with testosterone early in development altered the partner preference of the male experimental animals. The TP males spent more time in the ATD stimulus male chamber during the partner preferences tests than did the C males (Figure 3.4; [F(1,31) = 4.1, p=0.05]). The data for time spent in the male chamber were subjected to a square root transformation.

In both the initial and final partner preference test, the C males had positive preference scores statistically different than chance, indicating a preference for the female stimulus animal. The TP males, however, did not show a preference greater than chance for either stimulus animal (Table 3.1). Also, the duration of time spent in the ATD male chamber in

the final test was significantly less than chance for the C males, but not for the TP males (Table 3.1).

Social and sexual behavior also altered the partner preference of the experimental males regardless of hormone treatment. Across both hormone treatments, the average latency to enter the female chamber was significantly shorter for the final test compared to the initial one ($\bar{X}$ ± SEM: 20.1 ± 2.0 vs 55.5 ± 6.4; [$F(1,31) = 38.2$, $p<0.001$]). The latency to enter the ATD male chamber was also shorter for the final test than for the initial test ($\bar{X}$ ± SEM: 29.3 ± 3.4 vs 76.4 ± 18.6; [$F(1,31) = 5.8$, $p=0.02$]). The experimental males spent less time in the middle chamber during the initial partner preference test than during the final test ($\bar{X}$ ± SEM: 311.1 ± 21.3 vs 382.1 ± 24.9; [$F(1,31) = 5.6$, $p=0.02$]), and they spent more time in the ATD male chamber during the initial test compared with the final test ($\bar{X}$ ± SEM: 345.6 ± 22.2 vs 245.8 ± 29.0; [$F(1,31) = 14.7$, $p=0.001$]). Finally, the males had a lower preference score during the initial test than during the final test ($\bar{X}$ ± SEM: 197.6 ± 50.0 vs 323.9 ± 64.3; [$F(1,31) = 6.4$, $p=0.01$]).

The proportion of males that showed sexual behavior with the stimulus female during the partner preference test was analyzed using the Fisher's exact probability test. More C males showed mounts, intromissions, and ejaculations during the final test compared with the TP males (Table 3.2). No differences were seen in behaviors shown with the stimulus male.

Social and Sexual Behavior: Early postnatal testosterone treatment altered the expression of male sexual behavior when paired with a stimulus female. TP males showed fewer mounts, intromissions, and ejaculations during the test than did C males (Figure 3.6; Mann-Whitney). Also, the proportion of males that showed behaviors when paired with a female was higher in the C male group than in the TP male group (Table 3.3). No significant differences were seen behaviors shown when paired with the stimulus male.

Experiment 3 – Prenatal TP

Partner Preference: In initial and final partner preference tests, both the C males and the prenatal TP males had positive preference scores statistically different from chance, indicating a preference for the female stimulus animal (Table 3.4). Also, the duration of time spent in the male chamber in the initial and final tests for the C males, and the final test for the TP males was significantly less than chance (Table 3.4).

Social and sexual experience altered the partner preference of the experimental males regardless of hormone treatment. The latency to enter the female chamber was longer during the initial partner preference test compared to the final ($\bar{X}$ ± SEM: 51.0 ± 11.4 vs. 14.4 ± 3.1; [$F(1,12) = 24.9$, $p<0.001$]). The data for latency to enter the female chamber was subjected to a square root transformation. The duration of time spent in the female chamber was higher during the final test than during the initial ($\bar{X}$ ± SEM: 813.6 ± 51.5 vs. 655.7 ± 40.8; [$F(1,12) = 8.0$, $p=0.015$]). The duration of time spent in the male chamber also decreased from initial to final test ($\bar{X}$ ± SEM: 316.8 ± 24.8 vs. 100.7 ± 23.6; [$F(1,12) = 75.8$, $p<0.001$]). Finally, the males showed an increased preference score

from the initial test to the final test ($\bar{X}$ ± SEM: 338.8 ± 63.2 vs. 712.9 ± 71.9; [F(1,12) = 24.8, p<0.001]).

The latency to enter the stimulus male chamber was affected by a test by prenatal treatment interaction [F(1,12) = 14.2, p=0.003]. The Oil males decreased the latency to enter the chamber from initial to final test ($\bar{X}$ ± SEM: 79.8 ± 13.5 vs. 17.9 ± 6.3), but the TP males showed no change ($\bar{X}$ ± SEM: 23.3 ± 13.5 vs. 35.9 ± 6.3). Also, during the initial test, the Oil males took longer to enter the male chamber than did the TP males ($\bar{X}$ ± SEM: 79.8 ± 13.5 vs. 23.3 ± 13.5). The data for latency to enter the male chamber was subjected to a square root transformation

Prenatal testosterone treatment only had an effect on one sexual behavior measure during the partner preference tests. During the final test, the TP males showed fewer intromissions than did the Oil males (Mann-Whitney U; $\bar{X}$ ± SEM: 6.3 ± 2.8 vs. 19.4 ± 2.8).

Social and Sexual Behavior: No behaviors measured during the social and sexual behavior tests were affected by the prenatal testosterone treatment.

Table 3.1. One-sample t-test analyses of preference scores and duration of time spent in the male chamber during the partner preference tests (Experiments 1 and 2). This analysis was used to determine if the experimental males showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the stimulus male above chance (duration = 400 sec). In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. In both experiments, C males showed a preference for the stimulus female and spent less time in the male chamber than was expected by chance. *Significantly different than chance, $p \leq 0.02$. ** Significantly different than chance, $p \leq 0.001$.

	Experiment 1							
	Preference Score (Chance = 0)				**Duration in male Chamber (Chance = 400)**			
	Initial		**Final**		**Initial**		**Final**	
	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value
TP	11.4 (52.0)	0.2	30.3 (70.8)	0.4	411.0 (31.2)	0.4	342.2 (46.9)	-1.2
C	242.7 (62.1)	3.9*	330.2 (112.0)	2.9*	259.4 (30.9)	-4.5**	163.0 (44.9)	-5.3**
	Experiment 2							
	Preference Score (Chance = 0)				**Duration in male Chamber (Chance = 400)**			
	Initial		**Final**		**Initial**		**Final**	
	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value
TP	146.0 (71.6)	2.0	184.7 (92.8)	2.0	381.2 (32.7)	-0.6	326.5 (45.9)	-1.6
C	183.5 (71.6)	2.6*	440.1 (78.3)	5.6**	342.5 (34.9)	-1.6	160.6 (30.3)	-7.9**

Table 3.2. Proportion of experimental males that displayed sexual behavior with the stimulus female during the partner preference tests (Experiments 1 and 2). In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. C males were more likely to display male sexual behavior with the stimulus female than were TP males in both partner preference tests during Experiment 1, and in the final partner preference test during Experiment 2. *Significantly different from cholesterol, $p<0.02$, **Significantly different from cholesterol, $p<0.001$

	Experiment 1				
	Initial Test		**Final Test**		
Behavior	Mount	Intromission	Mount	Intromission	Ejaculation
Cholesterol	6/12	4/12	10/14	10/14	8/14
Testosterone*	1/17	0/17	3/18	2/18	1/18
	Experiment 2				
	Initial Test		**Final Test**		
Behavior	Mount	Intromission	Mount	Intromission	Ejaculation
Cholesterol	8/19	5/19	15.17	15/17	12/17
Testosterone	5/18	1/18	2/18**	2/18**	1/18**

Table 3.3. Proportion of experimental males that displayed sexual behavior with the stimulus female during the social and sexual behavior tests (Experiments 1 and 2). In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. C males were more likely to display male sexual behavior with the stimulus female than were TP males in both experiments. *Significantly different from cholesterol, $p<0.002$

	Experiment 1		
	Social and Sexual Behavior Test		
Behavior	Mount	Intromission	Ejaculation
Cholesterol	11/13	11/13	9/13
Testosterone*	5/18	4/18	2/18
	Experiment 2		
	Social and Sexual Behavior Test		
Behavior	Mount	Intromission	Ejaculation
Cholesterol	19/21	19/21	16/21
Testosterone*	6/20	6/20	5/20

Table 3.4. One-sample t-test analyses of preference scores and duration of time spent in the male chamber during the partner preference tests (Experiment 3). This analysis was used to determine if the experimental males showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the stimulus male above chance (duration = 400 sec). In Experiment 3, experimental males were exposed to prenatal treatments and stimulus males were untreated. Both TP and Oil males showed a preference for the stimulus female and spent less time in the male chamber than was expected by chance. *Significantly different than chance, p≤0.03. **Significantly different than chance, p≤0.001.

	Preference Score (Chance = 0)				**Duration in male Chamber (Chance = 400)**			
	Initial		**Final**		**Initial**		**Final**	
	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value
TP	311.9 (70.8)	4.4*	673.3 (103.5)	6.5**	339.9 (30.7)	-2.0	107.2 (35.8)	-8.2**
Oil	303.0 (62.8)	4.8**	714.0 (91.6)	7.8**	330.9 (28.1)	-2.4*	88.8 (23.6)	-13.2**

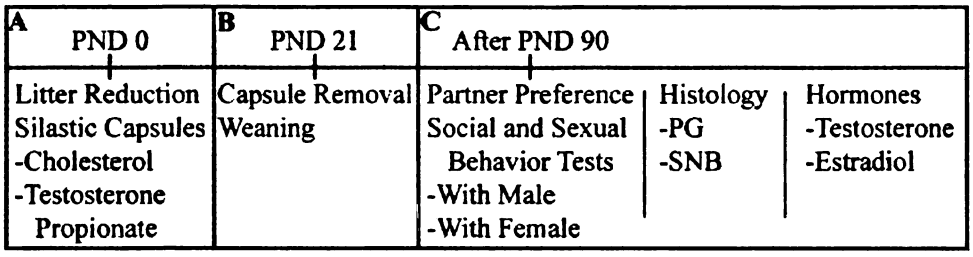

Figure 3.1. Treatment and testing schedule for the experimental males (Experiments 1 and 2). In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. (A) On the day of birth, PND 0, litters were reduced to four males and four females and received Silastic capsules of cholesterol or testosterone propionate (Experiment 1: TP n=18, C n=14; Experiment 2: TP n=19, C n=20). (B) At weaning on PND 21, the Silastic capsules were removed. (D) After the males reached 90 days of age, they were tested for partner preference and social and sexual behavior with both male and female stimulus animals. After testing was concluded, the pelvic ganglia (PG) and spinal nucleus of the bulbocavernosus (SNB) were removed. Blood samples were also taken to analyze plasma testosterone and estradiol concentrations.

A GD 16-20	B PND 0	C PND 21	D After PND 90
Injections -Oil -Testosterone Propionate	Litter Reduction	Weaning	Partner Preference Social and Sexual Behavior Tests -With Male -With Female

Figure 3.2. Treatment and testing schedule for the experimental males (Experiment 3). In Experiment 3, experimental males were exposed to prenatal treatments and stimulus males were untreated. (A) Pregnant dams were treated with either oil (n=13) or testosterone propionate (n=9) on GD 16-20. (B) On the day of birth, PND 0, litters were reduced. (C) Pups were weaned on PND 21. (D) After the males reached 90 days of age, they were tested for partner preference and social and sexual behavior with both male and female stimulus animals.

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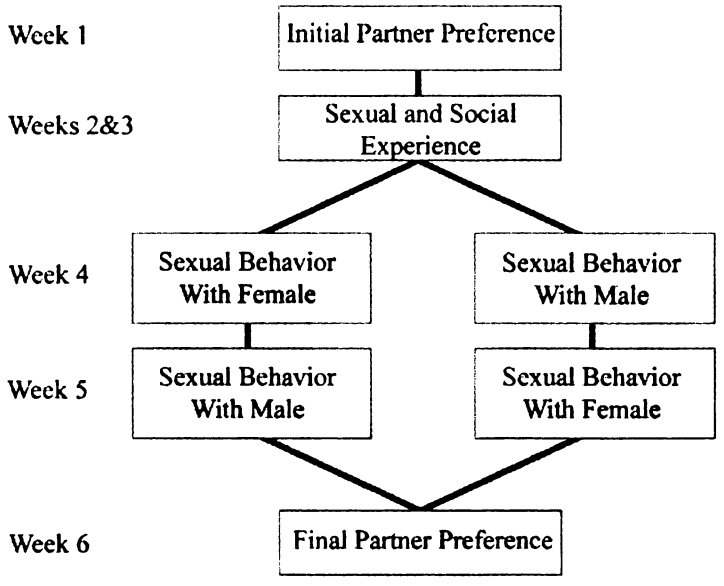

Figure 3.3. Behavioral testing schedule for the experimental males (Experiments 1-3). In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. In Experiment 3, experimental males were exposed to prenatal treatments and stimulus males were untreated. Data were not collected during weeks 2 and 3 during which the animals received sexual and social experience.

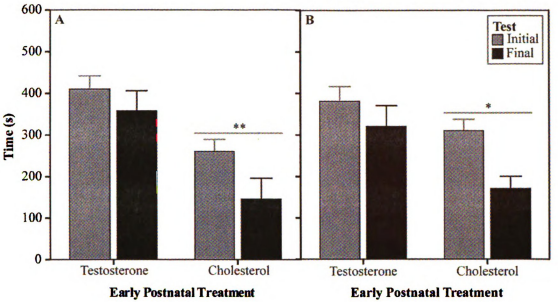

Figure 3.4. Time spent with in the stimulus male chamber during the partner preference tests (Experiments 1 and 2). (A) In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. (B) In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. In both experiments the TP males spent more time in the stimulus male chamber than did the C males. *Significantly different from TP males, $p<0.05$. **Significantly different from TP males, $p<0.001$.

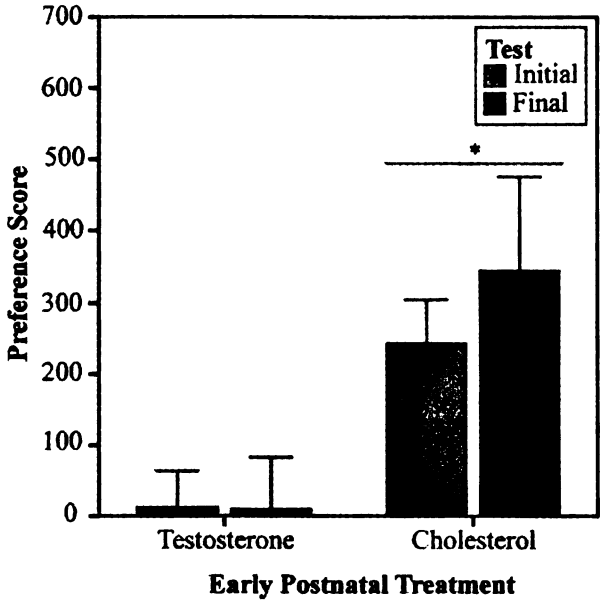

Figure 3.5. Preference scores of the experimental males during the partner preference tests (Experiment 1). In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. Preference score is calculated as time spent with stimulus female minus the time spent with stimulus male. The C males showed a higher preference score than did the TP males in the partner preference tests in Experiment 1. *Significantly different from TP males, $p<0.01$.

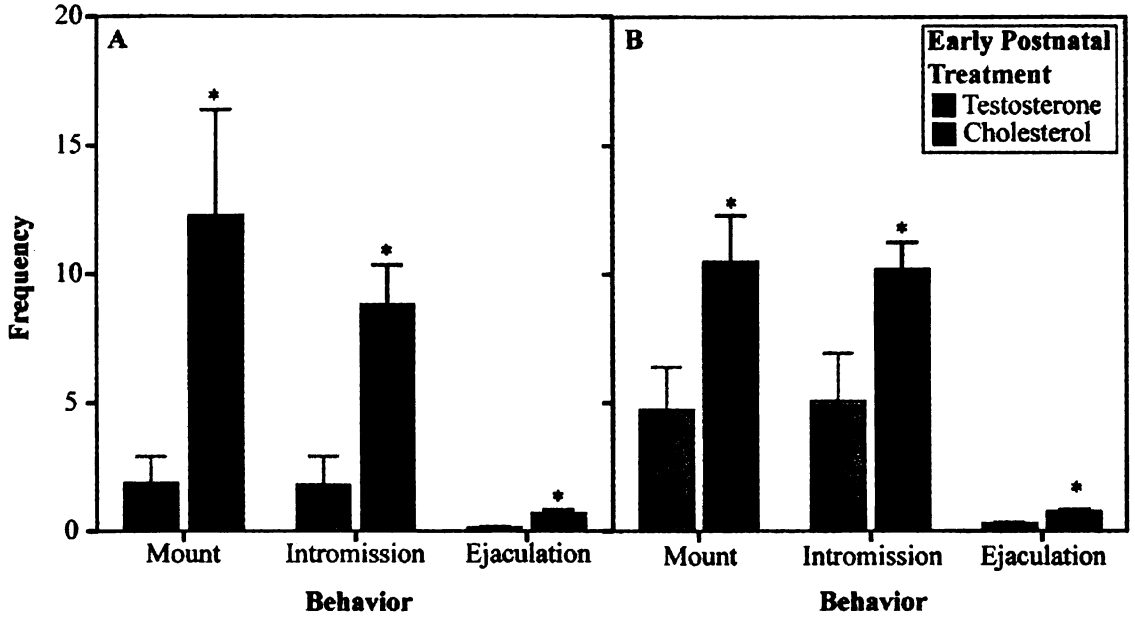

Figure 3.6. Frequencies of mounts, intromissions, and ejaculations displayed by the experimental males during the social and sexual behavior tests when paired with a stimulus female (Experiments 1 and 2). (A) In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. (B) In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. TP males showed fewer sexual behaviors compared to the C males. *Significantly different from TP males, $p<0.03$.

