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Fetal Ethanol Effects on Benzodiazepine Sensitivity Measured by Behavior on the Elevated Plus-Maze

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OSBORN, J. A., C. YU, K. GABRIEL AND J. WEINBERG. *Fetal ethanol effects on benzodiazepine sensitivity measured by behavior on the elevated plus-maze.* PHARMACOL BIOCHEM BEHAV **60**(3) 625–633, 1998.—Rodents prenatally exposed to ethanol demonstrate altered behavioral and hormonal responses to stressful environments. Prenatal ethanol exposure may also have long-term effects on the offspring's GABAergic system. Using the elevated plus-maze, the present study examined the sensitivity of adult Sprague–Dawley rat offspring from prenatal ethanol (E), pair-fed (PF) and ad lib-fed control (C) conditions to the effects of benzodiazepine (BZD) on plus-maze behavior and corticosterone (CORT) responses. At 60–90 days of age, E, PF, and C males and females were injected subcutaneously with either BZD or saline. Twenty minutes later animals were placed in an open field (OF) for a 5-min test and then on the plus-maze for a 5 min test; behaviors were recorded during testing and blood samples collected at the end of testing for CORT determinations. Overall, sex differences were observed in both OF and plus-maze behaviors. Females showed more ambulation and rearing in the OF than males, and exhibited increased exploratory behaviors and decreased fear-related behaviors compared to males on the plus-maze. Following BZD treatment, both males and females exhibited increased time on open arms, increased open arm entries, and decreased time on closed arms compared to saline-treated males and females, regardless of prenatal treatment. These differences did not appear to be due to altered activity levels, as BZD treatment had no effect on total ambulation in the OF. Importantly, although no significant differences in plus-maze behaviors were found among saline-injected E, PF, and C males or females, BZD treatment differentially affected E males and females compared to their PF and C counterparts. Both E males and females treated with BZD spent increased time on open arms and decreased time on closed arms compared to their PF and C counterparts, suggesting decreased fear. Further, BZD-treated E males exhibited decreased open and closed arm entries, spent significantly more time in the central area, and had lower CORT levels, another index of fear or stress, compared to BZD-treated PF and C males. These data support and extend previous work demonstrating that the plus-maze provides a reliable measure of anxiety/fear, and that plus-maze behavior is sensitive to anxiolytic agents such as BZD. Furthermore, these data suggest that prenatal ethanol exposure may alter sensitivity to the effects of BZD on plus-maze behavior and CORT responsiveness, and may do so differentially in male and females offspring. © 1998 Elsevier Science Inc.

Prenatal ethanol exposure Fear-related behavior Exploratory behavior γ-Aminobutyric acid (GABA)
Anxiolytic agents Elevated plus maze Sex differences

RODENTS prenatally exposed to ethanol (E) demonstrate many of the physical findings seen in children exposed to alcohol in utero, including growth deficiencies (4,24), changes in brain morphology (48,85), and soft tissue and skeletal abnormalities (1,24,74). Importantly, as in children exposed to alcohol in utero, cognitive (2) and behavioral alterations have also been seen in E offspring. Many of the behavioral changes observed appear to reflect hyperactivity and hyperresponsiveness and/or deficits in response inhibition. Increased open-field activity (13,14), increased wheel running (44), increased startle reactions (6), and increased exploratory behavior (15, 66), as well as deficits in passive avoidance learning (16,17, 64, 65), taste-aversion learning (66), reversal learning (40), and nose-poking behavior (67) have all been demonstrated in E offspring. In addition to altered performance and activity, rodents prenatally exposed to ethanol exhibit altered behavioral responses to stressors including increased stress-induced analgesia (54), increased stress-induced alcohol consumption (52),

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and an inability to adapt to a stressful swimming paradigm (76). E animals also demonstrate hypothalamic–pituitary–adrenal (HPA) hyperresponsiveness to stressors, manifested as increased or prolonged secretion of adrenocorticotropin (ACTH), β-endorphin (β-EP), and corticosterone (CORT). Increased HPA responsiveness to cardiac puncture (77), restraint (77,79), noise and shaking (77), novel environments (79), intermittent shock (53,55), ether (7,83), and cold stress (7,33) have been reported in E compared to control offspring.

