Pharmacology, Biochemistry and Behavior 90 (2008) 691–700

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Influence of hormonal status on behavioral responses to an acute ethanol challenge during ethanol withdrawal in male and female rats

Bikul Koirala [a], Paul E. Alele [b], Leslie L. Devaud [a,*]

[a] Department of Biomedical and Pharmaceutical Sciences, Idaho State University College of Pharmacy, Pocatello, ID 83209-8334, United States
[b] Department of Pharmacology and Therapeutics, Mbarara University of Science and Technology, Mbarara, Uganda

ARTICLE INFO

Article history:
Received 3 September 2007
Received in revised form 14 May 2008
Accepted 22 May 2008

Keywords:
Ethanol withdrawal
Sex differences
Ovariectomy
Seizures
Incoordination
$GABA_A$ receptors

ABSTRACT

We previously reported significant sex differences in ethanol withdrawal (EW) recovery as well as in sensitivity to $GABA_A$ receptor modulators during EW. The aim of the present study was to determine if hormonal status moderated behavioral responses to an acute ethanol challenge in EW animals comparing two different behaviors. An initial set of experiments explored motor-incoordinating effects of the acute ethanol injection during EW at either 1 day or 3 days of withdrawal. EW male, but not female, rats showed a decrease in coordination compared to controls that persisted through 3 days EW. Female rats displayed tolerance to the motor-incoordinating actions of the acute ethanol challenge at 1 day EW whereas tolerance was more evident in EW male rats at 3 days. In contrast, EW animals generally remained responsive to the anticonvulsant actions of ethanol, irrespective of hormonal status. While EW by itself did not significantly alter seizure latency, duration or severity, it increased seizure-induced mortality especially at 3 days EW. There was some evidence of tolerance to the anticonvulsant effect of the acute ethanol challenge at the lowest dose employed (0.62 g/kg), which varied by sex condition and time of EW. All sex conditions displayed marked sensitivity to the anticonvulsant effects of the ethanol challenge at the two higher doses studied. Overall, ovariectomized females showed the greatest response to the acute ethanol administration. These findings provide additional evidence of a divergence in behavioral responses during EW and suggest that multiple neuroadaptations moderate various responses to ethanol during EW, with minor contributions of hormonal status.

1. Introduction

It has been well established that there are important differences between men and women in patterns of drinking, physiological and pathological responses to alcohol, as well as differences in manifestations of alcohol abuse, dependence and alcoholism (Alcohol and Health, 2000; The NSDUH Report, 2007; Schenker, 1997). Alcohol (ethanol) withdrawal is a significant consequence of alcohol dependence. It results from abrupt discontinuation of ethanol consumption by those that have become dependent on ethanol. Ethanol withdrawal (EW) symptoms reflect the rebound hyperexcitability resulting from removal of this central nervous system depressant and include anxiety, agitation, insomnia, general dysphoria and tremors. The initial manifestation of tremors may progress to seizures in a subset of alcoholics, which can become life threatening (Ballenger and Post, 1978; Goldstein and Pal, 1971). Pre-clinical studies with laboratory rats and mice have shown them to be useful models for studying ethanol dependence and withdrawal. For example, several research groups have shown that EW animals show a significant increase in seizure susceptibility and severity during early EW (Becker et al., 1997; Devaud et al., 1995a, 1999; Finn et al., 1995; Veatch et al., 2007).

For a number of years, our lab has been interested in studying how the inherent hormonal environment may modulate responses to ethanol. We have focused on this perspective because women have been reported to display fewer and less severe signs of EW compared to men, even when controlled for equivalent intake (Deshmukh et al., 2003). This data supported a number of anecdotal reports that recovering alcoholic women rarely present with seizures, in contrast to male alcoholics. Women also show structural differences in ethanol-induced brain damage (Pfefferbaum et al., 2001). This may be due to certain progesterone derivatives (which occur at higher levels in females than males) that possess anxiolytic and anticonvulsant properties (Baulieu, 1991; Carboni et al., 1996), similar to behavioral effects of ethanol (Brot et al., 1995). As females have higher levels of ovarian steroids and their neuroactive derivatives, inherent differences in the hormonal milieu may contribute to differences in neurophysiological responses to ethanol between males and females. In our studies, we found that male and female rats display a different time course for recovery from EW with seizure risk for female rats returning to basal levels quicker than seen for male rats (Devaud and Chadda, 2001, Devaud et al., 2003). A recent report also observed an

* Corresponding author.
E-mail address: ldevaud@otc.isu.edu (L.L. Devaud).

