

Parental Responsiveness Is Feminized after Neonatal Castration in Virgin Male Prairie Voles, but Is Not Masculinized by Perinatal Testosterone in Virgin Females

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We previously found a large sex difference in the parental responsiveness of adult virgin prairie voles (*Microtus ochrogaster*) such that most males are spontaneously parental, whereas most females are not. Because this sex difference is independent of the gonadal hormones normally circulating in adult virgin voles, the present study examined whether perinatal hormones influence the development of this sex difference. Males were treated prenatally (via their pregnant dam) with both the androgen receptor blocker flutamide (5 mg/day/dam) and the aromatase inhibitor ATD (1 mg/day/dam), or oil, for the last 2 weeks of gestation. Half of the subjects from each group were castrated on the day of birth and the other half received a sham surgery. As adults, intact males were castrated and all males received a silastic capsule filled with testosterone. Prenatal treatment with flutamide and ATD had no effect on males' behavior toward pups, but neonatal castration significantly reduced the percentage of males acting parentally. In a second experiment, females were exposed to testosterone propionate (TP; 50 µg/day/dam) or oil via their dam during the last 2 weeks of gestation. For the first neonatal week, half of the females from each group were injected with TP (1 mg/day) and the other half oil. As adults, females were ovariectomized and half from each group received a testosterone-filled capsule and the other half received an empty capsule. None of the perinatal TP treatments increased females' parental responsiveness, although females from all groups that received testosterone capsules as adults were highly parental. Therefore, although postnatal testicular hormones are necessary for high parental responsiveness in males, the

behavior of females is not influenced by perinatal exposure to testosterone. © 2002 Elsevier Science (USA)

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Many sex differences in the behavior of adult rodents can be influenced by perinatal exposure to gonadal hormones and sex differences in parental responsiveness are no exception (for review see Lonstein and De Vries, 2000a). In rats, virgin males that are castrated soon after birth show more feminine responses toward pups during adulthood in that they are more likely to act parentally and less likely to attack pups than gonadally intact controls (Quadagno and Rockwell, 1972; McCullough, Quadagno, and Goldman, 1974; Rosenberg and Herrenkohl, 1976; Rosenberg, Denenberg, Zarrow, and Frank, 1971). Conversely, virgin female rats that are treated prenatally (Ichikawa and Fujii, 1982; Juarez, Rio-Portilla, and Corsi-Cabrera, 1999) or neonatally (Quadagno and Rockwell, 1972; Quadagno, McCullough, Ho, and Spevak, 1973; Rosenberg and Sherman, 1974) with testosterone propionate (TP) display reduced parental responsiveness on all, or at least some, measures when introduced to pups during adulthood. Although perinatal exposure to gonadal hormones influences parental responsiveness in adult virgin male and female rats, this may not be generally true across rodent species.

Virgin prairie voles (*Microtus ochrogaster*) are an interesting model to examine the factors influencing sex differences in parental behavior because, unlike rats, adult virgin males are much more parentally respon-

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sive than adult virgin females (Lonstein and De Vries, 1999a). Similar to rats, this sex difference is not altered by gonadectomy during adulthood (Lonstein and De Vries, 1999a), suggesting that perinatal exposure to gonadal hormones plays a role in generating this sex difference in virgin prairie vole behavior. However, neither prenatal or postnatal inhibition of estrogenic activity with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD), nor perinatal inhibition of androgenic activity with the androgen receptor antagonist flutamide, reduced the high parental responsiveness of adult males. Similarly, neither prenatal nor neonatal administration of TP increased the relatively low parental responsiveness of females (Lonstein and De Vries, 2000b). These results suggested that androgenic or estrogenic activity during a discrete prenatal or postnatal period was not responsible for sex differences in the parental responding of virgin prairie voles. However, it remained possible that androgenic and estrogenic activity *together* could influence later parental responding and that exposure to gonadal hormones during an extended period that includes *both* prenatal and postnatal life could affect parental behavior. To address some of these possibilities, males in the present experiment were treated prenatally with both ATD and flutamide and castrated on the day of birth. In the second experiment, females were exposed to testosterone both prenatally and during the first week of life. Parental responsiveness of these subjects was then examined during adulthood.

