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In Utero Alcohol Heightens Juvenile Reactivity[1]

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ANANDAM, N., W. FELEGI AND J. M. STERN. In utero *alcohol heightens juvenile reactivity.* PHARMAC. BIOCHEM. BEHAV. 13(4) 531–535, 1980.—The behavioral teratogenicity of ethanol was studied in a laboratory model of the fetal alcohol syndrome. Pregnant rats were placed in one of three groups: Ethanol (4 g ethanol/kg intubated twice daily; Purina Chow ad lib.); Sucrose (7 g sucrose/kg intubated instead of ethanol; Untreated (no intubations; Purina Chow ad lib.). Ethanol offspring did not differ from either control group in neonatal body weight or developmental measures. On Day 35, 2 female offspring per litter were tested for reactivity to acoustic startle stimuli. Activity was measured during the pre-stimulus foreperiod and during inter-stimulus intervals. Ethanol pups displayed heightened startle reactivity in the absence of hyperactivity or disrupted habituation. These data indicate that ethanol *in utero* produces hyperreactivity in the absence of morphological, body weight or developmental abberations.

Ethanol Prenatal Fetal Alcohol Syndrome Activity Acoustic startle reflex

THE fetal alcohol syndrome (FAS), found in many offspring of women who are chronically alcoholic during pregnancy, is characterized by increased perinatal mortality, deficient prenatal and postnatal growth, developmental delay, deformities of the head, face and limbs, fine motor dysfunction and varying degrees of mental retardation [18, 19, 35]. Many of these children are also hyperactive, jittery and irritable, with attentional disorders [35]. It has been suggested that consumption of only "3 ounces of alcohol per day or a one-time drinking binge during pregnancy" may place offspring at risk for fetal alcohol effects [33].

Alcoholism in pregnant women is often accompanied by poor nutrition, usage of other drugs, emotional stresses, and/or inadequate health care [24]. Furthermore, clinical studies on the FAS rely heavily on retrospective self-reports, making quantification of alcohol consumed difficult. Thus, animal models provide an opportunity to assess the effects of alcohol *per se,* administered in known quantities and at known times during gestation. To date, many of the morphological anomalies (except facial) and growth deficiencies found in the human FAS have been reproduced in mice [9,25] and rats [30,38].

More widespread and milder fetal alcohol effects may exist in the absence of dysmorphogenesis, consisting of behavioral dysfunctions such as reduced IQ and hyperactivity [32,35]. Some children born to alcoholic women do not have the FAS, but their behavioral profile and that of the non-FAS children born to moderate drinking mothers is only beginning to be studied. Animal models may serve to further identify the realm of alcohol-induced behavioral teratogenesis.

In rats, ethanol *in utero* has been related to decreased motor coordination [2], learning deficiencies [21, 27, 28] and hyperactivity [5, 6, 7, 22]. We now report that the behavioral effects of prenatal alcohol in rats also includes hyperreactivity.

METHOD

Animals

Eighteen virgin female Sprague-Dawley rats (Blue-Spruce Farms, Altamont, NY) weighing 250 to 300 g were assigned to one of three groups: E (ethanol), S (sucrose; pair-fed control) or U (untreated; ad lib control). Each animal in group E was matched for weight with one animal in group S and one in group U. All were mated and housed individually in a room maintained on a 12-hr light-dark cycle commencing at 0700 hours. Sighting sperm in vaginal smears was used to establish Day 1 of pregnancy.

Maternal Treatment

Beginning on gestation Day 6, E rats were intubated twice daily (0900 and 1700 hours) with 4.0 g ethanol/kg body weight as a 20% w/v solution in a liquid diet. Additional plain liquid diet was intubated at 1300 hours if an E rat's body weight was less than that of the previous day. Solid food (Purina Lab Chow) and water were available ad lib. Rats in group S received an amount of sucrose in liquid diet isocaloric (7.0 g/kg as a 40% solution) with the 4.0 g/kg ethanol dose. In addition, these rats were pair-fed the amount of solid food consumed by their weight-matched E rats, and received supplemental plain liquid diet when necessary. The liquid diet consisted of Nutrament (Mead-Johnson, Inc), supplemented with 1.8 g/100 ml casein hydrolysate, to which was