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doi:10.1016/j.pbb.2008.05.013

increased seizure severity in male, but not female mice, following chronic ethanol administration and withdrawal (Veatch et al., 2007), a finding that provides additional evidence for notable sex differences of EW. A focus on comparing and contrasting responses in both males and females is acquiring greater importance as an increasing number of studies suggest there are significant physiological differences between the sexes that broadly impacts health and disease (reviewed in Wizeman and Pardue, 2001).

The GABA (gamma-aminobutyric acid) neurotransmitter system is an important target for ethanol and neuroadaptations in this system caused by persistent ethanol exposure are likely to be involved in the development of dependence and manifestations of EW (Crews et al., 1996; Grant and Lovinger, 1995; Grobin et al., 1998). Furthermore, a number of brain adaptations of GABAergic systems engendered by chronic ethanol exposure have been found to vary between male and female rats as well as by brain region (Alele and Devaud, 2005; Cagetti et al., 2003; Charlton et al., 1997; Devaud et al., 1995b, 1998, 1999; Devaud and Alele, 2004; Mhatre and Ticku, 1994; Mehta and Ticku, 2005). A significant alteration linked to functional changes in receptor activity is the increased levels of the $GABA_A$ receptor α4 subunit protein that occurs with development of ethanol dependence and persists during expression of EW (Alele and Devaud, 2005; Cagetti et al., 2003; Devaud and Alele, 2004).

An important consequence of persistent ethanol exposure is the development of tolerance, i.e. the requirement for increasing levels of intake to achieve the same 'intoxicating' effects seen with initial consumption. Assessment of tolerance to behavioral actions of ethanol, such as ataxia (motor incoordination) and sedation/hypnosis (loss of righting), are common measures for determining physical dependence. Alterations in GABAergic neurotransmission have also been implicated as playing a role in the development of tolerance to ethanol. Early studies addressing this question showed that tolerance to the ataxic effects of ethanol developed rapidly (Gallaher et al., 1982) and that administration of the benzodiazepine antagonist, flumazenil, reversed tolerance in dependent mice (Buck et al., 1991). In concert with these findings, we observed that EW animals displayed cross-tolerance to the anticonvulsant actions of the benzodiazepine, diazepam, a $GABA_A$ receptor positive modulator (Devaud et al., 1996). In contrast, the GABAergic neuroactive steroid and progesterone derivative, allopregnanolone, provided an enhanced anticonvulsant action during EW compared to its actions in ethanol-naïve animals (Devaud et al., 1995a; Finn et al. 1995).

This divergence in $GABA_A$ receptor-mediated responses during EW led investigators to question how this same receptor type can be altered by the development of ethanol dependence such that it displays reduced responsiveness to one type of modulator but sensitization to another. Moreover, responses to the same modulator have been shown to vary by behavioral measure and genetic profile. For example, EW rodents displayed tolerance to the hypnotic effects of the anesthetic neuroactive steroid, alphaxalone, but sensitization to its anticonvulsant actions (Cagetti et al. 2003; 2004). Additionally, studies with various mouse lines found that sensitivity to the anticonvulsant effect of the GABAergic neuroactive steroid, allopregnanolone was increased in EW C57Bl/6 but not DBA/2 mice, with tolerance to the muscle relaxant effects of this neurosteroid observed in both lines (Finn et al., 2000). A more recent study found a divergence in actions of allopregnanolone between Withdrawal Seizure-Prone (WSP) versus Withdrawal Seizure-Resistant (WSR) mice during ethanol withdrawal in that WSP mice were cross-tolerant whereas WSR mice remained sensitive to its anticonvulsant actions (Finn et al., 2006). These reports indicate that the development of ethanol dependence and expression of withdrawal elicit complex neuroadaptations that can result in a separation between effects of modulators at $GABA_A$ receptors.

While it is clear that the development of tolerance to the intoxicating effects of ethanol plays a role in ethanol dependence, studies have not yet determined how long tolerance to ethanol persists during withdrawal, nor how it is expressed across various behavioral measures of EW. Therefore, the present set of experiments was designed to assay responses to an acute ethanol injection in male and female rats using two behavioral measures at two time points of withdrawal.

