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Postparturitional Testosterone Surge in Male Offspring of Rats Stressed and/or Fed Ethanol during Late Pregnancy

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Male offspring of rats exposed to restraint stress and/or alcohol during late pregnancy show aberrant patterns of sexual behavior masculinization and defeminization that vary as a function of treatment. The impact of these treatments on the postparturitional testosterone (T) surge that contributes to sexual behavior differentiation was investigated. Plasma T was measured using radioimmunoassay in individual males sampled on day 21 of gestation within 10 min of cesarean delivery or 1, 2, or 4 h thereafter. Neonatal T in the group exposed only to stress did not differ from that in the control group. T was lower than control levels at birth in both alcohol groups. The magnitude of the T surge that occurred during the first hour of birth in the control group was diminished by 50% in both alcohol groups, whose T pattern was very similar. There was no common alteration in postparturitional T associated with the increased lordotic behavior potential that males in all three treatment groups typically share, nor were there idiosyncratic endocrine abnormalities linked to the very different male copulatory pattern each exhibits. Exposure to an abnormal T milieu during fetal as well as neonatal ontogeny may underlie the etiology of the different sexual behavior patterns exhibited by males exposed to stress and/or alcohol. Possible unique effects each treatment exerts on perinatal plasma T and it's aromatization to estradiol in hypothalamic targets are discussed. © 2002 Elsevier Science (USA)

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Differentiation of sexual behavior potentials is altered in male offspring of rats fed ethanol or exposed to restraint stress during the latter part of pregnancy. Prenatal alcohol or stress, alone (Hård, Dahlgren, Engel, Larsson, Liljequist, Lindh, and Musi, 1984; Ward, 1972a, 1977; Ward and Reed, 1985; Ward, Ward, Winn, and Bielawski, 1994) or in combination (Ward *et al.*, 1994), lead to a similarly enhanced propensity in males to display lordosis, the female receptivity pattern. These prenatal treatments have more variable effects on the male sexual pattern. Male copulatory behavior was impaired in most (e.g., Dunlap, Zadina, and Gougis, 1978; Ward, 1972a, 1977; Ward, Bennett, Ward, Hendricks, and French, 1999; Ward and Reed, 1985), but not all (e.g., Dahlöf, Hård, and Larsson, 1977; Ward, Monaghan, and Ward, 1986; Ward *et al.*, 1994; Ward, Ward, Mehan, Winn, French, and Hendricks, 1996), studies evaluating the consequences of prenatal stress alone. Fetal exposure to alcohol alone is usually reported to have little effect on male sexual behavior in rats (Chen and Smith, 1979; Dahlgren, Eriksson, Gustafsson, Harthon, Hård, and Larsson, 1989; Hård *et al.*, 1984; Ward *et al.*, 1996, 1999), although minor decrements are occasionally found (McGivern, Handa, and Raum, 1998; Ward *et al.*, 1994). However, when prenatal stress and alcohol exposure are combined, there is a profound and highly reliable deficit in the ability of adult males to mate (Ward *et al.*, 1994, 1996, 1999), i.e., fewer than 25% copulate despite repeated testing with estrous females.

It is well known that the differentiation of male and suppression of female reproductive behavioral potentials is the result of exposure to testosterone (T) or its metabolites during critical stages of perinatal development (e.g., see review by Ward and Ward, 1985). In rats, males normally experience a surge in plasma T during days 18 and 19 of gestation (Weisz and Ward, 1980; Ward and Weisz, 1984), and again during the first few hours following birth (Baum, Brand, Ooms,

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Vreeburg, and Slob, 1988; Corbier, Kerdelhue, Picon, and Roffi, 1978; Lalau, Aubert, Carmignac, Gregoire, and Dupouy, 1990; Slob, Ooms, and Vreeburg, 1980). The current investigation focused on the neonatal elevation in T. Incomplete defeminization and masculinization of behavior occur when the postparturitional T surge is interrupted. Male rats castrated within 1 h of birth show higher levels of lordosis (Corbier, Roffi, and Rhoda, 1983; Thomas and Gerall, 1969) and less mounting of females (Roffi, Chami, Corbier, and Edwards, 1987) than those gonadectomized at 6 h.