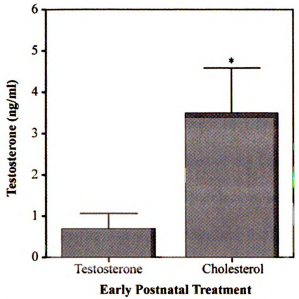

Figure 3.7. Levels of circulating testosterone in a subset of males from Experiments 1 and 2. In Experiment 1, the experimental males were exposed to early postnatal treatments and stimulus males were untreated. In Experiment 2, experimental males were exposed to early postnatal treatments and stimulus males were treated with ATD. C males had higher levels of plasma testosterone than did TP males. *Significantly different from TP males, p=0.012.

DISCUSSION

Early Postnatal Hormone Effects

Regardless of the treatment of the stimulus male (Experiments 1 and 2), early postnatal testosterone treatment increased the amount of time the TP males spent in the stimulus male chamber compared to that of the C males. The C males spent less time in the male chamber than was expected by chance, but the TP males did not. This resulted in the C males showing a distinct preference for the stimulus female, whereas the TP males did not show a preference for a stimulus animal in either Experiment 1 or 2.

In the human literature, the idea arises that hyper-androgen exposure early in development may lead to an increase probability of homosexuality (Rahman, 2005; Rahman and Wilson, 2003; Robinson and Manning, 2000; Williams et al., 2000). In the present study, a change in sexual preference for a stimulus female to a stimulus male by the TP males is not seen, but there is a disruption of the clear preference for the female shown by the C male controls. A preference for the female is not seen because the TP males are spending as much time with the stimulus males as they are with the stimulus females. The TP males also spent as much time with the female as did the cholesterol males. Thus, the TP males' interest in the female did not decline, but their interest in the male increased Therefore, in terms of preference, the early testosterone treatment appears to have caused the males to become bisexual in their preference.

Early postnatal TP treatment also disrupted male sexual behavior. In both Experiments 1 and 2 during the final partner preference test and during the social and sexual behavior

tests, the C males were more likely to show copulatory behavior with a stimulus female than were the TP males. The disruption of male sexual behavior is in agreement with other studies that find perinatal treatment of intact male rats (Diamond et al., 1973; Piacsek and Hostetter, 1984; Pollak and Sachs, 1975; Zadina et al., 1979), ferrets (Baum and Schretlen, 1975), and hamsters (Vomachka et al., 1981) with TP reduced male copulatory behavior in adulthood. It is difficult to determine from these data if the early hormone treatment disrupted normal male sexual behavior or if the TP males are less interested in the stimulus female. It is possible that the TP males do not show a preference for the female because they have copulatory deficits. However, it is equally likely that they are less motivated to copulate and, therefore, do not show a preference for the female.

Circulating testosterone levels were also affected by early postnatal hormone treatment in the present study. C males had higher levels of serum testosterone than did the TP males. In a previous study, male hamsters given TP early in development also showed a decrease in adult serum testosterone levels (Vomachka et al., 1981), and numerous studies have linked neonatal estrogen treatment to decreased adult testosterone production (for review, Delbes et al., 2007). One hypothesis concerning the effect of neonatal steroid treatment on adult hormone levels is that early estrogen may have a direct inhibitory effect on the enzymes that produce testosterone (Goyal et al., 2004; Goyal et al., 2003; Majdic et al., 1996; Tsaimorris et al., 1986). It also has been shown that early treatment with estrogens decreases AR-ir and ERa-ir in the testis in adulthood (Shibayama et al., 2001; Warita et al., 2006). While the mechanism concerning how early

steroids affect adult testosterone levels needs further elucidation, it appears that neonatal hormone treatment permanently alters the sensitivity of the hypothalamic-pituitary-gonadal axis and reduces the responsiveness of adult Leydig cells.

It is unlikely, however, that the behavioral differences in the display of partner preference and sexual behavior seen in the males treated with early postnatal testosterone are due to lower levels of serum testosterone that controls. In the present study, no correlation was seen between serum testosterone level and preference score. Also, plasma testosterone concentrations as low as 0.7 ng/ml have been shown to maintain male sexual copulatory measures in castrated adult rats (Damassa et al., 1977), which is the mean concentration of serum testosterone seen in the early testosterone-treated males in this study. Portillo et al (2006) showed that sexually sluggish male rats (duds) did not have testosterone concentrations different from copulating males and that both groups showed male-typical partner preferences. Finally, ATD males show a nocturnal rhythmicity in their partner preference, preferring a stimulus male early in the dark phase and an estrous female late in the dark phase, whereas control males prefer an estrous female throughout (Bakker et al., 1995). Adult castration and testosterone replacement did not affect partner preference expression indicating that the ATD male preference was not due to individual differences in circulating testosterone. Therefore, circulating testosterone levels do not appear to correlate with male behavior.

In the human literature, results from studies of sexual orientation and circulating hormone levels are inconsistent. Some studies find no effect of orientation on plasma testosterone

levels, some find testosterone levels to be higher in homosexual men than heterosexual men, and some find testosterone levels to be higher in heterosexual men than homosexual men (for review, Banks and Gartrell, 1995). No definite conclusions about the relationship between human circulating steroid levels and sexual orientation can be drawn at this time. In the present study low circulating testosterone levels were associated with altered partner preference behavior and reduced male sexual behavior; however, the extent to which these low levels contributed to this altered preference cannot be determined from these data.

Early postnatal testosterone treatment did not affect the number of motoneurons in the SNB or neurons in the PG. These areas appear not to be affected by increased levels of exogenous androgens postnatally.

Prenatal Hormone Effects

Prenatal TP treatment did alter a few behaviors measured during the behavior tests, but it did not have a large effect on adult partner preference or sexual behavior. Both the Oil and TP males showed a positive preference score during both tests, and during the social and sexual behavior tests, the prenatal TP treatment did not affect any of the behaviors measured.

In both partner preference tests the Oil males spent less time with the stimulus male than was expected by chance, but the TP males did so only during the final test. Also, Oil males decreased their latency to enter the male chamber from initial to final test, whereas

the TP males remained the same, and therefore, during the initial test, the Oil males took longer to enter the male chamber than did the TP males. These interactions may imply that the Oil males were less interested in the stimulus male than the TP males during the initial partner preference. However, no statistical differences were seen between the treatment groups when analyzing the amount of time spent with the male. Therefore, although the Oil males spent less time than chance with the stimulus male, the Oil males were not significantly different from the TP males. Finally, in the final partner preference test, the Oil males showed more intromissions with the stimulus female than did the TP males. However, no other sexual behavior measures were altered by the early hormone treatment, including ejaculation frequency during the final test or any measures during the sexual behavior test with a female.

Experience Effects

During the partner preference tests, regardless of perinatal hormone treatment, a number of measures were affected by the experience received during the social and sexual behavior tests. In Experiment 1, the males decreased the time to enter the female chamber and decreased the amount of time spent in the male chamber from initial to final test. Also, the TP male increased the amount of time spent in the middle chamber from initial to final test. In Experiment 2, the latency to enter both stimulus animal chambers decreased from initial to final test, as did the amount of time spent in the male chamber. The males increased the amount of time spent in the middle chamber from initial to final test and showed a higher preference score in the final test. In Experiment 3, the males decreased the time to enter the female chamber and decreased the amount of time spent in

the male chamber from initial to final test. They also increased the amount of time spent with the female and increased their preference score from initial to final test.

These changes can be explained by the experience the males received between the two partner preference tests. Sexual experience has been shown to lead to increased preference for a female over a male stimulus animal (Matuszczyk and Larsson, 1994). Therefore, in the present study, the sexual experience would lead to a decreased latency to enter the female chamber (Experiments 1, 2, 3), a reduced amount of time spent with the male (Experiments 1, 2, 3), an increased preference score (Experiments 2, 3), and increased time spent with the stimulus female (Experiment 3).

In experiments 1 and 2, the increase seen in the amount of time spent in the middle chamber from initial to final test is also probably due to the social and sexual experience received. In Experiment 1, we thought the tests with a stimulus male were probably aversive to the experimental males because in the final test, both treatment groups spent more time alone in the middle chamber than was expected by chance (data not shown). The change in stimulus males was an attempt to prevent this aversion. However, the experimental animals still spent more time alone during the final partner preference test in Experiment 2. The experience with the ATD stimulus males may have been aversive to both treatment groups in Experiment 2. It is also possible that they interaction with ATD males was aversive to the C males, whereas the sexual tests with a female were aversive to the TP males, which could also explain the increase in middle chamber duration seen in Experiment 1.

Selection of Experimental Males

It should be noted that the experimental males used in the present study were not selected randomly at birth. The males with the largest AGD were kept on PND 0 when the litter was reduced. The AGD is an indication of androgen exposure perinatally, and therefore the males used were selected on the basis of high androgen responsiveness.

Overall

The early postnatal testosterone treatment caused the males to increase the time spent with the stimulus male without affecting the time spent with the stimulus female. This resulted in the testosterone treated males showing no preference for either animal, and no sexual behavior was seen with either stimulus animal. Exogenous testosterone during early development also decreased adult levels of circulating testosterone compared to controls. Prenatal testosterone treatment did not significantly alter either partner preference or sexual behavior.

CHAPTER FOUR:

ESTROGEN IS NOT RESPONSIBLE FOR THE EFFECTS OF HYPER-ANDROGEN EXPOSURE DURING DEVELOPMENT ON ADULT PARTNER PREFERENCE AND REPRODUCTIVE BEHAVIOR IN GONADALLY INTACT MALE RATS

INTRODUCTION

In the previous chapter, I reported that early postnatal treatment of male rats with testosterone propionate altered the expression of adult partner preference. Males treated during early development with testosterone spent more time in the stimulus male chamber during the preference tests than did cholesterol-treated controls. Since testosterone can have its effects by acting directly on the androgen receptor, or by being metabolized into dihydrotestosterone and estradiol, further studies are needed to determine the mechanism by which the early testosterone treatment is changing the adult behavior.

Early postnatal estrogen treatment of males has effects on adult behavior that are similar in some ways to those seen after postnatal treatment with testosterone. For example, neonatal estradiol exposure, like testosterone treatment (Pollak and Sachs, 1975), has been shown to disrupt male sexual behavior in adulthood (Diamond et al., 1973; Harris and Levine, 1965; Whalen, 1964). Also, early estrogen exposure has been shown to decrease adult levels of circulating testosterone (Atanassova et al., 1999; Delbes et al., 2007; Goyal et al., 2004; Goyal et al., 2003; Shibayama et al., 2001; Warita et al., 2006),

a measure that was also altered by early testosterone administration in the previous study. As reported in Chapter Two, early postnatal estrogen masculinizes partner preference of adult females in the same manner as neonatal testosterone treatment (Meyerson and Lindstrom, 1973). From these comparisons, it is reasonable to suggest that the effects of early postnatal testosterone treatment, seen in the previous chapter, are due to the action of the estrogenic metabolite of testosterone. Therefore, to determine if the alteration of adult male partner preference by hyper-androgen exposure early in development is mediated by the estrogen metabolite, intact male rats were exposed to early postnatal estradiol treatments and adult behaviors were examined.

METHODS

Animals

Experimental Males: Time-mated pregnant Long-Evans rats (Charles River, Raleigh, NC) were housed individually with *ad lib* food and water in plastic cages (45.5 x 24 x 21 cm) in a 14:10-hr light dark cycle with lights on at 01:00. For nest building material thirty, one-inch paper towel strips were given to the dams on GD 20. A subset of the male offspring of these dams became the experimental males of this study (see below). On the day of birth, PND 0, the litter was reduced to four male and four female pups (Figure 4.1A). For litter reductions, the AGD for each pup was measured, and since the AGD is shorter in females than in males, the four shortest and the four longest were retained.

Stimulus Animals: Sexually experienced, gonadally intact, adult Long Evans rats (Charles River, Raleigh, NC) were used as stimulus animals for the behavioral tests (females at least 60 days old; males at least 90 days old).

Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experimental procedures were approved by the Michigan State University Animal Care and Use Committee.

Hormone Treatments

Postnatal hormone treatments were the same as those used in Chapter Two (Figure 4.1A,C). Only male pups were used in this Experiment. Behavioral tests began after the animals reached 90 days of age (Figure 4.1D). While these estradiol treatments are most likely outside the physiological range of estrogen exposure, this study was designed to provide further details about long-lasting effects of early exposure to estrogen on males. A number of studies show that early exposure to estrogenic environmental contaminants disrupts normal male sexual development (for review, Delbes et al., 2006).

Stimulus females hormone treatments were the same as those used in Chapter Two.

Maternal Licking and Grooming

Maternal behavior was analyzed as described in Chapter Two (Figure 4.1B).

Behavioral Testing

Partner Preference: Tests for partner preference were conducted as described in Chapter Two.

Social and sexual behavior: Tests for social and sexual behavior were conducted as described in Chapter Three.

Testing Schedule: Testing schedule was the same as described in Chapter Three (Figure 4.2).

Histology

Pelvic Ganglia and Spinal Nucleus of the Bulbocavernosus: Tissue harvesting, sectioning, staining and analysis were conducted as described in Chapter Three (Figure 4.1D). One male from each litter was used for analysis.

Hormone Analysis

Hormone Analysis was conducted as described in Chapter Three (Figure 4.1D).

Analysis

Maternal licking and grooming data were analyzed using a 3 x 4 (early postnatal treatment x day) ANOVA with repeated measures on the second factor. The data for the behavioral measures during the partner preference tests were analyzed using a 3 x 2 (early postnatal treatment x initial or final test) ANOVA with repeated measures on the

second factor. Preference scores and duration of time spent in the male chamber during the partner preference tests were also analyzed within each early postnatal treatment group using a one-sample t-test. This test was used to determine if the animals showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the male above chance (duration = 400 seconds). The data for the behavioral measures during the social and sexual behavior tests, histological cell counts, and hormones assays were analyzed using a one-way ANOVA by early postnatal treatment.

For some variables, the data did not meet homogeneity of variance assumptions, even after the prescribed transformations (i.e. square root). For these measures, nonparametric statistics were used for analysis. In these situations, data from the 5% and 10% males were collapsed into a early postnatal estradiol treatment group (EB males) because no significant differences were found between the two postnatal estradiol doses for any of the variables analyzed using nonparametric statistical tests. The Mann-Whitney U and the Fisher's exact probability tests were used for these analyses.

RESULTS

Maternal Licking and Grooming

No significant differences in maternal licking and grooming were seen among treatment groups (p=.292) or days (p=0.078), with no significant interaction (p=0.147; Figure 4.3).

Partner Preference

During the initial test the 5% males had a lower preference score than the other two treatment groups (Figure 4.4B), and they did not show a preference for either the male or

female stimulus animal. Both the C and 10% males, though, showed a preference score significantly higher than expected by chance, indicating a preference for the female (Table 4.1). During the final test all groups showed a preference for the female (Table 4.1) with both the 5% and C males showing a significant increase in preference score as indicated by an early postnatal treatment by week of test interaction [$F(2, 31) = 3.9$, $p=0.03$], (Figure 4.4B). In addition, the duration of time spent in the stimulus male chamber was significantly less than expected by chance for the 10% and C males in both the initial and final tests, and for the 5% males during the final test (Table 4.1).

The changes seen in preference score were the result of alterations in the duration of time spent with the stimulus male, which was also affected by an early postnatal treatment by week of test interaction [$F(2,31) = 11.2$, $p<0.001$]. Both the 5% males and the C males decreased the amount of time they spent in the stimulus male chamber from the initial to final test, but the 10% males remained the same ($\bar{X} \pm$ SEM: all measures are in seconds: C males: 235.3 ± 31.1 vs. 63.0 ± 21.8; 5% males: 399.6 ± 79.1 vs 155.5 ± 32.4; 10% males: 189.9 ± 55.7 vs 198.9 ± 43.3). During the initial test, the 5% males spent more time with the stimulus male than did the 10% males. During the final test, the 10% males spent more time with the stimulus male than did the C males (Figure 4.4A). Duration of time spent with the stimulus male was subjected to a square root transformation before analysis.

