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Anxiolytic effects of diazepam and ethanol in two behavioral models: comparison of males and females

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Abstract

The present study compared the anxiolytic effects of the benzodiazepine agonist diazepam and ethanol in adult male and female rats. Varying doses of diazepam (1–3 mg/kg) or ethanol (0.5–2.0 g/kg) were tested using both the elevated plus maze and defensive prod-burying models. Two time points following ethanol administration (10 and 30 min) were tested in the plus maze. Sex differences were seen in some anxiety-related behaviors, with females showing greater open arm time and reduced burying behavior than males. Although this suggests females displayed less anxiety-like behavior than males, the differences in the plus maze were not observed in all testing situations. Both diazepam and ethanol dose-dependently increased open arm times in the plus maze and reduced burying behavior in the defensive prod-burying task. The parallel nature of the dose–response curves suggests that both diazepam and ethanol have similar anxiolytic effects in males and females. No sex differences were seen in the brain levels of diazepam-like activity or blood alcohol levels with these treatments. A greater corticosterone response was observed in females than males with these two behavioral tests, but neither diazepam nor ethanol decreased this response. These results suggest a dissociation between the anxiety-reducing influences of these compounds and the changes in stress-related endocrine responses.

Keywords: Anxiety; Benzodiazepine; Ethanol; Sex differences; Elevated plus maze; Defensive prod burying; Rats; Corticosterone

1. Introduction

Gender differences are seen in a variety of psychiatric disorders, including the prevalence of depression and anxiety disorders (Yonkers and Hamilton, 1996; Yonkers and Ellison, 1996; Breslau et al., 1997; Sonne et al., 2003; Simonds and Whiffen, 2003). Panic with agoraphobia, generalized anxiety disorder (GAD) and posttraumatic stress disorder (PTSD) occur two to three times more frequently in women as in men (see Yonkers and Ellison, 1996, for a review). Although findings are inconsistent, animal studies also suggest that sex differences exist in fear and anxiety-related behaviors in the open-field test, the elevated plus maze, the social interaction test, the defensive prod-burying test, acoustic startle reflex, and conflict procedures (Beatty, 1979; Steenbergen et al., 1991; Fernandez-Guasti and Picazo, 1992, 1997; Zimmerberg and Farley, 1993; Frye et al., 2000; Stock et al., 2000; Palanza, 2001; Frye and Walf, 2002; Gulinello et al., 2003). Male rodents generally exhibit more fear-related behaviors than females, although some studies fail to see sex differences in specific tests (Frye et al., 2000; Marcondes et al., 2001) or have demonstrated the opposite sex difference in "anxiety-like" behaviors (Johnston and File, 1991; Fernandes et al., 1999). The ability of gonadal hormones, neurosteroids such as 3α5α tetrahydroprogesterone (3α5αTHP), and the estrous cycle to modulate responses in these anxiety models contribute to the notion that males and females show differing levels of anxiety-related behaviors based on their differing hormonal milieus (Johnston and File, 1991; Bitran et al., 1991b; Bitran et al., 1993; Frye et al., 2000; Marcondes et al., 2001; Frye and Walf, 2002; Gulinello and Smith, 2003). These sex differ-

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ences in anxiety-related behaviors are dependent upon the testing conditions, the age of the animals, and the model used in assessing the behavioral outcomes (Steenbergen et al., 1991; Johnston and File, 1991; Imhof et al., 1993; Meng and Drugan, 1993; Mora et al., 1996). In fact, studies suggest that the primary factors influencing behavioral endpoints in these tests may differ between males and females (Fernandes et al., 1999).

Endocrine status or gonadal hormone treatments can modulate benzodiazepine sensitivity in humans (Ellinwood et al., 1983; Kroboth and Mcauley, 1997; Sundstrom et al., 1997a,b), and both humans and rats show sex differences in the effects of diazepam on electroencephalogram (EEG) measures (Romano-Torres et al., 2002; Fernandez-Guasti et al., 2003). Although women also appear more susceptible to the toxic effects of ethanol, this may be related to gender differences in the metabolism of ethanol (see Lieber, 2002). Animal studies also suggest the gonadal hormone milieu modifies the actions of various anxiolytic agents that interact with the $GABA_A$ receptor, including the benzodiazepines, ethanol, and 3α5αTHP. The results from these reports, however, show inconsistent sex differences that are dependent on the pharmacologic effect and the animal model used. The anxiolytic effects of diazepam in the elevated plus maze (Bitran et al., 1991a), light–dark transition (Carey et al., 1992), and defensive prod-burying (Fernandez-Guasti and Picazo, 1990, 1997) tests differ depending on the stage of the estrous cycle and/or endocrine status in rodents. A few studies also indicate that there are sex-related differences or influences of gonadal status on the acute ataxic or hypnotic effects of ethanol (Webb et al., 2002; Tayyabkhan et al., 2002). The role of neurosteroids in ethanol effects (Morrow et al. 1999), combined with influences of gonadal status or sex on neurosteroid levels and effects (Finn and Gee, 1993; Wilson, 1996; Wilson and Biscardi, 1997; Fernandez-Guasti and Picazo, 1999), support the possibility that sex differences exist in ethanol's anxiety-reducing effects. The role of gonadal status and sex differences in the anxiolytic influences of ethanol remains relatively unexplored. Furthermore, these previous studies examining sex differences in diazepam effects have not systematically assessed if these differences were related to altered drug levels.

The observation of sex differences in anxiety-related behaviors and in responses to various anxiolytic compounds is dependent on the model used (Fernandes et al., 1999). The elevated plus maze takes advantage of the rodent's natural tendencies to avoid brightly lit, open, elevated spaces. Although this test can be confounded by changes in activity levels, it does not involve rewarding or noxious stimuli and relies on a passive avoidance response to detect anxiety behavior (i.e., avoidance of open arms). In contrast, the defensive prod-burying model relies on responses to a noxious stimulus. This test is less affected by locomotor changes and the index of anxiety involves an active behavioral response, specifically burying of a discrete object (the shock probe). Thus, these tests take advantage of different aspects of animal behavior while having a distinct complement of problems associated with interpretation (Dawson and Tricklebank, 1995).

Based on the distinctions between these tests, the anxiolytic effects of diazepam and ethanol were tested in intact adult male and female rats using both the elevated plus maze and defensive prod-burying models. The studies were designed to examine the central premise that sex differences would be observed in the anxiolytic effects of both these compounds based on their similar interactions with the GABAergic system. To insure that any differences were not related to sex differences in drug levels, brain diazepam-like activity and blood alcohol content (BAC) were determined after behavioral testing. Because of the variances in times and doses of ethanol used in previous studies (File, 1980; Lister, 1989; Prunell et al., 1994; Spanagel et al., 1995; Hall et al., 1998; Ferreira et al., 2000; Bertoglio and Carobrez, 2002; LaBuda and Fuchs, 2002; see Eckardt et al., 1998), two time points (10 and 30 min) after ethanol administration were also examined in the elevated plus maze. Diazepam-induced alterations in corticosterone responses associated with behavioral testing were assessed in both paradigms, along with ethanol-induced changes in corticosterone responses in the prod-burying test. Overall, the results suggest that although sex differences can be observed in certain anxiety-related behaviors, males and females show similar dose-dependent anxiolytic effects of diazepam and ethanol in these two models.

