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Androgen Threshold to Activate Copulation Differs in Male Rats Prenatally Exposed to Alcohol, Stress, or Both Factors

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Few male rats prenatally exposed to a combination of alcohol and stress copulate spontaneously. This study determined adult sensitivity to testosterone (T) in males prenatally exposed to alcohol, to stress, or to both factors. Sexually naive males were tested with receptive females following castration and implantation of 20-, 30-, or 45-mm Silastic T-filled capsules. Serum T levels provided by these implants were measured. The behavior shown by males exposed only to prenatal alcohol did not differ from untreated control animals at any T dosage. Prenatal stress alone diminished the copulatory potential below control levels only when the intermediate T dosage was provided. Few males exposed to both alcohol and stress copulated under the lowest or the intermediate dose of adult T replacement, but most ejaculated normally when the largest capsule was implanted. The threshold to the sexual behavior-activating-properties of adult T exposure was moderately raised by prenatal stress but was severely affected when prenatal stress was combined with alcohol. We conclude that a diminished sensitivity to androgen in adulthood underlies some copulatory deficits resulting from treatments that alter fetal T levels. Such deficits may be concealed when behavior is evaluated in gonadally intact animals.

Key Words: **sexual differentiation; male sexual behavior; ethanol; testosterone sensitivity; fetus; Silastic implant; testosterone metabolism.**

Prenatal exposure of male rats to ethanol, to the stress induced by maternal restraint, or to a combination of both factors results in an increased propensity to show female lordotic patterns in adulthood (Ward, Ward, Winn, and Bielawski, 1994). Moreover, exposure to the combination of alcohol and stress severely diminishes the probability that males will spontaneously display ejaculatory patterns. Deficits in male sexual behavior occur more reliably after prenatal exposure to both stress and alcohol than after alcohol or stress alone (Ward *et al.,* 1994; Ward, Ward, Mehan, Winn, French, and Hendricks, 1996).

The etiology underlying atypical sexual behaviors in prenatally stressed and alcohol-treated rats may involve alterations in circulating levels of testosterone (T) during perinatal ontogeny. For example, a species-specific surge in T normally seen on days 18 and 19 of gestation (Ward and Weisz, 1984; Weisz and Ward, 1980) is diminished in fetal males of rat dams exposed either to stress (Ward and Weisz, 1980, 1984) or to alcohol (McGivern, Handa, and Raum, 1998; McGivern, Raum, Salido, and Redei, 1988; Sinha, Halasz, Choi, McGivern, and Redei, 1997) during pregnancy. Further, prenatal alcohol-exposed males exhibit a reduction in the magnitude of the T surge that occurs within the first few hours after birth (McGivern, Handa, and Redei, 1993; see Ward, 1992 for review). This neonatal T surge has not been characterized in prenatally stressed males, nor has the effect of the combination treatment on perinatal T patterns been explored.

Abnormal levels of T during critical perinatal stages alter the organization of adult sexual behavior potentials (e.g., see review by Ward and Ward, 1985). In rats, both the fetal (Hoepfner and Ward, 1988) and the neonatal (Corbier, Roffi, and Rhoda, 1983; Roffi, Chami, Corbier, and Edwards, 1987; Thomas and Ger-

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all, 1969) T surges are important for the differentiation of sexual behaviors. Moreover, the fetal surge influences the development of several sexually dimorphic structures in the brain (Rhees, Shryne, and Gorski, 1990) and spinal cord (Ward, Wexler, Carlucci, Eckert, and Ward, 1996) that have been implicated in the control of male copulatory behavior.

Our previous research suggested that the deficient ejaculatory potential of animals prenatally exposed to alcohol combined with stress may reflect a low sensitivity to the activating effects of T in adulthood (Ward *et al.*, 1996a). While endogenous serum levels of adult T were no different between control males and those prenatally exposed to both alcohol and stress, the treated group copulated only after being injected with large doses of testosterone propionate (1000 μg daily for up to 3 weeks). Thus, it can be concluded that males exposed to the combination treatment during fetal life have a potential for ejaculatory behavior, but higher than normal adult levels of T are required for its activation.

The present study directly compared the amount of circulating T needed to activate sexual behavior in castrated adult males that had been prenatally exposed to stress, to alcohol, or to both treatments. The Silastic implant technique of Smith, Damassa, and Davidson (1977) was used to provide serum levels of T that were approximately (a) 50%, (b) 70%, or (c) equivalent to those found in untreated gonadally intact males.

METHODS

Subjects

Sprague–Dawley rats (colony 205, Madison, WI) were obtained at 45–50 days of age and allowed to adapt for at least 2 weeks before being bred. All animals were housed in temperature (21°C) and humidity (45–55% R.H.) controlled vivaria maintained on a reverse light / dark cycle (lights off 0700–1900 h). Water and Purina Lab Chow (No. 5012) were provided *ad libitum* when animals were not receiving liquid diet. Experimental males were housed in vivaria also containing females. All experimental procedures were approved by the Villanova University Institutional Care and Use Committee and were conducted in accordance with *NIH Guidelines for the Care and Use of Laboratory Animals* (DHEW Publication 80-23, revised 1985).