The latency to enter the stimulus female chamber [$F(1,31) = 6.0$, $p=0.02$] and time spent with the female [$F(1,31) = 5.6$, $p=0.025$] were both affected by a main effect of week of test. Regardless of early postnatal hormone treatment, the males decreased their latency

to enter the female chamber from initial to final test ($\bar{X}$ ± SEM: 59.0 ± 74.0 vs. 23.1 ± 14.1), and they also spent more time in the stimulus female chamber ($\bar{X}$ ± SEM: 475.2 ± 35.8 vs. 578.0 ± 36.1)

Non-parametric statistics (Mann-Whitney U) were used to compare the male sexual behaviors displayed during the partner preference tests by the EB males (combining the 5% and 10% groups as explained in Methods) and the C males. No significant differences were seen in the display of male sexual behavior in the initial partner preference test. In the final partner preference test mount, intromission, and ejaculation frequencies were affected by early postnatal estradiol treatment. EB males showed decreased sexual behavior with the stimulus female compared to the C males (Figure 4.5). Also, the proportion of C males that showed mounts, intromissions, and ejaculations during the final test was higher than that of the EB males (Table 4.2). No differences were seen in behaviors shown with the stimulus male.

Social and Sexual Behavior

Early postnatal estradiol treatment altered the expression of male sexual behavior when paired with a stimulus female. C males showed more intromissions and ejaculations during the test than did EB males (Figure 4.6; Mann-Whitney U). Also, the latency to show a mount or intromission were longer for the EB males that for the C males (Figure 4.7; Mann-Whitney U). Finally, the proportion of males that showed behaviors when paired with a female was higher in the C male group than in the EB male group (Table

4.2). No significant differences were seen in behaviors shown when paired with a stimulus male.

Histology

Early postnatal estradiol treatment altered the number of neurons seen in the PG of the experimental males [$F(2,18) = 5.1$, $p=0.02$]. Males treated postnatally with 10% estradiol had fewer cells in the PG than did males treated with cholesterol (Figure 4.8). Early postnatal estradiol treatment did not affect the number of motoneurons present in the SNB.

Hormone Assay

The amount of circulating testosterone was affected by the early postnatal estradiol treatment (Kruskel-Wallis, $p=0.001$). The C males had significantly higher circulating testosterone levels than did either the 10% or 5% males (Figure 4.9). Circulating levels of estradiol were not affected by the early hormone treatment.

Table 4.1. One-sample t-test analyses of preference scores and duration of time spent in the male chamber during the partner preference tests. This analysis was used to determine if the experimental males showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the stimulus male above chance (duration = 400 sec). The 10% and C males (initial and final test) and 5% males (final test only) showed a preference for the stimulus female and spent less time in the stimulus male chamber than was expected by chance. *Significantly different from chance, $p \leq 0.01$. ** Significantly different from chance, $p \leq 0.001$.

	Preference Score (Chance = 0)				**Duration in Male Chamber (Chance = 400)**			
	Initial		**Final**		**Initial**		**Final**	
	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value
10% EB	357.2 (99.3)	3.4*	368.8 (88.7)	4.2*	189.8 (55.7)	-3.8*	198.9 (43.3)	-4.6*
5% EB	-41.4 (121.8)	-0.3	408.4 (75.6)	5.4**	399.6 (79.1)	0.0	155.5 (32.4)	-7.6**
C	285.1 (62.6)	4.6**	539.6 (69.6)	7.8**	235.3 (31.1)	-5.3**	63.0 (21.8)	-15.4**

Table 4.2. Proportion of experimental males that displayed sexual behavior with the stimulus female during the final partner preference test and during the social and sexual behavior test. C males were more likely to display mounts, intromissions, and ejaculations than were EB males. *Significantly different from cholesterol, $p<0.01$. **Significantly different from cholesterol, $p<0.001$

	Final Partner Preference Test			**Social and Sexual Behavior Test**		
Behavior	Mount	Intromission	Ejaculation	Mount	Intromission	Ejaculation
Cholesterol	14/15	14/15	12/15	14/15	14/15	12/15
Estradiol	2/19**	2/19**	0/19**	8/17*	8/17*	1/17*

A PND 0	B PND 1,2,4,6	C PND 21	D After PND 90		
Litter Reduction Silastic Capsules -Cholesterol -5% EB-C -10% EB-C	Maternal Licking and Grooming	Capsule Removal Weaning	Partner Preference Social and Sexual Behavior Tests -With Male -With Female	Histology -PG -SNB	Hormones -Testosterone -Estradiol

Figure 4.1. Treatment and testing schedule for the experimental males. (A) On the day of birth, PND 0, litters were reduced to four males and four females and received Silastic capsules of cholesterol (C, n=15) or estradiol benzoate [EB either 5% (n=10) or 10% (n=9)]. (B) Maternal licking and grooming behavior was observed on PND 1, 2, 4, and 6. (C) At weaning on PND 21, the Silastic capsules were removed. (D) After the males reached 90 days of age, they were tested for partner preference and social and sexual behavior with both male and female stimulus animals. After testing was concluded, the pelvic ganglia (PG) and spinal nucleus of the bulbocavernosus (SNB) were removed. Blood samples were also taken to analyze plasma testosterone and estradiol concentrations.

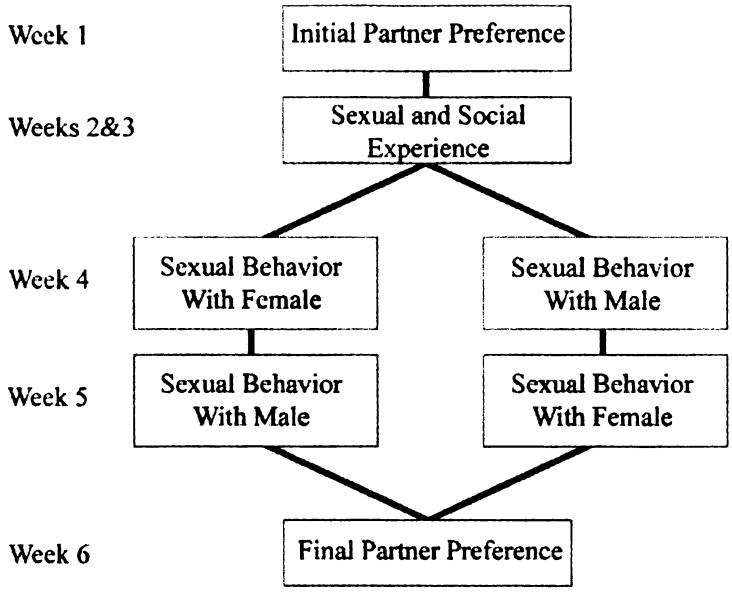

Figure 4.2. Behavioral testing schedule for the experimental males. Data were not collected during weeks 2 and 3 during which the animals received sexual/social experience.

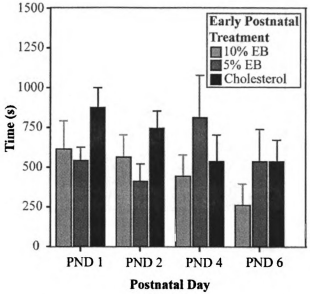

Figure 4.3. Time (in seconds) mothers of experimental males spent licking and grooming during the first postnatal week. No significant differences were seen among treatment groups or days, with no significant interaction.

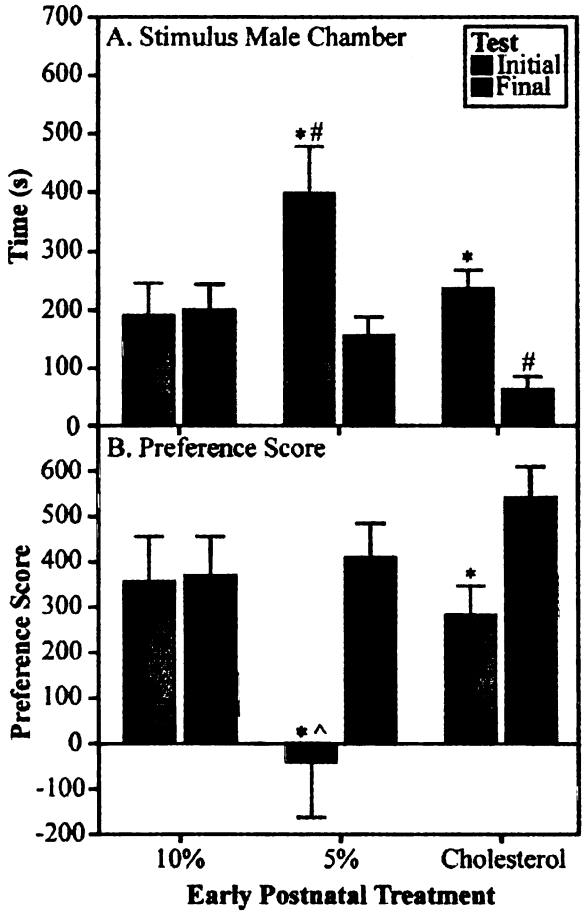

Figure 4.4. (A) Duration of time experimental males spent in the stimulus male chamber and (B) preference scores. Preference score is calculated as time spent with stimulus female minus the time spent with stimulus male. The 5% and C males increased their preference score and decreased the time spent with the male from initial to final test, while the 10% males remained the same. *Significantly different from final test (within treatment group). #Significantly different from 10% males (within test). ^Significantly different from 10% and C males (within test).

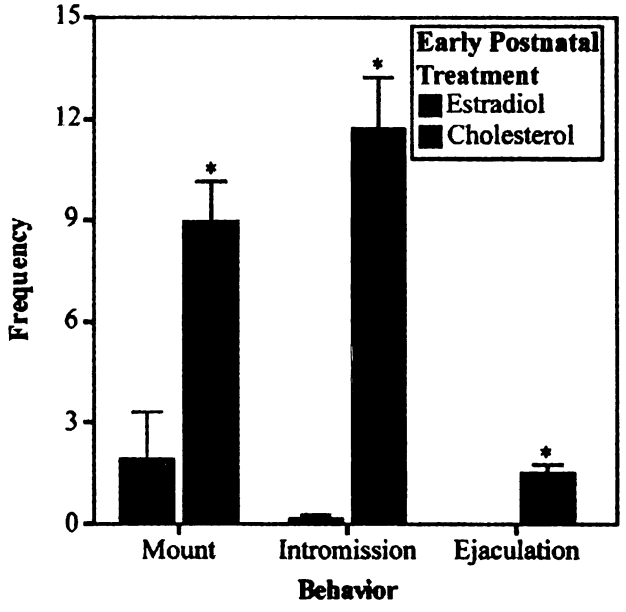

Figure 4.5. Frequencies of mounts, intromissions, and ejaculations displayed by the experimental males during the final partner preference test. EB males showed fewer sexual behaviors compared to the C males. *Significantly different from EB males, $p<0.001$.

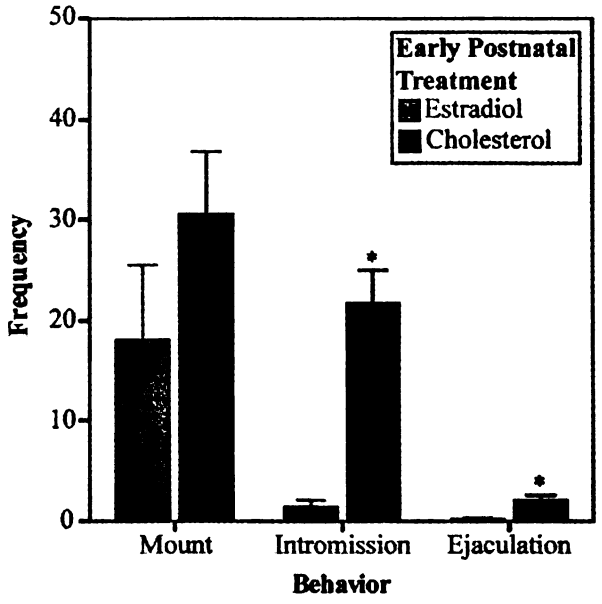

Figure 4.6. Frequencies of mounts, intromissions, and ejaculations displayed by the experimental males during the social and sexual experience test when paired with a female. EB males showed fewer intromissions and ejaculations compared to the C males. *Significantly different from EB males, $p<0.001$.

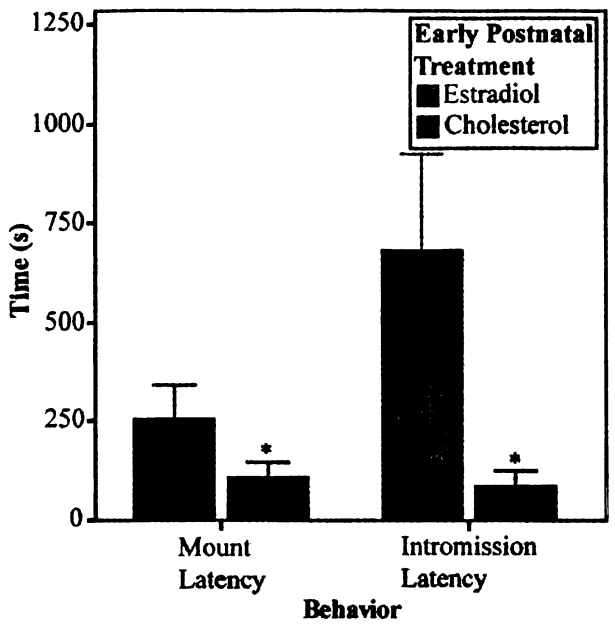

Figure 4.7. Mount and intromission latencies shown by the experimental males during the social and sexual experience test when paired with a female. EB males showed longer latencies to display a behavior than did C males. *Significantly different from EB males, $p<0.05$.

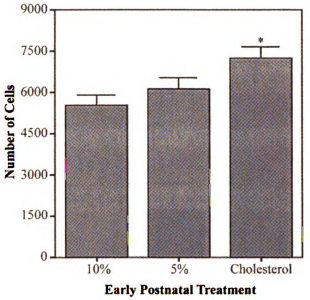

Figure 4.8. Number of neurons present in the PG of experimental males. C males had more PG neurons than did the 10% males. *Significantly different from 10% males, p=0.02.

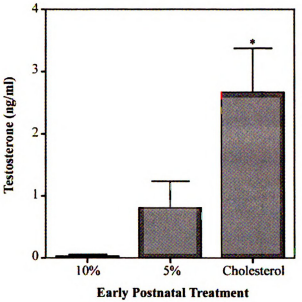

Figure 4.9. Levels of circulating testosterone in the experimental males. C males had higher levels of plasma testosterone than did the 10% or 5% males. *Significantly different from 10% and 5% males, p=0.001.

DISCUSSION

Early Postnatal Hormone Effects

Estradiol treatment during early development affected adult male partner preference in different ways depending on the dose used. The low dose of estradiol seemed to alter naïve partner preference, while the high dose of estradiol seemed to remove the effect of sexual experience on preference.

Control males showed a strong preference for the stimulus female in both partner preference tests, and this preference increased significantly from the initial to the final test. Males that received the high dose (10%) of EB also showed a preference for the female in both tests, but this preference did not increase over testing. In contrast, males receiving a low dose (5%) of EB during early development did not show an initial preference for either the male or female stimulus animal, but during the final test they displayed a preference for the stimulus female. The early postnatal treatments did not alter the amount of maternal licking and grooming received by the litters; therefore, it is unlikely that the early postnatal treatment group differences were mediated by changes in maternal care.

One explanation for this lack of effect of experience seen in the 10% males could be due to the fact that the 10% males did not copulate during the sexual behavior tests. However, the 5% males did not show any male sexual behavior, either, and they continued to display an increase in preference for the female in the final test. Another, and perhaps more likely explanation, could be the health of the 10% males. Prior to the start of the

behavioral studies, six males treated with the high dose of estrogen became ill and died. Upon examination of two of these males, it was found that the animals had severe kidney and bladder infections. These infections were only found in the males treated with the high dose of estradiol, and no other animals in the colony were found to be ill at that time. The early postnatal estrogen exposure likely had damaging effects on the male's urinary and renal systems as has been shown in other studies (Lehtimaki et al., 1996; Streng et al., 2001). Although the remaining males used in the study remained outwardly healthy, and their behavior seemed normal, there is a chance that the behavior seen in both the 5% and the 10% males was altered by subclinical conditions.

It seems that early postnatal estradiol treatment affected naïve adult partner preference in the 5% males, but these results do not correspond to those seen when males were treated during early development with testosterone. Therefore, the effects on adult male partner preference seen with early postnatal testosterone treatment most likely are due either to androgen effects alone, or to a combination of androgen and estrogen effects, but not estrogen effects alone.