2. Methods

2.1. Subjects

Male and female Long Evans rats were housed in groups of two to three on a 12:12 light–dark cycle with food and water available ad libitum. The animal facility is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and all animal procedures were approved by the University of South Carolina's Institutional Animal Care and Use Committee. Rats were 2–2.5 months of age at the onset of behavioral testing in elevated plus maze, and mean body weights at testing were 260–350 g for males and 200–250 g for females. Stage of the estrous cycle was assessed in females using daily vaginal lavage for at least two cycles (8 days) before each behavioral test, and males were handled similarly during the same time. Females were tested randomly throughout their cycle and all behavioral experiments were conducted during the early light portion of their light–dark cycle (lights on at 7:00 a.m.). Although randomly cycling animals were utilized, testing was held to early light periods to avoid confounding effects of shifts in baseline anxiety-related behaviors in females seen the afternoon of proestrus. To maximize animal usage for these studies, subjects tested in the prod-burying experiment had been previously tested in the elevated plus maze

with either saline vehicle or various ethanol doses. Animals were allowed at least 10 days for complete drug clearance between the two behavioral tests, and treatments received in the prod-burying experiment (saline, diazepam vehicle, ethanol, or diazepam) were completely randomized from the treatment received in the plus maze experiment.

2.2. Behavioral testing

2.2.1. Elevated plus maze

The anxiolytic actions of differing doses of diazepam and ethanol were tested in groups of male and randomly cycling females using the elevated plus maze. For the plus maze, rats were placed in the center square of the brightly lit (~ 20 fc) X-shaped maze modified from that described in Pellow et al. (1985) and videotaped for 5 min (Kang et al., 2000; Stock et al., 2000). Time spent in both open and closed arms, in addition to the number of entries into each arm, were later scored by an individual blind to the rat's treatment group. An entry was defined by all four paws crossing into the arm. Increased percent time or percent entries in the open arms are indicative of a reduced anxiety state, whereas total number of arm entries and closed arm entries can be used as measures of overall activity levels (Handley and McBlane, 1993).

For the diazepam experiment, animals were immediately transported to another room after the behavioral testing and sacrificed by decapitation. Trunk blood was collected for analysis of circulating corticosterone levels, and brains were harvested for analysis of central levels of diazepam-like activity using a radioreceptor assay. In ethanol experiments, animals were transported to another room for collection of duplicate 10 μl samples of blood from a tail vein puncture. Blood samples were placed into microcentrifuge tubes containing 190 μl of 0.53 N perchloric acid. After addition of 200 μl of 0.3 M potassium carbonate, samples were vortexed, centrifuged at $1000 \times g$ for 15 min and stored at − 20 °C until analysis of BAC.

2.2.2. Defensive prod-burying

The anxiolytic influences of diazepam and ethanol were also assessed in the defensive prod-burying task, as based on Treit et al., (1981, 1993), Treit (1985), and Gallo and Smith (1993). For 3 days before drug testing, rats were habituated to the Plexiglas prod-burying test chamber ($46 \times 30 \times 44$ cm) filled with 5-cm-deep bedding minus the prod for 30 min per day. For testing, rats were placed in the chamber with an electrified prod (6.5 cm long, 0.5 cm diameter) wrapped in copper wire positioned 2 cm above bedding. Rats received a single shock (Coulbourn Inst., Allentown, PA) upon touching the prod and the shock source was turned off after the initial shock. High shock levels (3 mA) were used to avoid potential confounds due to potential sex differences or drug-induced changes in nociception. From videotapes, blind observers subsequently scored the latency to bury following prod contact, the time burying the prod (duration of burying), and number of rears during the 15-min period following prod contact. Animals that failed to bury after shock were assigned a latency value of 900 s. Animals that did not touch the prod during a 15-min exposure were excluded from analysis. Following the prod-burying test, animals were transported to a separate room for sacrifice and blood collection. Circulating levels of corticosterone, plus estradiol and progesterone (in females), were analyzed using radioimmunoassay procedures. Uteri were removed and weighed for further verification of the estrous cycle stage.

2.2.3. Drug treatments

Groups of males and females received 1, 2, or 3 mg/kg diazepam or vehicle (10% ethanol, 40% propylene glycol) intraperitoneally, 30 min before testing in the plus maze. For testing in defensive prod-burying, diazepam (1 and 2 mg/kg ip) or vehicle (10% ethanol, 40% propylene glycol) was administered 30 min before placement in the testing chamber.

For examining ethanol effects in the plus maze, separate groups of male and female rats were tested at 10 or 30 min after ethanol administration. At the 10-min time point, ethanol doses of 0.5, 1.0, 1.5 g/kg ethanol (0.15 g/ml ethanol ip) or vehicle (sterile 0.9% saline) were tested, whereas doses of 0.5, 1.0, and 2.0 g/kg ethanol were used at the 30-min time point. The upper dose was reduced at 10 min because of the heavy sedation observed with the 2.0 g/kg dose at that time. In the prod-burying test, doses of 1 and 1.5 g/kg ethanol or saline vehicle were administered 10 min before testing.

2.3. Radioreceptor assay for benzodiazepine levels

The brain levels of benzodiazepine-like activity resulting from various diazepam doses were determined with the modified radioreceptor assay of Gallager et al. (1985), as described in Wilson and Biscardi (1992). Forebrain tissue was dissected and homogenized, extracted into ethyl acetate, and extracts evaporated to dryness. After resuspension in 25 mM K_2PO_4 buffer (pH 7.4) samples were incubated with 1 nM [^{3}H]flunitrazepam and a bovine brain P_2 membrane suspension for 30 min at 4 °C. Samples were filtered over glass fiber filters and radioactivity determined using liquid scintillation counting. Benzodiazepine-like activity in the samples was calculated from the standard curve (0.5–100 ng diazepam) with a log–logit transformation of the data and is indicative of all active drug and metabolites competing at the benzodiazepine receptor. This method will detect 3 pmol equivalents of diazepam per assay, and recovery of known diazepam samples was 98%.

2.4. Blood alcohol assay

BAC levels were determined based on the procedure of Dudek and Abbott (1984), as described previously (Wilson et al., 1996a). Briefly, triplicate 50 μl aliquots of each sample supernate were added to 450 μl of a mixture consisting of

0.750 μM nicotinamide adenine dinucleotide (NAD; Sigma, St. Louis, MO) and 4.46 units of alcohol dehydrogenase (ADH, Sigma) in 500 mM Tris buffer (pH 8.8). Standards ranging from 0 to 500 mg/100 ml were prepared similarly. After incubation of samples and standards for 1 h at room temperature, absorbance at 340 nm was measured in a spectrophotometer and the BAC content of samples was determined from comparison to the standard curve.

2.5. Radioimmunoassay for corticosterone, estradiol, and progesterone

The ability of diazepam and ethanol to modulate serum corticosterone levels induced by plus maze or prod-burying procedures was determined using the radioimmunoassay (RIA) described in Wilson et al. (1996b). Trunk blood was collected immediately (< 5 min) after the animal completed behavioral testing. In prod-burying experiments, circulating estradiol and progesterone levels were used, along with uterine weights, to confirm the stage of the estrous cycle in females. Levels were determined using the RIA described in Wilson and Biscardi (1992). These RIA procedures recover 87–90% of known samples with 11–20% interassay variability.