METHODS

Subjects

Subjects were F3 and F4 generation prairie voles (*M. ochrogaster*) born and raised in our colony, which was established in 1996 at the University of Massachusetts, Amherst, from breeding stock originating from offspring of voles captured in 1994 from Urbana, Illinois, provided by Dr. Betty McGuire (Smith College, Northampton, MA) and Dr. Zuoxin Wang (Emory University, Atlanta, GA). Animals were mated by socially isolating adult virgin female and male prairie voles for 3 days, after which females were placed in the cage of an unfamiliar male. Animals were maintained on a 14:10-h light:dark cycle with an ambient temperature of 21°C. At all ages, animals were housed in plastic cages (48 × 28 × 16 cm) containing wood chips, wood shavings, and a substantial hay covering. Water and a mixture of cracked corn, whole oats,

sunflower seeds, and Purina rabbit chow (ratio of 1:1:2:2) were available *ad libitum*. On the day of birth, litters were fostered to unmanipulated lactating prairie voles from our colony that had given birth to a litter within the previous 24 h. Subjects were weaned at 20 days of age and housed with their littermates in mixed-sex groups. When subjects were 30 days old, their anogenital distance was measured to the nearest millimeter and they were rehoused in single-sex groups of 2–4 animals per cage. Subjects remained housed with their same-sex littermates until behavioral testing when they were 90–95 days old.

Procedure

Treatment of males. To inhibit testosterone action prenatally, pregnant female prairie voles ($n = 19$) received daily subcutaneous (sc) injections of 5 mg of the androgen receptor blocker flutamide (α, α, α -trifluoro-2-methyl-4-nitro-*m*-propionotoluidide; Sigma, St. Louis, MO) dissolved in 100 μ l sesame oil immediately followed by a sc injection of 1 mg of the aromatase inhibitor ATD (1,4,6-androstatriene-3,17-dione; Steraloids Inc., Wilton, NH) dissolved in 100 μ l sesame oil beginning 1 week after pairing with a male. To generate control subjects, other pregnant females ($n = 24$) received two sc injections of 100 μ l of sesame oil each day. Doses were chosen based on the effectiveness of similar or even smaller doses to influence sexual differentiation in rats and other mammals (e.g., Clemens, Gladau, and Coniglio, 1978; Grisham, Casto, Kashon, Ward, and Ward, 1992; Houtsmuller, Brand, de Jonge, Joosten, Van der Poll, and Slob, 1994; Roy, 1991; Tate-Ostroff and Bridges, 1988; Tobet, Zahniser, and Baum, 1986).

Within 2–12 h after birth, pups were removed from their dam and sexed and males were cryoanesthetized on ice. With the use of a dissecting microscope, half of the males from each group received two lateral incisions of their ventrum and their testes were removed. The other half of the males from each group were cryoanesthetized and received a sham castration. Muscle and skin were closed with a single silk suture. To insure that at least one male from each litter would survive to adulthood and could be used in the experiment, two or three males within each litter received the same treatment. Pups were placed under a warm lamp until they were active, after which they were returned to their dam.

When 70–75 days old, subjects were anesthetized with an ip injection of a cocktail containing ketamine (62.5 mg/kg), xylazine (7.5 mg/kg), and aceproma-

zine (0.8 mg/kg). Gonadally intact males received a single midline incision to the scrotum and were castrated. Neonatally castrated males received a sham surgery. All males then received a single 2.5-cm silastic capsule filled with crystalline testosterone implanted subcutaneously in the nape of their neck. The final sample sizes were as follows: prenatal oil/neonatal sham, $n = 12$; prenatal oil/neonatal castration, $n = 12$; prenatal ATD + flutamide/neonatal sham, $n = 10$; prenatal ATD + flutamide/neonatal castration, $n = 9$. One, and in a few cases two, males per litter were used as subjects.

Treatment of females. To examine the effects of exposing females to exogenous androgen, pregnant female prairie voles received either a daily sc injection of 50 μg of testosterone propionate (TP) dissolved in 100 μl sesame oil ($n = 18$ dams) or 100 μl sesame oil ($n = 18$ dams) beginning 1 week after pairing with males until the birth of pups. This dose was chosen because a regimen of daily injections of higher doses of TP (75–100 μg) causes the majority of pregnant prairie vole dams to either abort their litters or commit infanticide at parturition (Lonstein and De Vries, 2000b; Roberts, Zullo, Gustafson, and Carter, 1996). Beginning on the day of birth and lasting for the next 6 days, half the litters from each prenatal treatment group were injected sc each day with 1 mg of TP in 50 μl sesame oil. The other half were injected each day with 50 μl sesame oil. All pups within the same litter received the same treatment.