Recently, we have shown that prenatal ethanol exposure alters behavioral and CORT responses to the elevated plus-maze (57). The plus-maze consists of an apparatus in the shape of a cross, with two open arms (i.e., no walls) and two arms enclosed by walls. This task has been shown to provide a valid and reliable measure of anxiety/fear as measured by behavioral, physiological, and pharmacological responses (39,58). The task is based on spontaneous behavior and does not require training of the animal, exposure to noxious stimuli, or manipulation of appetitive behaviors such as food deprivation. Behavior on the plus-maze is sensitive to anxiolytic agents, particularly agents that act via the γ-aminobutyric acid (GABA) system such as the benzodiazepines (BZD), without the interference of sedative side effects (39,58). It can be considered an aversive task in that it generates a conflict situation by simultaneously activating two natural tendencies, exploration of a novel environment, and avoidance of open spaces (22). It has been shown that control or undrugged animals prefer the closed arms of the maze, demonstrating decreased entries onto the open arms and decreased time spent on the open compared to the closed arms (39,58). In addition, animals confined to the open arms exhibit higher CORT levels, an index of stress (71), than animals confined to the closed arms (58). Previous studies in our laboratory (57) found that E males and females both showed an increase in exploration-related behaviors compared to control males and females when placed directly from the home cage onto the plus-maze. However, following behavioral activation by prior open-field (OF) exposure, a procedure that tends to increase overall activity on the plus-maze and increase the likelihood that the open arms are explored (58), E males and females demonstrated a decrease in exploration-related behaviors compared to control animals. In addition, we found that E females but not E males exhibited increased fear-related behaviors during plus-maze testing as well as an increase in CORT levels at the end of testing compared to control females, regardless of prior OF exposure, suggesting increased fear or arousal in E animals.

To date no studies have examined BZD sensitivity in E animals in an aversive or stressful situation. However, a number of studies (35,49,62) have demonstrated alterations in the GABAergic system in E animals. The present study was designed to examine the effects of prenatal ethanol exposure on anxiety/fear-related behaviors on the elevated plus-maze and to compare behavioral responses of E and control animals following administration of BZD.

METHOD

Animals and Mating

Sprague–Dawley males (n = 25) and females (n = 82) were obtained from Canadian Breeding Farms, St. Constant, Quebec. Both males and females were group housed for 1–2 weeks prior to breeding to allow for recovery from transportation and adaptation to the colony room. Males were then singly housed in stainless steel hanging cages with mesh front and floor (25 × 18 × 18 cm), and were maintained on standard laboratory chow (Ralston Purina of Canada, Woodstock, Ontario) and water. The colony room had controlled temperature (21°C) and lighting, with lights on from 0600 to 1800 h.

Females were then placed with a male and cage papers were checked daily for vaginal plugs. Day 1 of gestation (GD 1) was considered the day a plug was found.

Diets and Feeding

On GD 1, dams were rehoused into polycarbonate cages (24 × 16 × 46 cm) and randomly assigned to one of three groups: 1) ethanol (E): liquid ethanol diet (36% ethanol-derived calories), ad lib (n = 14); 2) Pair-fed (PF): liquid control diet (maltose–dextrin isocalorically substituted for ethanol), with each animal pair-fed the amount consumed by a female in the E group (g/kg body weight) on the same day of gestation (n = 14); 3) control (C): laboratory chow and water, ad lib (n = 15).

The diets used were previously developed by our laboratory to provide adequate nutrition to pregnant dams regardless of ethanol intake (78) and were prepared by BioServ, Inc., Frenchtown, NJ. Fresh diet was placed on the cages daily just prior to lights off to avoid a shift in the CORT circadian rhythm. It has been demonstrated that if animals receive a restricted amount of food (such as that received by the PF group), circadian rhythms will reentrain to the feeding time, thus shifting the CORT rhythm (23). Bottles from the previous day were removed and weighed at this time to determine the amount of diet consumed. Experimental diets were continued until GD 22 when they were replaced with laboratory chow and water ad lib.

Dams were undisturbed except for weighing and cage cleaning on GD 1, 7, 14, and 21. At birth, designated postnatal day 1 (PND 1), dam and pups were weighed, cages cleaned, and litters culled to 10 (five males and five females). Dam and pups were again weighed and cages cleaned on PND 8, 15, and 22. On PND 22, pups were weaned and group housed by sex and by litter until testing at 60–90 days of age.

Blood samples (0.4–0.6 ml) were obtained from the tail of three unanesthized dams at 1900 h on GD 14 for determination of blood ethanol levels [Sigma Diagnostic Kit 332-UV, based on Bonnischsen and Theorell (18)].

Apparatus and Scoring

Open-field. The OF consisted of a square apparatus (100 × 100 × 40 cm). The floor was divided into 16 squares. The field was illuminated by two 60-watt bulbs suspended 75 cm above the surface. OF behavior was scored by a single investigator. Ambulation (all four feet crossing into a square) and the number of rears were recorded.

Elevated plus-maze. The plus-maze was designed according to the specifications outlined by Pellow and File (59). It was constructed of black Plexiglas and consisted of two open arms (i.e., no walls) (50 × 10 cm) and two arms enclosed by walls (50 × 10 × 40 cm) such that the two enclosed arms were opposite each other. The maze was elevated 50 cm off the floor.