The severely attenuated male copulatory pattern that follows prenatal exposure to a combination of alcohol and stress suggests that its etiology may involve a more profound flaw in perinatal plasma T than results from either treatment alone. Abnormalities in the prenatal T pattern have already been observed in males exposed only to stress (Ward and Weisz, 1980, 1984) or only to alcohol (McGivern, Handa, and Raum, 1998; McGivern, Raum, Salido, and Redei, 1988a; Sinha, Halasz, Choi, McGivern, and Redei, 1997). Furthermore, the neonatal T surge is reduced by prenatal exposure to ethanol (McGivern, Handa, and Redei, 1993; Rudeen and Kappel, 1985). Possible effects of prenatal restraint stress on the neonatal T surge have not been investigated, and no information on any portion of the perinatal T pattern exists for males exposed to both alcohol and stress. The present study evaluated whether these prenatal treatments disrupt the timing or size of the postparturitional T surge differently, thereby possibly explaining their differential effects on behavioral masculinization.

METHODS

Subjects

Fifty-eight female rats (colony 205; Harlan Sprague–Dawley, Inc., Indianapolis, IN) were obtained at 45–50 days of age and allowed to adapt for about 2 weeks before being bred. The vivaria were temperature (21°C) and humidity regulated (50 ± 10% RH) and maintained on a reversed light/dark cycle (lights on 1900–700 h). Animals not on a liquid diet were given *ad libitum* access to water and Purina Lab Chow (No. 5012). These studies adhered to the standards on animal care and treatment specified by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23) and were approved by the Villanova University Institutional Animal Care and Use Committee.

Prenatal Treatments

Alcohol diet. Between 1130 and 1400 h, estrous rats were placed with a vigorous male until two ejaculations were observed (day 0 of pregnancy). Impregnated females were caged individually into hanging wire-mesh cages (24.5 × 17.5 × 17.5 cm). The animals were randomly assigned to one of four gestational treatments. Beginning on day 10 of pregnancy, a liquid diet delivered in drinking tubes (Product No. 9007; Bio-Serv Inc., Frenchtown, NJ) was substituted for chow and water. Half of the females were given a diet in which 36% of the calories were ethanol-derived (Diet No. 1265; Bio-Serv). The other half received a control diet in which alcohol was replaced by an isocaloric amount of maltose-dextrin (Diet No. 1264; Bio-Serv). Between 1000 and 1100 h, clean drinking tubes containing 120 ml of diet were weighed and attached to the cages. Each tube was reweighed 24 h later, providing a measure of daily intake. Animals on the ethanol and control diets were housed in separate vivaria to prevent exposing the control groups to alcohol fumes.

Stress. Beginning on day 14 of pregnancy, half of the dams in the alcohol and half in the control diet groups were stressed for 45 min daily (at 930, 1330, and 1730 h). Nonstress animals were not handled. Stress animals were placed into individual 13 × 5 × 8.3 cm Plexiglas restrainers (A. H. Thomas No. 1123-C30). The movable restraining shield was readjusted to the tightest setting the expanding body size of the pregnant animals would allow. Two 150-W floodlights were positioned to deliver approximately 2150 lm/m^2 of white light across the restrainers.

Yoked control. Rats have an aversion to the taste of alcohol. To control for reduced caloric intake in the alcohol groups and possible effects on fetal development, a yoked control group was included. The yoked group was not stressed but was given daily access to amounts of control diet equivalent to the calories spontaneously consumed by 6 randomly chosen animals in the alcohol-plus-stress group and by 8 animals in the alcohol-alone group.

The numbers of dams included were as follows: combination treatment, 15; alcohol-alone, 14; stress-alone, 15; yoked control, 14.

Neonatal Procedures

At 2130 h on the 21st day of gestation, the litters were delivered by cesarean section. The dam was stunned by a sharp blow to the head and decapitated.

