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# Aggressive Behavior Induced in Female Mice by an Early Single Injection of Testosterone Is Genotype Dependent

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*Early exposure of female mice to testosterone induces aggressive behaviors in adulthood as a function of strain. Inbred BALB/cBy females that receive a single injection of testosterone propionate (TP) (1 mg in 0.02 ml peanut oil) on Day 4 of life manifest attacking behavior toward a male attempting to mate, whereas C57BL/6By do not. Ovariectomy at weaning does not modify the effect of neonatal androgenization. Neither comparison of reciprocal $F_1$'s nor observations of inbred females from hybrid maternal environments suggest that the strain-dependent effects of TP are dependent on different maternal environment effects. Thus the difference in reactivity to a single early injection of TP between female BALB/cBy and female C57BL/6By has a purely genetic correlate.*

**KEY WORDS:** female aggression; early testosterone exposure; inbred strains; mice; ovarian transplantation.

## INTRODUCTION

Female mice routinely employed in laboratory studies are relatively nonaggressive compared to males (Scott, 1966). In contrast, when testosterone is administered neonatally (Bronson and Desjardins, 1968; Vale *et al.*, 1972), female mice exhibit aggressive behaviors toward the various opponents employed in these experiments. Previous findings have suggested that the effects of neonatal androgen upon adult behavior in females

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may be strain dependent (Vale *et al.*, 1972). These authors found a strain × exogeneous neonatal androgen interaction for aggressive behavior in three inbred strains of female mice (A/J, C57BL/6J, and BALB/cJ). The androgenized BALB/c females responded at a much higher rate than either the control BALB/c or the control and androgenized females of the other two strains. The originality of the work of Vale *et al.* lies not only in that it studied female mice having different genotypes, but in that the authors chose to administer a single injection of testosterone 3 days after birth without consecutive injections in adulthood as is the case in most studies. Vale and co-workers' study was restricted to a strain comparison, and their experiment was not designed to test for a possible maternal effect and/or interaction between genotype and maternal effects.

Experiment 1 attempts to replicate these differences in reactivity at adulthood to an early androgenization in BALB/cBy and C57BL/6By female mice, through indices of aggressive behavior. Supplementary controls were used to ensure that the observed behavioral modifications were the direct result of the single injection of testosterone and could not be attributed to ovarian dysfunction. The two methods presented in the second experiment were aimed at dissociating effects of genotype, maternal environment, and/or genotype × maternal environment interaction through comparison of the reciprocal $F_1$'s and an ovary transplant design.

## MATERIALS AND METHODS

*General Rearing Conditions.* All subjects used in this study were born in the laboratory from identified breeders supplied by the CSEAL, CNRS (Orleans, France), or IFFA-CREDO (Les Oncins, France). The rearing conditions were the following: temperature, 23 ± 0.5°C; photoperiod, 12:12 h with lights on at 0830; food (IM, UAR) and water, ad lib.; and bedding, dust-free sawdust.

*Early Testosterone Exposure Procedure.* Females visibly close to parturition were housed individually. The cages were visually inspected every day. A litter was stated to have been born on the day it was seen for the first time (Day 1 of life). The early exposure to testosterone (EET) consisted of a single subcutaneous injection of 1 mg of testosterone propionate (TP) diluted in 0.02 ml of peanut oil. Whole litters were injected in the morning of the fourth day of life. The control groups received an equal volume of peanut oil alone under the same conditions.

At weaning (Day 30), males were discarded and females were placed individually in cages (42 × 12 × 18 cm) until 80 ± 10 days of age. This isolation procedure has been used in studies on androgenized females (Edwards, 1968; Bronson and Desjardins, 1968; Howard *et al.*, 1981) to

parallel standard practices for increasing the probability of the occurrence of aggressive behavior in studies of male aggression (Ginsburg and Allee, 1942).