2.6. Statistics

For elevated plus maze, percent open arm time, closed arm entries, and corticosterone levels were compared using two way analysis of variance (ANOVA) with sex and dose (diazepam or ethanol) as factors. For prod-burying, the latency to bury after shock, the duration of burying, rears, and serum corticosterone were compared using two-way ANOVA, with sex and drug dose as factors. Specific differences between groups were analyzed using post hoc *t* tests with Bonferroni correction, once significant main effects were seen. Planned *t*-test comparisons between male and female vehicle groups were conducted in all tests. The ethanol and diazepam experiments were analyzed separately, although the control (vehicle) groups for the prod-burying experiments were combined because they were run during the same time frame and did not differ statistically. This was not done in the plus maze analyses due to a change in the testing facility between the two experiments. Individual levels of serum corticosterone, estradiol, and progesterone values were compared to behavioral measures using correlational analyses. Significance level was set at P=.05.

3. Results

3.1. Effects of diazepam and ethanol on elevated plus maze behaviors

As shown in Fig. 1, diazepam dose-dependently increased percent open arm time [$F(3,52) = 4.83$, $P < .005$ for diazepam effect] when administered 30 min before testing, but this anxiolytic effect did not differ significantly between males and females [$F(1,52) = 1.29$, P=.26 for sex effect]. Females

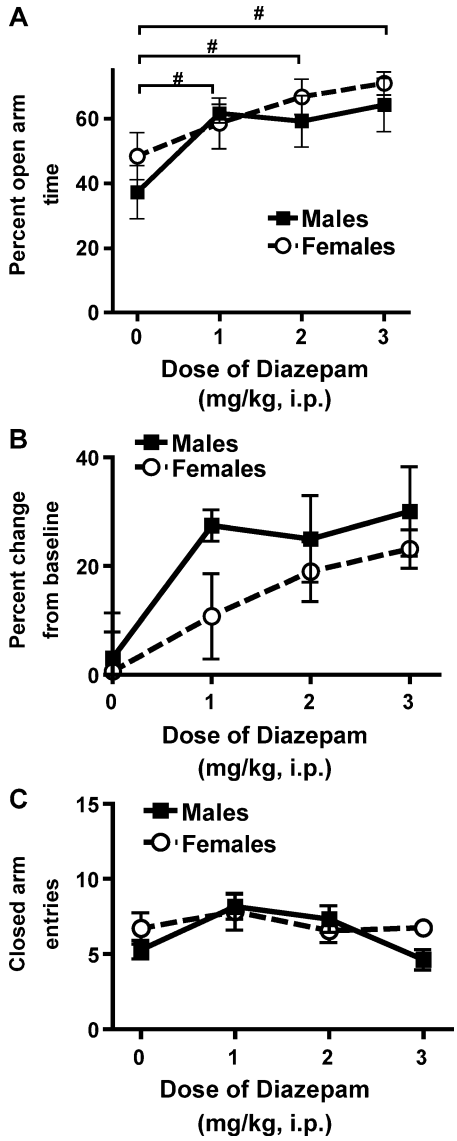

Fig. 1. The anxiolytic effects of diazepam in the elevated plus maze are similar in male and female rats ($F = 1.3$, P=.3 for sex effect). Diazepam was administered intraperitoneally 30 min before testing in the plus maze. The upper panel (A) shows significant increases in percent open arm time induced by diazepam ($F = 4.8$, $P < .005$); however, the parallel curves suggest a similar anxiolytic effect in both sexes. The # symbol indicates $P < .05$ versus vehicle, indicating that all three doses of diazepam increased percent open arm time over baseline. The middle panel (B) shows the effects of diazepam on open arm time expressed as a percent above baseline. Although the 1 mg/kg dose of diazepam appeared somewhat more effective in males than females, sex did not significantly modify the percent increase over baseline ($F = 2.7$, P=.11) and no interaction was observed ($F = 0.38$). The bottom panel (C) shows the closed arm entries in the plus maze, demonstrating that over this dose range a slight but significant ($F = 2.9$, $P < .045$) increase in this measure of activity was observed. Note that even at the highest dose, diazepam did not decrease in activity below control levels, and that males and females did not differ in the influences of diazepam on closed arm entries ($F = 1.1$, P=.31). Points represent the mean ± S.E.M. for Ns = 6–9 rats/point.

(48.4 ± 7.3%) showed slightly higher baseline levels of percent open arm time than males (37.8 ± 8.3%), but this difference did not attain statistical significance ($t = 1.2$). Because baseline values were slightly different, the results in males and females were also expressed as a percent change from the appropriate mean baseline response. These results, shown in Fig. 1 (middle panel), showed a significant effect of diazepam [$F(3,52) = 4.83$, $P < .005$]. Although the 1.0 mg/kg dose of diazepam appeared more effective in males than females, there was no statistically significant sex difference in diazepam responses when analyzed as percent above baseline ($F = 2.66$, $P = .11$) and no interaction was observed ($F = 0.38$). Diazepam modified activity levels, as indicated by closed arm entries [see Fig. 1, bottom panel; $F(3,52) = 2.89$, $P = .045$], but this measure did not show any sex differences [$F(1,52) = 1.07$, $P = .31$). The effect of diazepam in this dose range is to enhance activity, as indexed by closed arm entries. Even at the 3 mg/kg dose diazepam did not depress activity below control (vehicle) levels.

The effects of ethanol in the elevated plus maze were assessed at both 10 and 30 min after injection in separate groups of animals, and the results are shown in Fig. 2. At 10 min after injection doses of 0.5, 1.0, and 1.5 g/kg, ethanol produced similar dose-related increases in percent open arm time in both males and females [$F(3,74) = 3.2$, $P < .03$ for ethanol effect), indicating an anxiolytic effect in this test. No sex difference in anxiety-related behaviors were seen at this time point [$F(1,74) = 0.21$, $P = .64$ for sex effect), and there was no interaction between sex and ethanol. Several animals were tested after injection of 2.0 g/kg ethanol, but due to the heavy sedation seen at 10 min with this dose, the highest dose tested at the 10-min time point was decreased to 1.5 g/kg. Over this dose range (0–1.5 g/kg) ethanol significantly increased closed arm entries [$F(3,74) = 4.42$, $P < .01$) in a similar fashion in males and females, but no effects of sex on this measure of activity were observed [$F(1,74) = 0.13$, $P = .7$).