When 70–75 days old, females were anesthetized with an ip injection of ketamine cocktail and ovariectomized. Because the effects of perinatal TP treatment on the later parental (or infanticidal) responses of adult virgin female mice are only observed when subjects are given TP again as adults (e.g., Gandelman and Vom Saal, 1977), half of the subjects in each group received a subcutaneous implant of a single 2.5-cm silastic capsule filled with crystalline testosterone. The other half of the subjects received an empty capsule. In several cases two females from each litter were used, with one female assigned to the empty capsule group and the other assigned to the testosterone capsule group. The final sample sizes were 7–10 subjects per group for the adult empty capsule groups and 7–9 subjects per group for the testosterone-filled capsule groups.

Behavioral Testing

When 90–95 days old, subjects were removed from their home cage and placed in a clear Plexiglas cage of similar dimensions as their home cage containing

wood shavings, food, and water, and a small amount of hay. After a 15-min habituation period, two 1- to 4-day-old prairie vole pups obtained from lactating prairie voles from our colony were scattered in the cage diagonally from where the subject was sitting. Although prairie vole dams typically give birth to three to five pups per litter, only two pups were presented during behavioral testing to keep the number of pups attacked by potentially infanticidal subjects at a minimum. Behavior of the subject was continuously recorded for 10 min with the aid of a custom-made data acquisition system designed to provide data on latency, frequency, and duration of numerous behaviors as described previously (Lonstein and De Vries, 1999a,b, 2000b). Active behaviors directed toward pups included sniffing, licking, and carrying them from one position to another. Non-pup-oriented activities recorded included self-grooming, exploration away from the pups, digging or nest construction, and eating or drinking. Measures of huddling behavior included actively hovering over the pups while performing other activities, and being quiescently positioned over the litter in a nursing/huddling posture. If a subject did not make contact with a pup within 3 min after the beginning of the test, the test was briefly paused while the pups were placed approximately 1 inch in front of the subject. In our experience, many virgin prairie voles do not quickly find and initiate contact with pups in a relatively large testing arena, even though they may be highly parental when the pups are offered directly to them. These subjects were assigned a latency to contact pups of at least 180 s (180 s plus the time taken to contact the closely placed pups). Total activity was the sum of the durations of all active behaviors recorded, and total time with pups was sum of the durations of actively hovering over the litter and quiescent nursing/huddling. Total quiescence was the sum of the duration of time subjects spent in all three quiescent postures. In the few cases where subjects attacked and seriously injured a pup, the test was immediately terminated and the pup quickly euthanized. In other cases, pups were removed and returned to their parents after behavioral testing. A subject was considered parental if it spent longer than 100 s in physical contact with pups and was observed to lick them for longer than 30 s. Retrieval of the pups was not included in these criteria because neither virgin nor postpartum prairie voles of either sex readily retrieve pups and often attend to them individually unless the scattered pups crawl to the adult (Lonstein and De Vries, 1999a,b, 2000b).

Data Analyses

The percentages of subjects in each group acting parentally were compared with the G test of independence. This test often provides similar results as either the chi-square or Fisher's Exact Probability tests but was better suited for our data because although the number of subjects in each comparison was fixed, the number that could potentially be parental was not and could range from none to all of the subjects (Sokol and Rohlf, 1981). To avoid litter effects, the mean anogenital distance was calculated for males and females within each litter and the litter means were compared between groups with a two-way analysis of variance (ANOVA) using prenatal and postnatal treatment as factors followed by Fisher's least significant difference post-hoc tests. Because there were too few responding males in the neonatally castrated groups, behavioral data for males were collapsed across prenatal or postnatal treatment and analyzed with independent t tests instead of a two-way ANOVA. Behavioral data for females were analyzed with two-way ANOVAs using prenatal treatment (TP or oil) and postnatal treatment (TP or oil) as factors, followed by Fisher's least significant difference post-hoc tests.