Early postnatal estradiol also disrupted male sexual behavior in both the final partner preference test and the social and sexual experience tests when paired with a stimulus female. The outcomes of the present study are similar to those seen in Chapter Three, and therefore the disrupting effects of testosterone on male sexual behavior could be attributable to the action of the estrogen metabolite. This also agrees with results seen in other studies. In addition to the studies that show that perinatal testosterone treatment to

intact rodents reduces male sexual behavior (Baum and Schretlen, 1975; Diamond et al., 1973; Piacsek and Hostetter, 1984; Pollak and Sachs, 1975; Vomachka et al., 1981; Zadina et al., 1979), other studies have found similar outcomes after perinatal treatment with estrogen (Diamond et al., 1973; Harris and Levine, 1965; Whalen, 1964).

The PG is a sexually dimorphic, mixed sympathetic and parasympathetic ganglion that innervates the reproductive organs, bladder, and colon of the male rat (Dail et al., 1975; Keast, 1995; Keast et al., 1989; Keast and Degroat, 1989). Little is known, however, about the development of this autonomic ganglion. In the present study, the high dose of early postnatal estradiol decreased the number of cells present in the adult PG compared to controls. The estradiol could be affecting the PG directly during development, either preventing neurogenesis of the PG neurons or increasing the amount of cell death. The neurons in the pelvic ganglia have been shown to express both ERα and ERβ as well as the aromatase enzyme (Purves-Tyson et al., 2007). However, estrogen does not seem to play a role in the normal development of the PG. Studies show that males with the testicular feminizing mutation, a mutation of the androgen receptor, have fewer neurons than do controls (Fang, Breedlove, and Clemens, unpublished results). It was also reported in Chapter Two that early postnatal estrogen treatment did not increase PG cell number in adult females.

Another explanation for the effects on the PG could be an indirect action of the estradiol on PG cell number. Neonatal estrogen treatment has been shown to have significant effects on the male urogenital system, specifically treatment decreases penis size (Goyal

et al., 2004), testes weight (Atanassova et al., 1999), and prostate size (vomSaal et al., 1997). The PG is known to innervate all of these reproductive organs in the male. If the major target organs of the ganglion have been reduced in size, thereby decreasing the level of innervation, it is likely there would be fewer neurons remaining in the adult PG.

Circulating testosterone levels were also affected by early postnatal hormone treatment in the present study. C males had higher levels of serum testosterone than did the 5% and 10% males. Therefore, it seems likely that the change in adult plasma testosterone levels seen in Chapter Three after postnatal testosterone treatment, is mostly likely an effect of the estradiol metabolite. Numerous studies have linked neonatal estrogen treatment to decreased adult testosterone production (Atanassova et al., 1999; Delbes et al., 2007; Goyal et al., 2004; Goyal et al., 2003; Shibayama et al., 2001; Warita et al., 2006). As previously mentioned, early estrogen is thought to have a direct inhibitory effect on the enzymes that produce testosterone (Goyal et al., 2004; Goyal et al., 2003; Majdic et al., 1996; Tsaimorris et al., 1986), and it has been shown that early treatment with estrogens decreases AR-ir and ERa-ir in the testis in adulthood (Shibayama et al., 2001; Warita et al., 2006). The mechanism for how early steroids affects adult testosterone levels needs further elucidation, but it appears that neonatal hormone treatment permanently alters the sensitivity of the hypothalamic-pituitary-gonadal axis.

Experience Effects

Regardless of early postnatal hormone treatment, the experimental males decreased the time to enter the female chamber and increased the amount of time spent with the female

from initial to final partner preference test. Similar to the discussion of experience effects in Chapter Three, these changes can be explained by the sexual and social experience the males received between the two partner preference tests. Sexual experience has been shown to lead to increased preference for a female over a male stimulus animal (Matuszczyk and Larsson, 1994), which would explain the decreased latency to enter the female chamber, as well as the increase in time spent with the female.

Limitations

It should be mentioned that there were apparent limitations associated with the methods of the present study, which make interpretation of the data difficult. First, the doses of estrogen used were well above physiologically relevant levels and most likely higher than the level of exogenous estrogen seen after aromatization in the previous study with TP. However, the doses of estradiol used were based on developing a positive estrogen control for evaluating estrogenic environmental agents, such as polychlorinated biphenyls. Another issue that arose was the health status of the estradiol-treated males. The high dose of estradiol most likely disrupted the development of the urogenital system, which lead to some males becoming severely ill with kidney and bladder infections.

Overall

Early estradiol exposure affected the initial preference of males treated with a low dose, while reducing the effect of sexual experience on preference in males treated with a high dose. However, these results may be confounded by the health status of the males. The

changes in adult male partner preference observed in the present study are not comparable to the alteration seen after early postnatal testosterone treatment. Therefore, based on the present study, estradiol alone does not seem to be responsible for the hyper-androgenization effects on partner preference. However, the dosages of estradiol used in the current study were certainly super-physiological and led to sever disruption of genital physiology and anatomy. Quite possibly lower doses of estrogen that do not result in such severe disruptions might have more subtle masculinizing effects that parallel those seen with testosterone treatments.

CHAPTER FIVE:

NATURAL VARIATION IN MATERNAL LICKING AND GROOMING DOES NOT ALTER ADULT PARTNER PREFERENCE EXPRESSION IN EITHER MALE OR FEMALE RATS

INTRODUCTION

Rat dams have been shown to discriminate between male and female pups, showing more anogenital licking and grooming towards male pups in a litter (Moore and Morelli, 1979). Studies have shown that neonatal hormone treatments can alter the display of maternal behavior. Testosterone, estradiol, or dihydrotestosterone injections to female rat pups on the day of birth increased the amount of maternal licking and grooming the females received (Moore, 1982). The level of maternal licking and grooming was equivalent for treated female and male pups and greater than that of untreated females. Therefore, early postnatal hormone manipulations can alter the behavior of the mother.

Alterations in maternal behavior in the rat have been shown to influence a number of behavioral measures, endocrine responses, and neural development in offspring. Maternal licking and grooming, specifically, can affect a range of offspring measures including offspring maternal care (Champagne et al., 2001; Francis et al., 1999), sexual behavior (Cameron et al., 2008; Moore, 1984), cognitive ability (Liu et al., 2000), hypothalamic-pituitary-adrenal stress responses (Liu et al., 1997), motoneuron number (Moore et al., 1992), and neural receptor number and methylation (Caldji et al., 1998; Champagne et al., 2006; Liu et al., 1997; Weaver et al., 2004).

Natural variations in maternal care have been shown to affect steroid receptor number and feedback responses in offspring. Offspring raised by high licking and grooming dams show decreased corticosterone responses to restraint stress as adults compared to animals raised by low licking and grooming dams (Liu et al.). The high licking and grooming offspring also show decreased corticotropin-releasing hormone mRNA and increased glucocorticoid receptor mRNA in the hypothalamus. These changes lead to enhanced glucocorticoid feedback sensitivity and a decreased response to stress.

In our laboratory, recent data indicate that maternal licking and grooming do not play a role in the expression of adult partner preference (Cummings et al., 2008). Cummings et al (2008) found no correlation between amount of licking and grooming and preference score. However, these data were analyzed within groups of animals treated with PCBs. Although early postnatal estradiol treatment did not alter maternal licking and grooming in the earlier studies, the fact that variations in maternal care have been shown to have powerful effects on the offspring's behavior and neuroendocrine responses made it necessary to definitively rule out any possibility that results seen in the previous chapters could be attributed to changes in maternal care. Therefore, maternal licking and grooming was studied without other early postnatal treatments to determine if maternal care could alter adult partner preference in the laboratory rat. In this study, naturally occurring variations in licking and grooming displayed by rat dams were measured, and their effects on adult partner preference and reproductive behavior were analyzed.

METHODS

Animals

Experimental Animals: Time-mated pregnant Long-Evans rats (Charles River, Raleigh, NC) were housed individually with *ad lib* food and water in plastic cages (45.5 x 24 x 21 cm) in a 14:10-hr light dark cycle with lights on at 01:00. For nest building material thirty, one-inch paper towel strips were given to the dams on GD 20. A subset of the offspring of these dams became the experimental animals of this study (see below). On the day of birth, PND 0, the litter was reduced to four male and four female pups. For litter reductions, the AGD for each pup was measured, and since the AGD is shorter in females than in males, the four shortest and the four longest were retained. The pups were weaned on PND 21 and were then housed with same-sex littermates.

After animals reached 60 days of age, two females from each litter were ovariectomized and implanted with a Silastic capsule containing estradiol benzoate (Sigma; EB) mixed with cholesterol (Sigma; C) to achieve 25% EB-C mixtures. The capsules were implanted subcutaneously (s.c.) while the animals were anesthetized with isoflurane; the incision was closed with an Auto Clip and covered with First Aid Cream. After 4 weeks, the capsules begin to lose efficacy because of connective tissue growth around the capsule (personal observation). The capsules were removed and reimplanted via a new incision in the neck. For all tests the females were injected s.c. with 0.5 mg progesterone (Sigma; P, in sesame oil) four hours prior to data collection.

Two male offspring from each litter were tested after they reached 90 days of age. Males were tested gonadally-intact.

Stimulus Animals: Sexually experienced, gonadally intact, adult Long Evans rats (Charles River, Raleigh, NC) were used as stimulus animals for the behavioral tests (females at least 60 days old; males at least 90 days old).

Stimulus females were implanted with a Silastic capsule containing 25% EB-C mixture prior to testing but were not ovariectomized. It is the practice of this laboratory to not ovariectomize stimulus females for testing procedures. The hormone treatments used reliably induce sexual receptivity while avoiding the trauma of an unnecessary surgery. Stimulus females were injected s.c. with 0.5 mg P four hours prior to partner preference and sexual behavior testing.

Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experimental procedures were approved by the Michigan State University Animal Care and Use Committee.

Maternal Licking and Grooming

Maternal behavior was videotaped during the last hour of the light phase and the first hour of the dark phase of the light-dark cycle on PND 1, 2, 4, and 6. These recordings, as well as those for all other behavioral tests, were analyzed using The Observer 5.0 (Noldus), a behavioral data acquisition computer program. The amount of time the dam

spent licking and grooming (LG) the litter was determined. Experimental offspring were divided into 3 LG groups dependent on the level of LG received during development. Dams that fell below one standard deviation from the mean are referred to as Low LG mothers, whereas dams that were above one standard deviation from the mean are referred to as High LG mothers. All other dams are called Mid LG mothers.

Behavioral Testing

Partner Preference: Tests for partner preference were conducted as described in Chapter Two.

Female sexual behavior: When female offspring were tested for sexual behavior, a barrier with four holes (4.5 x 4.5 cm) was placed in a Plexiglas observation chamber (46 x 58 x 51 cm), which divided it into a female escape chamber (46 x 22 x 51 cm) and a male chamber (46 x 36 x 51 cm). The holes in the barrier were too small for the male to get through but gave the female free access to both chambers. The tests lasted 30 minutes or until the female exited and returned after an ejaculation, which ever occurred first. Behavioral testing took place under dim red light illumination in the middle part of the dark phase of the light-dark cycle. The test was videotaped, and frequency of male mounts, intromissions and ejaculations were scored, as was the latency to show these behaviors. The latency of the experimental female to approach the male, the amount of time the female spent in male chamber, and sexual receptivity were also recorded. Receptivity was measured using lordosis quotient (LQ), which was calculated for all females who received at least seven mounts. For the first 10 mounts (including

intromissions or ejaculations) female responses were scored as a 0 (no lordosis) or 1 (lordosis), from which a percentage of number of lordoses per test was calculated. Finally, the frequency of exits and latency to return to the male after mounts, intromissions, and ejaculations were calculated.

Male sexual behavior: Male offspring were tested for male sexual behavior in a Plexiglas observation chamber (46 x 58 x 51 cm). During the test, the experimental male had unrestricted access to the female stimulus animal. The tests lasted 30 minutes or until the first intromission after an ejaculation, whichever occurred first. Behavioral testing took place under dim red light illumination in the middle part of the dark phase of the light-dark cycle. Video recordings of these tests were analyzed to determine frequency of mounts, intromissions, and ejaculations shown by the experimental males and the latency to show these behaviors.

Testing Schedule: Experimental animals were tested once a week for 5 weeks. The initial partner preference of each animals was tested in week 1. Then each experimental animal received sexual experience (female offspring with intact, adult stimulus males; male offspring with hormonally primed stimulus females) during weeks 2 and 3, but data were not collected. During experience weeks, experimental animals were partnered with stimulus animals for 30 minutes during which time sexual behavior could occur. During week 4, the experimental animals were tested for sexual behavior (female offspring with intact, adult stimulus males; male offspring with hormonally primed stimulus females),

and the test was recorded and scored. In week 5, the animal's final partner preference was evaluated.

Analysis

The data for the behavioral measures during the partner preference tests were analyzed using a 3 x 2 (LG treatment group x initial or final test) ANOVA with repeated measures on the second factor. Preference scores and duration of time spent in the male chamber (for experimental female offspring) or female chamber (for experimental male offspring) during the partner preference tests were also analyzed within each LG treatment group using a one-sample t-test. This test was used to determine if the animals showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the stimulus animal above chance (duration = 400 seconds). The data for the behavioral measures during the sexual behavior tests were analyzed using a one way ANOVA by LG treatment group.

RESULTS

Female Offspring

Partner Preference: The time spent in the stimulus male chamber was affected by a week of test by LG treatment interaction (Figure 5.1; [$F(2,70) = 3.5$, $p<0.036$]). The Low LG females increased the amount of time spent with the stimulus male from initial to final test, but there was no change in duration for the Mid or High LG females. Also, in the final partner preference test, the Low LG females spent more time in the male chamber

than did the Mid LG females. The data for time spent in the male chamber were subjected to a square root transformation.

The sexual experience received between the partner preference tests affected the latency to enter the female chamber [$F(1,70)=14.6$, $p<0.001$], latency to enter the male chamber [$F(1,70)=21.5$, $p<0.001$], and the display by the stimulus male of mounts [$F(1,70)=7.2$, $p=0.009$], intromissions [$F(1,70)=32.2$, $p<0.001$], and ejaculations [$F(1,70)=55.9$, $p<0.001$]. Regardless of LG treatment, the female offspring decreased from initial to final test the time it took to enter both the female stimulus chamber ($\bar{X}$ ± SEM: all measures are in seconds: 24.5 ± 3.1 vs 12.7 ± 1.9) and the male stimulus chamber ($\bar{X}$ ± SEM: 20.2 ± 2.3 vs 8.8 ± 1.2). The stimulus male also increased from initial to final test the number of mounts ($\bar{X}$ ± SEM: 1.5 ± 0.2 vs 2.7 ± 0.4), intromissions ($\bar{X}$ ± SEM: 3.8 ± 0.4 vs 8.1 ± 0.9), and ejaculations ($\bar{X}$ ± SEM: 0.4 ± 0.1 vs 1.3 ± 0.1) shown with the experimental female offspring.

In both the initial and final partner preference tests, all three treatment groups had a negative preference score significantly different from chance, indicating a preference for the male stimulus animal (Table 5.1). Also, the duration of time spent in the male chamber was significantly greater than chance for the Mid LG and High LG females in both the initial and final tests, and greater than chance for the Low LG females in the final test (Table 5.1).

Female Sexual Behavior: Lordosis quotient was the only behavior measured in the female sexual behavior tests that was altered by maternal LG (Figure 5.2; [F(2,58)=10.2, p<0.001]). The Low LG females had a significantly lower LQ than did the Mid or High LG females, which did not differ from each other.

Male Offspring

Partner Preference: No behaviors measured were affected by maternal LG in the male partner preference tests. However, some measures, regardless of LG treatment, were affected by the sexual experience received between the partner preference tests. All males had a longer latency to enter the female chamber during the initial test than during the final test ($\bar{X}$ ± SEM: 29.3 ± 4.2 vs 14.2 ± 1.5; [F(1,56)=12.1, p=0.001]). They also increased the time spent in the stimulus female chamber ($\bar{X}$ ± SEM: 556.3 ± 20.1 vs 723.0 ± 31.8; [F(1,56)=29.6, p<0.001]) and decreased the time spent in the stimulus male chamber ($\bar{X}$ ± SEM: 331.0 ± 19.5 vs 139.5 ± 18.0; [F(1,56)=71.6, p<0.001]) from initial to final partner preference test. The preference score shown by the experimental male offspring increased from initial to final test ($\bar{X}$ ± SEM: 225.3 ± 35.7 vs 583.5 ± 45.9; [F(1,56)=55.9, p<0.001]). Finally, the male offspring increased the number of mounts ($\bar{X}$ ± SEM: 2.4 ± 0.5 vs 7.9 ± 1.0; [F(1,56)=37.9, p<0.001]), intromissions ($\bar{X}$ ± SEM: 6.0 ± 1.2 vs 16.2 ± 1.4; [F(1,56)=48.4, p<0.001]), and ejaculations ($\bar{X}$ ± SEM: 0.2 ± 0.1 vs 1.3 ± 0.1; [F(1,56)=56.2, p<0.001]) shown with the stimulus female from initial to final test.