*Behavioral Testing.* Vale *et al.* (1972) used three types of behavioral tests: spontaneous aggressive behavior between two females of the same genotype and treatment (repeated a total of three times), the dangler test (Scott, 1946) (two dangles and one free session over 3 consecutive days), and reactions toward a male attempting to mate (only one test). In the present study only this last test was used because it constitutes the only test where the opponent is not likely to initiate a fight. A male rarely attacks a female unless she has just received an androgenizing treatment (Mugford and Nowell, 1970). The aggressive behavior test using two females of the same genotype and neonatal treatment lacks this feature. It can provide only a score per pair of opponents and does not eliminate the problem of establishing who initiates the fight or the dominant–dominated relationship that can arise. In the study by Vale *et al.*, this test showed a treatment but not a strain effect. The dangler test, following the general procedure introduced by Scott (1946), where the female is bumped with the head and forepaws of a male held by the experimenter, is too subjective. These drawbacks can be minimized and reliability increased, however, by using a series of tests, but this introduces additional problems related to successive tests such as serial order effects and experience in winning and losing (Ginsberg and Allee, 1942; Lagerspetz and Lagerspetz, 1971).

Because the opponent in the present aggression test is a male attempting to mate, female attractiveness was produced by administering two intramuscular injections of 0.02 mg of estradiol benzoate in oil in the evening (19 h) approximately 66 and 42 h before the test. On the day of the test, approximately 36 h after the second injection, each female mouse was injected with 0.2 mg of progesterone in oil. The test took place 3–5 h after this last injection, during the light part of the cycle (between 10 and 13 h). The female was placed in an observation Makrolon cage (42 × 32 × 18 cm) containing clean sawdust first. A sexually vigorous B6D2F1 male was then introduced into the cage by the experimenter. The observation period began when the first sniffing took place and lasted 20 min. Female aggressive behavior was measured for the latency of the first attack and number of attacks. The latter variable was dependent upon the latency of the first attack. Since the test had a constant duration for all subjects, the number of attacks over a 1-min period was analyzed. The number of females attacking at least once/the total number of females was also recorded (incidence of attack).

## EXPERIMENT 1

Experiment 1 was designed to replicate the findings of Vale *et al.* (1972) which show that two strains of female mice differ in their reactivity to EET.

*Subjects and Procedure.* Thirty-five BALB/cBy (C) and 23 C57BL/6By (B6) female mice were administered an early testosterone treatment. The control groups consisted of 13 C and 11 B6 that received an injection of pure peanut oil. These four groups were isolated and observed according to the behavioral testing methods described above.

*Results.* The results appear in Fig. 1. The incidence of attack was significantly different in C (20/35) and B6 (0/23) ($\chi^2 = 17.61$, $P < 0.001$). B6 androgenized females do not differ from the controls (0/11), whereas C androgenized females do ($\chi^2 = 7.51$, $P < 0.001$). The partition of the $\chi^2$ (Snedecor, 1946) shows a treatment effect ($\chi^2 = 6.65$, $P < 0.05$), a strain effect ($\chi^2 = 17.77$, $P < 0.001$), and a strain $\times$ treatment interaction ($\chi^2 = 10.09$, $P < 0.001$).

With regard to attack number and latency, the skewness of the distribution of control groups B6 and C (one female C attacks and no B6) and the treated B6 (none of the B6 attack), an analysis of variance is inapplicable. However, the interaction effect can be deduced from the difference in reactivity to treatment in B6 and C. The mean values obtained for the treated C (latency of the first attack—$\bar{X} = 735.51$, SD = 430.41; and number of attacks per minute—$\bar{X} = 0.628$, SD = 0.781) were compared to hypothesized values (Mac Nemar, 1969) which are the medians for the C oil (latency of the first attack, med = 1200; number of attacks per minute, med = 0). This test shows a significant testosterone effect for C [latency of the first attack—$t(34) = 6.39$, $P < 0.001$; number of attacks per minute—$t(34) = 4.76$, $P < 0.001$]. The controls and treated B6 do not differ for either variable, since no females from either of the groups attack.

