HORMONES AND BEHAVIOR 12, 1–11 (1979)

Prenatal Exposure to Androgen Influences Morphology and Aggressive Behavior of Male and Female Mice

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To assess the effects of prenatal exposure to androgen on adult aggressiveness in mice, pregnant mice were given injections of 1.5 mg testosterone propionate (TP) or oil from Days 12 to 16 of pregnancy. All offspring were gonadectomized on the day of birth. Neonatal treatment occurred on the day following birth and consisted of one-half of the animals from each prenatal treatment group being injected with 100 μg TP while the other half were injected with oil, yielding four Prenatal/Neonatal treatment groups for each sex. On postnatal Day 60, all offspring were given subcutaneous implants of encapsulated testosterone (T) and tested for 10 min every other day against a male opponent until aggression was observed. Female offspring of TP-treated mothers were indistinguishable from males on external examination at birth. The duration of exposure to T required to induce aggression provides an index of the sensitivity of the neural substrate to T. When arranged from the most sensitive to the least sensitive to the aggression inducing action of T, the four Prenatal/Neonatal treatment groups of females were significantly different from each other: Group TP/TP > Group OIL/TP > Group TP/OIL > Group OIL/OIL. A similar pattern was observed for the male offspring. There were no differences in the proportion of animals per group that exhibited aggression (virtually all animals fought) or the intensity of aggression once exhibited. The results demonstrate that morphological and behavioral masculinization can occur in response to exposure to androgen during prenatal as well as neonatal life in mice.

Androgen exposure during early life has been found to profoundly alter the neuroendocrine regulation of reproductive function as well as behavior in rodents. For some time it was presumed that androgen exposure during a critical period, which began shortly after birth, was necessary to permanently alter the neural substrate mediating sex-related behaviors. Without exposure to androgen during this critical period, it was assumed that "masculine" behavior would not be exhibited later in life: males

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[2] This research was conducted in the laboratory of Dr. R. Gandelman and was supported by Grant HD06863 from NICHD, NIH.

0018-506X/79/010001-11$01.00/0

castrated at birth were "female-like" while females androgenized at birth were "male-like" in adulthood (c.f. Bronson and Desjardins, 1970; Edwards, 1969; Pfeiffer, 1936; Young, Goy, and Phoenix, 1964). Subsequent studies did not provide support for this model in that neonatal androgen exposure was found not to be a necessary prerequisite for adult male or female mice to exhibit aggression (normally categorized as a "masculine" behavior in mice) in response to chronic adult testosterone (T) exposure (vom Saal, Gandelman, and Svare, 1976a). However, male mice gonadectomized at birth were found to exhibit aggression after a shorter period of adult T exposure than females gonadectomized at birth, although all animals eventually exhibited aggression. This finding suggested that exposure by males to endogenously produced androgen during prenatal life had rendered them more sensitive than females to the aggression inducing action of T in adulthood (vom Saal, Svare, and Gandelman, 1976b).

The present experiment was designed to test the importance of exposure to androgen during prenatal life on the sensitivity of the neural substrate to the aggression inducing action of T in adulthood. Fetuses were exposed to testosterone propionate (TP) or oil prenatally via injections administered to pregnant females during the last trimester of gestation. In addition, the pups then were injected with TP or oil on the day after birth (utilizing a 2 × 2 design) to assess the relative effects of prenatal and neonatal androgen exposure on adult aggressive behavior. Both males and females from all treatment conditions were tested. The results revealed that while both prenatal and neonatal exposure to TP increased the sensitivity of the neural substrate to the aggression inducing action of T in adulthood, neonatal exposure to TP was more effective in this regard.