In the initial partner preference test, both the Mid and High LG males had a positive preference score significantly different from chance, indicating a preference for the female stimulus animal. In the final partner preference test, all three groups had a positive preference score significantly different from chance (Table 5.2). Also, the duration of time spent in the female chamber was significantly greater than chance for all three groups in both the initial and final tests (Table 5.2).

Male Sexual Behavior: Maternal LG did not affect any behaviors measured in the male sexual behavior test.

Table 5.1. One-sample t-test analyses of preference scores and duration of time spent in the male chamber during the female offspring partner preference tests. This analysis was used to determine if the female offspring showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the stimulus male above chance (duration = 400 sec). All three treatment groups showed a preference for the male stimulus animal. Also, the Mid LG and High LG females (initial and final tests) and the Low LG females (final test only) spent more time in the male chamber than was expected by chance. *Significantly different from chance, $p<0.01$. **Significantly different from chance, $p<0.05$.

	Preference Score (Chance = 0)				**Duration in Male Chamber (Chance = 400)**			
	Initial		**Final**		**Initial**		**Final**	
	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value
Low LG	-89.8 (23.3)	-3.8*	-285.3 (36.2)	-7.9*	430.7 (21.3)	1.4	561.7 (25.2)	6.4*
Mid LG	-154.1 (32.4)	-4.8*	-152.3 (33.8)	-4.5*	466.5 (24.7)	2.7**	453.7 (22.8)	2.3**
High LG	-177.5 (38.8)	-4.6*	-205.0 (53.4)	-3.8*	490.4 (29.8)	3.0*	505.6 (31.2)	3.4 *

Table 5.2. One-sample t-test analyses of preference scores and duration of time spent in the female chamber during the male offspring partner preference tests. This analysis was used to determine if the male offspring showed a preference for one stimulus animal above chance (preference score = 0) or spent more time with the stimulus female above chance (duration = 400 sec). The Mid LG and High LG males (initial and final test) and Low LG males (final test only) showed a preference for the stimulus female. All three treatment groups spent more time in the stimulus female chamber than expected by chance. *Significantly different from chance, $p<0.01$. **Significantly different from chance, $p<0.05$.

	Preference Score (Chance = 0)				**Duration in Female Chamber (Chance = 400)**			
	Initial		**Final**		**Initial**		**Final**	
	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value	Mean (SEM)	t value
Low LG	146.6 (83.7)	1.8	527.1 (116.1)	4.5*	499.3 (40.3)	2.5**	669.5 (81.2)	3.3*
Mid LG	349.9 (49.1)	7.1*	610.1 (52.0)	11.7*	623.7 (30.4)	7.4*	738.8 (37.2)	9.1*
High LG	195.5 (53.2)	3.7*	613.4 (83.2)	7.4*	556.6 (29.4)	5.3*	760.7 (53.7)	6.7*

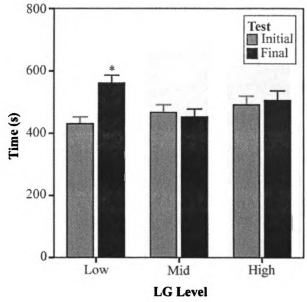

Figure 5.1. Time female offspring spent in the stimulus male chamber during the partner preference tests. The Low LG females increased the amount of time spent with the male from initial to final test. *Significantly different from Low LG initial test and Mid LG final test.

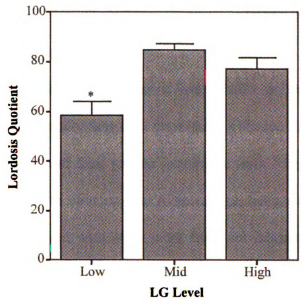

Figure 5.2. Lordosis quotient of the female offspring during the female sexual behavior test. Low LG females displayed a lower LQ than did Mid or High LG females. *Significantly different from Mid and High LG.

DISCUSSION

Female Offspring

All females, regardless of level of maternal care they received, showed preference scores significantly less than expected by chance, indicating a preference for the male in both initial and final partner preference tests. Therefore, maternal behavior does not seem to affect the development of partner preference in female offspring. This lack of effect is in agreement with other work from our laboratory. Cummings et al (2008) did not find a correlation between amount of LG and female partner preference scores when analyzed within PCB treatment groups.

However, the level of maternal licking and grooming did affect one measure in the female offspring partner preference test. Females that received low levels of LG increased the amount of time spent in the stimulus male chamber from initial to final test, and during the final test the Low LG females spent more time with the male than did the Mid LG females. The Low LG females also did not spend more time than was expected by chance with the stimulus male during the initial test. The Mid and High LG females did not increase time spent with the male over testing, and spent more time than expected by chance with the stimulus male in both preference tests.

It seems that the sexual behavior experience during the female sexual behavior tests affected the amount of time the Low LG females spent with the stimulus male. It is interesting to note that the Low LG females showed less receptivity with the stimulus male during the female sexual behavior test (see below), yet the experience led to an

increase in the time spent with the male during the final partner preference test. It appears that even though the LG females were less likely to display lordosis in response to the stimulus males, the females still found the experience to be rewarding, leading to increased time spent with the male. Increases in the amount of time spent with a male after sexual experience has been seen in other studies (Clark et al., 2004; Matuszczyk and Larsson, 1991), and in the present study the experience received seem to affect the behavior of the Low LG females.

Maternal licking and grooming also altered female offspring receptivity to a sexually active male. Female offspring of Low LG mothers exhibited a lower lordosis quotient during the female sexual behavior test than did offspring of either Mid or High LG mothers, who did not differ from each other. In contrast to our findings, Cameron et al (2008) reported that female offspring from High LG dams displayed lower lordosis quotients than Low LG female offspring. However, there are significant methodological differences between the two studies. Cameron et al (2008) measure maternal behavior for five 75-minute daily observations on PND 1 through 6, whereas in the present study, maternal behavior is observed for last hour of the light phase and the first hour of the dark phase of the light-dark cycle on PND 1, 2, 4, and 6. Also, we observed female sexual behavior after ovariectomy and hormone replacement. Cameron et al (2008) tests for female sexual behavior in intact animals. These methodological differences make comparisons between two studies difficult.

The experience received between the two partner preference tests also affected a number of measures. Regardless of level of maternal LG received during development, all females decreased their latency to enter the stimulus female and stimulus male chambers from initial to final test. The females also received more mounts, intromissions, and ejaculations from the stimulus male during the final test than during the initial. As mentioned earlier, sexual experience has been shown in other studies to affect behaviors seen in partner preference tests (Clark et al., 2004; Matuszczyk and Larsson, 1991). Also, since the female offspring were sexually naïve in the initial partner preference test, it is apparent that sexual experience for the females was necessary for the display of full copulatory behavior.

Male Offspring

No behaviors measured in the male offspring partner preference tests were altered by the level of maternal licking and grooming during development. Also, in both preference tests, all three groups spent more time with the stimulus female than was expected by chance. In the initial test, the Mid and High LG males showed preference scores significantly higher than chance, indicating a preference for the female, and during the final test all three groups showed preference for the female. Maternal LG, therefore, does not appear to play a role in the development of adult partner preference in male offspring.

Maternal LG also did not affect any measures of male sexual behavior. This is in agreement with other studies that show no effect of maternal LG on male sexual behaviors (Cameron et al., 2008).

However, regardless of level of maternal LG received during development, a number of measures in the partner preference test were altered by an effect of experience. The male offspring decreased their latency to enter the female chamber, increased the amount of time spent with the stimulus female, decreased the time spent with the stimulus male, and thereby increased their preference scores from initial to final test. The male offspring also showed an increase in sexual behavior from initial to final partner preference test. The males displayed more mounts, intromissions, and ejaculations in the final test. As mentioned in previous chapters, these changes can be explained by the sexual experience the males received between the two partner preference tests. Sexual experience has been shown to lead to increased preference for a female over a male stimulus animal (Matuszczyk and Larsson, 1994). Similar to the females, the male offspring were sexually naïve during the initial partner preference tests, and sexual experience must have been necessary for the males to show copulatory behavior.

Overall

Maternal licking and grooming had little effect on the display of partner preference in female and male offspring. Regardless of level of LG, female offspring continued to show a preference for the stimulus male, and male offspring continued to show a preference for the stimulus female. Maternal LG also did not alter any male sexual behavior measure. However, females that received low levels of LG did show a reduced lordosis quotient when paired with a stimulus male. Therefore, it appears that maternal licking and grooming plays no role in the development of adult partner preference in the laboratory rat.

CHAPTER SIX:

GENERAL DISCUSSION

In the studies reported in this dissertation, early postnatal hormone treatments altered adult partner preference in the laboratory rat. In females, early postnatal exogenous estradiol masculinized female-typical partner preference and sexual behavior. Females treated early in development with estradiol preferred to spend more time with an estrous stimulus female and less time with a stimulus male compared to cholesterol-treated females. Compared to control females, females exposed to estradiol postnatally also showed increased male-like sexual behavior when paired with a stimulus female. In males, treatment with testosterone during the early postnatal period led to an altered partner preference in which the testosterone-treated males spent more time with a stimulus male than did cholesterol-treated males. Early postnatal testosterone treatment also disrupted the display of normal male sexual behavior. The effect of early postnatal estradiol treatment in males is difficult to analyze due to the impaired health status of the animals. However, all postnatally estradiol-treated males did show disrupted male sexual behavior. Finally, these postnatal hormone changes in adult partner preference were not mediated by maternal behavior changes since altered maternal licking and grooming did not affect sex-typical adult partner preference expression. These novel findings increase the significance of the broadening literature on the role of perinatal hormones on the development of partner preference. As reviewed in Chapter One, perinatal hormones play a role in the development of adult partner preference in a number of mammalian species (i.e. rat, ferret, mouse, pig, hamster, dog, human).

The development of human sexual orientation is obviously a more complicated process than that seen in animal models. However, research on laboratory animals can still provide insight and be applicable to the human. Understanding the endocrine components and neural factors that play a role in sexual motivation and partner preference across mammalian species, can also have implications for understanding the basis of human orientation.

The masculinizing effects of early estradiol on the display of adult partner preference in the female rat can be associated with the effect of prenatal DES on the increased incidence of homosexuality and bisexuality in exposed women (Ehrhardt et al., 1985; Meyer-Bahlburg et al., 1995). However, the organizational effects of estrogen on human sexual orientation needs further evaluation since XY individuals who carry a dysfunctional ERα or aromatase gene self report as heterosexual males (for review, Baum, 2006). The effects of early postnatal testosterone on the expression of partner preference in the male rat lends further support to the idea that hyper-androgenization during early development can lead to a male-oriented sexual preference. Having older brothers increases a male's chance of being homosexual (Blanchard and Bogaert, 1996) and also leads to digit length ratios that are more masculine than controls (Williams et al., 2000). It appears that males with older brothers are exposed to increased levels of androgen prenatally.

In addition to the notion that hyper-androgenization affects adult sexual orientation, there are alternative explanations for the fraternal birth order effect that should be discussed.

For example, one explanation may be that a younger brother has an increased chance of being homosexual due to the psychosocial environment in which he grows up. Perhaps the influence of older brothers leads to a male-oriented preference in a younger brother, either due to teasing, fighting, or some other measure. However, it has been shown that the fraternal birth order effect applies only to brothers who share the same mother, i.e. not brothers with the same father only and not step-brothers (Bogaert, 2006). In addition, biological brothers with the same mother who are raised separately also show the fraternal birth order effect on homosexuality (Bogaert, 2006). If an increased probability of homosexuality in younger brothers was due to a social cause, it should be seen in boys who have older step-brothers as well and not seen in younger brothers who are raised apart from older brothers.

Parental age effects have also been suggested as an alternative explanation. Parents are naturally older when younger sons are born compared to older sons, and homosexuality may not be associated with birth order but with parental age. However, statistical analyses indicate that the fraternal birth order effects still remain even after controlling for both paternal and maternal age (Blanchard and Bogaert, 1996).

One further alternate explanation is the maternal immunization hypothesis. It has been suggested that a mother carrying a son could have an immune reaction to a Y-linked minor histocompatibility antigen, known as H-Y antigen (Blanchard, 2001; Blanchard and Bogaert, 1996). Once the mother is exposed to the male-specific proteins, she produces antibodies against the H-Y antigen. Subsequent births cause stronger immune

reactions, and later sons are exposed (through the placenta) to higher levels of the maternal antibodies (Blanchard, 2001; Blanchard and Bogaert, 1996). These maternal antibodies could then bind the fetal H-Y antigens, possibly preventing neural development in a male-typical fashion (Blanchard, 2001).

The maternal immunization hypothesis would provide an explanation for why only older brothers, and not older sisters, have an effect on the orientation of younger brothers. Since female fetuses do not produce any proteins foreign in nature to the mother, no maternal antibodies would be made. However, the H-Y antigen theory needs to be examined further as it does not provide an explanation for why homosexual men are hyper-masculine on some measures such as digit length ratio (Rahman, 2005; Rahman and Wilson, 2003; Robinson and Manning, 2000), genitalia size (Bogaert and Hershberger, 1999), and auditory evoked potentials (McFadden and Champlin, 2000). Much still remains to be determined about the mechanisms underlying the fraternal birth order effect.

Neural Control

The medial preoptic area (MPOA) of the hypothalamus is an essential brain area for the expression of male sexual behaviors (Hull et al., 2002; Meisel and Sachs, 1994). Lesions directed to the MPOA lead to deficits in male copulatory behavior in a number of species (e.g. rat, mice, guinea pig, gerbil, monkey, dog, cat; for review, Hull et al., 2002). Electrical stimulation of the MPOA, on the other hand, increases the frequency of ejaculations in a 30 min test, as well as decreases latency to ejaculation, the

postejaculatory interval, and the number of intromissions prior to an ejaculation in male rats (Rodriguez-Manzo et al., 2000).

The MPOA may also be critical for the display of adult partner preference behavior. Studies in sheep have provided an interesting animal model in which to study neural control of partner preference because a small percentage (7-9%) of rams are innately male-oriented, and no hormone manipulations are needed for the males to display a same-sex preference (Roselli et al., 2004). Male-oriented rams appear to have received levels of testosterone perinatally equivalent to those of female-oriented rams. In ewes, an estradiol injection will cause a surge of luteinizing hormone (LH), but in rams, regardless of orientation, no estradiol-mediated LH surge is seen(Perkins et al., 1995). This implies that defeminization has occurred in the male-oriented rams. Also, male-oriented rams do not show any female-like sexual behaviors even after adult castration and treatment with ovarian hormones (Stormshak et al., 2008).

Studies in sheep may implicate specific areas of the brain in the expression of sex-typical partner preference, as there are some anatomical and functional difference in neural circuitry between male- and female-oriented rams. The ovine sexually dimorphic nucleus (oSDN) of the MPOA is two times larger in female-oriented rams than male-oriented rams (Roselli et al., 2004). Also, in the oSDN aromatase activity (Resko et al., 1996) and aromatase mRNA (Roselli et al., 2004) have been shown to be lower in male-oriented rams than in female-oriented rams. The male-oriented rams, therefore, may experience

lower than normal levels of estrogenic exposure during developmental critical periods compared with female-oriented rams.

In addition to the sheep, the MPOA also seems important for the display of sex-typical adult partner preference in other animal models. In ferrets, electrolytic lesions of the male's MPOA led to female-like approach behavior to stimulus males, and approach latencies decreased in a dose-dependent manner to increasing levels of adult estradiol treatment (Cherry and Baum, 1990). Also, lesions in the male ferret MPOA caused males to prefer to spend time with a stimulus male over a stimulus female after adult estradiol treatment (Kindon et al., 1996; Paredes and Baum, 1995) and to prefer male odors over estrous female odors (Baum, 2006).

Similar findings are seen when examining rat behavior. Male rats castrated in adulthood, treated with either testosterone or estradiol, and given bilateral MPOA lesions prefer to approach a stimulus male over an estrous female (Paredes et al., 1998). Other studies of males with MPOA lesions also show a decrease in the preference for a female (Edwards and Einhorn, 1986; Edwards et al., 1996; Paredes et al., 1993). Sham-operated control males continue to show a male-typical preference for the female. In addition, males administered lidocaine into the MPOA show a decreased preference for the female (Hurtazo et al., 2008). Also, in male rats treated neonatally with ATD, SDN-POA volume correlates positively with male-typical copulatory behavior and partner preference for a female (Houtsmuller et al., 1994). Further indication that the MPOA is important for the expression of partner preference can be found in the maternal behavior literature. Prenatal

stress leads to a smaller SDN volume in male offspring (Kerchner et al., 1995), and prenatal stress has also been shown to decrease preference for a stimulus female compared to that shown by unstressed controls (Wang et al., 2006).