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TABLE 1
EFFECTS OF ETHANOL DURING PREGNANCY ON MATERNAL AND OFFSPRING MEASURES

Measure	Gestational treatment: Ethanol Mean*	Ethanol SE	Sucrose Mean*	Sucrose SE	Untreated Mean*	Untreated SE
Maternal measures						
Weight gain (g)	95.6	15.6	89.8	7.3	122.9	10.1
Retrieval latency (sec)	11.6	1.8	11.2	1.8	12.9	2.5
Crouch latency (min)	3.4	0.6	4.1	0.5	2.9	0.2
Neonatal measures†						
Litter size	10.4	0.7	12.0	0.7	10.8	1.1
Birth weight (g)	6.2	0.5	6.7	0.6	6.8	0.5
Male A-G distances (mm)	3.4	0.1	3.4	0.2	3.3	0.3
Adrenals (mg/100g BW)‡	24.1	3.6	22.7	1.0	28.5	1.2
Testes (mg/100g BW)‡	81.6	5.8	82.6	4.5	73.9	3.8
Developmental measures (day)						
Earflap uncurling	2.8	0.4	2.7	0.2	2.0	0.0
Righting	3.6	0.5	3.2	0.3	2.7	0.2
Eye opening	15.8	0.2	15.3	0.2	15.7	0.4

*Means are based on 5, 7 and 6 litters from groups E, S and U respectively, unless otherwise noted.
†Neonatal measures were obtained 12–24 hr after birth.
‡Means are based on 3 litters from each of groups E and S, and 4 litters from group U.

added 95% ethanol or sucrose and water. Food intake and body weight were monitored daily. The intubations were terminated after the morning of Day 21. U rats were given Purina Lab Chow and water ad lib and were left undisturbed except for body weight and food intake measures.

Blood alcohol level (BAL) and rate of alcohol elimination were measured on gestation Days 6, 11 and 21 in 4 other rats given the same treatment as the E dams. Approximately 200 μl of blood was collected from the tail vein into heparinized vials at 45 min intervals beginning at 2 hours after the morning intubation. Three to five samples were obtained on each rat on each sampling day. BAL was determined spectrophotometrically, using a modification of the yeast-ADH method previously described [20].

Neonatal and Developmental Measures

On the morning after birth (Day 1), litters were assessed for number of pups, sex ratio, body weight and presence of morphological anomalies. The litters were culled to 8 (4 male and 4 female when possible) and each pup was identified with a paw-mark. The live offspring were returned to their mothers. Fostering was not employed in the present study because it was our intent to assess the maternal behavior of treated dams. The culled male pups were sacrificed; adrenals and testes were weighed.

The maternal behavior of the lactating dams was observed for 15 min daily during the first 7 days postpartum. Every morning between 0900 and 1100 hours litters were removed from their dams, weighed, and then placed under a heat lamp while the dams were allowed to settle in their cages. The pups were replaced in their home cage at the corner farthest from the nest after 15–30 min of separation. Onset and termination of retrieval, and latency to crouch over all the pups were noted.

Litter weights were obtained on Days 1 through 7, 14 and 21. The pups were observed for the following indices of development: day of ear flap uncurling, righting on a flat surface and eye opening. The litters were checked daily until all members of the litter had both pinnae uncurled and both eyes completely open. Presence of the righting reflex was checked by placing the pup on its dorsal side on a flat surface. If the pup righted itself within 5 sec, the reflex was said to be present. The day when the entire litter displayed each index of development was taken as the measure for analysis. Litters were weaned and housed in pan cages by sex and litter on Day 21.

Activity and Reactivity Measures

On Day 30, 12 females per group (usually 2 per litter) were housed individually in a nearby laboratory (Dr. P. Carlton, College of Medicine and Dentistry of New Jersey, Piscataway, NJ). On Day 35, each rat was tested for pre-stimulus activity and and acoustic startle reactivity in a modification of the procedure and apparatus described previously [40]. The acoustic startle reflex has been used extensively as a behavioral index of reflex reactivity, habituation and sensitization [10, 11, 12, 14].