Apparatus and General Procedures

Two sets of 44 females were mated within 48 h of each other. The second set served as foster mothers for the offspring of the first (the experimental dams). Mating took place between 1300 and 1600 h. The day a female was observed to receive two ejaculations was designated as gestational day 0.

Pregnant rats were housed individually. Foster mothers were placed into Plexiglas cages (45 × 24 × 20 cm) with beta-chip bedding that was replaced weekly. Experimental dams were housed in hanging wire mesh cages (24.5 × 17.5 × 17.5 cm). Shredded paper was provided as nesting material on gestational day 21.

Prenatal Treatments

From days 10 to 21 of gestation, water and chow were removed from the experimental dams and replaced with *ad libitum* access to a liquid diet. Half received a diet in which ethanol (5% wt / vol) made up 36% of the calories (Diet 1265 from Bio Serv, Inc., Frenchtown, NJ). The other half received a control diet in which ethanol had been replaced by an isocaloric amount of maltose-dextrin (Diet 1264, Bio Serv). The diets were presented in drinking tubes that were weighed, cleaned, refilled, and reweighed daily. Weight of diet consumed was used as the measure of intake. Animals receiving ethanol diet were housed in a separate vivarium to avoid exposing the other groups to alcohol fumes. Foster mothers were maintained on *ad libitum* chow and water throughout the study.

Half of the rats in each of the two dietary groups were stressed from days 14 to 21 of gestation. Stressed animals were placed into 13 × 5 × 8.3 cm Plexiglas restrainers (A. H. Thomas 1123-C30), three times daily (900, 1300, and 1700 h) for 45 min. Two 150-W floodlights delivered approximately 2150 L / m^2 across the surface of the restrainers. Nonstress dams were not handled.

The experimental females were assigned randomly to the following groups: (1) stress and alcohol diet (N = 12); (2) stress and control diet (N = 12); (3) alcohol diet and no stress (N = 13); (4) yoked control group (N = 7). Rats have an aversion to the taste of alcohol, causing dams given alcohol diet to consume fewer calories than those given control diet (Ward, *et al.*, 1994, 1996a). Therefore, a nonstressed group whose access to control diet was restricted to the calories voluntarily consumed by the alcohol groups was in-

cluded. The amount of diet available to this control group was yoked to the intake of four randomly chosen animals in the alcohol group and three rats in the alcohol plus stress group. The spontaneous intake of the stress plus alcohol and the alcohol alone groups is the same (Ward *et al.*, 1994, 1996a); therefore, one yoked group sufficed. Similarly, because the behavior of males in the yoked control group does not differ from males from nonstressed mothers that had received *ad libitum* control diet, the latter group was not incorporated into the design.

Within 6 h after birth, all experimental litters were transferred to foster mothers that had delivered within the preceding 48 h. Fostering was intended to control for possible deficits in maternal care caused by the procedures utilized during pregnancy. The litters were not culled because the treatments do not alter litter size (Ward *et al.*, 1994, 1996a). The pups were weaned, weighed, earmarked, and housed in same-sex and treatment triads when 21 days old.

Adult Castration and Hormone Replacement

At 55–60 days of age, the males were castrated under ketamine (80 mg/kg) and xylazine (10 mg/kg) anesthesia. After a 2- to 3-week recovery period, all were given a 20-min screening test with an estrous female. None copulated.

At 70–80 days of age, the males from the four prenatal treatments were randomly assigned to one of four Silastic tubing implant groups. No more than two males from any one litter were given the same implant. T-containing Silastic implants were 20, 30, or 45 mm long. A control group consisting of 6 yoked control, 5 alcohol alone, 4 stress alone, and 6 combination treatment males were given 30-mm empty implants. The number of males in each of the 12 groups implanted with T is presented in Table 1. The Silastic capsules were prepared as described by Smith *et al.* (1977). Briefly, segments of Silastic Medical Grade Tubing (1.57 mm i.d., 3.18 o.d.; Dow Corning Corp., Midland, MI) were cut 10 mm longer than the desired length of the capsule. A 5-mm wooden dowel was inserted into one end of the tubing. T crystals (Sigma Chemical Co., St. Louis, MO) were packed into the tubing until only 5 mm remained empty. Another wooden dowel was placed into the other end and trimmed to be even with the end of the tubing. Both ends of the capsule were sealed with two coats of Silastic Medical Grade Elastomer (382, Dow Corning Corp.). Each coat was allowed to cure for 24 h. The capsules were incubated for 24 h in 0.01 M phosphate buffered saline at 37°C. A similar procedure was used to make the empty control implants. Males were anesthetized with ketamine and xylazine and the capsules were inserted subcutaneously through a small incision in the middle of the back that was closed with a wound clip.

Sexual Behavior Testing

One week after implantation, tests for male sexual behavior were initiated. Males were allowed to adapt to individual semicircular Plexiglas arenas ($53 \times 33 \times 23$ cm) for 3 min, after which an estrous female was added. The females had been ovariectomized and were brought into heat by intramuscular injections of 0.01 mg of estradiol benzoate and 0.2 mg of progesterone, given 48 and 4 h, respectively, before each test. The tests were 30 min long. If an ejaculation occurred, the test continued until the first mount of the next copulatory sequence to measure the postejaculatory interval (PEI). Twice weekly testing continued for 3 weeks or until a male ejaculated, whichever occurred first. Each mount, intromission, and ejaculation was recorded, along with the latency to the first mount, length of the copulatory sequence (time from first mount to ejaculation), and PEI.