*Discussion.* When assessed on one aggression test in adulthood, strains B6 and C differ in reactivity to a single injection of TP administered on Day 4 of life. However, it cannot be taken for granted that this difference in reactivity at adulthood is entirely due to a difference in the early sensitivity of these two strains to EET 4 days after birth. Early androgenization also dramatically alters ovarian function (Barraclough and Leathem, 1954). The behavioral modifications observed in adulthood in strain C could have resulted from an ovarian dysfunction, for example, ovarian tumors producing chronic androgenic stimulation. Aggressive behavior could also be accounted for by an interaction between eventual endogenous steroids and steroids administered before the behavioral test-

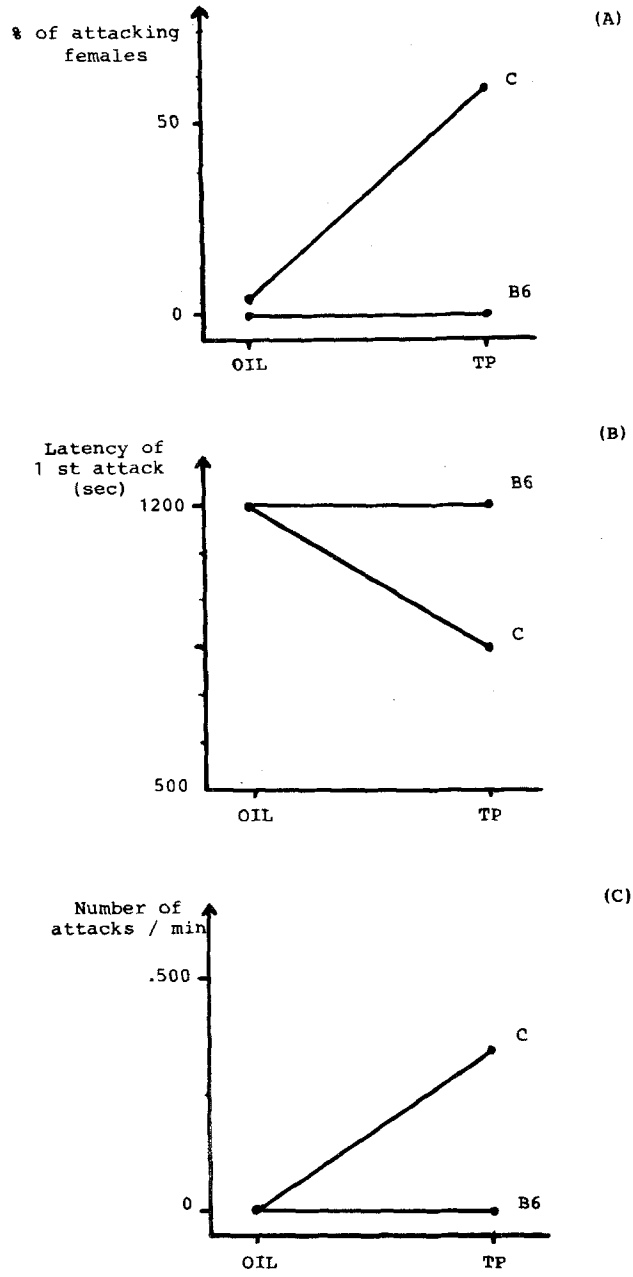


**Fig. 1.** Strain × early exposure to testosterone interaction for three variables: (A) incidence of attack (percentage of attacking females); (B) latency of the first attack (median in seconds); and (C) number of attacks per minute (median).

ing. Finally, the presence of ovaries during development may mask the effects of neonatal TP treatment (Blizard and Denef, 1972) and should also be controlled.

### Effect of Ovariectomy

This complementary experiment was designed to test whether EET effects on aggressive behavior on C female mice were ovarian dependent.

*Materials and Methods.* Thirteen female C mice administered the testosterone treatment previously described were ovariectomized at the age of 30 days (sodium pentobarbital anesthesia). They were then isolated and observed under conditions as described above.