The plus-maze behavior was videotaped. Each videotape was independently scored by two individuals and a mean of the two scores was used in analyses; intra- and interrater coefficients of variance were 2.6 and 4.2%, respectively. Behaviors scored included time spent on the open arms, the closed arms, and in the center portion of the maze, number of full entries (all four feet) onto open and closed arms, number of partial (one or two feet) entries onto the open arms and number of rears on the closed arms. Time on open and closed arms is re-

ported as percent time, calculated as time spent on the open (or closed) arms divided by total time spent on the open plus closed arms.

Testing and Blood Sampling Procedures

Animals were group housed until testing at 60–90 days of age. One week prior to testing, adult offspring were singly housed and randomly assigned to adult experimental treatment groups (i.e., BZD or saline injection). No more than one male and one female from any litter were assigned to any adult treatment group. Testing order throughout the experiment was counterbalanced across prenatal treatment (E, PF, C), sex, age, and adult experimental treatment (BZD/saline) (n = 5–7 for each of E, PF, and C males and females for each experimental condition). All testing occurred at 0730–1100 h on the test day. White noise (40 dB) was used during both OF and plus-maze testing to mask any extraneous background noises.

Animals were taken from the colony room to an adjacent holding room and injected subcutaneously (SC) with either 0.15 mg/kg body wt. Diazemuls (diazepam injectable emulsion, Kabi Pharmacia Inc., Quebec) (BZD) or saline (0.9%), in an injection volume of 2.0 ml. Twenty minutes later, animals were taken from the holding room to an adjacent testing room and were exposed to the OF for a 5-min test followed by a 5-min test on the plus-maze.

Following testing, animals were returned to their home cages without water for 10 min [we have shown that consummatory behavior can reduce CORT levels in animals placed in a novel environment (84)], after which they were quickly and lightly anesthetized with Metofane (Janssen Pharmaceutica, Mississauga, Ontario) and blood samples (0.5 cc) taken by cardiac puncture using heparinized syringes. The 10-min delay following testing and prior to blood sampling was based on pilot studies indicating that peak CORT levels were obtained 20 min after the initiation of behavioral testing. The entire blood sampling procedure was completed within 2 min of touching the animal's cage, which is rapid enough to obtain a reliable measure of CORT at the time of sampling (20 min after the initiation of behavioral testing), without any effect of disturbance or anesthesia (20). Blood samples were centrifuged and plasma collected and stored at −70°C.

All experimental protocols involving animals were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Canadian Council on Animal Care, and were approved by the UBC Animal Care Committee.

Radioimmunoassay

Total corticosterone (bound plus free) was measured by radioimmunoassay in plasma extracted in absolute ethanol (1:10 v/v), using our adaptation (82) of the method of Kaneko et al. (31). Antiserum was obtained from Immunocorp, Montreal, Quebec; tracer, [1,2,6,7-^{3}H]-corticosterone, was obtained from Dupont, New England Nuclear, Mississauga, Ontario; unlabeled corticosterone for standards was obtained from Sigma Chemical Co., St. Louis, MO. Dextran coated charcoal was used to absorb and precipitate free steroids after incubation. Samples were counted in Formula 989, Dupont. The intra- and interassay coefficients of variation were 3 and 3.9%, respectively.

Statistical Analyses

Data were analyzed by appropriate analyses of variance (ANOVA) for the factors of sex, prenatal treatment, and adult experimental treatment (BZD/saline). Significant main and interaction effects were analyzed by Newman–Keuls paired comparisons.

RESULTS

Developmental Data

Ethanol intake of pregnant dams was consistently high throughout gestation, averaging 9.2 ± 0.4, 11.6 ± 0.4, 11.5 ± 0.2 g/kg body wt./day for weeks 1, 2, and 3 of gestation, respectively. Blood alcohol levels were consistent with levels previously reported (78), averaging 159.5 ± 19.6 mg/dl.

A repeated-measures ANOVA on maternal weight gain during gestation revealed significant main effects of group ($p < 0.001$) and days ($p < 0.001$), as well as a group × days interaction ($p < 0.001$). Post hoc tests indicated that E and PF dams weighed significantly less than C dams on GD 7, 14, and 21 ($ps < 0.01$). During the postnatal period, a group × days interaction ($p < 0.001$) was also seen. E dams weighed significantly less than C dams on PND 1 ($p < 0.01$). There were no significant differences among E, PF, and C dams on PND 8, 15, or 22.

There were no significant differences among groups for litter size or number of stillborn pups. Analysis of body weights for pups showed a significant group × days interaction ($p < 0.05$). E and PF pups weighed significantly less than C pups on PND 1 and 8 ($ps < 0.01$). There were no significant differences in pup weights on PND 15 and 22 and no significant differences in weight at the time of testing at 60–90 days of age.