Hormone Assays

Within 2 weeks after the last test, the males were weighed, stunned by a sharp blow to the head, and decapitated using a guillotine. Trunk blood was collected, allowed to clot for about 30 min at room temperature, and then centrifuged for 15 min. The separated serum was stored at −20°C. The Silastic implants were removed and visually inspected. All data from two alcohol exposed males with a 20-mm implant were deleted because the capsule could not be found at the time of autopsy.

To determine T and LH values in comparably aged control males that had not been castrated or implanted with Silastic capsules, serum was also collected from 16 gonadally intact, sexually experienced males.

Testosterone and LH levels were measured in the same sample using radioimmunoassay. Testosterone assays were conducted on 25-μl aliquots of unextracted serum. The antibody in the coated-tube assay system (Bio-Rad Diagnostics Group, Hercules, CA) cross-reacted with 10-hydroxytestosterone (28.7%), methyltestosterone (15.1%), and 5α-dihydrotestosterone (6.6%). ED10 and ED90 for the T assay were 1.963 and 3.2 pg, respectively. Performance of the T assays

was monitored by the repeated assay (in triplicate) of a pool of rat serum. The mean (±SEM) intra-assay coefficient of variation was 3.5 ± 0.8% ($N = 5$) and the inter-assay coefficient of variation was 7.6%.

LH concentrations in 200-μl aliquots of serum were measured in an assay that used antiserum (NIDDK-rLH-S-11) and a standard reference preparation (NIDDK-rLH-RP-3) elaborated by Dr. A. F. Parlow and made available through the NHPP, NIDDK, NICHHD, and USDA. ^{125}I-radiolabeled rLH was acquired from Corning-Hazleton (Vienna, VA). Precipitating second antibody (goat anti-rabbit) was acquired from Antibodies Inc. (Davis, CA). ED10 and ED90 for the LH assay were 6.1 and 0.1 ng, respectively. Samples containing high titers of LH (below ED10) were reassayed at a volume of 100 μl, and data for these samples were entered into the parametric statistical analyses only if the result was above ED10. Samples containing low LH titers (above ED90) were not reassayed or used in the parametric analyses. The intra-assay coefficient of variation (based on duplicate sample agreement for samples that fell between ED10 and ED90) was 8.1 ± 1.3% ($N = 124$). The inter-assay coefficient of variation based on assay of a sample of pooled rat serum in each of three assay runs was 24.1%. LH was not measured in one sample, for which a T value had been obtained, from a single male in the stressed-alcohol diet group.

RESULTS

A two-way (stress condition × days) analysis of variance (ANOVA) performed on the amount of alcohol diet consumed by the dams during pregnancy indicated significant differences across days ($F_{(10,253)} = 6.88$, $P < 0.0001$), but no difference between stressed and nonstressed rats ($F_{(1,253)} = 0.06$, $P < 0.8$). The interaction of stress condition with days was not significant. Intakes averaged 69.3 (±3.5) g of diet/day in the alcohol alone rats and 68.2 (±4.0) g in the alcohol plus stress subjects during the day-16 to day-20 period of gestation. Body weights were not taken during pregnancy in the current study; therefore the amount of alcohol consumed per kilogram of body weight cannot be reported. However, in our previous studies (Ward *et al.*, 1994, 1996a) in which the same experimental procedures were used and maternal body weights were presented, alcohol intake was virtually identical to that in the current study and equaled about 12–13 g of alcohol/kg of body weight.

There were no group differences in litter size (mean = 12–13 pups in all groups) at birth. A three-way (stress condition × diet × Silastic capsule length) ANOVA revealed no significant differences among the 16 treatment groups in adult body weight (data not shown).

Sexual Behavior

None of the males in any treatment group implanted with empty capsules ejaculated. The cumulative percentages of T implanted males that ejaculated across the six tests are presented in Fig. 1. For the stress alone, alcohol alone, and control males given the smallest (20 mm) T implant, approximately 30% began to copulate by the third test, and slightly less than half copulated by the end of testing. In contrast, only 1 of 14 males prenatally exposed to both stress and alcohol copulated during the first five tests, with 1 additional male starting on the sixth test. Binomial tests on the proportion that had ejaculated by the last test indicated a significantly lower proportion ($P < 0.02$) of animals prenatally exposed to both alcohol and stress ejaculated compared to the yoked control group, or to males exposed only to alcohol, or only to stress.

The T provided by 30-mm implants was sufficient to activate the ejaculatory pattern in 70–75% of the males in the yoked control and alcohol alone groups. In contrast, only 40% of the males in the prenatal stress group and 21% from the combined stress and alcohol group showed the ejaculatory pattern with this size T implant. A significantly lower proportion of rats prenatally exposed to stress alone ($P < 0.04$) or to the combination treatment ($P < 0.001$) ejaculated compared to the yoked control group.

When the largest (45 mm) implant of T was provided, nearly half of the males in all groups ejaculated by the third test. There were no significant group differences, with at least two-thirds of the males in all groups ejaculating by the end of testing. Thus, the T levels provided by the largest implants were required to activate copulation in the majority of the males from the stress and stress plus alcohol groups.