The hypothalamus also may be involved in human sexual orientation, but the data are slightly more complicated than those found in the animal model literature. The interstitial nuclei of the anterior hypothalamus region 3 (INAH3) has been found to be sexually dimorphic with men having a larger volume than women (Allen et al., 1989; Levay, 1991). It was also discovered that homosexual men have a smaller INAH3 than do heterosexual men (Levay, 1991). Another study (Byne et al., 2001) tried to replicate these findings, however, and only found a non-significant trend for homosexual men to have a smaller INAH3 than heterosexual men. One criticism of the Levay (1991) study is that nearly all of his homosexual subjects had HIV/AIDS, and therefore he could not rule out the possibility that the disease had an effect on INAH3 size (Baum, 2006). However, in the follow-up study by Byne (2001), it was determined that HIV/AIDS did not have an effect on the size of the region in either homosexual or heterosexual subjects. The INAH1 region is also sexually dimorphic, but no anatomical differences were seen between homosexual and heterosexual men in this region (Swaab and Fliers, 1985). However, other studies were unable to replicate the finding that there exists a sexual dimorphism in the INAH1 (Allen et al., 1989; Levay, 1991).

Future Directions

As noted earlier, the MPOA is a critical brain region for the display of male sexual behavior, and possibly for the expression of male-typical partner preference. However, the majority of these studies are based on correlations between size of the nucleus or effect of lesion on behavior. Further research is needed to provide a direct indication that the MPOA is involved in preference, especially since some data are conflicting. Although many studies show an alteration in interest in a stimulus female after MPOA lesion, this is not always the case (Everitt and Stacey, 1987; Hughes et al., 1990). It should also be noted, however, that MPOA lesions tend to decrease a male's preference for a female but not eliminate it completely (Dominguez and Hull, 2005; Everitt, 1990). One possible way to further link the MPOA to partner preference would be to measure dopamine in the MPOA.

Within the MPOA, the neurotransmitter dopamine (DA) may be critical for the expression of male sexual behavior, particularly in the rat, and may also facilitate the display of partner preference. Systemic administration of a DA receptor agonist increases copulatory behavior in male rats, whereas administration of a DA antagonist has been shown to decrease mount, intromission and ejaculation frequency (for review, Dominguez and Hull, 2005). Also, after administration of a DA antagonist, male rats placed in a bilevel chamber, which can be used to determine sexual motivation, are less likely to display anticipatory behavior (e.g level changes) prior to the introduction of a stimulus female (Pfaus and Phillips, 1991).

DA agonists administered directly into the MPOA have effects similar to systemic administration, as do antagonists. Apomorphine, a DA agonist, administered in the MPOA increases the frequency of ejaculations while decreasing the latency to an ejaculation and the postejaculatory interval (Hull et al., 1986). DA antagonists administered directly into the MPOA, however, impair male sexual behavior (Pehek et al., 1988) and decrease a male's preference for an estrous female in an X-maze goal box paradigm (Moses et al., 1995; Warner et al., 1991). DA in the MPOA has also been shown to increase *in vivo* when a male is allowed to see, hear, and smell a female (Hull et al., 1995). This increase in central DA was not seen when the male was exposed to a male or a running wheel (Hull et al., 1995), indicating DA release occurs in response to only a female.

I suggest analyzing DA release in the MPOA of early estrogenized females to possibly provide further support for the idea that the MPOA influences partner preference. As noted above, a normal male will show an increase in extracellular DA concentration in the MPOA after being presented with a female (Hull et al., 1995). A normal female, however, does not appear to show any precopulatory rise in DA in the MPOA (Matuszewich et al., 2000). The early estrogenized female show a clear partner preference for a stimulus female. It is likely that the early estrogenized females would show an increase in DA release in the MPOA when exposed to a receptive female, but not when exposed to a stimulus male, similar to a control male, indicating their brain and their preference have been masculinized. If however, there is no change in DA release in

the MPOA in these female, that may imply that the MPOA is not a critical brain region for partner preference.

Based on the assumption that DA concentration would increase in the MPOA of early estrogenized females after presentation of a stimulus female, further tests could be undertaken. The hyper-androgenized males did not show a clear preference for either stimulus animal, so there are a number of possible outcomes for an MPOA DA test. 1) The males could show DA release in response to both stimulus animals, indicating a bisexual preference and provide an explanation for why no preference was seen in the partner preference paradigm. 2) The males could show DA release in response to the male stimulus animal, but for some reason preference was not clear in the partner preference paradigm. 3) The males could show DA release in response to the female stimulus animal, but for some reason preference was not clear in the partner preference paradigm. 4) The males could show no DA release in response to both stimulus animals. This outcome could indicate one of two things: a) The males are not interested in either stimulus animal (asexual), or b) The males may have a feminized MPOA DA system, and as mentioned above, normal females do not show a pre-copulatory rise in DA when exposed to a male.

Non-dopamine studies would also be useful to further understand the role of the MPOA in the display of partner preference. The studies in sheep and ATD-treated rats, imply that the estrogenic organization of the MPOA during development leads to male-typical partner preference behavior in adulthood. Male-oriented rams have lower aromatase

activity in the oSDN (Resko et al., 1996; Roselli et al., 2004), thereby receiving less estrogen during early life.

Male-oriented rams also have a smaller oSDN than female-oriented rams (Roselli et al., 2004). It is acceptable to assume that the estrogenized females would have a larger SDN than control females given that other studies have shown that neonatal estrogen treatment increases the size of the SDN in female rats (Tarttelin and Gorski, 1988), and neonatal estrogen antagonist treatment decreases the size of the SDN in male rats (Dohler et al., 1986). However, it is not known what super-physiological levels of testosterone may do to the size of and aromatase activity level in the SDN of hyper-androgenized males. The effect of testosterone on aromatase activity is not well understood because the data are conflicting; testosterone has been shown to increase, decrease, or have no effect on aromatase activity (for review, Negri-Cesi et al., 2008). Environmental contaminants with steroid hormone-mimicking effects have been shown to alter aromatase in different ways depending on the dose used (Andrade et al., 2006). Perhaps physiological male-typical levels of testosterone lead to normal masculine aromatase activity, but super-physiological levels of testosterone down-regulate activity in the MPOA. Similar to the male-oriented rams that have lower aromatase activity in the MPOA than female-oriented rams (Resko et al., 1996; Roselli et al., 2004), perhaps the male-oriented hyper-androgenized males also have lower aromatase levels than control males.

It should be noted here that Tfm male rats, which do display a masculine partner preference (Hamson, personal communication), also have a smaller neuronal soma size

(Morris et al., 2005) and lower aromatase activity (Roselli et al., 1987) in the SDN than their wild-type brothers. However, these males have also been shown to have similar estrogen binding in the hypothalamus to their wild-type brothers (Olsen and Whalen, 1982). Tfm males also show higher levels of circulating estrogen than their wild-type brothers (Roselli et al., 1987), unlike the sheep (see Roselli et al., 2004) or the hyper-androgenized rats in the previous chapters, which both show normal levels of circulating estrogen. It is likely that the Tfm male rats show a male-typical partner preference because they are exposed to sufficient levels of estrogen during development, whereas male-oriented rams and hyper-androgenized male rats are exposed to lower levels.

It is likely that the MPOA is not the only brain region involved in regulating partner preference. However, few other regions have been studied. Further investigation of differences in the brain circuitry of male- and female-oriented rams may provide useful information into other areas that control preference. In the rat, lesions in the dorsolateral tegmentum (DLT) and central tegmental field (CTF) have no effect on the display of male partner preference, indicating these regions can be excluded from possible sites of control (Romero-Carbente et al., 2006; Romero-Carbente et al., 2007). The DLT and CTF regions are reciprocally connected with the MPOA and are implicated in the motor and sensory function of male sexual behavior, respectively (Hull et al., 2002). Lesions in the central zona incerta, a region with connections with the DLT that has been shown to be important for the display of male sexual behavior (Hull et al., 2002), also do not affect the expression of male partner preference (Edwards and Isaacs, 1991). In addition, dopamine antagonist administration into the nucleus accumbens, an area involved in the

reward pathway, do not affect male partner preference display (Moses et al., 1995). Many other regions remain to be studied. For example, Bodo and Rissman (2008) suggest that Tfm male mice may display a female-typical partner preference due to alterations in the accessory olfactory system, which mediates pheromonal cues. It is also possible that one brain region is not solely involved in the control of partner preference, and that a full neural circuit is necessary for the expression of sex-typical preference.

In addition to studies investigating the neural control of partner preference, more endocrine manipulation studies would also be useful. Studying the effects of a lower dose of early postnatal estrogen on male partner preference could proved insight into the effects of environmental contaminants on the sexual behavior of animals in the wild. Also, attempting to replicate the early postnatal testosterone studies in other species would lend more support to the partner preference altering effects of hyper-androgenization seen here.

Overall

A great deal is still unknown about the development of partner preferences in mammalian species; however, steroid hormone exposure during development is arguably an important factor. More research is needed both in animals models and humans to tease apart the neuroendocrine basis of preference. Humans, ultimately, stand separate from other animal models as more than simply preference needs to be taken into account (e.g. gender identity), but the importance of understanding the mechanisms of the development of partner preference in other animal models cannot be understated.

REFERENCES

Adkins-Regan, E., 1988. Sex hormones and sexual orientation in animals. Psychobiology. 16, 335-347.

Adkins-Regan, E., Orgeur, P., Signoret, J. P., 1989. Sexual differentiation of reproductive behavior in pigs - defeminizing effects of prepubertal estradiol. Hormones And Behavior. 23, 290-303.

Allen, L. S., Hines, M., Shryne, J. E., Gorski, R. A., 1989. Two Sexually Dimorphic Cell Groups In The Human-Brain. Journal Of Neuroscience. 9, 497-506.

Andrade, A. J. M., Grande, S. W., Talsness, C. E., Grote, K., Chahoud, I., 2006. A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): Non-monotonic dose-response and low dose effects on rat brain aromatase activity. Toxicology. 227, 185-192.

Atanassova, N., McKinnell, C., Walker, M., Turner, K. J., Fisher, J. S., Morley, M., Millar, M. R., Groome, N. P., Sharpe, R. M., 1999. Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, sertoli cell number, and the efficiency of spermatogenesis in adulthood. Endocrinology. 140, 5364-5373.

Bakker, J., 2003. Sexual differentiation of the neuroendocrine mechanisms regulating mate recognition in mammals. Journal Of Neuroendocrinology. 15, 615-621.

Bakker, J., Brand, T., Vanophemert, J., Slob, A. K., 1993a. Hormonal regulation of adult partner preference behavior in neonatally ATD-treated male rats. Behavioral Neuroscience. 107, 480-487.

Bakker, J., Honda, S., Harada, N., Balthazart, J., 2002. Sexual partner preference requires a functional aromatase (Cyp19) gene in male mice. Hormones And Behavior. 42, 158-171.

Bakker, J., Honda, S., Harada, N., Balthazart, J., 2004. Restoration of male sexual behavior by adult exogenous estrogens in male aromatase knockout mice. Hormones And Behavior. 46, 1-10.

Bakker, J., Vanophemert, J., Slob, A. K., 1993b. Organization of partner preference and sexual behavior and its nocturnal rhythmicity in male rats. Behavioral Neuroscience. 107, 1049-1058.

Bakker, J., vanOphemert, J., Slob, A. K., 1996. Sexual differentiation of odor and partner preference in the rat. Physiology & Behavior. 60, 489-494.

Bakker, J., Vanophemert, J., Timmerman, M. A., Dejong, F. H., Slob, A. K., 1995. Endogenous reproductive hormones and nocturnal rhythms in partner preference and sexual behavior of ATD-treated male rats. Neuroendocrinology. 62, 396-405.

Banks, A., Gartrell, N. K., 1995. Hormones and sexual orientation - a questionable link. Journal Of Homosexuality. 28, 247-268.

Barraclough, C. A., Gorski, R. A., 1962. Studies on mating behaviour in androgen-sterilized female rat in relation to hypothalamic regulation of sexual behaviour. Journal Of Endocrinology. 25, 175-182.

Baum, M. J., 1979. Differentiation Of Coital Behavior In Mammals - Comparative-Analysis. Neuroscience And Biobehavioral Reviews. 3, 265-284.

Baum, M. J., 2006. Mammalian animal models of psychosexual differentiation: When is 'translation' to the human situation possible? Hormones And Behavior. 50, 579-588.

Baum, M. J., Erskine, M. S., Kornberg, E., Weaver, C. E., 1990. Prenatal And Neonatal Testosterone Exposure Interact To Affect Differentiation Of Sexual-Behavior And Partner Preference In Female Ferrets. Behavioral Neuroscience. 104, 183-198.

Baum, M. J., Schretlen, P., 1975. Neuroendocrine effects of perinatal androgenization in the male ferret. Progress in Brain Reserach. 42, 343-355.

Baum, M. J., Tobet, S. A., 1986. Effect Of Prenatal Exposure To Aromatase Inhibitor, Testosterone, Or Antiandrogen On The Development Of Feminine Sexual-Behavior In Ferrets Of Both Sexes. Physiology & Behavior. 37, 111-118.

Beach, F. A., Holz, A. M., 1946. Mating Behavior In Male Rats Castrated At Various Ages And Injected With Androgen. Journal Of Experimental Zoology. 101, 91-142.

Beach, F. A., Johnson, A. I., Anisko, J. J., Dunbar, I. F., 1977. Hormonal-Control Of Sexual Attraction In Pseudohermaphroditic Female Dogs. Journal Of Comparative And Physiological Psychology. 91, 711-715.

Beach, F. A., Noble, R. G., Orndoff, R. K., 1969. Effects Of Perinatal Androgen Treatment On Responses Of Male Rats To Gonadal Hormones In Adulthood. Journal Of Comparative And Physiological Psychology. 68, 490-497.

Bixo, M., Backstrom, T., 1990. Regional Distribution Of Progesterone And 5-Alpha-Pregnane-3,20-Dione In Rat-Brain During Progesterone-Induced Anesthesia. Psychoneuroendocrinology. 15, 159-162.

Blanchard, R., 2001. Fraternal birth order and the maternal immune hypothesis of male homosexuality. Hormones And Behavior. 40, 105-114.

Blanchard, R., Bogaert, A. F., 1996. Homosexuality in men and number of older brothers. American Journal Of Psychiatry. 153, 27-31.

Blaustein, J. D., 2008. Neuroendocrine regulation of feminine sexual behavior: Lessons from rodent models and thoughts about humans. Annual Review Of Psychology. 59, 93-118.

Bodo, C., Rissman, E. F., 2007. Androgen receptor is essential for sexual differentiation of responses to olfactory cues in mice. European Journal Of Neuroscience. 25, 2182-2190.

Bodo, C., Rissman, E. F., 2008. The androgen receptor is selectively involved in organization of sexually dimorphic social behaviors in mice. Endocrinology. 149, 4142-4150.

Bogaert, A. F., 2006. Biological versus nonbiological older brothers and men's sexual orientation. Proceedings Of The National Academy Of Sciences Of The United States Of America. 103, 10771-10774.

Bogaert, A. F., Hershberger, S., 1999. The relation between sexual orientation and penile size. Archives Of Sexual Behavior. 28, 213-221.

Brand, T., Kroonen, J., Mos, J., Slob, A. K., 1991. Adult Partner Preference And Sexual-Behavior Of Male-Rats Affected By Perinatal Endocrine Manipulations. Hormones And Behavior. 25, 323-341.

Brand, T., Slob, A. K., 1991a. Neonatal Organization Of Adult Partner Preference Behavior In Male-Rats. Physiology & Behavior. 49, 107-111.

Brand, T., Slob, A. K., 1991b. On The Organization Of Partner Preference Behavior In Female Wistar Rats. Physiology & Behavior. 49, 549-555.

Breedlove, S. M., Arnold, A. P., 1980. Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord. Science. 210, 564-566.

Breedlove, S. M., Arnold, A. P., 1983. Hormonal control of a developing neuromuscular system 2. sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. Journal Of Neuroscience. 3, 424-432.

Breedlove, S. M., Jacobson, C. D., Gorski, R. A., Arnold, A. P., 1982. Masculinization of the female rat spinal cord following a single neonatal injection of testosterone propionate but not estradiol benzoate. Brain Research. 237, 173-181.