2. Methods

2.1. Animals

Male and female SD rats (Simonsen Labs, Gilroy CA) approximately 50 days old at the start of experiments were made ethanol-dependent by administration of 6% ethanol, v/v in a nutritionally complete liquid diet (Invitrogen-MP Biomedical, Costa Mesa, CA) for 14–16 days as previously described (Alele and Devaud, 2005, 2007). Prior to liquid diet administration, ovariectomies (OVX) were performed on half of the female rats used in the pentylenetetrazol Influence of hormonal status on behavioral responses to an acute ethanol challenge during ethanol withdrawal in male and female rats(PTZ) bolus experiments. Ovariectomies were performed under ketamine/xylazine anesthesia and the animals allowed at least 4 days to recover from surgery before initiation of liquid diet administration. Control animals were pair-fed the same liquid diet but with dextrose substituted isocalorically for the ethanol to ensure equivalent caloric intake and comparable nutritional status. The amount of liquid diet consumed was recorded daily. All animals gained weight during the course of the study, with male rats weighing from 240–310 g, females weighing 190–235 g and OVX weighing 200–265 g at the conclusion of this set of experiments.

The estrous cycle of intact females was monitored by daily collection of vaginal smears and histological examination of epithelial cell types; near confluence of female cycles was achieved by the end of the experiment. There was some evidence of disruption of estrous cyclicity, with estrus often persisting for more than 1 day in most intact female rats although they continued to cycle every 4–5 days. All animals were handled briefly each day for habituation to behavioral testing and to keep handling consistent across sex conditions.

Animals consumed the expected average of 10–13 g/kg ethanol per day during the final 7 days of ethanol diet administration, with males drinking less than both groups of females. Ethanol consumption for this set of experiments was 10.8±0.6 g/kg for males, 12.1±0.4 g/kg for intact females and 12.7±0.5 g/kg for OVX females. Studies by our group and others have verified that this level of consumption reliably produces ethanol dependence. Previous investigations using this liquid diet methodology determined that amount consumed per day was a more reliable indicator of ethanol dependence than assay of blood ethanol levels (BEC) as animals drink in bouts with this paradigm, resulting in highly variable BEC among animals at any point in time (Devaud et al., 1995a,b). As all tests were conducted at 1 day or 3 days EW, there would not be any measurable BEC at this time as it is undetectable by 6 hr of EW (Finn and Crabbe, 1999). Both groups of female rats drank more ethanol per kg body weight than males, consistent with previous reports (Lancaster and Spiegel, 1992) and previous studies in our lab.

After the 2 weeks of liquid diet administration, the liquid diet was removed and regular lab chow provided ad libitum to all animals to maintain equivalent diet conditions. Testing was scheduled at 1 day or 3 days EW and for when intact female rats were in late estrus or diestrus 1 (when progesterone and estradiol levels are low). All procedures were conducted in accordance with approved Idaho State University Animal Welfare Protocols and NIH guidelines for the humane care and use of animals and in an AAALAC-accredited facility.

2.2. Materials

Pentylenetetrazol (PTZ) from Sigma-Aldrich (St. Louis, MO) was dissolved in normal saline. Ethanol was diluted to 20% v/v in

normal saline for acute administration. All drugs were administered intraperitoneally (IP). The doses of ethanol employed in this study were 0.62, 1.25 or 2.5 g/kg.

2.3. Accelerating rotorod

Rats were trained on the accelerating rotorod 4 days before the experiment (while all animals were still consuming the liquid diet) with the speed set at a constant 5 rpm. The criterion for training was that each animal needed to remain on the rod for 30 sec. Animals were briefly removed from their home cage for training. All animals easily acquired the ability to remain on the rotorod for 30 sec with only 1–2 trials. On the day of the experiment, each animal was given an IP injection of ethanol (1.2 or 2.5 g/kg at 20% in saline v/v) or the saline vehicle 15 min prior to being placed on the rotorod apparatus. Animals were run in groups of 4, with four different treatment conditions represented during each run. The rod was turned on with initial speed at 5 rpm; acceleration commenced after 20 sec. Data was collected as time remaining on the rotorod during the acceleration phase. Each animal was tested once only, with separate groups used for the 1 day EW and the 3 days EW time points in all experiments.