A male was defined to be a "copulator" if he displayed more than five intromissions on any one test. Only two males, both yoked control animals with 30-mm T implants, copulated without eventually also ejaculating.

Three-way ANOVAs (stress × diet × capsule length) were performed on each of the several quantitative and temporal measures taken on tests on which responding animals first displayed the ejaculatory pattern. The data for the animals which ejaculated

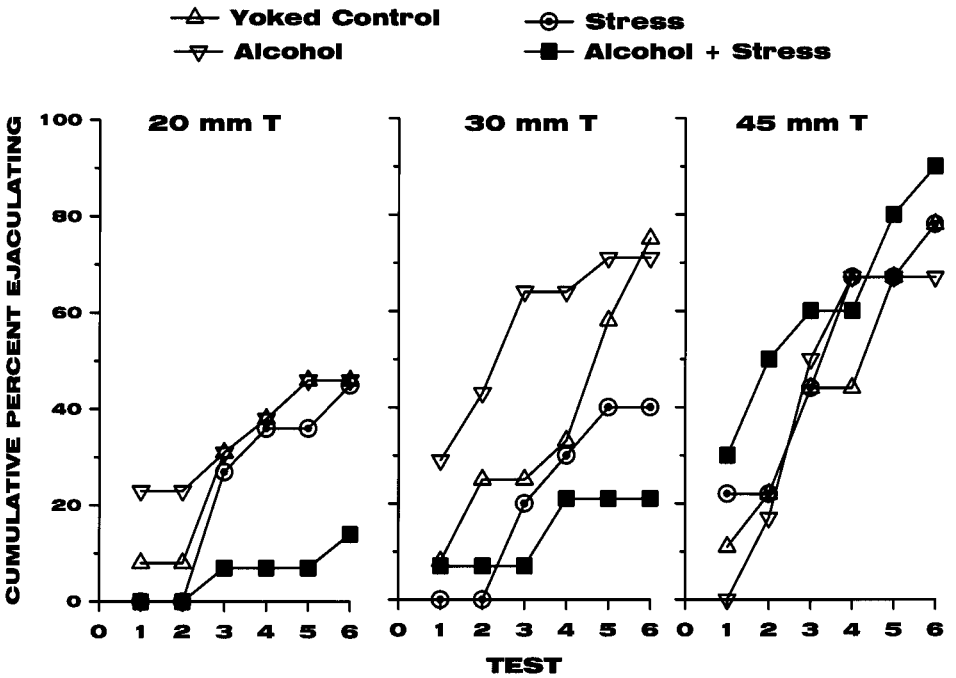

FIG. 1. Cumulative percentage of males ejaculating over a series of six tests with estrous females. Males had been castrated in adulthood and implanted with a 20-, 30-, or 45-mm length of Silastic tubing filled with testosterone. The animals were derived from mothers exposed to alcohol, or to stress, or to alcohol plus stress during pregnancy and compared to the offspring of yoked control dams.

are summarized in Table 1. Due to equipment failure the data characterizing the ejaculatory sequence of five animals were lost. These were three alcohol alone males, two implanted with 20-mm capsules and one with a 30-mm capsule. Also lost were the data from two combination treatment males, one with a 30-mm implant and one with a 45-mm implant. The ANOVAs revealed that males exposed to alcohol displayed a

TABLE 1

Proportion of Ejaculating Males, the Mean (±SEM) Number of Mounts (M) and Intromissions (I) Exhibited on the Test when Ejaculation Occurred, the Duration (s) of the Copulatory Sequence and Postejaculatory Interval (PEI)

Treatment	Proportion ejaculating	Number of M	Number of I	Sequence duration	PEI
Yoked control					
20 mm	6/13	46.3 (11.9)	18.3 (2.2)	1224 (178)	389.8 (14.9)
30 mm	9/12	34.9 (3.7)	16.6 (2.9)	1233 (93)	358.8 (18.9)
45 mm	7/9	34.4 (4.5)	17.9 (3.6)	1124 (121)	337.1 (6.7)
Alcohol alone					
20 mm	6/13	30.3 (7.9)	10.3 (1.9)	911 (179)	366.3 (12.0)
30 mm	10/14	29.1 (4.4)	9.8 (1.4)	1158 (180)	357.7 (14.0)
45 mm	4/6	18.0 (7.9)	10.8 (4.8)	694 (224)	402.2 (25.4)
Stress alone					
20 mm	5/11	33.4 (8.2)	14.6 (3.4)	941 (164)	339.0 (22.1)
30 mm	4/10	32.3 (5.8)	16.3 (2.5)	998 (176)	324.0 (14.7)
45 mm	7/9	41.0 (6.0)	12.6 (2.0)	982 (127)	317.0 (19.4)
Stress + alcohol					
20 mm	2/14	34.0 (6.0)	8.5 (3.5)	972 (240)	396.0 (10.0)
30 mm	3/14	13.0 (5.0)	7.7 (2.5)	520 (265)	365.0 (14.0)
45 mm	9/10	27.3 (4.7)	13.3 (1.8)	963 (127)	353.0 (16.6)

significantly smaller number of mount ($F_{(1,55)} = 7.37$, $P < 0.01$) and intromission patterns ($F_{(1,55)} = 10.70$, $P < 0.01$) before ejaculating than did males not exposed to alcohol during fetal ontogeny. Also, the mean duration of the copulatory sequence of alcohol-exposed males was significantly shorter ($F_{(1,55)} = 4.07$, $P < 0.05$) and the PEI was significantly longer ($F_{(1,55)} = 5.87$, $P < 0.05$) than those of males not exposed to alcohol. No other main effect or interaction was significant in any variable. There were no significant differences in the latency to the first mount or in the number of tests required before ejaculation occurred.