The uterus was excised. All pups were quickly removed and cleaned. As soon as all were breathing, the litter was placed on a heating pad (about 37°C) which produced an ambient temperature of 31°C, in a high humidity chamber. Male pups were randomly designated to be killed at 0 (within 10 min of delivery), 1, 2, or 4 h postparturition. At least one male pup was bled at each sampling time in 88% of the litters. In addition, the female littermates of the yoked control males were sampled at each time point. No more than two males and two females were sampled at any one time point from a single litter. Individual pups were decapitated and blood was drawn into heparinized capillary tubes (Oxford No. 8889-301209). The tubes were centrifuged (IEC MB Microhematocrit Centrifuge) for 5 min at 11,500 rpm and plasma harvested from each pup was stored in individual cryogenic vials at −60°C. Sex was determined by visual inspection of the anogenital distance or, when there was doubt, by exposing the gonads under a dissecting microscope after blood collection. Two yoked control, three alcohol, and two alcohol-plus-stress litters were killed at 930 h on the 22nd day of pregnancy. Their data were combined with those of comparably treated litters killed 12 h earlier because they did not differ. The following number of pups contributed useable data: combination treatment, 78; alcohol-alone, 77; stress-alone, 93; yoked control males, 90; yoked control females, 81.

Testosterone was measured in 25-μl samples taken from individual pups using radioimmunoassay (RIA). All but 4% of the 419 samples were assayed in duplicate. Samples with a high coefficient of variation were eliminated as unreliable (about 6% of the male samples). A T antibody (ICN Diagnostics, Costa Mesa, CA) that has low cross-reactivity with other androgens (5-α-dihydrotestosterone, 7.8%; 11-oxotestosterone, 2.0%; all other steroids $<$1.0%) was used. Although steroids were not chromatographically separated prior to assay, given the high specificity of the antibody for T, the data are referred to as "plasma T." For statistical purposes, samples yielding T values below the standard curve were assigned a value of 0.14 ng/ml, the lower limit of sensitivity in the assay. Most male samples (94%) were within the detectable range.

Testosterone titers were assessed in five assay runs. Samples from animals in various treatments collected at different time points were distributed randomly across assay runs. At least three male pups were included in each assay run for all time points and treatment conditions. Variation among assays was assessed by measuring, in duplicate, a sample of pooled neonatal rat plasma in each assay run. The interassay coefficient of variation based on the pool concentrations was 11.3%. Within-assay variation was determined with reference to the variation between the duplicate pool determinations in each assay. The average intra-assay coefficient of variation for these duplicate pools was 1.4%.

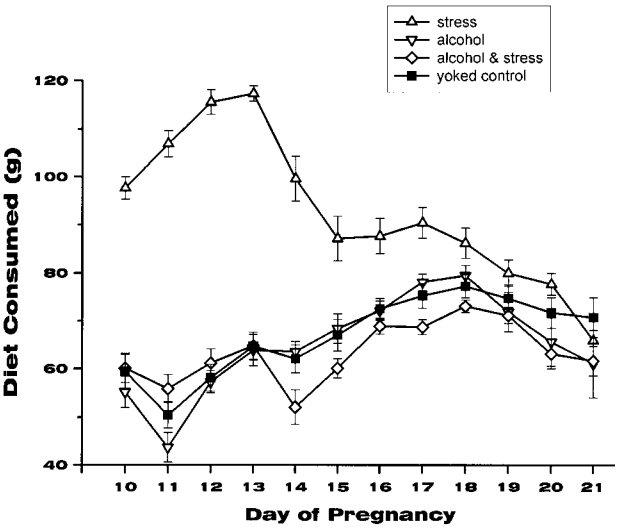

FIG. 1. Mean daily (g ± SE) intake of a liquid control diet or a diet containing ethanol by pregnant rats, half of which had been exposed to light/restraint stress beginning on day 14 of pregnancy.

RESULTS

There were no significant differences among the treatment groups in average litter size (12.2 pups) or proportion of male (52%) to female pups at delivery.