The rat was weighed and placed in a small, clear, 1/4" Plexiglas box (18×8×8 cm) which was mounted onto a platform in a light and sound attenuated chamber for a 30 min fore-period (FP). A constant 82 dB white noise was present throughout the session. Activity was accumulated and printed every 60 sec. On min 31, the first of 60 startle stimuli, consisting of a 100 msec presentation of a 110 dB, 8 kHz tone with a rise-decay time of <0.1 msec, was presented. The inter-stimulus interval (ISI) was 60 sec. The ballistic movement of the startle reflex induced a current in an accelerometer on top of the platform; a peak-reading digital voltmeter

read and printed the maximum voltage as well as the activity accumulated during the preceding ISI.

Statistics

One-way analyses of variance were performed on neonatal and developmental data, and repeated measures analyses of variance on maternal behavior data and on offspring activity and reactivity measures. Analysis of offspring data was performed either on litter means or with litter as the error term for treatment (cf. [8]).

RESULTS AND DISCUSSION

Intubation of 4.0 g ethanol/kg produced observable behavioral intoxication in the pregnant rats. Maternal BAL peaked around 140 mg/100 ml on all three days of measurement, and the alcohol was eliminated in 6 to 7 hours after intubation. All E rats needed supplemental diet on the first 5 days of ethanol administration. Thereafter, 3 out of 6 E rats required the supplement occasionally between gestation Days 11 to 14, and only one E rat was given the supplement after gestation Day 14.

Table 1 presents the maternal and offspring developmental data. Rats which had received ethanol exhibited weight gain during pregnancy similar to the two control groups, indicating that nutritional requirements were adequately met. Maternal weight gain was slightly, but not significantly, higher in group U than in groups S or E, $F(2,15)=2.67$, $p=0.10$. That gross malnutrition did not result from ethanol administration was further indicated by the normal litter size, pup birth weight and growth. Ethanol treatment did not result in reduced litter size ($p=0.9$), altered sex ratio ($p=0.7$) or reduced birth weight ($p=0.3$). Prenatal ethanol did not alter neonatal adrenal weights ($p=0.4$), indicating that the fetuses were not unduly stressed [8]. Similarly, our treatments did not affect neonatal testicular weights ($p=0.2$) or ano-genital distance ($p=0.9$), suggesting that exposure to alcohol during gestation does not grossly interfere with the sexual differentiation of male fetuses even though chronic alcohol consumption interferes, in adult males, with reproductive physiology [3,39].

Differences in maternal behavior, as measured by the latency to retrieve and crouch, were not significant, $F(2,15)=0.21$, $p=0.81$ and $F(2,15)=1.92$, $p=0.18$, respectively. The normal maternal behavior of ethanol treated dams suggests that behavioral changes in the offspring were primarily due to prenatal drug treatment. This is further suggested by the normal postnatal growth and development of E pups. Figure 1 shows mean pup weights from birth until weaning. There was no treatment effect, $F(2,15)=0.34$, $p=0.8$, and no treatment by days interaction was evident ($p=0.6$). There were no developmental delays in ear flap uncurling ($p=0.1$), righting reflex ($p=0.2$) or eye opening ($p=0.2$).

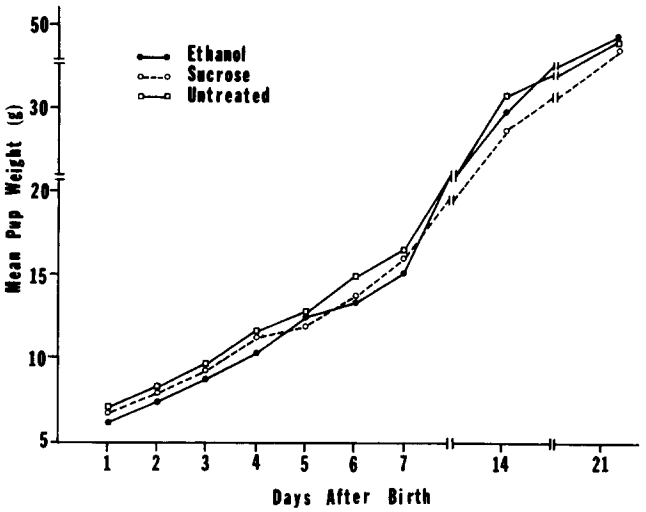

FIG. 1. Mean pup weight during postpartum Days 1 to 7, 14 and 21. Each mean is based on 5 to 7 litters, with 4 males and 4 females representing each litter. Squares and open circles represent untreated and pair-fed controls respectively; closed circles represent the ethanol group.