Ethanol (0.5, 1.0 and 2.0 g/kg doses) administered 30 min before testing also induced dose-dependent increases in percent open time [$F(3,39) = 5.69$, $P < .003$ for ethanol effect], indicating an anxiolytic effect. Sex also had a significant main effect [$F(1,39) = 6.06$, $P < .02$] at 30 min because females showed higher percent open arm times (36.3 ± 5.2%) compared to males (21.6 ± 5.7%) at all doses, although baseline values between males and females did not differ significantly ($t = 1.5$, $P = .08$). Ethanol effects showed parallel dose–response curves in males and

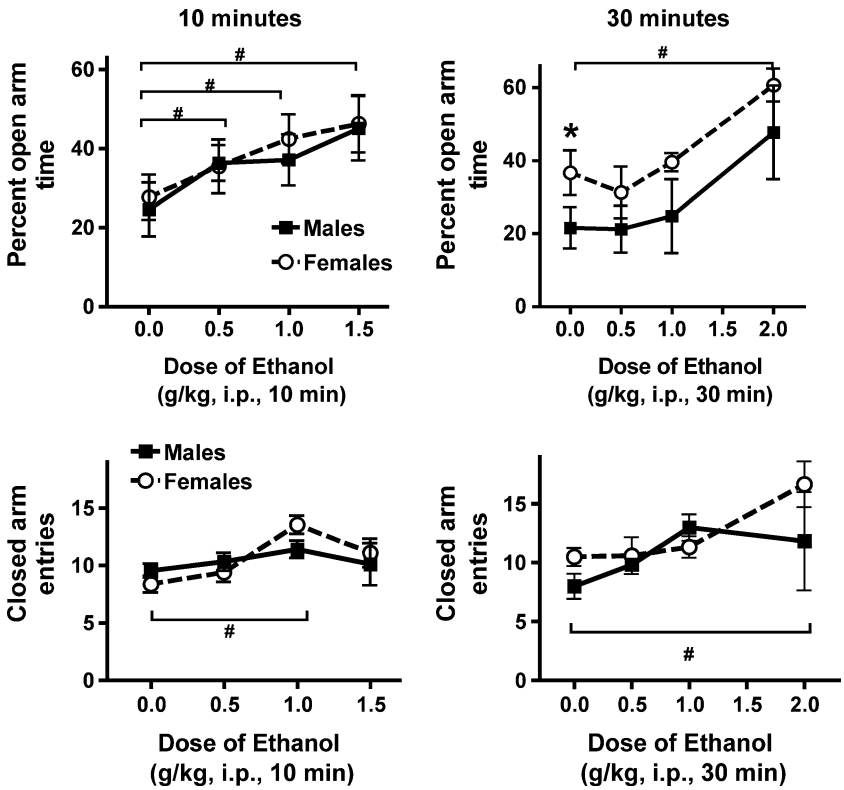

Fig. 2. The anxiolytic effects of ethanol were tested at 10 min (left panels) and 30 min (right panels) after injection in male and female rats. Upper panels show percent open arm time, whereas lower panels show ethanol effects on closed arm entries. Ethanol significantly increased percent open arm time at both 10- ($F = 3.2$, $P < .03$) and 30-min time points ($F = 5.7$, $P = .003$), but these effects did not differ between males and females. A main effect of sex was seen in percent open arm time at 30 ($F = 6.1$, $P < .02$, denoted by *), but not 10 min ($F = 0.21$, $P = .64$), suggesting this difference may be modified by the testing situation. Closed arm entries were increased by ethanol administration ($F = 4.42$, $P < .01$ at 10 min and $F = 2.84$, $P = .05$ at 30 min), but no sex differences were seen in this measure of activity at either time point ($F = .13$ and 0.12; $P = .7$). All doses of ethanol were effective in increasing open arm time at 10 min (see # demonstrating $P < .05$ vs. vehicle), but only the 2.0 g/kg dose was effective when administered 30 min before testing. Points represent the mean ± S.E.M. for Ns = 9–11 rats/point at 10 min and $N = 6$ rats/point at 30 min. The * indicates $P < .05$ for male–female difference, whereas # indicates $P < .05$ at each dose versus vehicle.

females, although the curve was shifted upward in females compared with males. The similarity of the ethanol effects was supported by the lack of any interaction between ethanol and sex effects ($F = 0.05$, $P = .99$). Analysis of the data as a percent increase above baseline values also failed to show any sex difference [$F(1,39) = 0.12$, $P = .7$] in ethanol's anxiolytic effects in the plus maze once baseline differences were factored out (data not shown). Ethanol increased closed arm entries [$F(3,39) = 2.84$, $P = .05$), but no sex effect or interaction was seen in this measure of activity in the plus maze.

3.2. Effects of diazepam and ethanol on defensive prod-burying behaviors

The effects of ethanol and diazepam in the prod-burying test are seen in Fig. 3. Because the control (vehicle-treated) rats in these two experiments did not differ statistically, the vehicle-treated animals were combined. The duration of burying showed a significant ($P < .05$) sex difference in baseline (vehicle) values, although mean latency measures in vehicle-treated rats failed to show any significant sex difference at baseline. Interestingly, if the vehicle-treated