Brown, W. M., Hines, M., Fane, B. A., Breedlove, S. M., 2002. Masculinized finger length patterns in human males and females with congenital adrenal hyperplasia. Hormones And Behavior. 42, 380-386.

Byne, W., Tobet, S., Mattiace, L. A., Lasco, M. S., Kemether, E., Edgar, M. A., Morgello, S., Buchsbaum, M. S., Jones, L. B., 2001. The interstitial nuclei of the human anterior hypothalamus: An investigation of variation with sex, sexual orientation, and HIV status. Hormones And Behavior. 40, 86-92.

Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P. M., Meaney, M. J., 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proceedings Of The National Academy Of Sciences Of The United States Of America. 95, 5335-5340.

Cameron, N. M., Fish, E. W., Meaney, M. J., 2008. Maternal influences on the sexual behavior and reproductive success of the female rat. Hormones And Behavior. 54, 178-184.

Cantor, J. M., Blanchard, R., Paterson, A. D., Bogaert, A. F., 2002. How many gay men owe their sexual orientation to fraternal birth order? Archives Of Sexual Behavior. 31, 63-71.

Champagne, F., Diorio, J., Sharma, S., Meaney, M. J., 2001. Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. Proceedings Of The National Academy Of Sciences Of The United States Of America. 98, 12736-12741.

Champagne, F. A., Meaney, M. J., 2006. Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. Biological Psychiatry. 59, 1227-1235.

Champagne, F. A., Weaver, I. C. G., Diorio, J., Dymov, S., Szyf, M., Meaney, M. J., 2006. Maternal care associated with methylation of the estrogen receptor-alpha 1b promoter and estrogen receptor-alpha expression in the medial preoptic area of female offspring. Endocrinology. 147, 2909-2915.

Cherry, J. A., Baum, M. J., 1990. Effects of Lesions of a Sexually Dimorphic Nucleus in the Preoptic Anterior Hypothalamic Area on the Expression of Androgen-Dependent and Estrogen-Dependent Sexual Behaviors in Male Ferrets. Brain Research. 522, 191-203.

Cihak, R., Gutmann, E., Hanzliko.V, 1970. Involution and hormone-induced persistence of muscle sphincter (levator) ani in female rats. Journal of Anatomy. 106, 93-&.

Clark, A. S., Kelton, M. C., Guarraci, F. A., Clyons, E. Q., 2004. Hormonal status and test condition, but not sexual experience, modulate partner preference in female rats. Hormones And Behavior. 45, 314-323.

Clemens, L. G., 1970. Androgen And Development Of Progesterone Responsiveness In Male And Female Rats. Physiology & Behavior. 5, 673-678.

Clemens, L. G., Gladue, B. A., 1978. Feminine Sexual-Behavior In Rats Enhanced By Prenatal Inhibition Of Androgen Aromatization. Hormones And Behavior. 11, 190-201.

Cummings, J. A., Clemens, L. G., Nunez, A. A., 2008. Exposure to PCB 77 affects partner preference but not sexual behavior in the female rat. Physiology & Behavior. 95, 471.

Cummings, J. A., Nunez, A. A., Clemens, L. G., 2005. A cross-fostering analysis of the effects of PCB 77 on the maternal behavior of rats. Physiology & Behavior. 85, 83-91.

Dail, W. G., Evan, A. P., Eason, H. R., 1975. Major ganglion in pelvic pexus of male rat - histochemical and ultrastructural study. Cell And Tissue Research. 159, 49-62.

Damassa, D. A., Smith, E. R., Tennent, B., Davidson, J. M., 1977. Relationship Between Circulating Testosterone Levels And Male Sexual-Behavior In Rats. Hormones And Behavior. 8, 275-286.

Davidson, J. M., Levine, S., 1969. Progesterone And Heterotypical Sexual Behaviour In Male Rats. Journal Of Endocrinology. 44, 129-130.

Dejonge, F. H., Eerland, E. M. J., Vandepoll, N. E., 1986. The influence of estrogen, testosterone and progesterone on partner preference, receptivity and proceptivity. Physiology & Behavior. 37, 885-891.

Dejonge, F. H., Muntiewerff, J. W., Louwerse, A. L., Vandepoll, N. E., 1988. Sexual-Behavior And Sexual Orientation Of The Female Rat After Hormonal Treatment During Various Stages Of Development. Hormones And Behavior. 22, 100-115.

Delbes, G., Duquenne, C., Szenker, J., Taccoen, J., Habert, R., Levacher, C., 2007. Developmental changes in testicular sensitivity to estrogens throughout fetal and neonatal life. Toxicological Sciences. 99, 234-243.

Delbes, G., Levacher, C., Habert, R., 2006. Estrogen effects on fetal and neonatal testicular development. Reproduction. 132, 527-538.

Diamond, M., Llacuna, A., Wong, C. L., 1973. Sex Behavior After Neonatal Progesterone, Testosterone, Estrogen, Or Antiandrogens. Hormones And Behavior. 4, 73-88.

Dohler, K. D., Coquelin, A., Davis, F., Hines, M., Shryne, J. E., Sickmoller, P. M., Jarzab, B., Gorski, R. A., 1986. Prenatal And Postnatal Influence Of An Estrogen Antagonist And An Androgen Antagonist On Differentiation Of The Sexually Dimorphic Nucleus Of The Preoptic Area In Male And Female Rats. Neuroendocrinology. 42, 443-448.

Dominguez, J. M., Hull, E. M., 2005. Dopamine, the medial preoptic area, and male sexual behavior. Physiology & Behavior. 86, 356-368.

Edwards, D. A., Einhorn, L. C., 1986. Preoptic And Midbrain Control Of Sexual Motivation. Physiology & Behavior. 37, 329-335.

Edwards, D. A., Isaacs, S., 1991. Zona Incerta Lesions - Effects On Copulation, Partner-Preference And Other Sociosexual Behaviors. Behavioural Brain Research. 44, 145-150.

Edwards, D. A., Walter, B., Liang, P., 1996. Hypothalamic and olfactory control of sexual behavior and partner preference in male rats. Physiology & Behavior. 60, 1347-1354.

Ehrhardt, A. A., Meyerbahlburg, H. F. L., Rosen, L. R., Feldman, J. F., Veridiano, N. P., Zimmerman, I., McEwen, B. S., 1985. Sexual Orientation After Prenatal Exposure To Exogenous Estrogen. Archives Of Sexual Behavior. 14, 57-77.

Everitt, B. J., 1990. Sexual motivation: a neural and behavioral analysis of the mechanisms underlying appetitive and copulatory responses of male rats. Neuroscience And Biobehavioral Reviews. 14, 217-232.

Everitt, B. J., Stacey, P., 1987. Studies Of Instrumental Behavior With Sexual Reinforcement In Male-Rats (Rattus-Norvegicus).2. Effects Of Preoptic Area Lesions, Castration, And Testosterone. Journal Of Comparative Psychology. 101, 407-419.

Ford, J. J., 1983. Postnatal Differentiation Of Sexual Preference In Male Pigs. Hormones And Behavior. 17, 152-162.

Francis, D., Diorio, J., Liu, D., Meaney, M. J., 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science. 286, 1155-1158.

Frye, C. A., Bayon, L. E., Pursnani, N. K., Purdy, R. H., 1998. The neurosteroids, progesterone and 3 alpha,5 alpha-THP, enhance sexual motivation, receptivity, and proceptivity in female rats. Brain Research. 808, 72-83.

Frye, C. A., Walf, A. A., 2004. Estrogen and/or progesterone administered systemically or to the amygdala can have anxiety-reducing, fear-reducing, and pain-reducing effects in ovariectomized rats. Behavioral Neuroscience. 118, 306-313.

Gooren, L., 2006. The biology of human psychosexual differentiation. Hormones And Behavior. 50, 589-601.

Goyal, H. O., Braden, T. D., Williams, C. S., Dalvi, P., Williams, J. W., Srivastava, K. K., 2004. Exposure of neonatal male rats to estrogen induces abnormal morphology of the penis and loss of fertility. Reproductive Toxicology. 18, 265-274.

Goyal, H. O., Robateau, A., Braden, T. D., Williams, C. S., Srivastava, K. K., Ali, K., 2003. Neonatal estrogen exposure of male rats alters reproductive functions at adulthood. Biology Of Reproduction. 68, 2081-2091.

Greenwood, D., Coggeshall, R. E., Hulsebosch, C. E., 1985. Sexual dimorphism in the numbers of neurons in the pelvic ganglia of adult rats. Brain Research. 340, 160-162.

Harris, G. W., Levine, S., 1965. Sexual Differentiation Of Brain And Its Experimental Control. Journal Of Physiology-London. 181, 379-400.

Herbst, A. L., Ulfelder, H., Poskanze.Dc, 1971. Adenocarcinoma Of Vagina - Association Of Maternal Stilbestrol Therapy With Tumor Appearance In Young Women. New England Journal Of Medicine. 284, 878-&.

Hetta, J., Meyerson, B. J., 1978. Sex-specific orientation in the male rat. A methodological study. Acta Physiologica Scandinavica. Suppl. 452, 5-27.

Houtsmuller, E. J., Brand, T., Dejonge, F. H., Joosten, R., Vandepoll, N. E., Slob, A. K., 1994. Sdn-Poa Volume, Sexual-Behavior, And Partner Preference Of Male-Rats Affected By Perinatal Treatment With Atd. Physiology & Behavior. 56, 535-541.

Hughes, A. M., Everitt, B. J., Herbert, J., 1990. Comparative Effects Of Preoptic Area Infusions Of Opioid-Peptides, Lesions And Castration On Sexual-Behavior In Male-Rats - Studies Of Instrumental Behavior, Conditioned Place Preference And Partner Preference. Psychopharmacology. 102, 243-256.

Hull, E. M., Bitran, D., Pehek, E. A., Warner, R. K., Band, L. C., Holmes, G. M., 1986. Dopaminergic Control Of Male Sex Behavior In Rats - Effects Of An Intracerebrally-Infused Agonist. Brain Research. 370, 73-81.

Hull, E. M., Du, J. F., Lorrain, D. S., Matuszewich, L., 1995. Extracellular Dopamine In The Medial Preoptic Area - Implications For Sexual Motivation And Hormonal-Control Of Copulation. Journal Of Neuroscience. 15, 7465-7471.

Hull, E. M., Meisel, R. L., Sachs, B. D., Male sexual behavior. Hormones, Brain and Behavior. Academic Press, 2002, pp. 3-137.

Hurtazo, H. A., Paredes, R. G., Agmo, A., 2008. Inactivation of the medial preoptic area/anterior hypothalamus by lidocaine reduces male sexual behavior and sexual incentive motivation in male rats. Neuroscience. 152, 331-337.

Jaffe, R. B., 1969. Testosterone Metabolism In Target Tissues - Hypothalamic And Pituitary Tissues Of Adult Rat And Human Fetus, And Immature Rat Epiphysis. Steroids. 14, 483-498.

Jansen, H. T., Cooke, P. S., Porcelli, J., Liu, T. C., Hansen, L. G., 1993. Estrogenic And Antiestrogenic Actions Of Pcbs In The Female Rat - Invitro And Invivo Studies. Reproductive Toxicology. 7, 237-248.

Jenkins, W. J., Becker, J. B., 2003. Dynamic increases in dopamine during paced copulation in the female rat. European Journal Of Neuroscience. 18, 1997-2001.

Johnson, W. A., Tiefer, L., 1972. Sexual Preferences In Neonatally Castrated Male Golden-Hamsters. Physiology & Behavior. 9, 213-217.

Keast, J. R., 1995. Visualization and immunohistochemical characterization of sympathetic and parasympathetic neurons in the male rat major pelvic ganglion. Neuroscience. 66, 655-662.

Keast, J. R., Booth, A. M., Degroat, W. C., 1989. Distribution of neurons in the major pelvic ganglion of the rat which supply the bladder, colon or penis. Cell And Tissue Research. 256, 105-112.

Keast, J. R., Degroat, W. C., 1989. Immunohistochemical characterization of pelvic neurons which project to the bladder, colon, or penis in rats. Journal Of Comparative Neurology. 288, 387-400.

Kerchner, M., Malsbury, C. W., Ward, O. B., Ward, I. L., 1995. Sexually Dimorphic Areas In The Rat Medial Amygdala - Resistance To The Demasculinizing Effect Of Prenatal Stress. Brain Research. 672, 251-260.

Kindon, H. A., Baum, M. J., Paredes, R. J., 1996. Medial preoptic/anterior hypothalamic lesions induce a female-typical profile of sexual partner preference in male ferrets. Hormones And Behavior. 30, 514-527.

Lehtimaki, J., Makela, S., Viljamaa, J., Yagi, A., Paranko, J., Santti, R., 1996. Neonatal estrogenization of the male mouse results in urethral dysfunction. Journal Of Urology. 156, 2098-2103.

Levay, S., 1991. A Difference In Hypothalamic Structure Between Heterosexual And Homosexual Men. Science. 253, 1034-1037.

Levine, S., Mullins, R., 1964. Estrogen Administered Neonatally Affects Adult Sexual Behavior In Male And Female Rats. Science. 144, 185-187.

Liu, D., Diorio, J., Day, J. C., Francis, D. D., Meaney, M. J., 2000. Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nature Neuroscience. 3, 799-806.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P. M., Meaney, M. J., 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science. 277, 1659-1662.

Maclusky, N. J., McEwen, B. S., 1978. Estrogen Modulates Progestin Receptor Concentrations In Some Rat-Brain Regions But Not In Others. Nature. 274, 276-278.

Maclusky, N. J., McEwen, B. S., 1980. Progestin Receptors In Rat-Brain - Distribution And Properties Of Cytoplasmic Progestin-Binding Sites. Endocrinology. 106, 192-202.

Majdic, G., Sharpe, R. M., Oshaughnessy, P. J., Saunders, P. T. K., 1996. Expression of cytochrome P450 17 alpha-hydroxylase/C17-20 lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. Endocrinology. 137, 1063-1070.

Mani, S. K., Allen, J. M. C., Clark, J. H., Blaustein, J. D., Omalley, B. W., 1994. Convergent pathways for steroid hormone-induced and neurotransmitter-induced rat sexual behavior. Science. 265, 1246-1249.

Martin, J. T., Baum, M. J., 1986. Neonatal Exposure Of Female Ferrets To Testosterone Alters Sociosexual Preferences In Adulthood. Psychoneuroendocrinology. 11, 167-176.

Matuszczyk, J. V., Fernandezguasti, A., Larsson, K., 1988. Sexual Orientation, Proceptivity, And Receptivity In The Male-Rat As A Function Of Neonatal Hormonal Manipulation. Hormones And Behavior. 22, 362-378.

Matuszczyk, J. V., Larsson, K., 1991. Role Of Androgen, Estrogen And Sexual Experience On The Female Rats Partner Preference. Physiology & Behavior. 50, 139-142.

Matuszczyk, J. V., Larsson, K., 1994. Experience Modulates The Influence Of Gonadal-Hormones On Sexual Orientation Of Male-Rats. Physiology & Behavior. 55, 527-531.

Matuszczyk, J. V., Larsson, K., 1995. Sexual preference and feminine and masculine sexual behavior of male rats prenatally exposed to antiandrogen or antiestrogen. Hormones And Behavior. 29, 191-206.

Matuszewich, L., Lorrain, D. S., Hull, E. M., 2000. Dopamine release in the medial preoptic area of female rats in response to hormonal manipulation and sexual activity. Behavioral Neuroscience. 114, 772-782.

McAbee, M. D., Doncarlos, L. L., 1999. Estrogen, but not androgens, regulates androgen receptor messenger ribonucleic acid expression in the developing male rat forebrain. Endocrinology. 140, 3674-3681.

McEwen, B. S., Lieberburg, I., Chaptal, C., Krey, L. C., 1977. Aromatization - Important For Sexual Differentiation Of Neonatal Rat-Brain. Hormones And Behavior. 9, 249-263.

McFadden, D., Champlin, C. A., 2000. Comparison of auditory evoked potentials in heterosexual, homosexual, and bisexual males and females. Jaro. 1, 89-99.

McFadden, D., Loehlin, J. C., Breedlove, S. M., Lippa, R. A., Manning, J. T., Rahman, Q., 2005. A reanalysis of five studies on sexual orientation and the relative length of the 2nd and 4th fingers (the 2D: 4D ratio). Archives Of Sexual Behavior. 34, 341-356.

Meisel, R. L., Sachs, B. D., The physiology of male sexual behavior. In: E. Knobil, J. D. Neill, (Eds.), The Physiology of Reproduction. Raven Press, New York, 1994, pp. 3-105.

Mermelstein, P. G., Becker, J. B., 1995. Increased extracellular dopamine in the nucleus accumbens and striatum of the female rat during paced copulatory behavior. Behavioral Neuroscience. 109, 354-365.