METHODS

Prenatal manipulations. Beginning on Day 60 following birth, virgin female Rockland–Swiss (R–S) mice, housed in 27 × 17 × 12-cm polypropylene cages, were timed-mated. Food and water were available *ad lib.* Animal rooms were maintained at 23 ± 1° on a 12/12 light/dark cycle, with lights on at 0600 hr. Subsequent to mating (verified by the presence of a vaginal plug), the females were isolated, with that date being recorded as Day 0 of pregnancy. The females then were randomly assigned to one of three prenatal treatment groups: Undisturbed, Oil-injected, or TP-injected (see Table 1). Mothers in the undisturbed group were not removed from their cages throughout the 19-day gestation period to control for any stressful effects of the handling and injection procedures on adult aggressiveness (c.f. DeFries, Weir, and Hegmann, 1967). Mothers in the oil group were given a subcutaneous injection of 0.06 cc sesame oil at 1800 hr on Days 12 through 16 of gestation. Mothers in the TP group received injections of 1.5 mg TP dissolved in 0.06 cc sesame oil on the same

TABLE 1

Group name[a]	Sex	*N*	Prenatal Treatment of dams	Neonatal Treatment of pups	Adult Treatment
NON/OIL	Male	20	Undisturbed	Oil injection	T implant
	Male	18	Oil injection	Oil injection	T implant
	Female	24	Undisturbed	Oil injection	T implant
	Female	17	Oil injection	Oil injection	T implant
NON/TP	Male	15	Undisturbed	TP injection	T implant
	Male	13	Oil injection	TP injection	T implant
	Female	16	Undisturbed	TP injection	T implant
	Female	23	Oil injection	TP injection	T implant
TP/OIL	Male	22	TP injection	Oil injection	T implant
	Female	24	TP injection	Oil injection	T implant
TP/TP	Male	15	TP injection	TP injection	T implant
	Female	27	TP injection	TP injection	T implant
CON	Male	35	Undisturbed	No injection No gonadectomy	No implant

[a] All groups tested in the experiment. All animals except those in Group CON were gonadectomized at birth.

schedule. Each injected mother thus received five injections of either oil or TP.

The normal period of gestation for R–S mice is 19 days. The TP injection regime was begun on the evening of Day 12 of gestation since it has been reported that androgen production by male mouse fetuses commences at about this time (Block, Lew, and Klein, 1971). There were two reasons for terminating the TP injections on Day 16 of gestation. First, Gerall and Ward (1966), working with rats, reported that the time period but not the dose of TP administration during gestation determined whether parturition would occur on time. It appears that if high levels of TP are present in the plasma at term, parturition is inhibited and fetal death occurs. Second, TP probably is active for a number of days following administration in oil.

Neonatal manipulations. Between 0800 and 0900 hr on Day 19 of pregnancy, females were checked, and those that had not yet delivered or were not exhibiting maternal behavior were discarded. At this time all pups were removed from their mothers and placed under heat lamps. Anogenital distance (as measured by the distance in millimeters from the caudal aspect of the genital papilla to the rostral aspect of the anus) was measured for each animal. Anogenital distance is a sensitive bioassay for exposure to androgen during prenatal life. All pups then were gonadectomized under ether anesthesia; the skin was punctured, the gonads were removed with forceps, and the wounds were covered with an antiseptic adhesive (Newskin). In addition, the pups were marked for individual identification using a toe-marking procedure. It has been reported (Gandelman and vom Saal, 1977) that exposing a female mouse to TP induces pup killing. This necessitated the use of foster mothers, each receiving five to six pups, to insure that all experimental animals received adequate, and comparable, postnatal care.

At 0900 hr on the day following birth, the gonadectomized pups were subdivided into two neonatal treatment groups. One-half of the pups from each prenatal treatment group received a subcutaneous injection of 0.02 cc sesame oil, and the remaining pups received an injection of 100 μg TP in 0.02 cc sesame oil.

Some males (Group CON) from mothers in the undisturbed group served as intact control animals. These males were not anesthetized or injected following birth, but they were individually marked and anogenital distance measures were recorded. Fostering and housing procedures were the same as above.

Adult treatment and testing. The animals were weaned on Day 21 following birth and were maintained in foster-litter groups until 60 days old. At this time all animals (with the exception of the intact males in Group CON) were anesthetized with ether and implanted subcutaneously with a 10-mm length of Silastic tubing (Dow Corning, 0.062 id) containing 5 mg T in 0.02 ml oil. Following surgery, anogenital distance and body

weight were again recorded, and each animal was housed individually. Intact males were weighed and anogenital distance was measured. However, they then were housed individually without being anesthetized or implanted with the T-containing capsule.