Open-Field

Analysis of OF behavior revealed a significant main effect of sex ($p < 0.001$). Overall, females had higher ambulation scores and made significantly more rears than males ($ps < 0.001$) (Fig. 1). There were no significant effects of adult ex-

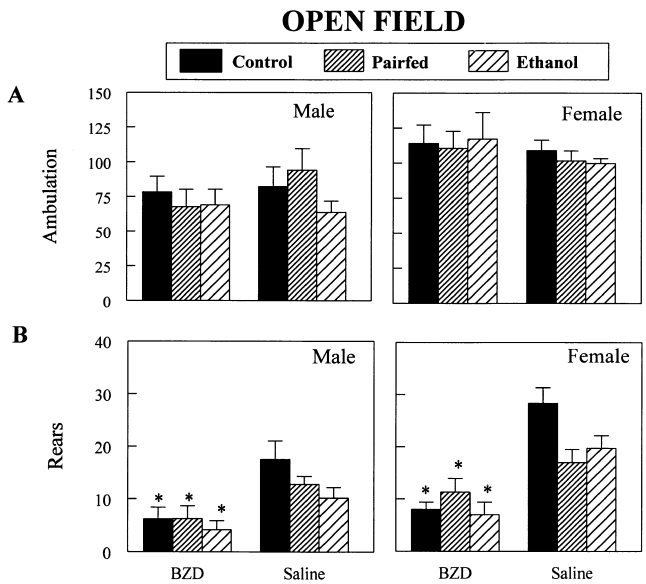

FIG. 1. Ambulation (A) and rearing (B) on the open field for males and females (mean + SEM). Ambulation: no significant difference among E, PF, or C males and females. Rearing: for both males and females, (*)main effect of adult experimental (BZD/saline) treatment, $F(1, 32) = 24.2$, $p < 0.001$, and $F(1, 33) = 46.3$, $p < 0.001$, respectively; BZD < saline, $ps < 0.001$.

perimental treatment (BZD/saline) on ambulation (Fig. 1). However, a significant main effect of adult BZD/saline treatment on rearing ($p < 0.001$) indicated that both males and females injected with BZD reared significantly less than males and females injected with saline (Fig. 1). There were no significant differences in ambulation or number of rears among E, PF, and C males or females.

Plus-Maze

Main effects of sex were observed for five of the behavioral measures ($ps < 0.01$): females spent increased time on open arms, decreased time on the central area, and made more open-arm entries, more closed-arm entries, and more closed-arm rears ($ps < 0.01$) compared to males (Figs. 2 and 3).

For both males and females, prenatal treatment × adult BZD/saline treatment ANOVAs revealed significant main effects of adult treatment for all behavioral measures ($ps < 0.05$), with the exception of partial open-arm entries for males. Overall, both males and females treated with BZD had increased time on open arms ($ps < 0.01$), made more open-arm entries ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 2), had decreased time on closed arms ($ps < 0.01$), made fewer closed-arm entries ($p < 0.05$ and $p < 0.01$, respectively) and made fewer closed-arm rears ($ps < 0.01$) (Fig. 3) compared to their saline-treated counterparts. Females treated with BZD also made fewer partial open-arm entries ($p < 0.01$) (Fig. 2) and spent decreased time on the central area ($p < 0.01$) compared to saline-treated females.

ANOVAs further indicated that BZD treatment differentially affected E, PF, and C males and females ($ps < 0.05$) compared to their PF and/or C counterparts. BZD-treated E