Diet Consumption

The mean daily intake of alcohol or control diet consumed by dams in the four treatment groups is shown in Fig. 1. A 4 treatment × 12 day repeated measures analysis of variance (ANOVA) yielded significant effects for treatment ($F(3, 54) = 66.48$, $P < 0.001$), for days ($F(11, 594) = 13.37$, $P < 0.001$), and for the interaction ($F(33, 594) = 15.47$, $P < 0.001$). Dams given *ad libitum* control diet (stress-alone group) ate nearly twice as much prior to the onset of the stress treatment (days 10–14) as did the two groups exposed to the alcohol diet ($P < 0.001$, Tukey HSD tests). Further, planned comparisons showed that imposition of restraint stress significantly suppressed consump-

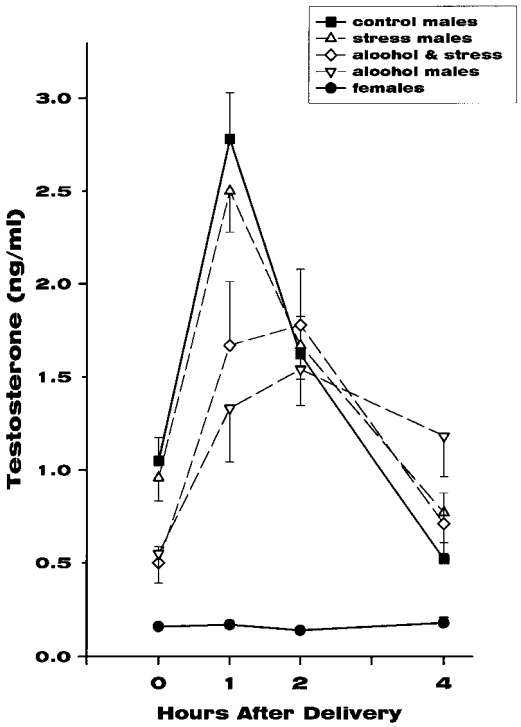

FIG. 2. Mean plasma testosterone levels (ng/ml ± SE) of the offspring of dams exposed to stress and/or ethanol during pregnancy. Litters were delivered by cesarean section on day 21 of gestation and individual male pups were sampled at delivery or 1, 2, or 4 h later. Both male and female pups were sampled in the yoked control group.

tion in the dams given the control diet ($P < 0.001$), but not the ethanol diet, so that by the last 3 days of pregnancy, there were no significant differences in intake among the groups. There were no significant differences in intake among the yoked control, stress-plus-alcohol, and alcohol-alone dams at any time.

Plasma T Levels

Mean plasma T found at each sampling time in the four male groups and the female control group is shown in Fig. 2. A two-way ANOVA applied to the male data revealed significant effects associated with treatment ($F(3, 322) = 3.13$, $P < 0.026$), sampling time ($F(3, 322) = 34.67$, $P < 0.001$), and the interaction ($F(9, 322) = 3.34$, $P < 0.001$). Tukey tests indicated that, in comparison to the yoked control group, plasma T was significantly lower at delivery (0 h) in the alcohol-alone (53% of control, $P < 0.04$) and in the combined stress-and-alcohol (48% of control, $P < 0.02$) groups. T continued to be lower at the 1-h sampling time in both the alcohol-alone group (48% of control, $P < 0.003$) and in the combined treatment group (60% of control, $P < 0.03$). There were no significant group differences in the 2-h or the 4-h samples.

Plasma T in the stress-alone group did not differ significantly from the yoked control group at any sampling time. The yoked control and stress-alone groups exhibited a sharp surge in T between delivery (0 h) and 1 h later ($P < 0.001$). Thereafter, T levels dropped sharply between the 1-h peak and the 2-h sampling time in the control ($P < 0.001$) and stress-alone ($P < 0.02$) groups. Both groups displayed a second significant decline between the second- and fourth-hour samples ($P < 0.01$). By the 4th h, T levels were not significantly different from those at delivery (0 h) in either group.