Aggression testing was begun on the day following isolation and continued with one 10-min test session being conducted every day for the first 4 days, after which testing was conducted on alternate days for a maximum of 50 days (27 tests). An aggression test consisted of an olfactory-bulbectomized male intruder being placed into the home cage of the test animal (Denenberg, Gaulin-Kremer, Gandelman, and Zarrow, 1973). The intruder was left in the cage for a maximum of 10 min if no fight occurred. If an attack occurred within this 10-min period, the bulbectomized male was left in the cage for 10 min after the first attack occurred. Therefore, following the onset of aggressive behavior, the number of attacks as well as the total duration of time spent fighting could be recorded for a constant amount of time for all animals regardless of the latency to exhibit the first attack from the start of the session. The latency (LATENCY: in sec) to exhibit the first attack following introduction of the intruder, the number of attacks (NUMBER), and total duration of time (DURATION: in sec) spent biting and chasing the intruder were recorded. An individual attack consisted of the test animal chasing and biting the bulbectomized male and was considered terminated if the test animal exhibited some other behavior, such as grooming. If the test animal had a score on the total duration of fighting measure which exceeded 5 sec, the session was scored as a fight, and the test animal was terminated from the experiment. At this time, the number of days (DAYS) of T exposure (or days of isolation for the intact male in Group CON) was also recorded. Upon termination from the experiment, the experimental animals were autopsied to verify complete removal of the ovaries and testes. Animals which did not exhibit aggression within the 50-day test period were not included in the data analyses. Post hoc comparisons were performed utilizing Duncan's new multiple range test ($p < 0.05$).

RESULTS

Preliminary analyses of the data for animals from undisturbed vs oil-treated mothers revealed that the prenatal injection procedure had no significant effect on any of the dependent measures, so these data were combined. For descriptive purposes these combined groups will be referred to as Prenatally NON-treated Groups, designated by the prefix NON/ (see Table 1).

Morphological Characteristics

At birth, normal male and female mice can be easily distinguished since males have a substantially larger space between the anus and the genital papilla than do females. Prenatal exposure to TP did not affect the anogen-

ital distance of males at birth (see Table 2). However, females exposed to TP prenatally were significantly different from nontreated females and indistinguishable from either TP-treated or nontreated males on this measure. Vaginal canalization did not occur in the prenatally TP-exposed females; positive identification of sex was made at autopsy in adulthood by the presence of a vaginal pouch and uterus. These females also had large seminal vesicles.

None of the groups of gonadectomized animals differed on the body weight in adulthood measure. The average body weight for all gonadectomized animals on Day 60 was 28.6 g.

Aggression Measures

The data for males and females were analyzed separately. Post hoc comparisons of the means for the females on the DAYS measure (see Fig. 1) revealed that exposure to TP either prenatally (Group TP/OIL) or neonatally (Group NON/TP) significantly decreased the duration of T exposure required to induce aggression in adulthood relative to nontreated females (Group NON/OIL), although the neonatal TP injection was significantly more effective in this regard. However, the females exposed to TP prenatally and neonatally (Group TP/TP) required the shortest exposure period to T to exhibit aggression. It should be remembered that males normally are exposed to endogenous androgen during prenatal life. Therefore, there was no group of males that had not been exposed to androgen prenatally. However, as was the case for the females, the single neonatal TP injection significantly decreased the duration of adult T exposure required to induce aggression (Groups NON/OIL and TP/OIL vs Groups NON/TP and TP/TP). Finally, it should be noted that the difference observed between the males and females in Group NON/TP on the variable DAYS is a function of the dose of TP administered during the neonatal period. Previously, vom Saal *et al.* (1976b) found no difference on the DAYS measure between males and females gonadectomized at birth and injected with 300 μg TP on either Day 0, 3, or 6 following birth.

Analysis of variance performed on the variables LATENCY, DURATION, and NUMBER revealed no differences between any of the groups of gonadectomized animals on these measures of aggression.