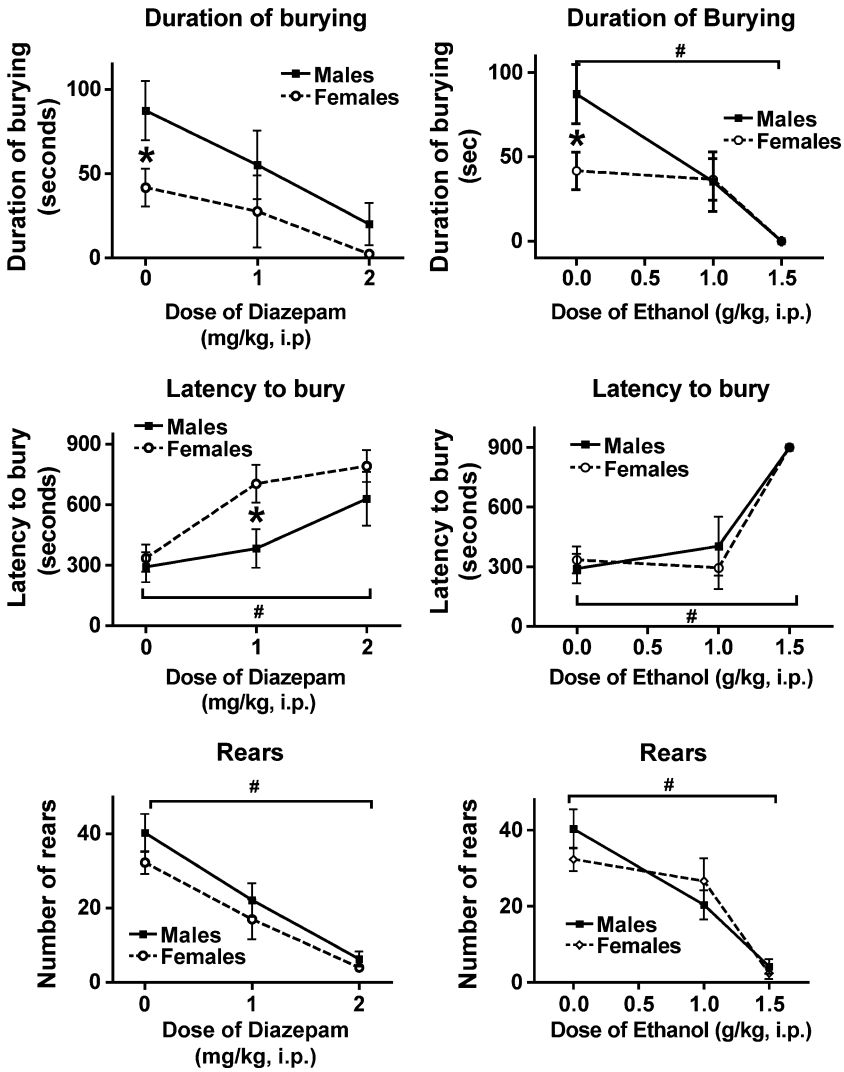

Fig. 3. The anxiolytic effects of diazepam (left panels) and ethanol (right panels) in the defensive prod-burying test are shown. Upper panels are the duration of burying, middle panels show latency to bury the prod, and lower panels show rearing behavior. The control (vehicle) groups for the ethanol and diazepam experiments did not differ and were combined for analysis (control $N = 17–18$). Vehicle-treated females showed a significantly lower amount of burying behavior than males (* denotes $P < .05$), although mean latencies did not differ at baseline. Main effects of sex were seen in duration of burying ($F = 4.4$, $P = .04$) and latency to bury ($F = 5.4$, $P = .02$) with diazepam (* denote $P < .05$), but not ethanol (F values = 1.0 and 0.3, respectively; $P > .3$). Both diazepam and ethanol dose-dependently decreased burying behavior (top panels), increased latency to bury the prod (middle panels), and reduced rearing behaviors (lower panels). Duration, latency, and rearing values only differed from vehicle values (# denotes $P < .05$ vs. vehicle) after the highest dose of diazepam (2.0 mg/kg, 30 min) and ethanol (1.5 g/kg, 10 min). These effects all suggest anxiolytic actions in this test. Although sex differences were seen in baseline burying responses, diazepam (intraperitoneal, 30 min) or ethanol (intraperitoneal, 10 min) effects did not differ between males and females, and no significant interactions were seen. Points represent the mean ± S.E.M. with Ns of 8–11 for the diazepam doses and 5–8 for the ethanol doses. The asterisk (*) indicates $P < .05$ for male–female difference at each point, whereas # indicates $P < .05$ versus vehicle.

animals that never buried (e.g., had latency values of 900 s) were removed from analysis (three males, two females), then vehicle-treated females showed significantly greater latency to bury than vehicle-treated males [male latency = 132 ± 21 s vs. female latency = 264 ± 53 s, $t(28) = 2.1$, $P < .04$]. This was not done for statistical analysis of diazepam and ethanol effects, because these anxiolytics increase the number of animals not burying at high doses. These findings suggest that females show less anxiety-like behavior than males, displayed as a reduction in the duration of burying and increased latency to bury compared with males. The results were not likely to be influenced by estrous cycle stage, because the variance in the female groups was not significantly greater than that of the male groups. Most of the females tested were in diestrus or metestrus (all but three females in this study), and no correlations were seen with the circulating estrogen levels, progesterone levels, or the estrogen/progesterone ratio at the time of testing.

As seen in Fig. 3, diazepam administered 30 min before testing dose-dependently decreased the duration of burying [$F(2,66) = 4.5$, $P < .02$], increased the latency to bury the prod [$F(2,66) = 9$, $P < .0003$], and decreased rears [$F(2,66) = 15$, $P < .0001$]. Diazepam effects were also seen in the number of animals that failed to show burying behavior during the 15-min time period after the shock, with 5 of 8 males and 6 of 8 females failing to bury at the 2.0 mg/kg dose. The overall main effects of sex showed females had reduced duration of burying [$F(1,66) = 4.4$, $P < .04$) and enhanced latencies to bury [$F(1,66) = 5.4$, $P < .02$) compared to males. In contrast, rearing behavior did not show a significant sex difference ($F = 0.1$, $P = .3$). None of the sex by diazepam–dose interactions was significant, and none of the post hoc t tests showed significant differences between males and females (see Fig. 3). This supports the lack of sex differences in the pattern of diazepam-induced changes in anxiety-related behaviors in the prod-burying task.

Ethanol administered 10 min before testing also dose-dependently decreased the duration of burying [$F(2,56) = 5.8$, $P < .005$], increased the latency to bury the prod [$F(2,56) = 19$, $P < .0001$], and decreased rears [$F(2,47) = 22$, $P < .0001$]. As seen in Fig. 3, no sex differences were observed in the influences of ethanol on prod-burying behavior. Of note is the rather steep dose–response curve for ethanol effects in this task with the 1.5 g/kg dose eliminating burying behavior after the shock in both males and females (none of the rats in these groups showed any burying). The 1.0 g/kg dose did not modulate latency measures in either sex, and due to the low baseline levels of duration of burying in females, failed to modulate duration measures in females. Thus, although females showed an overall reduction in anxiety-like behaviors in the defensive prod-burying task compared to males, diazepam and ethanol produced similar dose-dependent anxiety-reducing effects in males and females.

3.3. Drug levels

As seen in Fig. 4, diazepam administration dose-dependently increased brain levels of diazepam-like activity assessed using a radioreceptor assay [$F(2,35) = 16.3$, $P < .001$], but no sex differences were seen in brain drug levels [$F(1,35) = 0.84$, $P = .37$ for sex effect].

Analysis of BAC after testing showed dose-dependent increases at both 10 min [$F(1,54) = 13.4$, $P < .0001$] and 30 min [$F(1,27) = 6.34$, $P < .006$] after testing in the plus maze (see Fig. 5). No sex differences in BAC were seen at either time point.

3.4. Corticosterone responses

Stress-related increases in corticosterone were assessed immediately following testing in the plus maze paradigm in association with diazepam administration and after testing in the prod-burying test with both diazepam and ethanol. As seen in Fig. 6, behavioral testing in these paradigms yielded corticosterone levels well above basal levels (~ 170 ng/ml in males, 200 ng/ml in females; see Wilson et al., 1996b) seen when similarly handled animals are analyzed without maze or prod exposure. This response to maze exposure was more marked in females than males in the plus maze test [$F(1,52) = 37$, $P < .0001$ for the sex effect in upper panel]. Diazepam dose-dependently increased circulating corticosterone levels observed after plus maze testing [$F(3,52) = 3.94$, $P < .02$], but this effect did not appear to differ between males and females and there was no interaction between sex and diazepam on corticosterone levels. No effect of diazepam was seen in corticosterone levels after the prod-burying test ($F = 0.26$, $P = .80$; middle panel, Fig. 6), perhaps due to the lower diazepam dose range tested in this paradigm compared to that used in the plus maze test.

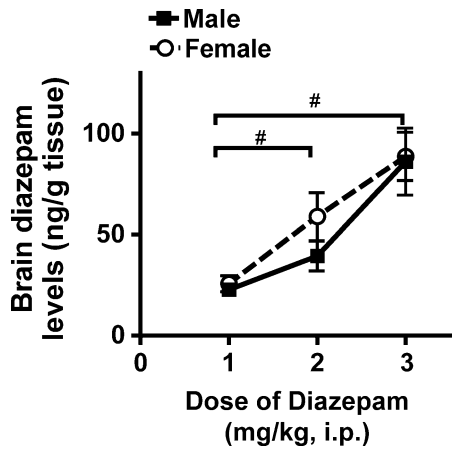

Fig. 4. Brain levels of diazepam-like activity were increased in a dose-dependent manner ($F = 16$, $P < .0001$), but they did not differ between males and females ($F = 0.8$, $P = .4$). Levels were assessed using a radioreceptor assay, and points represent the mean ± S.E.M. of Ns of 6–8 per point. The # indicates significant differences between doses of diazepam ($P < .05$).