Table 2 presents the data relevant to activity and acoustic startle reactivity. The groups did not differ in body weight on Day 35, $F(2,15)=0.07$, $p=0.9$. Offspring activity was analyzed in blocks of 3 trials and reactivity in 12 blocks of 5 trials. Groups E and S had somewhat greater activity than group U, but this difference was not significant, $F(2,15)=1.68$, $p=0.2$ and $F(2,15)=2.49$, $p=0.1$ for FP and ISI respectively. All groups exhibited a decrease in activity over trials during the FP, $F(9,135)=7.0$, $p=0.001$, and during the ISI, $F(19, 285)=2.1$, $p=0.001$. The lack of group by block interaction (FR: $p=0.8$; ISI: $p=0.7$) indicates that the decreases were similar in all groups.

An F-Max test performed on the startle data revealed heterogeneity among group variances; a square-root transformation was applied to these data to alleviate this problem. A repeated measures analysis of variance and Duncan's Multiple Range Test (at $\alpha=0.05$) indicated that the mean startle amplitude of group E was significantly higher than either control group, $F(2,15)=5.52$, $p=0.016$. Mean startle amplitudes over trials are presented in Fig. 2. There was no

TABLE 2

BODY WEIGHT, ACTIVITY COUNTS AND ACOUSTIC STARTLE REFLEX AMPLITUDE AFTER PRENATAL ETHANOL EXPOSURE

	Body weight	FP activity	ISI activity	Startle*
Ethanol	112.8 ± 7.4†	136 ± 3	126 ± 2	42 ± 6‡
Sucrose	110.4 ± 3.2	134 ± 5	128 ± 5	29 ± 2
Untreated	112.7 ± 4.6	125 ± 4	117 ± 3	30 ± 2

*Untransformed data.
†Mean ± standard error.
‡Significantly different from controls ($p=0.016$).

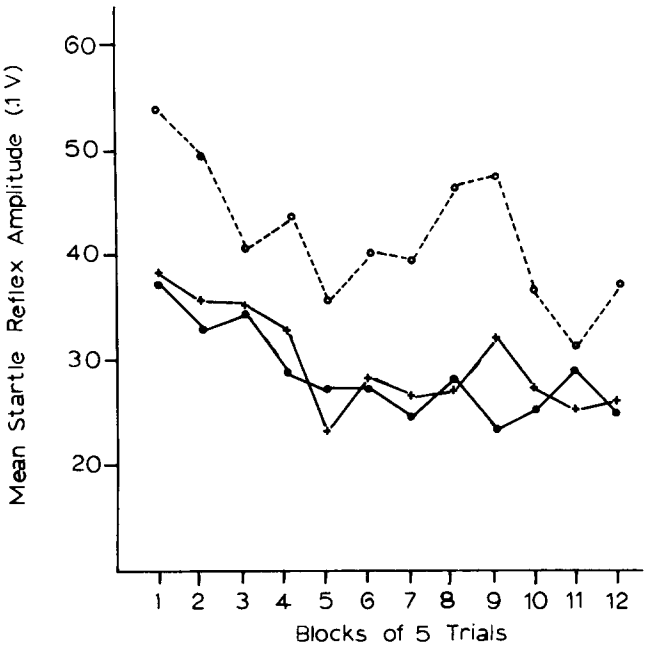

FIG. 2. Mean acoustic startle reflex amplitudes presented as a function of trials. Reflex amplitudes from 60 trials are presented in blocks of 5 trials. The intensity of the startle reflex was measured by the peak voltage induced in an accelerometer attached to the animal platform. Closed circles and crosses represent the pair-fed and untreated groups respectively; open circles represent the ethanol group.

group by trial interaction ($p=0.9$), indicating that response decrement over trials was similar in all groups.