Intact Males Compared to All Other Groups

All groups of animals gonadectomized at birth had significantly smaller anogenital distances in adulthood than the intact males. The intact males also were significantly heavier than the gonadecotomized animals on Day 60. None of the groups administered TP neonatally differed significantly from the intact males with respect to the number of days to the onset of aggression (DAYS). On the other hand, all of the groups administered oil

TABLE 2

Group[a]	Sex	*N*	AGB[b]	AGA	BWA	Days	Latency	Duration	Number
NON/OIL	M	38	1.63 ± 0.02	9.3 ± 0.1	28.1 ± 0.5	15.5 ± 1.4	192 ± 25	18.2 ± 2.3	9.3 ± 1.2
	F	41	0.85 ± 0.02	6.1 ± 0.1	27.8 ± 0.5	22.6 ± 1.7	180 ± 27	14.0 ± 1.2	7.9 ± 0.7
NON/TP	M	28	1.59 ± 0.01	9.1 ± 0.1	28.9 ± 0.3	3.9 ± 0.5	213 ± 28	17.9 ± 2.2	10.6 ± 1.2
	F	39	0.86 ± 0.02	6.4 ± 0.1	28.7 ± 0.3	7.4 ± 1.0	240 ± 30	21.3 ± 2.1	11.5 ± 1.1
TP/OIL	M	22	1.66 ± 0.02	10.1 ± 0.2	28.6 ± 0.7	12.7 ± 1.1	126 ± 30	17.2 ± 2.7	6.5 ± 1.1
	F	24	1.60 ± 0.02	9.5 ± 0.2	29.0 ± 0.5	13.0 ± 1.4	171 ± 35	15.9 ± 1.6	8.1 ± 0.8
TP/TP	M	15	1.67 ± 0.02	9.5 ± 0.1	28.7 ± 0.8	5.2 ± 0.9	171 ± 36	15.6 ± 3.0	8.2 ± 1.9
	F	27	1.66 ± 0.02	9.7 ± 0.1	29.4 ± 0.7	4.6 ± 0.8	246 ± 28	16.7 ± 2.3	9.0 ± 1.2
CON	M	35	1.64 ± 0.02	15.4 ± 0.2	32.2 ± 0.5	4.6 ± 1.5	204 ± 26	30.7 ± 3.2	11.1 ± 1.2

[a] Means and standard errors for all groups on the seven dependent measures. See Table 1 for a description of the groups.

[b] M = male; F= female; AGB = anogenital distance at birth in millimeters; AGA = anogenital distance in adulthood in millimeters; BWA = body weight in adulthood in grams; DAYS = the number of days of exposure to T required to induce aggression; LATENCY = the latency (in sec) to the first attack during a test session; DURATION = the total duration of time (in sec) spent fighting during a test session; NUMBER = the number of discrete biting bouts during a test session.

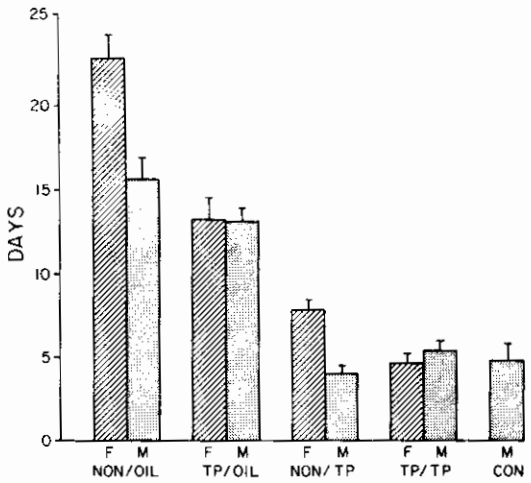

FIG. 1. The mean number of days of exposure to testosterone (DAYS) required to induce aggression for all animals which fought. Groups are identified by PRENATAL/NEONATAL treatment (See Table 1). M = males; F = females.

neonatally required a significantly longer period of isolation and exposure to T to exhibit aggression than the intact males. None of the T-implanted groups differed from the intact males on the variables LATENCY and NUMBER. However, the intact males spent significantly more time fighting (DURATION) than any of the other groups (Dunnett's test, $p < 0.05$; Winer, 1971).

Only 9 of the 278 animals tested in the experiment did not exhibit aggression within the 50-day limit following isolation in adulthood, with no more than 2 animals from any single group not exhibiting fighting behavior.

DISCUSSION

The results both replicate and extend the finding of vom Saal *et al.* (1976b) that males gonadectomized at birth exhibit aggression after a shorter period of exposure to T in adulthood than similarly treated female mice. Importantly, the administration of TP to pregnant females from Days 12 to 16 of gestation had no significant effect on the morphology or behavior of male offspring. However, females exposed to TP during prenatal life were indistinguishable from males castrated at birth both in their external appearance (e.g., large anogenital space and no external vaginal canal) and in their sensitivity to the aggression inducing action of T in adulthood. Thus, exposure to androgen during prenatal life influences adult morphology and aggressive behavior in mice.