*Results and Discussion.* The performances of ovariectomized C female mice (incidence of attack—7/13; latency of the first attack—$X$ = 670.08, SD = 495.88; number of attacks—$X$ = 0.658, SD = 0.783) do not differ from those of intact C female mice on any of the behavioral variables [incidence of attack, $\chi^2 = 0.015$; latency of the first attack, $t(46) = 0.449$; and number of attacks per minute, $t(46) = 0.116$].

These results override any necessity of controlling for an effect of the operation per se or the effects of an eventual interaction among EET, ovariectomy, and steroid injections before the test. These results show that EET effects on aggressive behavior are not dependent on the presence of ovaries in adulthood.

## EXPERIMENTS 2a AND 2b

These experiments were designed to identify the genetic and/or maternal sources of the difference in sensitivity to testosterone in B6 and C as measured by the presence of attacking behavior in the adult female mouse. The effects of three components were tested: the genotypic component, the global maternal environment, and the genotype × maternal environment interaction component. Two methods were used successively.

In experiment 2a the performances of genetically identical subjects, carried by mothers having different genotypes (i.e., the reciprocal $F_1$'s), were compared. This estimate of the environmental component in the heterozygotic $F_1$'s could be biased by the fact that $F_1$'s do not exhibit the same reactivity to environmental modifications as the homozygotic subjects (Falconer, 1960).

In experiment 2b, the performances of inbred females carried by females of the same genotype (hybrid mothers) were compared. These subjects were obtained through an ovarian transplant design. The surgical

**Table I.** Scores for $F_1$ Female Mice and B6 and C Females Born to Ovarian-Transplanted Mothers

| Genotype | Incidence of attack | Latency of first attack | | Number of attacks/min | |
|---|---|---|---|---|---|
| | | $X$ (s) | SD | $X$ | SD |
| CB6 | 30/42 | 584.17 | 420.44 | 1.092 | 1.158 |
| B6C | 33/46 | 516.74 | 455.94 | 0.963 | 0.885 |
| $C_oC.C$ | 11/16 | 627.00 | 437.75 | 0.854 | 0.812 |
| $C_oB6C.C$ | 13/23 | 717.44 | 440.59 | 0.489 | 0.791 |
| $B6_oB6.B6$ | 0/16 | 1200 | 0 | 0 | 0 |
| $B6_oB6C.B6$ | 4/25 | 1125 | 221.99 | 0.157 | 0.385 |

procedure is described by Palm (1961) and its application to behavior-genetic analysis is presented by De Fries *et al.* (1967). This method tests for the effects of the maternal component on homozygotes, as well as the effects of genetic and genotype × maternal environment components.

*Subjects (Expt 2a).* Forty-six female B6CF1 and 42 female CB6F1 mice were injected, isolated and tested as previously described.

*Results and Discussion.* The results are shown in Table I. A comparison of reciprocal female $F_1$'s shows no significant difference for incidence of attack ($\chi^2 = 0.041$), latency of the first attack [$t(86) = 0.719$], or number of attacks per minute [$t(86) = 0.59$]. Whether the female $F_1$'s had a B6 or a C maternal environment, both reacted in the same way to EET.

*Subjects, Surgery, and Electrophoretical Control Procedures (Expt 2b).* B6CF1 female recipients 6 weeks of age were anesthesized (sodium pentobarbital, Nembutal). Host ovaries were removed and ovaries from inbred donors, either B6 (females designated B6oB6CF1) or C (females designated CoB6CF1), were grafted bilaterally. The control groups for transplant effects consisted of host female mice from each of the two strains grafted with ovaries from donors of the same genotype designated, respectively, B6oB6 and CoC. At the age of 8 weeks, the female mice were mated with an appropriate male in order to obtain inbred offspring which could have been carried by either hybrid or inbred mothers. This procedure required controlling the origin of each individual in the litter. During ovarian transplant, fragments of host ovaries may remain, produce ova, and thus be responsible for all or part of the litter. The reliability of the ovarian transplant procedure was checked by coat color and two electrophoretic markers tested postmortem (*Mup-1*, major urinary protein, mapped on chromosome 4; *Hbb*, hemoglobin $\beta$-chain, mapped on chromosome 7) (Green, 1981). Two isodominant alleles of *Mup-1* are known:

*Mup-1a* determines the presence of a slow-migrating band occurring in C; *Mup-1b* determines the presence of a faster band occurring in B6. The $F_1$'s present both bands. The electrophoretical procedure was as follows: tissue sample, urine; migration, 20 min; voltage, 220 V; buffer, Tris barbital, pH 8.8; staining, Ponceau; and destaining, acetic acid. Three isodominant alleles are known for *Hbb*. *Hbbs* produces an electrophoretically single hemoglobin and is found in B6. *Hbbd* produces a diffuse electrophoretic pattern and is found in C. The $F_1$'s are indistinguishable from C except if specific reagents are employed in the buffer. The electrophoretical procedure was the following: tissue sample, hemolysate; migration, 30 min; voltage, 350 V; buffer, Tris EDTA borate (+cystamine + DTE); staining, Ponceau; and destaining, acetic acid. Details of the electrophoretic procedure are available from the authors.

Two litters with inappropriate coat color were discarded. However, electrophoretic identification did not detect any subjects with an inappropriate genotype. Twenty-three C and 25 B6 females from the same prenatal uterine and postnatal environment were observed and their performances were analyzed. The control groups were made up of 16 C carried by CoC and 16 B6 carried by B6oB6. All the subjects were administered the same testosterone treatment, isolated, and tested as previously described.

*Genetic Design.* The following groups were thus available for analysis:

B6oB6CF1.B6, group 1;
CoB6CF1.C, group 2;
B6oB6.B6, group 3; and
CoC.C, group 4.

The comparison of groups 1 and 2 provides a means of testing the effect of the genetic component. The effect of the maternal environment (hybrid vs. inbred) can be shown by a comparison of groups 1 and 3 and also by groups 2 and 4.

Given the distribution curve for groups 1 and 3 (null or very low variance), an ANOVA was not applicable. As above, a partial comparison technique was adopted. The genotype × maternal environment interaction ($F_1$'s vs. inbred) on TP-treated mice was tested for the variable "incidence of attack" as follows: $\chi^2_a$ = the value of the $\chi^2$ corresponding to the differences associated with maternal environments for the pooled genotypes of the offspring (B6oB6CF1.B6 + CoB6CF1.C vs. B6oB6.B6 + CoC.C); $\chi^2_b$ = the value of the $\chi^2$ corresponding to the maternal environment effect on B6 (B6oB6CF1.B6 vs. B6oB6.B6); and $\chi^2_c$ = the value of the $\chi^2$ corresponding to the maternal environment effect on C (CoB6CF1.C vs. CoC.C). The genotype × maternal environment inter-

action is significant if $\chi^2_a - (\chi^2_b + \chi^2_c)$ is significantly different from zero.

*Results and Discussion.* An absence of a transplant effect per se for all the variables tested was shown by a comparison of control groups (B6oB6.B6) and (CoC.C), respectively, to B6.B6 and C.C from experiment 1. No effect was observed for C [incidence of attack, $\chi^2 = 0.229$; latency of the first attack, $t(49) = 0.831$; number of attacks per minute, $t(49) = 0.949$] or for B6. No effect was observed for the $F_1$ maternal environment versus the inbred strain maternal environment. The C females' performances are identical whether they were born to an $F_1$ mother or a C mother [incidence of attack, $\chi^2 = 0.191$; latency of the first attack, $t(37) = 0.632$; number of attacks per minute, $t(37) = 1.404$]. A median test (Mann–Whitney) was used to test for this effect on the B6 and for comparisons including B6 groups. B6 female mice from an $F_1$ mother or a B6 mother do not differ on any of the parameters (incidence of attack, $\chi^2 = 1.31$; latency of the first attack, $z = 0.885$; number of attacks, $z = 0.855$). However, when B6 and C females mice are born to $F_1$ mothers (e.g., $F_1$ having received "inbred" ovaries), these behavioral differences persist [incidence of attack—$\chi^2 = 6.92$, $P < 0.01$; latency of the first attack—$z(46) = 2.73$, $P < 0.01$; number of attacks—$z(46) = 2.19$, $P < 0.05$]. In contrast, the effect of the genotype × maternal environment interaction is not significant: $\chi^2_a = 0.0206$, $\chi^2_b = 1.31$, and $\chi^2_c = 0.191$; yielding a $\chi^2$ for interaction = 1.48 (ns). Thus the maternal environment has no significant effect on the differences between B6 and C female mice.