RESULTS

Effects of Hormonal Manipulations on the Behavior of Males

There was no effect of prenatal treatment with ATD and flutamide on the percentage of males acting parentally during adulthood ($G^2 = 0.35$, $P > 0.5$). However, when collapsed across prenatal treatment, fewer neonatally castrated males were parental during adulthood compared with neonatal males that received a sham surgery ($G^2 = 4.00$, $P < 0.05$; Fig. 1a). There were no significant effects of prenatal or postnatal treatment on any measure of the behavior of responding males toward pups (data not shown); there were too few parentally responding males in the neonatally castrated groups to analyze for prenatal by postnatal interaction effects.

Prenatal treatment with ATD and flutamide reduced anogenital distance compared with that of oil-treated controls [4.7 ± 0.4 vs 5.7 ± 0.5 mm; $F(1, 32) = 6.61$, $P < 0.009$], as did neonatal castration compared with neonatal sham surgery [3.7 ± 0.2 vs 6.8 ± 0.3 mm; $F(1, 32) = 66.97$, $P < 0.0001$]. There was no significant prenatal by postnatal interaction effect on anogenital distance [$F(1, 32) = 0.18$, $P > 0.5$].

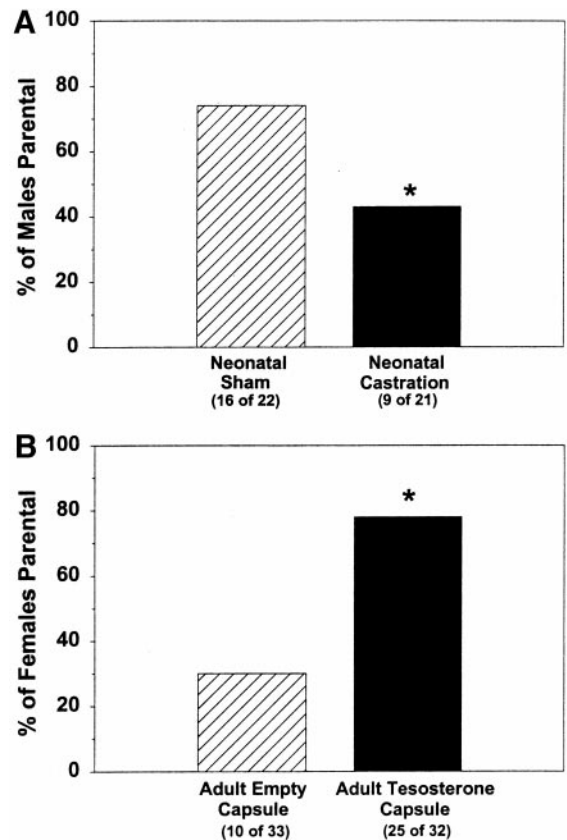


FIG. 1. Percentage of adult virgin prairie voles acting parentally after (A) males received a neonatal sham surgery or neonatal castration. Data collapsed across males' prenatal treatment with ATD + flutamide or oil, which had no effects on their behavior and (B) females received an empty capsule or capsule filled with testosterone 3 weeks prior to testing. Data collapsed across females' perinatal treatment with TP or oil, which had no effects on this measure. * $P \leq 0.05$.

Effects of Hormonal Manipulations on the Behavior of Females

There were no significant effects of either prenatal or postnatal TP treatment on the percentage of virgin female prairie voles acting parentally. Indeed, for females receiving an empty silastic capsule during adulthood, relatively few (20–43%) of the subjects in each group acted maternally, regardless of their perinatal treatment ($G^2 = 1.33$, $df = 3$, $P > 0.72$). Conversely, most (56–89%) females that received a silastic capsule filled with testosterone acted maternally, regardless of their perinatal treatment ($G^2 = 3.49$, $df = 3$, $P > 0.32$). Collapsed across perinatal treatment, the comparison between the percentage of females that acted parentally after receiving an empty capsule or a

TABLE 1

Latencies (Mean \pm SEM, in Seconds) for Female and Male Prairie Voles to Contact and Hover over Pups after Perinatal Hormone Manipulations