GENERAL DISCUSSION

Intermittent exposure to ethanol on gestation Days 6 to 21 produced offspring hyperreactivity in the absence of hyperactivity in a small, novel environment. It has been reported that rats prenatally exposed to ethanol are hyperactive in the open-field [5, 6, 7] and in a running wheel [22]. This descrepancy between results may arise from differences in dose and duration of ethanol treatment. Alternatively, prenatal ethanol exposure may predispose rats to hyperactivity detectable by some activity measures (cf. [23]) but not to the one used here.

Ethanol *in utero* increased startle amplitude but did not abolish habituation. Habituation, operationally defined as response decrement due to repeated stimulation, involves inhibitory processes [14]. Recent evidence suggests that prenatal ethanol exposure in rats may delay development of central inhibitory systems [27,28]. Because our juvenile E rats habituated, such deficits in inhibitory processes may have declined with age [27,28] or perhaps they are restricted to more complex tasks.

Maternal and fetal ethanol levels equilibrate rapidly [16], but the extent and mechanism of alcohol's deleterious effects on the fetus are not known. Gross brain dysmorphogenesis, including cortical lissencephalia and corpus callosum agenesis, were found in an FAS infant who died shortly after birth [18]. Prenatal ethanol may heighten startle reflex amplitude by acting on the differentiating neurons which mediate it. Lesions of the anterior reticular formation [15] or hippocampus [10] result in high startle amplitudes without abolishing habituation. Ethanol-induced CNS malformations occur in chick [31], rat [30] and mouse [9,25] embryos. Chronic alcohol exposure in the preweanling rat diminishes brain growth, including Purkinje cell loss [4], and longterm ethanol consumption in the adult rat results in loss of dendritic spines in hippocampal cells [29]. Central neurotransmitter manipulations modulate startle reflex amplitude [12, 13, 34], and prenatal ethanol exposure may alter neurotransmitter development in rodents [26,37]. Alternatively, prenatal ethanol may affect arousal mechanisms rather than mechanisms specific to the startle reflex, producing hyperreactivity via an interaction between heightened arousal to background noise and the acoustic startle stimulus [11,17].

Our data indicate that intoxicating doses of ethanol during the last two-thirds of pregnancy in the rat produce enhanced reactivity in the absence of obvious dysmorphogenesis, deficient growth and development, or even hyperactivity. Because offspring were raised by their natural mothers, it is conceivable that the hyperreactivity in the E rats was influenced by postpartum interaction with their previously intoxicated dams. This possibility appears unlikely since there was no evidence of altered maternal behavior or lactation, indicated by direct behavioral observations and pup growth. Abel [1] reported that rats which received 2.0 g ethanol/kg daily throughout pregnancy required more time to retrieve pups than did pair-fed controls on Day 1 postpartum. This increase in retrieval latency could reflect a residual effect of alcohol as the behavior was observed within 24 hours after the last alcohol administration. In contrast to Abel's procedure, our first observation was at least 48 hours after the last alcohol administration, and our observations continued for 7 days postpartum.

Heightened reactivity to sensory stimuli may be relevant to the behavioral problems, such as hyperactivity, jitteriness, irritability and hyperdistractibility, observed in many FAS children [24,35]. The incidence of a clinical parallel, i.e., children with psychological problems but no overt morphological anomalies, may be substantial [32]. Others have reported behavioral effects of prenatal alcohol in rats unaccompanied by morphological changes [2, 21, 27, 28], but these typically entail birth weight deficits and/or developmental delay which may affect behavior directly or indirectly. In only one experiment [28] of previous studies which reported birth weight or developmental measures, did pups exposed to ethanol prenatally display normal birth weight, morphology and growth. The present study provides a model for investigating the behavioral effects of prenatal ethanol exposure in the absence of confounding nutritional variables, and extends the realm of ethanol behavioral teratogenesis to hyperreactivity.

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