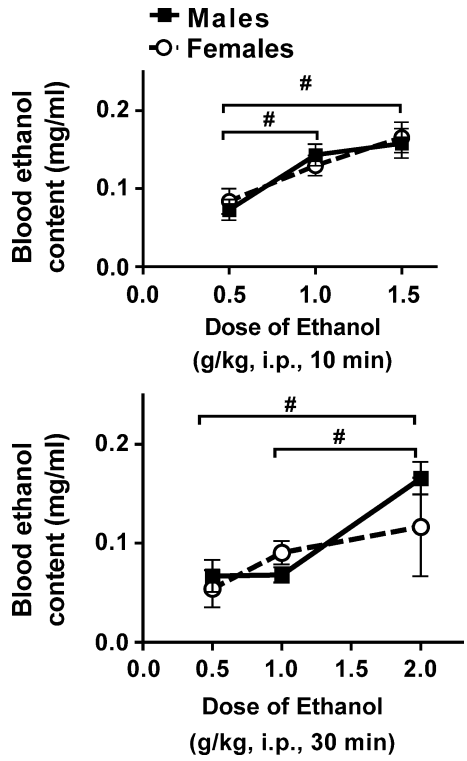

Fig. 5. Blood alcohol levels increased with increasing doses of ethanol ($F = 13$ and 6 for 10- and 30-min time points, respectively, $P < .006$), but no sex differences were seen in ethanol levels at either the 10-min (top; $F = .01$; $P = .9$) or 30-min (bottom; $F = 0.46$, $P = .5$) test. Blood samples were taken from tail vein puncture after behavioral testing in the plus maze. Points represent the mean ± S.E.M. with Ns of 8–11 at 10 min and $N = 5–6$ at 30 min. The # indicates significant differences between doses of ethanol ($P < .05$).

Ethanol also failed to significantly alter corticosterone levels associated with defensive prod-burying at doses up to 1.5 g/kg ($F = 1.53$, $P = .23$). A sex difference was seen in corticosterone responses following prod-burying [$F(1,47) = 13.7$, $P = .0006$], and this difference was accentuated by a heightened corticosterone level after 1.5 g/kg ethanol dose in females compared with males ($P < .05$).

4. Discussion

These studies assessed sex differences in the anxiolytic effects of the benzodiazepine agonist diazepam and ethanol using the elevated plus maze and the defensive prod-burying test. Although no marked sex differences were observed in the anxiolytic effects of either diazepam or ethanol in these models, slight differences were seen in baseline behavioral responses in these tests. The results generally support the notion that females show less anxiety-related behavior than males, but highlight the importance of the animal model and testing conditions used in assessing sex differences in anxiety behaviors in rodents.

Several studies have demonstrated sex differences in basal anxiety-related behaviors in these two models, although most often proestrus/estrus females differ from males, and differences are not observed when metestrus or diestrus females are used for comparisons (Johnston and

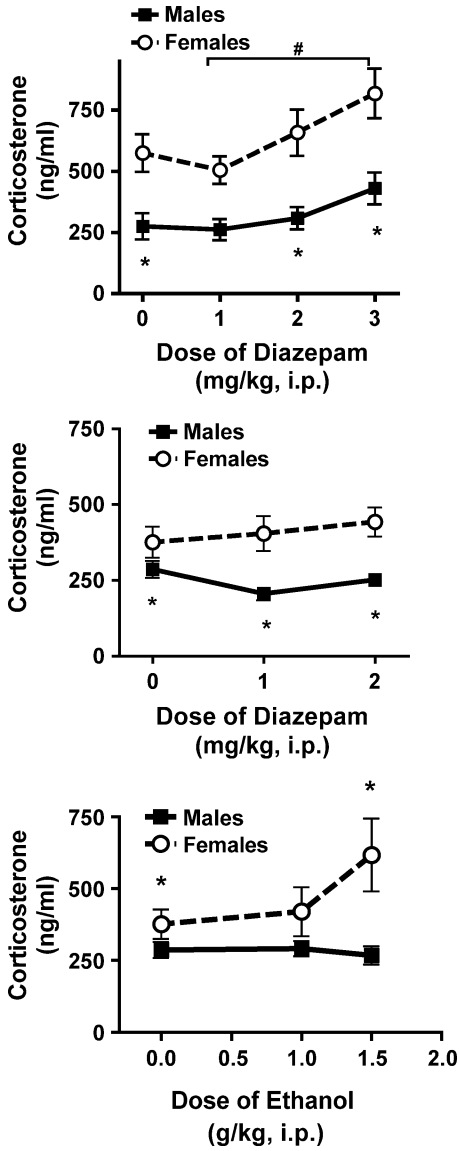

Fig. 6. Circulating corticosterone levels were determined after diazepam administration in the plus maze (upper panel; 30 min), diazepam administration in the prod-burying test (middle panel; 30 min), and ethanol administration in the prod-burying test (bottom panel; 10 min). Corticosterone responses were greater in females than males in the plus maze ($P < .05$ after vehicle; $F = 37$, $P < .0001$), and diazepam increased corticosterone release in this test (upper panel; $F = 3.9$, $P < .02$ for diazepam effect). Diazepam did not significantly alter corticosterone levels in the prod-burying test ($F = .2$, $P = .8$), probably due to the lower doses required in this test, but there was a significant main effect of sex on corticosterone levels ($F = 11$, $P < .002$). Ethanol did not significantly alter corticosterone levels ($F = 1.5$, $P = .2$), although females showed an increase in corticosterone levels at the highest (1.5 g/kg, 10 min) ethanol dose, inducing a significant (* denotes $P < .05$) sex difference at this dose ($F = 14$, $P < .0005$ for sex effect). Points represent the mean ± S.E.M., with Ns of 6–9 (top), and 4–14 (middle and bottom panel). The asterisk (*) indicates $P < .05$ for male–female difference at each point, whereas # indicates $P < .05$ between individual doses and vehicle values.

File, 1991; Fernandez-Guasti and Picazo, 1992; Zimmerberg and Farley, 1993; Imhof et al., 1993; Mora et al., 1996; Fernandes et al., 1999; Frye et al., 2000; Stock et al., 2000; Marcondes et al., 2001). Our results in the plus maze showed that percent open arm times were slightly greater in females than males, yielding an overall main effect of sex in some of our analyses. Although this generally supports the notion that females show less "anxiety-related behavior" than males, the sex differences in values seen after vehicle administration failed to be statistically significant using the post hoc *t* tests, (ethanol administration 30 min before testing, Fig. 2). Secondly, these marginally significant sex differences were only seen when the injections were done 30 min before testing, and no sex difference was observed in the ethanol analysis at the 10-min time point. This could suggest that the stress of injection closer to testing influenced the observation of the basal difference in open arm time. This is supported by several studies suggesting that prior stressors have differential sex-dependent influences in tests of anxiety-related behaviors (Steenbergen et al., 1990, 1991; Tayyabkhan et al., 2002). This stress-related effect might also explain the discrepancy between the two experiments testing diazepam versus ethanol effects 30 min after injection, because our testing facility was moved to a closer location that obviated animal transport before testing between these two experiments. The additional stress of transport in animals tested with diazepam vehicle (Fig. 1) could have also diminished the baseline sex difference observed under somewhat less stressful conditions in the ethanol experiment (Fig. 2 at 30 min). As expected, because females were not tested the afternoon of proestrus, this was likely to have diminished sex differences in basal states seen by some groups using proestrus/estrus animals. Some reports similarly fail to see sex differences in these tests or the dependence of sex differences on specific testing conditions (Nomikos and Spyraki, 1988; Ostaszewski and Pisula, 1994; Mora et al., 1996).