Merryman, W., Boiman, R., Barnes, L., Rothchild, I., 1954. Progesterone Anesthesia In Human Subjects. Journal Of Clinical Endocrinology And Metabolism. 14, 1567-1569.

Meyer-Bahlburg, H. F. L., Ehrhardt, A. A., Rosen, L. R., Gruen, R. S., Veridiano, N. P., Vann, F. H., Neuwalder, H. F., 1995. Prenatal Estrogens And The Development Of Homosexual Orientation. Developmental Psychology. 31, 12-21.

Meyer-Bahlburg, H. F. L., Gruen, R. S., New, M. I., Bell, J. J., Morishima, A., Shimshi, M., Bueno, Y., Vargas, I., Baker, S. W., 1996. Gender change from female to male in classical congenital adrenal hyperplasia. Hormones And Behavior. 30, 319-332.

Meyerson, B. J., Eliasson, M., Hetta, J., Sex-specific orientation in female and male rats: Development and effects of early endocrine manipulation. In: A. M. Kaye, M. Kaye, (Eds.), Development of responsivenss to steriod hormones: Advances in the biosciences. Pergamon Press, Oxford, England, 1980, pp. 451-460.

Meyerson, B. J., Lindstrom, L. H., 1973. Sexual motivation in the female rat. A methodological study applied to the investigation of the effect of estradiol benzoate. Acta Physiologica Scandinavica. Suppl. 389, 1-80.

Moore, C. L., 1982. Maternal-Behavior Of Rats Is Affected By Hormonal Condition Of Pups. Journal Of Comparative And Physiological Psychology. 96, 123-129.

Moore, C. L., 1984. Maternal Contributions To The Development Of Masculine Sexual-Behavior In Laboratory Rats. Developmental Psychobiology. 17, 347-356.

Moore, C. L., Dou, H., Juraska, J. M., 1992. Maternal stimulation affects the number of motor neurons in a sexually dimorphic nucleus of the lumbar spinal cord. Brain Research. 572, 52-56.

Moore, C. L., Morelli, G. A., 1979. Mother Rats Interact Differently With Male And Female Offspring. Journal Of Comparative And Physiological Psychology. 93, 677-684.

Morris, J. A., Jordan, C. L., Dugger, B. N., Breedlove, S. M., 2005. Partial demasculinization of several brain regions in adult male (XY) rats with a dysfunctional androgen receptor gene. Journal Of Comparative Neurology. 487, 217-226.

Moses, J., Loucks, J. A., Watson, H. L., Matuszewich, L., Hull, E. M., 1995. Dopaminergic Drugs In The Medial Preoptic Area And Nucleus-Accumbens - Effects On Motor-Activity, Sexual Motivation, And Sexual Performance. Pharmacology Biochemistry And Behavior. 51, 681-686.

Mullins, R. F., Levine, S., 1968. Hormonal Determinants During Infancy Of Adult Sexual Behavior In Female Rat. Physiology & Behavior. 3, 333-338.

Negri-Cesi, P., Colciago, A., Pravettoni, A., Casati, L., Conti, L., Celotti, F., 2008. Sexual differentiation of the rodent hypothalamus: Hormonal and environmental influences. Journal Of Steroid Biochemistry And Molecular Biology. 109, 294-299.

Nesaretnam, K., Corcoran, D., Dils, R. R., Darbre, P., 1996. 3,4,3',4'-Tetrachlorobiphenyl acts as an estrogen in vitro and in vivo. Molecular Endocrinology. 10, 923-936.

Nordeen, E. J., Nordeen, K. W., Sengelaub, D. R., Arnold, A. P., 1985. Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science. 229, 671-673.

Olsen, K. L., Whalen, R. E., 1982. Estrogen Binds To Hypothalamic Nuclei Of Androgen-Insensitive (Tfm) Rats. Experientia. 38, 139-140.

Paredes, R. G., Baum, M. J., 1995. Altered Sexual Partner Preference In Male Ferrets Given Excitotoxic Lesions Of The Preoptic Area Anterior Hypothalamus. Journal Of Neuroscience. 15, 6619-6630.

Paredes, R. G., Highland, L., Karam, P., 1993. Sociosexual Behavior In Male-Rats After Lesions Of The Medial Preoptic Area - Evidence For Reduced Sexual Motivation. Brain Research. 618, 271-276.

Paredes, R. G., Tzschentke, T., Nakach, N., 1998. Lesions of the medial preoptic area anterior hypothalamus (MPOA/AH) modify partner preference in male rats. Brain Research. 813, 1-8.

Pehek, E. A., Warner, R. K., Bazzett, T. J., Bitran, D., Band, L. C., Eaton, R. C., Hull, E. M., 1988. Microinjection Of Cis-Flupenthixol, A Dopamine Antagonist, Into The Medial Preoptic Area Impairs Sexual-Behavior Of Male-Rats. Brain Research. 443, 70-76.

Perkins, A., Fitzgerald, J. A., Moss, G. E., 1995. A Comparison Of Lh-Secretion And Brain Estradiol Receptors In Heterosexual And Homosexual Rams And Female Sheep. Hormones And Behavior. 29, 31-41.

Pfaus, J. G., Damsma, G., Wenkstern, D., Fibiger, H. C., 1995. Sexual activity increases dopamine transmission in the nucleus accumbens and striatum of female rats. Brain Research. 693, 21-30.

Pfaus, J. G., Phillips, A. G., 1991. Role Of Dopamine In Anticipatory And Consummatory Aspects Of Sexual-Behavior In The Male-Rat. Behavioral Neuroscience. 105, 727-743.

Phoenix, C. H., Goy, R. W., Gerall, A. A., young, W. C., 1959. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology. 65, 369-382.

Piacsek, B. E., Hostetter, M. W., 1984. Neonatal Androgenization In The Male-Rat - Evidence For Central And Peripheral Defects. Biology Of Reproduction. 30, 344-351.

Pollak, E. I., Sachs, B. D., 1975. Masculine Sexual-Behavior And Morphology - Paradoxical Effects Of Perinatal Androgen Treatment In Male And Female Rats. Behavioral Biology. 13, 401-411.

Portillo, W., Diaz, N. F., Retana-Marquez, S., Paredes, R. G., 2006. Olfactory, partner preference and Fos expression in the vomeronasal projection pathway of sexually sluggish male rats. Physiology & Behavior. 88, 389-397.

Purves-Tyson, T. D., Arshi, M. S., Handelsman, D. J., Cheng, Y., Keast, J. R., 2007. Androgen and estrogen receptor-mediated mechanisms of testosterone action in male rat pelvic autonomic ganglia. Neuroscience. 148, 92-104.

Rahman, Q., 2005. Fluctuating asymmetry, second to fourth finger length ratios and human sexual orientation. Psychoneuroendocrinology. 30, 382-391.

Rahman, Q., Wilson, G. D., 2003. Sexual orientation and the 2nd to 4th finger length ratio: evidence for organising effects of sex hormones or developmental instability? Psychoneuroendocrinology. 28, 288-303.

Reddy, D. S., Apanites, L. A., 2005. Anesthetic effects of progesterone are undiminished in progesterone receptor knockout mice. Brain Research. 1033, 96-101.

Reiner, W. G., Kropp, B. P., 2004. A 7-year experience of genetic males with severe phallic inadequacy assigned female. Journal Of Urology. 172, 2395-2398.

Resko, J. A., Perkins, A., Roselli, C. E., Fitzgerald, J. A., Choate, J. V. A., Stormshak, F., 1996. Endocrine correlates of partner preference behavior in rams. Biology Of Reproduction. 55, 120-126.

Rissman, E. F., Wersinger, S. R., Taylor, J. A., Lubahn, D. B., 1997. Estrogen receptor function as revealed by knockout studies: Neuroendocrine and behavioral aspects. Hormones And Behavior. 31, 232-243.

Robinson, S. J., Manning, J. T., 2000. The ratio of 2nd to 4th digit length and male homosexuality. Evolution and Human Behavior. 21, 333-345.

Rodriguez-Manzo, G., Pellicer, F., Larsson, K., Fernandez-Guasti, A., 2000. Stimulation of the medial preoptic area facilitates sexual behavior but does not reverse sexual satiation. Behavioral Neuroscience. 114, 553-560.

Romero-Carbente, J. C., Camacho, F. J., Paredes, R. G., 2006. The role of the dorsolateral tegmentum in the control of male sexual behavior: A reevaluation. Behavioural Brain Research. 170, 262-270.

Romero-Carbente, J. C., Hurtazo, E. A., Paredes, R. G., 2007. Central tegmental field and sexual behavior in the male rat: Effects of neurotoxic lesions. Neuroscience. 148, 867-875.

Roselli, C. E., Larkin, K., Schrunk, J. M., Stormshak, F., 2004. Sexual partner preference, hypothalamic morphology and aromatase in rams. Physiology & Behavior. 83, 233-245.

Roselli, C. E., Salisbury, R. L., Resko, J. A., 1987. Genetic-Evidence For Androgen-Dependent And Independent Control Of Aromatase-Activity In The Rat-Brain. Endocrinology. 121, 2205-2210.

Ryan, K. J., Reddy, V., Petro, Z., Naftolin, F., Flores, F., 1972. Estrogen Formation In Brain. American Journal Of Obstetrics And Gynecology. 114, 454-460.

Seegal, R. F., Brosch, K. O., Okoniewski, R. J., 2005. Coplanar PCB congeners increase uterine weight and frontal cortical dopamine in the developing rat: Implications for developmental neurotoxicity. Toxicological Sciences. 86, 125-131.

Selye, H., 1941. Anesthetic effect of steroid hormones. Proceedings Of The Society For Experimental Biology And Medicine. 46, 116-121.

Sengelaub, D. R., Arnold, A. P., 1986. Development and loss of early projections in a sexually dimorphic rat spinal nucleus. Journal Of Neuroscience. 6, 1613-1620.

Shibayama, T., Fukata, H., Sakurai, K., Adachi, T., Komiyama, M., Iguchi, T., Mori, C., 2001. Neonatal exposure to genistein reduces expression of estrogen receptor alpha and androgen receptor in testes of adult mice. Endocrine Journal. 48, 655-663.

Signoret, J. P., 1970. Reproductive behavior in pigs. Journal Of Reproduction And Fertility. 105-117.

Slob, A. K., Deklerk, L. W. L., Brand, T., 1987. Homosexual And Heterosexual Partner Preference In Ovariectomized Female Rats - Effects Of Testosterone, Estradiol And Mating Experience. Physiology & Behavior. 41, 571-576.

Stockman, E. R., Callaghan, R. S., Baum, M. J., 1985. Effects Of Neonatal Castration And Testosterone Treatment On Sexual Partner Preference In The Ferret. Physiology & Behavior. 34, 409-414.

Stormshak, F., Estill, C. T., Resko, J. A., Roselli, C. E., 2008. Changes in LH secretion in response to an estradiol challenge in male- and female-oriented rams and in ewes. Reproduction. 135, 733-738.

Streng, T., Launonen, A., Salmi, S., Saarinen, N., Talo, A., Makela, S., Santti, R., 2001. Nontraumatic urethral dyssynergia in neonatally estrogenized male rats. Journal Of Urology. 165, 1305-1309.

Suzuki, M., Nishihara, M., 2002. Estrogen affects gene expression of estrogen receptors, androgen receptor, and aromatase in the neonatal rat hypothalamus. Journal Of Reproduction And Development. 48, 17-23.

Swaab, D. F., Fliers, E., 1985. A Sexually Dimorphic Nucleus In The Human-Brain. Science. 228, 1112-1115.

Tarttelin, M. F., Gorski, R. A., 1988. Postnatal Influence Of Diethylstilbestrol On The Differentiation Of The Sexually Dimorphic Nucleus In The Rat Is As Effective As Perinatal Treatment. Brain Research. 456, 271-274.

Titus-Ernstoff, L., Perez, K., Hatch, E. E., Troisi, R., Palmer, J. R., Hartge, P., Hyer, M., Kaufman, R., Adam, E., Strohsnitter, W., Noller, K., Pickett, K. E., Hoover, R., 2003. Psychosexual characteristics of men and women exposed prenatally to diethylstilbestrol. Epidemiology. 14, 155-160.

Tobet, S. A., Shim, J. H., Osiecki, S. T., Baum, M. J., Canick, J. A., 1985. Androgen Aromatization And 5-Alpha-Reduction In Ferret Brain During Perinatal-Development - Effects Of Sex And Testosterone Manipulation. Endocrinology. 116, 1869-1877.

Tsaimorris, C. H., Knox, G., Luna, S., Dufau, M. L., 1986. Acquisition Of Estradiol-Mediated Regulatory Mechanism Of Steroidogenesis In Cultured Fetal-Rat Leydig-Cells. Journal Of Biological Chemistry. 261, 3471-3474.

Vomachka, A. J., Ruppert, P. H., Greenwald, G. S., Clemens, L. G., 1981. Adult Sexual-Behavior Deficits And Altered Hormone Levels In Male Hamsters Given Steroids During Development. Physiology & Behavior. 26, 461-466.

vomSaal, F. S., Timms, B. G., Montano, M. M., Palanza, P., Thayer, K. A., Nagel, S. C., Dhar, M. D., Ganjam, V. K., Parmigiani, S., Welshons, W. V., 1997. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proceedings Of The National Academy Of Sciences Of The United States Of America. 94, 2056-2061.

Wang, C. T., Shui, H. A., Huang, R. L., Tai, M. Y., Peng, M. T., Tsai, Y. F., 2006. Sexual motivation is demasculinized, but not feminized, in prenatally stressed male rats. Neuroscience. 138, 357-364.

Warita, K., Sugawara, T., Yue, Z. P., Tsukahara, S., Mutoh, K. I., Hasegawa, Y., Kitagawa, H., Mori, C., Hoshi, N., 2006. Progression of the dose-related effects of estrogenic endocrine disruptors, an important factor in declining fertility, differs between the hypothalamo-pituitary axis and reproductive organs of male mice. Journal Of Veterinary Medical Science. 68, 1257-1267.

Warner, R. K., Thompson, J. T., Markowski, V. P., Loucks, J. A., Bazzett, T. J., Eaton, R. C., Hull, E. M., 1991. Microinjection Of The Dopamine Antagonist Cis-Flupentixol Into The Mpoa Impairs Copulation, Penile Reflexes And Sexual Motivation In Male-Rats. Brain Research. 540, 177-182.

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., Meaney, M. J., 2004. Epigenetic programming by maternal behavior. Nature Neuroscience. 7, 847-854.

Weisz, J., Ward, I. L., 1980. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology. 106, 306-316.

Wersinger, S. R., Rissman, E. F., 2000. Oestrogen receptor alpha is essential for female-directed chemo-investigatory behaviour but is not required for the pheromone-induced luteinizing hormone surge in male mice. Journal Of Neuroendocrinology. 12, 103-110.

Wersinger, S. R., Sannen, K., Villalba, C., Lubahn, D. B., Rissman, E. F., De Vries, G. J., 1997. Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. Hormones And Behavior. 32, 176-183.

Whalen, R. E., 1964. Hormone-Induced Changes In Organization Of Sexual Behavior In Male Rat. Journal Of Comparative And Physiological Psychology. 57, 175-182.

Whalen, R. E., Edwards, D. A., 1967. Hormonal determinants of the development of masculine and feminine behavior in male and female rats. The Anatomical Record. 157, 173-180.

Whalen, R. E., Gladue, B. A., Olsen, K. L., 1986. Lordotic Behavior In Male-Rats - Genetic And Hormonal-Regulation Of Sexual-Differentiation. Hormones And Behavior. 20, 73-82.

Whalen, R. E., Nadler, R. D., 1963. Suppression Of Development Of Female Mating Behaviour By Estrogen Administered In Infancy. Science. 141, 273-274.

Williams, T. J., Pepitone, M. E., Christensen, S. E., Cooke, B. M., Huberman, A. D., Breedlove, N. J., Breedlove, T. J., Jordan, C. L., Breedlove, S. M., 2000. Finger-length ratios and sexual orientation. Nature. 404, 455-456.

Zadina, J. E., Dunlap, J. L., Gerall, A. A., 1979. Modifications Induced By Neonatal Steroids In Reproductive-Organs And Behavior Of Male-Rats. Journal Of Comparative And Physiological Psychology. 93, 314-322.

Zucker, K. J., Bradley, S. J., Oliver, G., Blake, J., Fleming, S., Hood, J., 1996. Psychosexual development of women with congenital adrenal hyperplasia. Hormones And Behavior. 30, 300-318.

Zuloaga, D. G., Puts, D. A., Jordan, C. L., Breedlove, S. M., 2008. The role of androgen receptors in the masculinization of brain and behavior: What we've learned from the testicular feminization mutation. Hormones And Behavior. 53, 613-626.