Serum T

Serum T levels in all males given blank implants were undetectable. The mean serum T values obtained in the groups carrying T capsules are shown in Fig. 2. On the average, serum T levels of males receiving 20-mm capsules were 44% of those receiving 45-mm capsules, while 30-mm capsules yielded 65% of the T levels provided by the largest capsules. A three-way ANOVA indicated a significant effect attributable to the T implant size ($F_{(2,119)} = 41.06$, $P < 0.0001$). Planned contrasts revealed that serum T levels in the 20-mm group were significantly lower than in either the 30-mm ($P < 0.001$) or the 45-mm ($P < 0.001$) groups and that the 30-mm group had lower T titers than the 45-mm ($P < 0.001$) group. There was an unexpected significant main effect of stress ($F_{(1,119)} = 5.97$, $P < 0.016$). Circulating T levels were slightly higher in prenatally stressed males than in nonstressed males. Neither the main effect of alcohol nor any of the interactions were significant. The mean T level of the males that ejaculated (1.50 ng/ml $\pm$ 0.07) was identical to that of the nonejaculators (1.50 ng/ml $\pm$ 0.09). Figure 2 also shows the serum T titers of the gonadally intact males. In order to compare animals bearing Silastic T implants with the intact control group, the implant data were collapsed across the prenatal treatments and analyzed using a one-way ANOVA ($F_{(3,143)} = 32.2$, $P < 0.0001$). Subsequent comparisons revealed that serum T levels in the gonadally intact group did not differ from the 45-mm Silastic implant group, but were significantly higher than either the 20- or the 30-mm groups.

Serum LH

For three-quarters of the castrated males implanted with blank capsules, serum LH levels were so high that they fell outside the 10–90% range of the standard curve. In contrast, the majority of the LH values in both the 30- and the 45-mm Silastic T implant groups were below the range of the standard curve. Therefore, only the 20-mm T implant group provided data that allowed LH comparison to be made across the prenatal treatment groups. In the 20-mm implant group, 41 of the 49 subjects had LH values that fell within the acceptable range. A two-way ANOVA on the 20-mm data indicated there were no significant differences due to prenatal diet or stress condition or to the interaction between them. Thus, these limited data provided no indication that negative feedback from T is altered by prenatal exposure either to stress or to alcohol. The LH values for the 20-mm implant groups were collapsed across prenatal conditions and compared to those in the intact controls. The mean LH titers of the 20-mm T implant animals (6.2 ng/ml $\pm$ 0.8) was significantly higher ($F_{(1,56)} = 14.5$, $P < 0.0004$) than that of the intact controls (1.3 ng/ml $\pm$ 0.1). A Kruskal–Wallis analysis of variance by ranks ($H = 114.2_{(2)}$, $P < 0.0001$) followed by multiple pairwise comparisons using Dunn's method was used to compare all the groups. Castrated males given blank implants had higher LH values than all other groups and the 20-mm T implant males and the intact males had higher LH than the 30- and 45-mm T implant males.

DISCUSSION

Overview

This study provides a direct demonstration that maternal stress in combination with alcohol consumption during pregnancy dramatically alters the sensitivity of the male offspring to T in adulthood. Only 21% of adult male rats exposed both to prenatal stress and alcohol copulated when given amounts of T (30-mm Silastic capsule) sufficient to activate ejaculation in 75% of the control males. However, the stress plus alcohol males did possess a potential for the ejaculatory pattern. When the size of the Silastic T implant was increased to 45 mm, the proportion of ejaculating males (90%) no longer differed from any of the other groups. The most parsimonious explanation for these data probably involves fetal patterns of circulating T that are altered by prenatal stress (Ward and Weisz, 1984; Weisz and Ward, 1980) or alcohol (McGivern *et al.*, 1988, 1993) exposure. If the combination of stress and alcohol induced an even greater disruption of T during critical periods of perinatal ontogeny than either factor alone, the later capability of the male's CNS