In contrast, sex differences were seen in the duration of burying in the defensive prod paradigm, with females burying less than males in this test. Although latency to bury did not show a striking sex difference, if animals that failed to bury were deleted from analysis, females showed longer latencies to bury than males. Latency to bury is thought to be more directly related to reactivity (Treit et al., 1981; Fernandez-Guasti and Picazo, 1990), and is increased by 3α5αTHP and diazepam in males but not females (Fernandez-Guasti and Picazo, 1997, 1999). Overall, our results continue to support the notion that females show less anxiety-like behaviors than males, although the differences are related to the specific behavioral model and testing conditions. The more robust sex differences observed in the prod-burying task could be related to sex differences in nociception (for reviews, see Aloisi, 2003; Craft, 2003a,b). Although a relatively high intensity shock stimulus was used to negate such differences and ensure the stimulus was sufficiently noxious to both sexes, and corticosterone responses support the shock was noxious in both males and females, sex differences in shock sensitivity cannot be discounted (Beatty, 1979). It is also possible, however, that more robust sex differences were seen in the prod-burying task due to sex-dependent adaptations induced by prior testing in the plus maze and/or ethanol administration. Although animals were allowed more than 10 days between testing to allow for drug elimination, and the animals were completely randomized for the prod-burying experiments, it remains a possibility that earlier exposure to the plus maze and/or ethanol could have modified prod-burying responses. The induction of changes in the $GABA_A$ receptor system could have also modified responses to the anxiolytic effects of ethanol or diazepam in these tasks, and could also explain some of the differences between our results and those seen in other studies.

No sex differences were seen in the anxiolytic effects of diazepam in either the plus maze or defensive prod-burying task. The lack of sex differences in the anxiolytic effects of diazepam are similar to previous results in the plus maze using other diazepam administration protocols (Stock et al., 2000). Although there was no significant interaction in the plus maze to support significant sex differences in the effects of diazepam, it is intriguing that the shape of the dose–response curve for males and females differed in this test. This is particularly interesting since these anxiolytic effects are seen within a very low dose range, well below the sedating influences of the drug, which could confound the results in this paradigm. The 1 mg/kg dose was more effective in males than females, suggesting a slightly increased sensitivity to low doses of diazepam in the plus maze. In males, but not females, this dose markedly increased percent open arm time above baseline levels. A similar pattern was seen in a prior study comparing the acute anxiolytic actions of diazepam in males and ovariectomized (OVX) females. Males also showed a significant and nearly maximal increase in open arm behaviors in response to 1 mg/kg diazepam, whereas OVX females showed less response to the low dose but a more progressive dose–response relationship (Stock et al., 2000). This would argue that this effect is not completely dependent upon the hormonal milieu at testing. Similar results have also been seen in the prod-burying test, with males being more sensitive than females to the actions of 1 mg/kg diazepam on burying behavior (Fernandez-Guasti and Picazo, 1990, 1997). Interestingly, however, we did not observe any sex differences in the effects of diazepam on burying behavior, and females actually showed a slightly greater effect of diazepam in the latency to bury than males in this test (see Fig. 3, middle left panel). These results also differ from earlier studies indicating that diazepam increased latency to bury in males but not females (Fernandez-Guasti and Picazo, 1997). The discrepancies between these studies might be related to testing parameters because

our animals are tested in a familiar testing arena (as in (Treit et al., 1981, 1993; Treit, 1985; Gallo and Smith, 1993)). This might again support the suggestion that sex differences in stress and/or habituation might influence the sex differences observed in these models (Steenbergen et al., 1990, 1991; Tayyabkhan et al., 2002). Alternatively, part of the influences of ethanol and diazepam in this task could be related to their antinociceptive properties. Both ethanol (Gatch, 1999; Gatch and Lal, 1999) and diazepam (Pang et al., 2001) in these dose ranges can show antinociceptive effects, and sex differences are observed in the analgesic effects of many compounds (see Craft, 2003a). Furthermore, there might be sex differences over a different dose range, and sex differences in anxiolytic effects might be more robust with lower doses of diazepam, and/or might be observed in the sedating effects of the drug at higher doses. Overall, however, our results suggest that sex differences in the effectiveness of these anxiolytics are dependent on the model used.

Several studies have indicated that the gonadal hormone milieu modulates responses to diazepam in males and females. In women, differences are seen in the effects of diazepam over the menstrual cycle, and these differences appear more robust in women with premenstrual syndrome (Sundstrom et al., 1997a). In rodents, estrous cyclicity modifies the effects of diazepam in the plus maze (Bitran and Dowd 1996), in prod-burying behavior (Fernandez-Guasti and Picazo, 1997), in active avoidance acquisition (Diaz-Veliz et al., 2000), and in light–dark transitions (Carey et al., 1992), although some studies fail to see differences in diazepam effects over the estrous cycle or following ovariectomy (Nomikos and Spyraki, 1988). Estrogen–progesterone administration and subchronic exposure to progesterone or 3α5αTHP also alter the anxiolytic actions of benzodiazepines (Gulinello and Smith, 2003). Recent studies suggest an important role for testosterone and androgen metabolites in modulating anxiety-related behaviors (Frye and Seliga, 2001; Fernandez-Guasti and Martinez-Mota, 2003) and the anxiolytic effects of diazepam (Fernandez-Guasti and Martinez-Mota, 2003). Our study used randomly cycling females, but avoided testing the afternoon of proestrus to minimize the changes in baseline anxiety levels seen at this time during the cycle (Fernandez-Guasti and Picazo, 1992; Frye et al., 2000; Marcondes et al., 2001; Frye and Walf, 2002). Although sex differences in diazepam's anxiolytic effects might have been observed by testing exclusively proestrus females with males, the results would have been confounded by significant differences in baseline anxiety-related behaviors (Fernandes et al., 1999). Moreover, it is equally plausible that diazepam's effectiveness might be similarly influenced by endocrine factors in both males and females, thus diminishing sex differences in anxiolytic responsiveness. This would not negate the potential influences of the reproductive cycle or hormone administration in females on the anxiolytic responses to benzodiazepines or ethanol, but rather suggest that androgenic hormones exert similar influences in males.