The difference between strain B6 and strain C for reactivity to EET, as measured by the occurrence of aggressive behaviors in adult female mice, has an exclusively genetic correlate.

## GENERAL DISCUSSION

This study successfully demonstrates that an androgen treatment procedure identical to the one used by Bronson and Desjardins (1968) and Vale *et al.* (1972) can modify the social behavior of adult female mice. A single injection of a large amount of TP (1 mg) produces an interaction between strain and early exposure to testosterone for attacking behavior in BALB/cBy and C57BL/6By female mice. The results of Vale *et al.* (1972) are partially replicated. These authors obtained a higher percentage of females attacking a male attempting to mate at least once (4/20 for B6 and 17/20 for C). They, however, report no significant TP effect for the B6 strain. A genetic reason may explain this disparity: the BALB/cBy and C57BL/6By strains were observed here, whereas Vale *et al.* (1972) studied BALB/cJ and C57BL/6J. Furthermore, there were some differ-

ences in the experimental procedure: in the present study the pups were approximately 24 h older when they were injected with TP and only one aggression test was used. Nevertheless, none of these hypotheses can validly explain the discrepancy between the results obtained here and those reported by Michard (1984). This study was conducted on an initial group in 1983 and 1984. At that time I obtained 45% of B6 attacking females and 90% of C. I have no explanation for the decrease in percentages. It should be noted, however, that the difference between the two strains is still present and is of the same magnitude.

The aggressive behaviors observed in adulthood are not dependent on maintenance of hormonal stimulation through ovarian dysfunction created by early androgenization (Expt 1). The comparison of the reciprocal $F_1$ females (Expt 2a) and the inbred females born to hosts with ovarian transplants (Expt 2b) demonstrates an absence of pre- and postnatal maternal environment effects. Thus there is a true genotype $\times$ EET interaction. The B6 and C genotypes react differently to an identical dose of testosterone.

What physiological mechanisms can explain this interaction? Several authors have reported the effect of intrauterine position on the sexual characteristics of female mice (Vom Saal and Bronson, 1980). It could be assumed that prenatal sensitization, which is a function of the social environment *in utero* (a female can be placed between two females or between two males or between one male and one female), might influence sensitivity to an early postnatal treatment. If such a phenomenon can account for the withingroup variability (28% of $F_1$ and 43% of C do not attack), clearly it cannot explain the difference between the two inbred strains, since the sex ratio is approximately the same in both strains and no study has shown that the sex distribution in the uterine horns varies according to the genotype of the pups! Two nonexclusive hypotheses may nevertheless be put forward for this interaction: C and B6 may differ in postnatal sensitivity period and/or there may be a lesser permanent sensitivity in B6 strain. The behaviors exhibited by B6 and also by nonattacking C and $F_1$ female mice do not negate either of these hypotheses. The nonattacking subjects manifest minimal sexual receptivity. Most actively resist copulatory attempts by back-kicking and avoidance. When an experienced male succeeds in mounting, the female mouse curves its hindquarters inward in a position prohibiting mating, lifts its head slightly, or—rarely—exhibits a lordotic response. These female mice at times exhibit threat behaviors, without, however, attacking the male.

This series of experiments has shown that a single injection of 1 mg of TP 4 days after birth, in the absence of any restimulation at adulthood by a prolonged treatment, induces extremely clear genotype-dependent

modifications in females' social behavior 3 months later. The effect of neonatal androgen treatment cannot be investigated independently of the genotype.

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