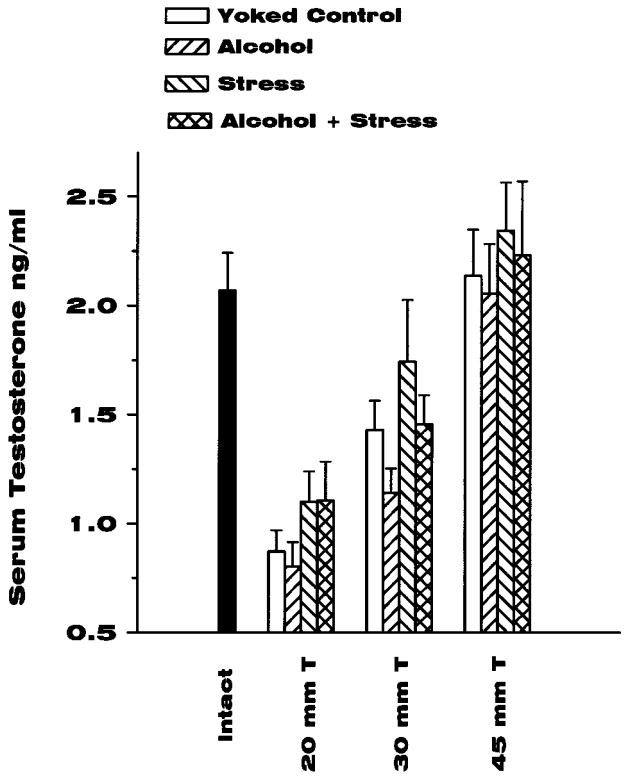

FIG. 2. Mean (±SEM) testosterone levels (ng/ml of serum) of adult gonadally intact males and of castrated males implanted with a 20-, 30-, or 45-mm length of Silastic tubing filled with testosterone. The castrated males were derived from mothers exposed to alcohol, or to stress, or to alcohol plus stress during pregnancy and compared to the offspring of yoked control dams.

to respond normally to T also would be more substantially altered. Thus, one component of the normal process of sexual behavior differentiation in male rats may involve imparting onto relevant CNS targets characteristics that make these structures sensitive to T in adulthood.

Silastic Implant Size, Serum T, and Behavioral Activation

The largest (45 mm) Silastic capsule was effective in stimulating copulation in all of the treatment groups, yet this implant size did not produce serum T titers higher than those found in the gonadally intact group. In our previous studies (Ward *et al.*, 1994, 1996a), very few males prenatally exposed to the combination of stress and alcohol copulated when tested while gonadally intact, despite the fact that their serum T levels were no different from control males (Ward *et al.*, 1996a). There has been a previous suggestion that mean T level delivered by Silastic implants may be behaviorally more effective than are equivalent T levels derived from testicular output (Damassa, Smith, Tennent, and Davidson, 1977). The expression of male sexual behavior in rats normally requires sustained occupation of androgen receptors (ARs) by T for at least 21 h/day (McGinnis, Mirth, Zebrowski, and Dreifuss, 1989). Silastic implants of T or daily injections of large doses of testosterone propionate suspended in oil (Ward *et al.*, 1996a) probably result in a more sustained occupation of ARs than is achieved by the episodic surges of T that the gonads normally deliver (Ellis and Desjardins, 1982; Leal and Moreira, 1997; Steiner, Bremner, Clifton, and Dorsa, 1984). Thus, stable physiological levels of T exogenously administered may lead to a greater activation of neural target sites than occurs from the normal variable pattern of endogenously released T. These findings may explain why combination treatment males ejaculate when control levels of T are delivered in 45-mm T capsules, but fail to copulate when gonadally intact despite normal, albeit fluctuating, levels of T (Ward *et al.*, 1996a).

The current study is the first to use Silastic capsules to investigate the amount of adult T required to activate copulation in sexually naive males prenatally exposed to factors known to alter fetal T levels. In most previous studies utilizing Silastic T implants in adult males, sexually experienced animals that had not been perinatally manipulated were castrated, and implant size required to maintain or restore ejaculatory behavior was determined. A 10-mm Silastic T capsule, implanted at the time of castration, provides approximately one-quarter of the T levels of intact males and is effective in maintaining ejaculatory behavior (Damassa *et al.*, 1977; Hsu, Hsu, Yu, and Peng, 1986). Silastic capsules 20 mm long are frequently used to restore ejaculation in previously castrated male rats (McGinnis and Dreifuss, 1989; McGinnis and Mirth, 1986; McGinnis *et al.*, 1989), although a number of tests may be required before the pattern is fully reestablished. However, in the present study, which involved activation of behavior in sexually inexperienced males, even by the end of six tests, less than half of the control males with 20-mm implants had copulated. In order to activate copulatory behavior in naive control males castrated several weeks before testing began, 30-mm implants were required. Indeed, the 20-mm implants did not provide enough T to suppress serum LH to the levels found in intact males, but 30-mm implants did. As Fig. 1 shows, 45-mm implants were required to activate copulation in animals that

had less than optimal levels of prenatal T exposure. Thus, the strategy of controlling amounts of circulating T available in adulthood with Silastic implants appears to be a valuable one for revealing alterations in behavioral masculinization.