Group	Contact pups	Hover over pups from start of test	Hover over pups from first contact with them
Females			
Oil/oil ($n = 6$)	87 \pm 25 ^a	105 \pm 36 ^a	18 \pm 12
Oil/TP ($n = 8$)	72 \pm 34 ^a	85 \pm 35 ^a	13 \pm 3
TP/oil ($n = 5$)	160 \pm 45 ^b	198 \pm 36 ^b	38 \pm 20
TP/TP ($n = 6$)	159 \pm 41 ^b	197 \pm 43 ^b	39 \pm 18
Males			
Prenatal oil ($n = 13$)	88 \pm 28	101 \pm 27	13 \pm 5
Prenatal flutamide + ATD ($n = 12$)	101 \pm 31	112 \pm 32	11 \pm 5
Neonatal sham surgery ($n = 16$)	112 \pm 30	126 \pm 29	14 \pm 5
Neonatal castration ($n = 9$)	62 \pm 17	72 \pm 19	9 \pm 2

Note. All nonresponders excluded from statistical analyses. Data from males collapsed across prenatal or postnatal treatment. Significant main effects within each sex indicated by different superscript letters ($P \leq 0.05$). Note that although neonatal TP treatment increased latencies in females, neonatal castration did not significantly reduce them in males.

testosterone-filled capsule during adulthood was highly significant ($G^2 = 15.62$, $df = 1$, $P < 0.0001$; Fig. 1b).

There were too few maternally responsive females in the empty capsule groups to compare the details of their behavior. A comparison of the behavior of the maternally responsive females in the testosterone capsule groups revealed that females exposed prenatally to TP were less parentally responsive than controls on one measure, taking almost twice as long as control females to make contact with the pups from the beginning of the test [$F(1, 21) = 4.7$, $P < 0.05$; Table 1]. Because females exposed prenatally to TP took longer to initiate contact with pups, they also took longer to begin hovering over them when measured from the beginning of the test [$F(1, 21) = 7.18$, $P < 0.05$]. If the latency to hover over the pups is calculated from the time that females first contacted them, however, there was no effect of prenatal treatment [$F(1, 21) = 2.9$, $P > 0.84$]. There were no significant postnatal treatment effects and no significant prenatal by postnatal interaction effects on any measure ($P_s > 0.1$).

Prenatal treatment of females with TP significantly increased anogenital distance compared to oil-treated controls [3.5 ± 0.2 vs 3.0 ± 0.1 mm; $F(1, 32) = 7.81$, $P < 0.05$], as did neonatal TP treatment [3.7 ± 0.2 vs 2.9 ± 0.1 mm; $F(1, 32) = 21.39$, $P < 0.0001$]. There was no significant prenatal by postnatal interaction effect on anogenital distance [$F(1, 32) = 0.00$, $P > 0.90$].

DISCUSSION

The present results demonstrate an interesting sex difference in the timing that gonadal hormones can influence parental responsiveness in prairie voles. Neonatal castration of males, but not prenatal inhibition of androgenic and estrogenic activity, significantly reduced the percentage that displayed parental behavior. Conversely, parental behavior in females appeared to be rather insensitive to perinatal exposure to testosterone because it did not increase their parental responding. Females from all perinatal treatment groups were more parental, however, when ovariectomized and treated with testosterone during adulthood, which is in contrast to the inability of adult castration or castration followed by testosterone or estrogen treatment to affect the behavior of males (Lonstein and De Vries, 1999a).

Exposure to testicular hormones during postnatal life is necessary for the masculinization of parental responsiveness in male prairie voles because neonatal castration significantly reduced the normally high levels typically displayed by males with intact gonads from birth until adulthood. The fact that neonatal castration reduced parental responsiveness, whereas the pharmacological inhibition of either androgenic or estrogenic activity during the first week after life does not (Lonstein and De Vries, 2000b), suggests that a combination of both androgenic and estrogenic activity at this time is responsible for the normal development of their parental responsiveness. In light of this,