Acute ethanol administration produced anxiolytic actions in both the plus maze and defensive prod-burying tests, although the effects were not as robust as those seen with the benzodiazepine agonist diazepam. Ethanol administration increased percent open arm time in the plus maze, while decreasing burying and increasing latency to bury in the prod-burying task. These effects were seen at doses that were nonsedating, and in fact tended to enhance measures of activity in the plus maze. Similar results have been seen with ethanol in the plus maze in both rats and mice over this low dose range (File, 1980; Lister, 1989; Prunell et al., 1994; Spanagel et al., 1995; Hall et al., 1998; Ferreira et al., 2000; Bertoglio and Carobrez, 2002; LaBuda and Fuchs, 2002; see Eckardt et al., 1998), although the anxiolytic influences of ethanol in this test are strain dependent (Stewart et al., 1993; Boehm et al., 2002). No sex differences were observed in the anxiolytic effects of ethanol in either test of anxiety or at either time point. Although the 1 g/kg ethanol dose appeared less effective in reducing burying behavior in females compared to males, this difference is difficult to interpret due to the lowered baseline in females and the narrow range of effective doses for ethanol in this test. Others have similarly failed to find sex differences in the anxiolytic influences of ethanol in certain strains of mice (Boehm et al., 2002), although sex differences in the acute anxiolytic effects of ethanol have not been extensively analyzed. Sex differences have been seen in ethanol-induced ataxia and loss of the righting reflex, although these effects are in a higher dose range (3 g/kg, (Webb et al., 2002; Tayyabkhan et al., 2002). Although we may have observed sex differences using higher doses of the compound, this would require the use of alternate models of anxiety. In our studies, we were unable to test ethanol effects at the 10-min time point in the plus maze with 2 g/kg or greater doses because of heavy sedation observed with this dose.

The lack of sex differences does not seem to be attributable to differences in drug levels. Brain levels of diazepam-like activity measured using a radioreceptor assay were similar in males and females. This is similar to our prior studies examining the acute anticonvulsant effects of diazepam (Wilson, 1992) and previous studies in mice (Carey et al., 1992). Blood alcohol levels assessed at both time points (10 and 30 min) after ethanol administration were also similar in males and females. Although sex differences in body weights, or brain/body mass ratios, could have modified drug distribution and responses, the similarity of drug levels in blood or brain after behavioral testing suggest our results are not completely attributable to different body size. Others have similarly failed to see differences in blood or brain alcohol levels between the sexes (Ogilvie and Rivier, 1997; Crippens et al., 1999; Boehm et al., 2002), although some studies report sex differences in blood levels or elimination of ethanol

depending on the administration route (Webb et al., 2002; Robinson et al., 2002).

Corticosterone levels were assessed after the plus maze test in association with diazepam administration and the prod-burying test. Levels of corticosterone were higher in females than males, consistent with other studies showing greater responses of the hypothalamic–pituitary–adrenal axis (HPA) following stress in females compared with males (see Wilson and Biscardi, 1994; Rivier, 1999, for references). Importantly, females showed greater HPA responses than males following the prod-burying paradigm, supporting the notion that despite sex differences in shock sensitivity (Beatty, 1979), both sexes found the shock stimulus noxious. Despite this, the level of difference between males and females in HPA responses was not of the magnitude observed with other stressors, including the plus maze (compare top and lower panels in Fig. 6). Thus, although a robust shock stimulus was used in this test to diminish the possibility that sex differences in shock sensitivity might alter responses in the prod-burying task, this possibility cannot be entirely discounted as an explanation for the reduced burying behavior in females compared to males in this task. Other studies, however, suggest that nociceptive differences do not influence responses in this task (Treit, 1985).

Acute diazepam administration enhanced corticosterone release associated with the plus maze, but not the prod-burying task. This might have been due to the different dose ranges used, because only the highest diazepam dose (3 mg/kg) had this effect in the plus maze. Interestingly, longer term diazepam administration (3 days or 3 weeks) diminished corticosterone or adrenocorticotropin (ACTH) responses to both plus maze exposure (Stock et al., 2000) and swim stress (Wilson et al., 1996b). Others have seen that rapid tolerance develops to the corticosterone elevating effects of high benzodiazepine doses (Lahti and Barsuhn, 1975; Torrellas et al., 1980). Although the influences of ethanol were not tested after plus maze exposure, there was no main effect of ethanol administration on corticosterone responses in the prod-burying task. At the highest dose tested, however, it appeared that females were showing an increase in corticosterone levels compared to males (Fig. 6). Studies generally report increases in corticosterone release with ethanol administration at higher doses ((Rivier, 1993, 1999 Ogilvie and Rivier, 1997; Boehm et al., 2002); see Eckardt et al., 1998). Our results suggest a sex difference in ethanol-induced effects on corticosterone responses to stress and support prior work demonstrating that females show heightened corticosterone release in response to ethanol administration at low doses (<3 g/kg) compared with males (Ogilvie and Rivier, 1997; Rivier, 1999). More critically, these studies suggest a dissociation between the effects of these drugs on HPA responses, and anxiety-reducing activity. At effective anxiolytic doses, diazepam actually increased corticosterone responses associated with plus maze exposure, rather than decreasing such responses. Neither ethanol nor diazepam decreased HPA responses associated with these two paradigms, suggesting that reductions in HPA stress responses do not mediate the anxiety-reducing influences of these compounds.

The lack of sex differences in the acute effects of ethanol and diazepam in these two tests of anxiolytic action is perhaps not surprising, given that several studies have failed to find sex differences in $GABA_A$/benzodiazepine receptor sites or many of their responses (see Wilson, 1996, for discussion and references). Despite this lack of sex differences in acute anxiolytic responses, however, changes induced during chronic treatments with both benzodiazepines and ethanol show reliable sex-dependent differences. These studies suggest that even with similar behavioral outcomes, the neural mechanisms underlying processes related to tolerance and dependence with these drugs may differ in males and females. With chronic benzodiazepine exposure, males and females show distinct changes in $GABA_A$ receptor responses, levels of corticotropin-releasing factor (CRF), and neurosteroid levels (Wilson, 1996; Wilson et al., 1996b; Wilson and Frye, 1999; Sloan et al., 2000). Similarly, ethanol withdrawal induces sex-dependent changes in the $GABA_A$ receptor system and neurosteroid levels (Janis et al., 1998; Devaud et al., 1999). Sex differences are also seen with chronic exposure and withdrawal from progestins, where progesterone withdrawal up-regulates the alpha4 subunit of the $GABA_A$ receptor in the amygdala of females but not males (Gulinello et al., 2003). Moreover, because these studies examined anxiolytic effects in ‘‘normal’’ animals, it is possible that even the acute anxiolytic influences of these compounds may differ during states of heightened anxiety. This is supported by human studies showing altered benzodiazepine sensitivity in patients with premenstrual syndrome (Sundstrom et al., 1997b), and animal studies using various models of heightened anxiety (Gulinello et al., 2002, 2003).

In summary, although some task-specific sex differences are observed in anxiety-like behaviors in these two models, the anxiolytic effects of both diazepam and ethanol are similar in males and females in the plus maze and defensive prod-burying tasks. After acute administration of these two compounds, similar dose–response curves are observed, although the curves are shifted because of baseline sex differences in the behavioral endpoints. The only sex difference appeared to be slightly increased sensitivity to low doses of diazepam in the plus maze in males compared to females, although a similar difference was not observed in the prod-burying test. No sex differences in drug levels were observed. Interestingly, neither diazepam nor ethanol decreased corticosterone responses induced by these behavioral paradigms, suggesting a dissociation between the anxiolytic effects of these compounds and HPA responses. Although no sex differences were seen in the anxiolytic effects of these compounds in ‘‘normal’’ rats, it remains an intriguing possibility that these drugs might show sex

differences in effectiveness during heightened anxiety states.

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