The neonatal T patterns of the alcohol-alone and the combined stress-and-alcohol groups differed from that of the control group. Compared to levels found at delivery, the alcohol-alone group showed a blunted rise in T at 1 h which was only marginally significant ($P = 0.06$). T continued to be elevated above birth levels at the 2-h sampling time ($P < 0.02$). No other differences among sampling times were significant within the alcohol-alone group. Within the combined stress-and-alcohol group, T had risen significantly between delivery (time 0) and 1 h later ($P < 0.01$) and remained at that level through hour 2 ($P < 0.003$). There were no significant differences in T between the combined alcohol-plus-stress and the alcohol-alone groups at any time.

Only 15% of the plasma samples harvested from females contained sufficient T to be detectable by the assay system. As Fig. 2 clearly shows, there was no postnatal T surge in the females. Mean plasma T in the females was well below male values at all sampling times.

DISCUSSION

Prenatal Stress Alone

Previous investigators have reported that the neonatal T surge is suppressed in males from dams exposed to glucocorticoids (Lalau *et al.*, 1990) or intermittent hypoxia (Hermans, Longo, and McGivern, 1994) from days 15 to 21 of gestation. However, in the present study, males from dams exposed only to light-restraint stress on fetal days 14–21 showed no attenuation in neonatal T. This finding is of particular in-

terest because prenatally stressed males typically exhibit an enhanced female behavioral potential (Ward, 1972a, 1977; Ward and Reed, 1985; Ward *et al.*, 1994). Complete elimination of the T surge by orchidectomy (Corbier *et al.*, 1983; Thomas and Gerall, 1969) or ether administration (Vega Matuszczyk, Silverin, and Larsson, 1990) within the first hour following birth is sufficient to increase lordosis in males. However, the current results suggest that while suppression of the postparturitional surge may be sufficient to augment female sexual behavior, it is not required. Further, prenatally stressed males in many studies show a partial failure in the masculinization of behavior (Dunlap *et al.*, 1978; Ward, 1972a, 1977; Ward *et al.*, 1999; Ward and Reed, 1985). As with defeminization, behavioral masculinization can be attenuated by eliminating the postparturitional T surge (Roffi *et al.*, 1987). Yet the present data indicate that the reported alterations in masculinization observed in prenatally stressed animals probably occurred despite normal neonatal T exposure.

The enhanced lordotic potential in prenatally stressed males may be due entirely to known disruptions in prenatal T. Prenatally stressed males show lower than normal levels of plasma T on days 18 and 19 of gestation, a time when control males experience a marked elevation (Ward and Weisz, 1980, 1984). Exposure of fetal male rats to the androgen antagonists flutamide and cyproterone acetate enhances their ability to exhibit lordosis (Gladue and Clemens, 1978; Ward, 1972b).

Similarly, behavioral masculinization of rats can be partially disrupted by suppressing T solely during prenatal ontogeny. Males exposed to cyproterone acetate on days 13–22 (Nadler, 1969), 17–19 (Perakis and Stylianopoulou, 1986), or 10–19 (Vega Matuszczyk and Larsson, 1995) show attenuated levels of intromissions and ejaculations. Thus, the incomplete masculinization that characterizes prenatally stressed males might result from abnormalities in the prenatal hormonal milieu, rather than to T circulating during later neonatal stages, when sexual differentiation is completed in the rat.

Prenatal Alcohol Alone

Although males prenatally exposed to alcohol share an increased propensity to display lordosis with prenatally stressed males, they show little evidence of a failure in behavioral masculinization (Chen and Smith, 1979; Dahlgren *et al.*, 1989; Hård *et al.*, 1984; Ward *et al.*, 1996, 1999). Thus, it is surprising that it is the fetal alcohol males that have significantly lower T levels at birth and a blunted surge 1 h later. This finding is in agreement with previous reports (McGivern, Handa, and Redei, 1993; Rudeen and Kappel, 1985). The abnormally low postparturient T surge could have contributed to the enhanced lordotic potential of alcohol-exposed males. However, one important ramification of these data is that normal behavioral masculinization can occur in the face of a neonatal T surge that is reduced by approximately 50%.