it was surprising that parental responsiveness was not even partially masculinized in females by treating them prenatally and/or postnatally with TP (some of which was presumably aromatized to estradiol). Perinatal treatment with TP influences other aspects of development in female prairie voles, however, because it significantly lengthened their anogenital distance. As far as we are aware, this is the only example in a rodent species of a demasculinization of a sexually dimorphic behavior in males following neonatal castration that is not matched by even a partial masculinization of the behavior of females following prolonged perinatal treatment with TP. This phenomenon apparently extends to at least one sexual dimorphism in the prairie vole brain because we recently found that although neonatal castration of male prairie voles feminizes extrahypothalamic arginine vasopressin (AVP) mRNA and peptide expression, perinatal treatment with TP does not at all masculinize it in females (Lonstein, Rood, Vas, and De Vries, in preparation). The inability of perinatal TP to masculinize and defeminize parental behavior in female prairie voles is reminiscent of the inability of perinatal hormone manipulations to equate the sexual behavior potentials of males and females in some nonrodent species such as rabbits (Campbell, 1965) and ferrets (Baum, 1976; Baum, Gallagher, Martin, and Damassa, 1982). In addition, a conceptually opposite phenomenon, such that the brain and behavior of females can be masculinized by perinatal administration of gonadal hormones but that males cannot be demasculinized or feminized by perinatal blockade of gonadal hormone activity, occurs in some species of birds (Arnold and Schlinger, 1993; Wade, 1999).

With regard to the present experiments, there are many possible explanations for the apparently incompatible effects of neonatal castration and perinatal TP on the parental responding of virgin male and female prairie voles, respectively. One of the simplest explanations may be that the effects of neonatal castration on paternal behavior may not have been due to the removal of testicular testosterone. The testes secrete other hormones, including androstenedione, dehydroepiandrosterone, inhibin, anti-Müllerian hormone, and estrogen itself. The presence or absence of any or all of these hormones in the appropriate concentrations may influence behavioral development in prairie voles. It is also possible that the neonatal testes, but not TP alone, influence other hormone systems (e.g., the HPA axis) in a manner that contributes to sexual differentiation of parental and other behaviors in voles (Roberts, Zullo, and Carter, 1997).

Although perinatal treatment with TP did not increase parental behavior in virgin females, chronic adult exposure to testosterone promoted high levels of parental responsiveness regardless of the females' perinatal treatment. Parental behavior in adult female rats can also be stimulated with chronic testosterone treatment (Bridges and Russell, 1981). Concurrent administration of an aromatase inhibitor with testosterone eliminates this effect in rats (Bridges and Russell, 1981), indicating the importance of testosterone's aromatization to estradiol for the behavioral change. This aromatization is also probably necessary in virgin female prairie voles, as evidenced by the tendency of adult treatment with estradiol alone to reduce infanticide and promote parental responsiveness (Lonstein and De Vries, 1999a). The influence of testosterone on parental behavior somewhat differs between female rats and female voles, however, because testosterone only stimulates parental responding in virgin rats if administered along with progesterone (Bridges and Russell, 1981), whereas testosterone alone stimulated high levels of parental responding in female prairie voles. This is consistent with other data indicating a relatively minor role for progesterone in the facilitation of reproductive behaviors in prairie voles (Dluzen and Carter, 1979) and other induced-ovulating species such as rabbits (Hudson, Gonzales-Mariscal, and Beyer, 1990) and ferrets (Baum *et al.*, 1986). The generally similar behavior of females from different perinatal treatment groups that received testosterone as adults was not unexpected considering that exposing perinatal male or female rats to testosterone has no effect on the later ability of estrogen, progesterone, and prolactin to stimulate parental responding if subjects are tested as adults in a relatively stress-free home cage environment (Bridges, Zarrow, and Denenberg, 1973).

Because many induced ovulating species are relatively unresponsive to masculinizing effects of perinatal TP on some reproductive behaviors (e.g., Campbell, 1965; Baum, Gallagher, Martin, and Damassa, 1982; present results), it has been suggested that testosterone masculinizes as well as defeminizes the brain only in species that require both neural estrogen and progesterone activity for female reproductive behaviors (see Baum, 1979). However, this apparently cannot be generalized across all induced ovulators, or even across the genus *Microtus*, because sexual behavior in the biparental, monogamous, and induced-ovulating pine vole (*Microtus pinetorum*) (Fitzgerald and Madison, 1983; Oliveras and Novak, 1986; Sawrey and Dewsbury, 1985; Taylor, Salo, and Dewsbury, 1992)

can be both masculinized and defeminized by even a brief neonatal testosterone treatment (a single 0.5-mg TP injection on day of birth) (Wekesa and Vandenberg, 1996). Therefore, even within closely related species of *Microtus* there is a diverse array of hormonal mechanisms affecting sexual differentiation of reproductive behaviors, emphasizing the value of using Microtine rodents as a model for studying the effects of hormones on behavioral development in mammals.

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