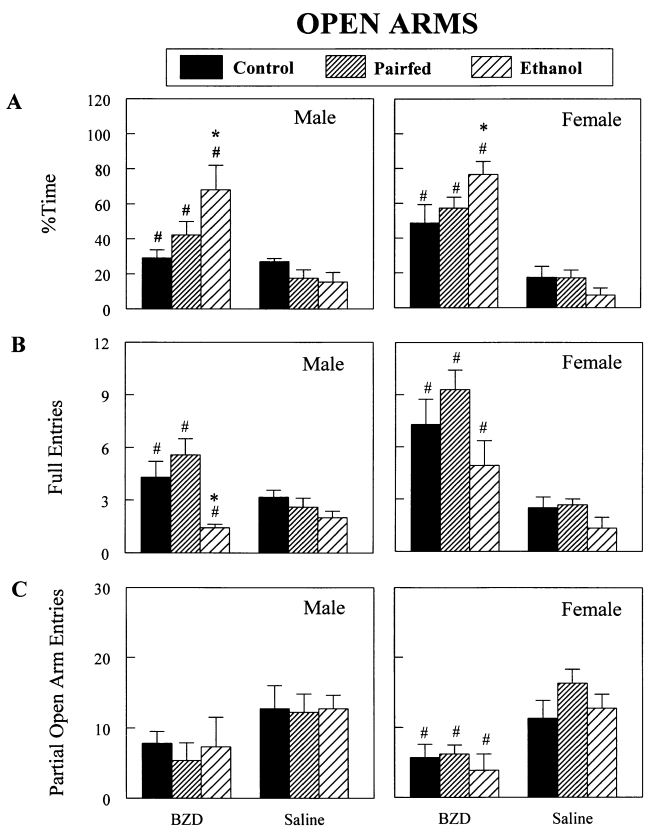

FIG. 2. Percent time (A) on the open arms and number of full (B) and partial (C) entries onto the open arms for males and females (mean + SEM). Percent time: for both males and females: (#)main effect of adult BZD/saline treatment, $F(1, 32) = 16.1$, $p < 0.01$, and $F(1, 33) = 64.8$, $p < 0.01$, respectively; BZD > saline, $ps < 0.01$. (*)prenatal treatment × adult experimental treatment interaction, $F(2, 32) = 5.1$, $p < 0.05$, and $F(2, 33) = 3.9$, $p < 0.05$, respectively; for BZD-treated males, E > PF = C, $ps < 0.05$; for BZD-treated females, E > C, $p < 0.05$, E > PF, $p < 0.10$. Full entries: for both males and females, (#)main effect of experimental treatment, $F(1, 32) = 5.2$, $p < 0.05$, and $F(1, 33) = 32.3$, $p < 0.05$, respectively; BZD > saline, $p < 0.05$, and $p < 0.01$, respectively. For males, (*)prenatal treatment × adult BZD/saline treatment interaction, $F(2, 32) = 4.2$, $p < 0.05$; for BZD, E < PF = C, $ps < 0.05$. Partial entries: for females, (#)main effect of adult BZD/saline treatment, $F(1, 33) = 10.6$, $p < 0.01$; BZD < saline, $p < 0.01$.

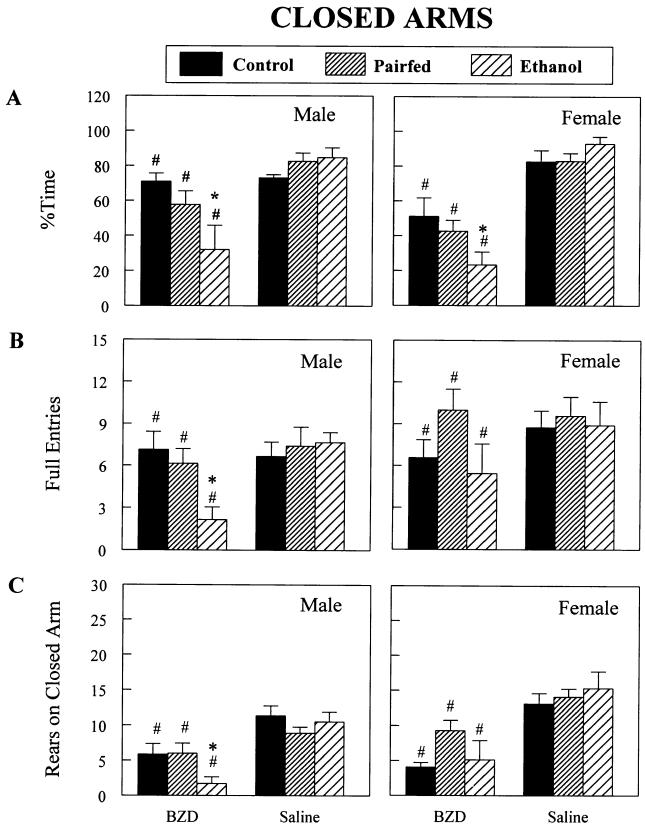

FIG. 3. Percent time (A) on the closed arms, and number of full entries (B) and rears (C) on the closed arms (mean + SEM). Percent time: for both males and females: (#)main effect of adult BZD/saline treatment, $F(1, 32) = 16.1$, $p < 0.01$, and $F(1, 33) = 64.8$, $p < 0.01$, respectively; BZD < saline, $ps < 0.01$. (*)prenatal treatment × adult BZD/saline treatment interaction, $F(2, 32) = 5.1$, $p < 0.05$, and $F(2, 33) = 3.87$, $p < 0.05$, respectively; for BZD-treated males, E < PF = C, $ps < 0.05$; for BZD-treated females, E < C, $p < 0.05$; E < PF, $p < 0.10$. Full entries: for both males and females, (#)main effect of adult BZD/saline treatment, $F(1, 32) = 5.5$, $p < 0.01$ and $F(1, 33) = 16.2$, $p < 0.01$, respectively; BZD < saline, $ps < 0.01$. For males, (*)prenatal treatment × adult BZD/saline treatment interaction, $F(2, 32) = 5.2$, $p < 0.05$; for BZD, E < PF = C, $ps < 0.05$. Rearing: for both males and females, (#)main effect of adult BZD/saline treatment, $F(1, 32) = 29.5$, $p < 0.01$, and $F(1, 33) = 26.3$, $p < 0.01$, respectively; BZD < saline, $ps < 0.01$. For males, (*)prenatal treatment × adult BZD/saline treatment interaction, $F(2, 32) = 7.95$, $p < 0.05$; for BZD, E < PF = C, $ps < 0.05$.