Effects of Prenatal Stress Alone

Males derived from dams that received stress, but not alcohol, also required more T than controls to activate copulatory behavior. With 30-mm implants, less than half of the stress alone males began to copulate. However, they were not as severely affected as the males exposed prenatally to both stress and alcohol. Significantly more of the stress alone than combination stress plus alcohol males copulated when the 20-mm T capsule was implanted. While a number of studies have reported that most prenatally stressed male rodents fail to copulate when gonadally intact (Anderson, Fleming, Rhees, and Kinghorn, 1986; Crump and Chevins, 1989; Dunlap, Zadina, and Gougis, 1978; Harvey and Chevins, 1984; Rhees and Fleming, 1981; Rhees, Badger, and Fleming, 1983; Ward, 1972, 1977; Ward and Reed, 1985), others have found a normal incidence of copulators when testing under endogenous hormone conditions (Dahlöf, Hård, and Larsson, 1977; Götz and Dörner, 1980; McLeod and Brown, 1988; Ward and Stehm, 1991; Ward *et al.*, 1994, 1996; Ward, Monaghan, and Ward, 1986). Further, circulating adult T levels in prenatally stressed males have been described to be elevated (Del Cerro, Pérez-Laso, Rodríguez, Ortega, Barona, Haba, Guillamón, and Segovia, 1998; Ward *et al.*, 1996a), suppressed (Anderson *et al.*, 1986), or unchanged (Crump and Chevins, 1989), in relation to control males. In the present study, endogenous T levels in intact control males were 50% higher than the levels required to activate copulation in castrated control animals. When prenatally stressed males are tested while gonadally intact, their endogenous T levels could be high enough to activate copulation even though the T threshold is elevated. Changes in target tissue sensitivity may be concealed if males exposed to teratogenic treatments are evaluated when gonadally intact.

Alterations in Testosterone Metabolism

Despite deficient copulatory behaviors in stressed males, serum T levels in castrated prenatally stressed animals implanted with T capsules were higher than in castrated control males receiving the same size implant. This finding is consistent with previous reports in gonadally intact prenatally stressed males (Del Cerro *et al.*, 1998; Ward *et al.*, 1996a), and raises the interesting possibility that prenatal stress exposure suppresses adult metabolic clearance of T, possibly by altering hepatic function. Prenatally stressed males lack the prenatal (Ward and Weisz, 1980) and perhaps postparturitional T surge found in control males. Males deprived of normal perinatal T exposure by antiandrogens or neonatal castration have reduced adult levels of some hepatic enzymes involved in T metabolism. For example, the activity of 16α-hydroxylase, a sexually dimorphic enzyme (e.g., Gustafsson and Stenberg, 1974), was reduced by 60% in adult males exposed to cyproterone acetate from the 13th day of gestation through the 21st day postpartum (Gustafsson, Ingelman-Sundberg, Stenberg, and Neumann, 1975). Further, males castrated neonatally, but not prepuberally at 4 weeks (Chung and Chao, 1980) or 7 weeks of age (Einarsson, Gustafsson, and Stenberg, 1973), had levels of 16α-hydroxylase activity in adulthood that were no higher than those of females. Prenatal stress, which also reduces perinatal T exposure, may cause levels of T catabolizing enzymes to be sufficiently suppressed in the adult liver to have consequences on the metabolic clearance rate of T, thereby explaining the slightly elevated plasma levels found in stressed males. It is of interest to note a recent report by McCormick and Mahoney (1999) that exposure of male rats to the androgen receptor blocker, flutamide, either during late (days 15–20) gestation or postnatally starting within 4 h of birth also led to elevated adult T levels. The suppression of androgenic activity during perinatal ontogeny by stress, castration, or pharmacological means may be linked to changes in T metabolism that endure into adulthood.

Possible Involvement of the Medial Preoptic

The nature of the changes that underlie the increased T thresholds in males prenatally exposed to stress or to both alcohol and stress is unknown. One possibility is that the threshold changes involve a diminished concentration of neural androgen receptors, particularly within the medial preoptic area (MPOA). The MPOA has been identified as a pivotal site within the brain circuitry controlling male copulatory patterns (see review by Meisel and Sachs, 1994) and c-fos expression in this site is diminished in prenatally stressed males exposed to estrous females in adulthood (Humm, Lambert, and Kinsley, 1995). The display of male sexual behaviors in the rat requires activation of neural androgen receptors (Vagell and

McGinnis, 1998). The medial (Simerly, Chang, Muramatsu, and Swanson, 1990) and caudal (Lisciotto and Morrell, 1994) portions of the rat MPOA are particularly heavily endowed with ARs and androgen receptors within the MPOA have been found to be especially important for the expression of male sexual behavior (McGinnis and Kahn, 1997; McGinnis, Williams, and Lumia, 1996).

Possible Mediating Role of ARs in the SDN-MPOA

Prenatal exposure to stress (Anderson *et al.*, 1986; Kerchner and Ward, 1992) or to alcohol (Ahmed, Shryne, Gorski, Branch, and Taylor, 1991; Barron, Tieman, and Riley, 1988) has been shown to feminize (decrease) the size of the sexually dimorphic nucleus of the medial preoptic area (SDN-MPOA), a subunit of the MPOA. The SDN-MPOA may be linked to the expression of male sexual behavior in rats. Lesions placed into the SDN-MPOA of sexually naive males (DeJonge, Louwerse, Ooms, Evers, Endert, and van de Poll, 1989) or into females (Turkenburg, Swaab, Endert, Louwerse, and van de Poll, 1988) suppressed male copulatory patterns, at least in the initial postoperative test. No suppression was found by Arendash and Gorski (1983) in males that were sexually experienced prior to lesioning. The majority of cells comprising the SDN-MPOA are born during days 18–19 of gestation (Bayer and Altman, 1987; Jacobsen, Davis, and Gorski, 1985; Jacobsen and Gorski, 1981; Jacobsen, Shryne, Shapiro, and Gorski, 1980) and are most sensitive to the masculinizing effects of T at this early developmental stage (Rhees *et al.*, 1990). The surge in circulating levels of T that normally occurs on days 18–19 of gestation (Weisz and Ward, 1980; Ward and Weisz, 1984) is depressed by prenatal ethanol (McGivern *et al.*, 1998; McGivern *et al.*, 1988; Sinha *et al.*, 1997) or stress exposure (Ward and Weisz, 1980, 1984).