The etiology of the suppressed postparturitional T surge in the alcohol groups may involve effects ethanol has on enzymes involved in both steroid synthesis and catabolism. Prenatal ethanol leads to a significant reduction in testicular activity of the steroidogenic enzyme 17α-hydroxylase immediately following birth (Kelce, Rudeen, and Ganjam, 1989). Suppressed gonadal synthesis would explain the low plasma T already present in alcohol-treated pups within minutes of delivery. Normally the initial rise in plasma T following birth probably is triggered by a brief increase in testicular steroidogenesis, but the magnitude of the increase and its duration have been attributed to low levels of T metabolism and clearance that ensue when the newborn is first separated from the placenta and maternal circulation (Baum *et al.*, 1988; Slob *et al.*, 1980). There appears to be a brief transitional period during which the neonate becomes dependent on its own liver to fully metabolize circulating androgens. The continued suppression of the postparturitional surge in prenatal alcohol-exposed males may reflect the effect ethanol had on the activity of hepatic enzymes. There is evidence that fetal alcohol exposure increases the rate at which T catabolizing enzymes are synthesized by the neonatal liver. Hepatic cytochrome P450 is increased on the day of birth in the offspring of dams fed alcohol throughout pregnancy (Rovinski, Hosein, Lee, Hin, and Rastogi, 1987). Accelerated liver metabolism of T would offset loss of the maternal contribution to T clearance during the first hours following birth. The end result would be to dampen the normal buildup of plasma T in newborns suddenly made entirely dependent on their own immature livers.

Prenatal Alcohol-plus-Stress

The neonatal T pattern offers few clues as to why stress-plus-alcohol males show such a severe failure in behavioral masculinization. Like alcohol-alone males, the stress-plus-alcohol group had significantly lower T

levels than the control group at delivery (0 h) and only a blunted T surge followed. At no time did plasma T differ between stress-plus-alcohol and alcohol-alone males. Thus, very similar aberrant neonatal T patterns were associated with quite different effects on the masculinization of adult sexual behaviors.

The pronounced deficiencies in sexual differentiation that characterize the stress-plus-alcohol group may involve the entire prepuberal T pattern. Combination treatment males probably experienced the suppressed T surge that occurs on gestational days 18 and 19 in males exposed only to stress (Ward and Weisz, 1980, 1984) or only to alcohol (Sinha *et al.*, 1997), leading to the increased lordotic potential shared by all three treatments. However, blood levels of T alone do not explain the very different male sexual patterns.

Behavioral masculinization in rats involves estrogenic metabolites of T (see review by Meisel and Sachs, 1994). Prenatal exposure to alcohol or to stress alters hypothalamic aromatization of androgens into estrogens in critically different ways during the stages when sexual differentiation is ongoing in the rat. Stress suppresses aromatase activity in hypothalamic-amygdaloid tissue of 18- and 19-day-old male fetuses (Weisz, Brown, and Ward, 1982), while alcohol increases aromatase activity in the hypothalamus on fetal days 18 and 19, as well as on the day of birth (McGivern, Roselli, and Handa, 1988b). Thus, in alcohol-alone males, increased brain aromatization may offset the reduced availability of substrate (T) during the time that the fetal and neonatal T surges occur in the control groups. Such a compensatory mechanism may lead to induction of the male sexual behavior potential, explaining why alcohol-alone males copulate normally with estrous females. Fetal males exposed to stress alone incur a reduction in both substrate and aromatase activity, but do experience a normal postparturitional T surge. Thus the male copulatory potential in stress-alone males is attenuated but not eliminated.

In males exposed to both alcohol and stress, the pattern of substrate availability and aromatase activity may be different from that in either single treatment group. The postparturitional T surge was attenuated in both alcohol groups. If both alcohol groups also experienced a failure in the prenatal T surge, one only need hypothesize that the stress treatment experienced by the combination group blocked the increase in brain aromatase activity normally associated with alcohol exposure. Thus combination treatment animals may develop under conditions of low substrate availability during both fetal and neonatal ontogeny, coupled with low hypothalamic aromatase activity. Such conditions are minimally conducive to normal differentiation of the male copulatory potential.

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