males had increased time on open arms ($ps < 0.05$) and made fewer open-arm entries ($ps < 0.05$) (Fig. 2), had decreased time on closed arms ($ps < 0.05$), and made fewer closed-arm entries ($ps < 0.05$) (Fig. 3), and had increased time on the central area ($ps < 0.01$) compared to BZD-treated PF and C males. There were no significant differences among saline-treated E, PF, and C males on any plus-maze measure. Similar to the data on males, BZD treatment differentially affected E, PF, and C females ($ps < 0.05$). BZD-treated E females had increased time on open arms ($p < 0.05$) (Fig. 2) and decreased time on closed arms ($p < 0.05$) (Fig. 3) compared to C females, and showed similar trends compared to PF females ($ps < 0.10$). There were no significant differences among saline-treated E, PF, and C females on any plus-maze measure.

Corticosterone

CORT levels obtained following testing are consistent with CORT levels measured previously in our lab (57,79,83) and by others (53,55,77). As expected (34), a main effect of sex ($p < 0.0001$) indicated that females had higher CORT levels than males (Fig. 4). For males, there was a significant main effect of adult BZD/saline treatment ($p < 0.05$) and a significant prenatal treatment × adult treatment interaction ($p < 0.05$) (Fig. 4). Post hoc tests indicated that BZD-treated E males had lower CORT levels than BZD-treated PF and C males ($ps < 0.05$), whereas there were no differences in CORT levels among E, PF, and C saline-treated males. For females, there were no significant effects of either prenatal treatment or adult treatment on CORT levels.

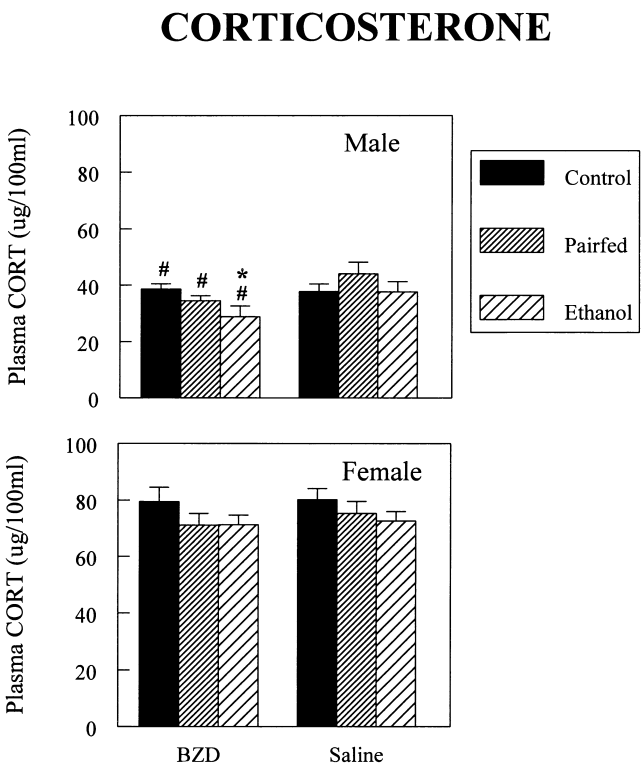

FIG. 4. Plasma corticosterone levels. For males: (#)main effect of adult BZD/saline treatment, $F(1, 32) = 10.5$, $p < 0.05$; BZD < saline, $p < 0.05$; (*)prenatal treatment × adult BZD/saline treatment interaction, $F(2, 32) = 3.6$, $p < 0.05$; for BZD, E < PF = C, $ps < 0.05$.

DISCUSSION

The plus-maze has been validated as a test of anxiety for both rats (58,59) and mice (39). Controversy exists, however, regarding interpretation of the anxiety/fear state generated by the plus-maze. Although the plus-maze has been shown to be sensitive to anxiolytic compounds that act on the GABA system (39,58,59), novel anxiolytic compounds, which act on the serotonergic or noradrenergic systems, produce variable results (69). Buspirone, for example, has been demonstrated to have both anxiogenic (19,51) and anxiolytic (21,37) properties. In addition, prior exposure to stressors has been shown to increase (58), decrease (73), or not affect the animal's activity level on the plus-maze (22). Thus, it has been suggested that plus-maze behavior is not an indicator of generalized anxiety but is a measure of situation-dependent anxiety/fear (22). It was on this basis that the plus-maze was chosen for the present study as an aversive task to examine both behavior and CORT responses in a stressful situation, as well as possible differential effects of BZD on behavioral and hormonal responses of animals prenatally exposed to ethanol compared to their control counterparts.