It is well documented (c.f. Edwards, 1969) that exposure to androgen during the neonatal period is important for mice to be maximally sensitive

to the aggression inducing action of T in adulthood. The single injection of 100 μg TP on the day after birth resulted in the animals exhibiting aggression after a period of isolation and T exposure which was not significantly different from the period of isolation needed to establish fighting behavior in intact males. This finding could lead one to conclude that the neonatal TP treatment was sufficient to completely masculinize gonadectomized males and females behaviorally. In fact, this was not the case. The intensity of the aggression exhibited by the intact males was much greater than the intensity of aggression exhibited by any of the other groups, with the intact males spending almost twice as much time as the gonadectomized animals biting and chasing the male intruder.

It has been reported that aggression in intact male mice was first observed between Days 30 and 35 of life, shortly after plasma androgen levels began increasing sharply. In addition, as both the levels of androgen and age of the animals increased, the intensity of the aggression exhibited increased, although the proportion of animals fighting remained constant (virtually all animals fought) after Day 40 (Barkley and Goldman, 1977a; McKinney and Desjardins, 1973). Additionally, Owen, Peters, and Bronson (1973) reported that while virtually all males castrated on either Day 10 or 50 of life exhibited aggression following exposure to TP, the aggression exhibited by the Day 50 castrates was significantly more intense than that exhibited by the Day 10 castrates. One possible interpretation of these findings is that androgen has a dual influence on aggressiveness: (1) Exposure to androgen prior to adulthood (during gestation, neonatal life, or adolescence) (Edwards, 1970; vom Saal *et al.*, 1976a) or possibly even in adulthood (Barkley and Goldman, 1977b) influences the sensitivity of the neural substrate to the aggression inducing action of T later in life. (2) The duration of exposure to androgen throughout development influences the intensity of aggression once it is elicited in adulthood. Alternatively, since dominance status and body weight of males are correlated (Barkley and Goldman, 1977a), and in the present study the intact males were heavier than the gonadectomized animals, the observed difference in the intensity of aggression between these groups may have been due to differences in body weight.

In the present study, all animals were exposed to T in adulthood. Numerous previous studies have demonstrated that in the absence of androgen exposure in adulthood, male or female mice will not attack a male opponent (c.f. vom Saal, *et al.*, 1976a,b). It should be noted, however, that extensive experience fighting while intact increases the likelihood that a male mouse will continue to exhibit aggression subsequent to castration (unpublished observation). While circulating T is necessary to induce a naive mouse (male or female) to attack a male opponent, aggression between females is not uncommon in some strains of mice and is not dependent on concurrent exposure to exogenous androgen.

While the present results could be interpreted as indicating that prenatal androgen exposure acts to increase the sensitivity of the neural substrate to the aggression-inducing action of T in adulthood, a recently completed series of studies raises doubts about this interpretation. Specifically, vom Saal and Bronson (1978) have found that female mice that develop between two males in the uterus have larger anogenital spaces at birth, go through puberty sooner when housed in groups, are less sexually attractive, and are highly aggressive in a variety of situations relative to female mice that develop *in utero* contiguous to other female fetuses.

Of particular relevance to the present study is the finding that females that develop between males *in utero* have larger anogenital spaces, which strongly suggests that androgen produced by contiguous males may somehow be the mediator of this phenomenon. These females were also highly aggressive toward other females in adulthood whether or not they were implanted with a T-containing capsule as was done in the present study. Taken together, these findings suggest that prenatal exposure to androgen may produce permanent changes in behavior as well as stimulus characteristics of females which are independent of exposure to androgen in adulthood. The model that the behavioral effects of exposure to androgen during prenatal and neonatal life are latent in that the neural substrate is sensitized to the behavioral inducing action of androgen later in life thus may apply only to the case in which the aggression eliciting stimulus is a male mouse.

In conclusion, exposure to androgen during both the prenatal and neonatal periods appears to interact with adult circulating levels of T and the sex of the opponent in determining whether a mouse will exhibit aggression.

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