Circulating levels of T have not been measured in perinatal male rats treated with both alcohol and stress, nor has the size of their SDN-MPOA been established in adulthood. However, the central (Simerly *et al.*, 1990) and caudal (Lisciotto and Morrell, 1994) parts of the MPOA have been shown to contain fewer ARs in females than in males. Simerly and his colleagues (1990) attributed the sex difference in ARs to the much smaller size of this sexually dimorphic component of the brain in females than males. It is possible that perinatal T levels are more severely reduced by the combined alcohol and stress treatment than by exposure to stress or to alcohol alone. Reductions in adult T sensitivity may be proportional to the degree to which teratogenic factors diminish testicular activity during very early stages of life. If that is the case, the SDN-MPOA is probably even smaller in the combination males than in those exposed to only one of the treatments. A greater reduction in tissue area should be accompanied by a proportionally greater loss in ARs, resulting in decreased neural activation of the SDN-MPOA. Fewer ARs would explain the reduced sensitivity to T and diminished neural activity within this component of the copulatory circuit would explain the impaired ability to ejaculate.

Possible Effects on Estrogenic Mechanisms

T also influences copulation in adults through estrogenic mechanisms (see review by Meisel and Sachs, 1994). Estradiol, produced by the intraneuronal aromatization of T taken from the circulation, activates estrogen receptors (ERs). Gonadally intact male rats showed deficits in mounting and ejaculation when the aromatase inhibitor, Fadrozole, was placed directly into the MPOA (Clancy, Zumpe, and Michael, 1995). Sexual behaviors also are impaired in male mice lacking the αER (Ogawa, Lubahn, Korach, and Pfaff, 1997; Wersinger, Sannen, Villalba, Lubahn, Rissman, and De Vries, 1997). It is possible that prenatal exposure of male rats to alcohol and/or stress influences central estrogenic activity in adulthood. While there are no data linking alcohol to altered ER receptor number, a transient elevation in hypothalamic ER density was reported in 3-day-old prenatally stressed male rats (Henry, Arsaut, Arnauld, and Demotes-Mainard, 1996). This elevation may reflect upregulation resulting from abnormally low perinatal blood levels of T. At later stages, i.e., in juvenile (day 12) or adult (90 days) life, ER number in stressed males did not differ from control levels. However, even if adult ER density is normal in prenatal stress and/or alcohol-exposed males, aromatase enzyme activity may not be. Roselli and Klosterman (1998) have shown that perinatal T exposure increases (masculinizes) aromatase activity in a number of brain areas of adult female rats. Steroid aromatase activity was attenuated in hypothalamic–amygdaloid tissue taken from prenatally stressed fetal rats on days 18, 19, and 20 of gestation (Weisz, Brown, and Ward, 1982). If remnants of the fetal aromatase deficit persist into adulthood, it may be reflected in local estradiol production, leading to lower levels of ER activation. In contrast to stress, prenatal exposure to alcohol has been reported to elevate hypothalamic aromatase activity on days 18 and 19 of gestation and

day 1 postpartum (McGivern, Roselli, and Handa, 1988). Given the opposing effects of stress and alcohol on fetal aromatase, and in the absence of data on adults, it is difficult to speculate as to possible effects on ER activation lingering into adulthood of the combined prenatal stress and alcohol treatment. Further research is needed to explore this intriguing topic.

Effects of Prenatal Alcohol Alone

Prenatal exposure to alcohol alone had no effect on the copulatory threshold or adult serum T titers of male rats. The alcohol diet used induces a mean consumption of approximately 12 g of ethanol / kg of body weight / day by the dam, yielding maternal blood alcohol levels of about 150 mg / dl (Ward *et al.*, 1996a). Our findings are in agreement with other studies that report that gonadally intact males derived from dams exposed to alcohol during pregnancy ejaculate normally (Chen and Smith, 1979; Dahlgren, Eriksson, Gustafsson, Harthon, Hård, and Larsson, 1989; Hård, Dahlgren, Engel, Larsson, Liljequist, Lindh, and Musi, 1984; McGivern *et al.*, 1998; Ward *et al.*, 1996a). It is in combination with stress that alcohol exposure has a consistently depressant effect on male copulatory behavior.

In summary, the strategy of controlling the amount of circulating T available in adulthood with Silastic implants provides insights into one possible mechanism underlying incomplete behavioral masculinization. The impaired ejaculatory behavior found after prenatal exposure to teratogenic agents that alter perinatal T levels may be the result of decreased target tissue sensitivity to androgenic hormones that activate sexual behavior in adulthood, rather than to a complete failure for the ejaculatory potential to be differentiated.

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