Data suggest that the open arms of the plus-maze are more fear- or stress-inducing than the closed arms (39,58). Undrugged animals spend less time on the open arms, make fewer open-arm entries (39,58), and demonstrate more anxiety/fear-related behaviors (freezing, immobility, defecation) on the open than on the closed arms (50,58). Animals confined to the open arms also exhibit higher CORT levels than those confined to the closed arms (50,57,58). Moreover, comparison of plus-maze behavior with exploration and locomotor activity in the holeboard task has demonstrated that open arm entries are not correlated with holeboard exploration or locomotion (58). Pellow et al. (58) reported that anxiolytic agents such as diazepam increase both time on open arms and number of open-arm entries on the plus-maze while reducing exploration and motor activity on the holeboard. In contrast, sedative agents such as haloperidol have no effect on time spent on the open arms, but reduce total arm entries on the plus-maze as well as both exploratory and locomotor activity on the holeboard. Together, these studies suggest that behavior on the open arms is likely a measure of fear or situation-dependent anxiety that is independent of exploration, whereas behavior on the closed arms and in the central area is likely a measure of exploration.

The results of the present study support previous work demonstrating that BZD treatment significantly decreases anxiety/fear on the plus-maze (39,58,59). Overall, BZD-treated males and females exhibited increased time on open arms, increased open-arm entries, and decreased time on closed arms compared to saline-treated males and females, regardless of prenatal treatment. These differences were not due to altered activity levels, as BZD treatment had no effect on total ambulation in the OF. In addition, consistent with the data from previous studies (5,25,27,38,47,68,72,73,88), the present data demonstrate sexually dimorphic responses to both the OF and plus-maze. Females showed increased exploratory behaviors on both the OF and plus-maze as well as decreased fear-related behaviors on the plus-maze compared to males. BZD treatment decreased fear-related behaviors in both males and females from all prenatal treatment groups, resulting in increased time on open arms and, as a consequence, decreased closed-arm exploration and activity compared to saline-treated animals.

Importantly, BZD treatment also differentially affected E males and females compared to their PF and C counterparts. Although no significant differences in plus-maze behaviors were seen among E, PF, and C males and females treated with saline, E males and females treated with BZD spent increased time on open arms and decreased time on closed arms compared to PF and C males and females, suggesting decreased fear. BZD-treated E males also exhibited decreased open and closed arm entries, spent significantly more time in the central area, and had lower CORT levels, another index of fear or stress (71), than BZD-treated PF and C males.

Together, these data support and extend previous work demonstrating that responses on the plus-maze provide a reliable measure of anxiety/fear (and at the same time, of exploration), and that plus-maze behavior is sensitive to anxiolytic agents such as BZD. Further, these data suggest that prenatal ethanol exposure may increase sensitivity to the effects of BZD on plus-maze behavioral and CORT responsiveness and may do so differentially in males and females.

It is well documented that in rodents sex differences occur in a variety of nonreproductive behaviors (9). The results from the present study support previous studies that demonstrate that females show greater ambulation and rearing in the OF (5,8,10,12,45,47,72) as well as decreased levels of fear and increased exploratory behaviors on the plus-maze (25,27, 29,38,68,73,88) compared to males. Sexually dimorphic responses to novel and aversive tasks have also been demonstrated following prenatal ethanol exposure. For example, E females but not E males showed a deficit in CORT response inhibition in a consummatory task and in CORT recovery toward basal levels during a 1-h restraint stress compared to their control counterparts (79). In contrast, during an extended period (4 h) of restraint stress, E males but not E females showed prolonged CORT elevations compared to controls (81). We have demonstrated previously (57) that prior exposure to an aversive environment (i.e., OF) may have differential effects on the behavior of E males and females on the elevated plus-maze. E males and females both exhibited higher levels of exploration when placed directly on the plus-maze from their home cages. Following activation of behavior by prior OF exposure, E males and females both showed a decrease in exploratory behaviors compared to controls, and E females showed increased CORT levels following plus-maze testing. Furthermore, E females but not E males showed an increase in fear-related behaviors compared to controls, regardless of prior OF exposure. In the present study, animals were similarly exposed to an aversive environment, the OF, prior to plus-maze testing. Although there were no significant differences in plus-maze behavior among E, PF, and C animals treated with saline, BZD treatment resulted in decreased fear-related behaviors in E males and females compared to their control counterparts, as well as differential behavioral and CORT responsiveness in E males compared to E females. These data suggest that prenatal ethanol exposure may alter possible interactive effects of stressors and BZD in a sexually dimorphic manner.

The sexual dimorphism in OF and plus-maze behavior observed in the present study may be mediated at least partially by the gonadal steroids. Estrogen and progesterone have been shown to have both organizational effects in neonates and activational effects in adults on plus-maze behavior (38, 88), whereas, testosterone appears to have organizational effects prenatally (25) and neonatally (61,75) but to lack activational effects in the adult (88). Thus, the finding that females overall showed more ambulation and rearing in the OF as well as increased exploratory behaviors and decreased fear-related behaviors on the plus-maze compared to males may be explained in part by differential organizational and/or activational effects of the gonadal steroids. It is also possible that the differential behavioral and hormonal responses of E males and females compared to their PF and C counterparts may be mediated partially by the gonadal steroids (26). Prenatal ethanol exposure has been shown to alter hypothalamic–pituitary–gonadal activity in both male and female offspring (30,32, 46, 63,80). Suppression of the prenatal and postnatal testosterone surges, deficits in testicular steroidogenic enzyme activity, delayed sexual maturation, alterations in plasma levels of male and female sex hormones and altered sexual behavior have been reported in E animals. Thus, it is possible that the sexually dimorphic responses in plus-maze behavior observed in E males and females in the present study may be mediated partially by differential gonadal steroid exposure during development and/or during adulthood.

Sex differences in plus-maze behavior in the present study may also be mediated partially by differential effects of ligands acting at the GABA receptor. BZD has been shown to bind to a BZD site on the $GABA_A$ receptor and to potentiate GABA-mediated inhibitory functions (56). In the present experiment, BZD may have acted by potentiating the inhibitory effects of GABA and thus reducing fear on the plus-maze. It has recently been demonstrated that females are less sensitive than males to the activity-suppressing effects of the BZD inverse agonist FG 7142, suggesting that there may be a sex difference in the response of the GABAergic system (70). The mechanism for this sexual dimorphism is unknown, but may be related to differential effects of neuroactive steroids (neurosteroids) that are known to bind to the $GABA_A$ receptor and alter the binding affinity for its ligand (43). Inhibitory (GABA-agonistic) steroid metabolites, such as 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and tetrahydrodeoxycorticosterone (THDOC), mimic and enhance the anxiolytic effects of GABA, whereas excitatory (GABA-antagonistic) neurosteroids, such as pregnenolone sulfate and dehydroepiandrosterone sulfate, antagonize $GABA_A$ function and thus increase arousal (11,42). Moreover, it has been shown that estrogen, progesterone, and CORT may have differential effects on GABA binding sites (41,86). Thus, it is possible that the overall sex differences in behavior on the OF and plus-maze may be modulated by differential levels of neurosteroids and/or interactive effects of the neurosteroids, gonadal steroids, and/or glucocorticoids at the GABA receptor.

Prenatal ethanol-induced alterations in the GABAergic system and/or in the interactions of gonadal steroids, CORT, and the neurosteroids at the GABA receptor may also underlie the differential behavioral and CORT responses to BZD seen in E males and females compared to their control counterparts during plus-maze testing. Previous studies have demonstrated alterations in the GABAergic system and in neurosteroid sensitivity following prenatal ethanol exposure. In utero ethanol exposure was shown to decrease sensitivity to allopregnanolone in neonates, as measured by ultrasonic vocalization (87), to alter GABA levels in a number of brain areas in 3-week-old pups (35), and to alter thermogenic responses to pentobarbitol and BZD in adult animals (3). Prenatal ethanol exposure was also shown to enhance both the positive modulatory effects of the BZD flunitrazepam and the negative modulatory effects of pregnenolone sulfate while attenuating the effect of the BZD inverse agonist FG 7142 on GABA-stimulated chloride ion flux into hippocampal membrane vesicles (70). Similarly, increased cerebral GABA con-

tent was found in neonates suckling on ethanol-consuming dams (62), and maternal ethanol consumption prior to pregnancy altered GABA turnover in adult offspring (36). Together these results suggest that prenatal ethanol exposure may alter CNS GABA content and/or turnover as well as responsiveness to GABA agonists and antagonists, and that effects may vary, depending upon the alcohol exposure regimen, age at testing, and site studied.

Finally, these data may have clinical implications. BZD receptor antagonists and inverse agonists have been shown to modulate learning and memory (28). Anxiolytic agents that enhance GABA binding impair memory, whereas anxiogenic agents that inhibit GABA binding enhance memory (28). It has been shown that BZD impairs learning and memory in animals prenatally exposed to ethanol to a greater extent than in control animals (60). Thus, it is possible that alterations in the GABAergic system, either directly or through altered neurosteroid activity, may play a role in the behavioral and/or cognitive alterations seen in children prenatally exposed to alcohol.

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