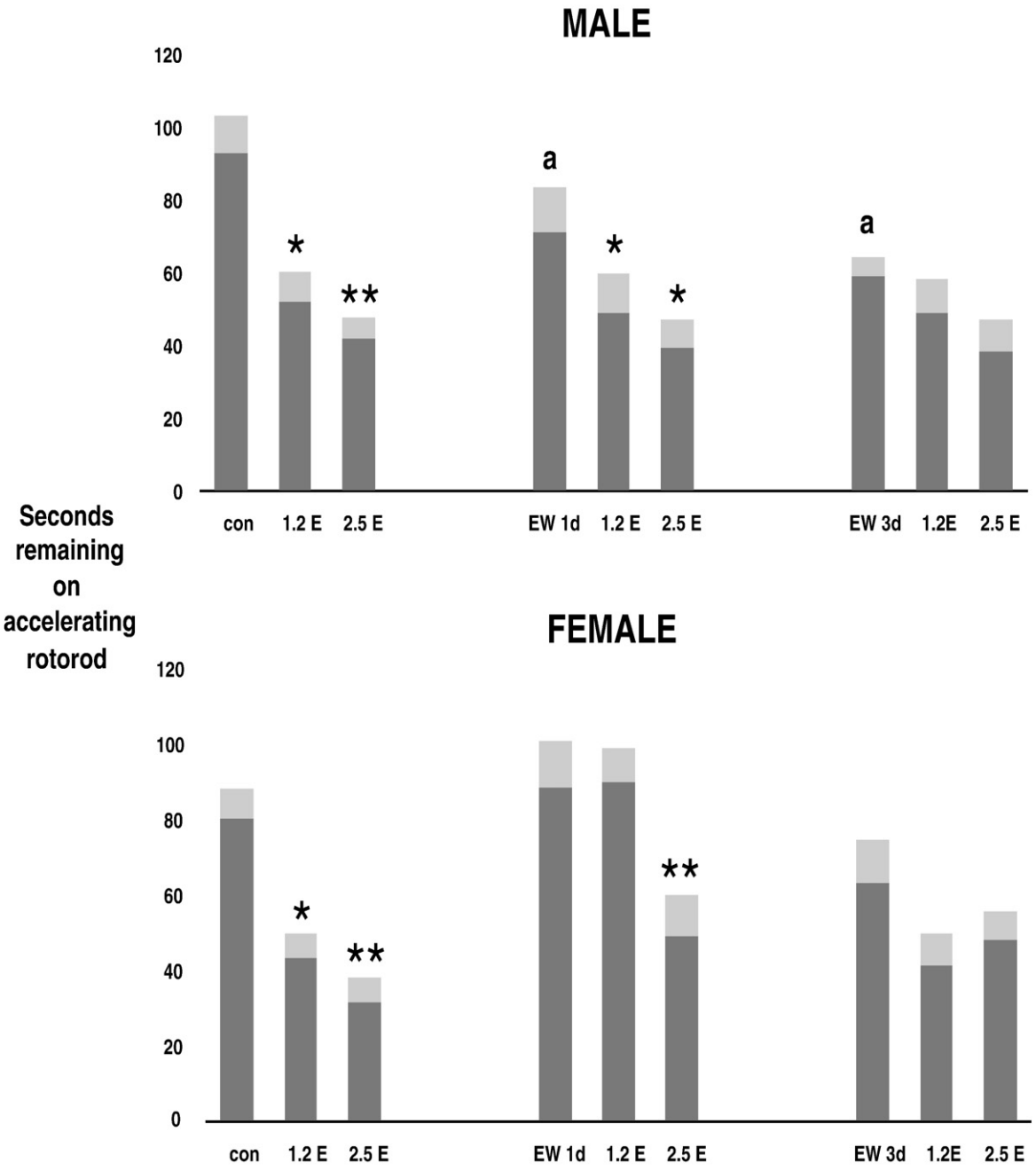

Fig. 1. Effects of acute ethanol (20% v/v) administered at 1 or 3 day EW on time spent on an accelerating rotorod in male and female rats. Con = control (pair-fed control diet) and EW = ethanol withdrawn at either 1 day or 3 days of withdrawal. 1.2 E = the 1.25 g/kg ethanol dose, 2.5 E = the 2.5 g/kg ethanol dose. The con, EW 1 day and EW 3 days conditions received vehicle (0 ethanol) injections. $^{*}P<0.05$ and $^{**}P<0.01$ compared to respective vehicle-treated conditions. $^{a}P<0.05$ compared to vehicle-treated ethanol naïve control values. Values are expressed as the mean (darker segment of each bar) ± S.E.M (lighter segment of each bar). $N = 16–19$ animals for control (vehicle-treated) conditions and 6–7 animals for each remaining treatment condition.

2.4. Seizure induction by bolus injection of PTZ

Animals were injected IP with the 20% v/v ethanol solution (0.62, 1.2 or 2.5 mg/kg) or saline vehicle 15 min prior to the bolus injection of PTZ (36 mg/kg), administered on the opposite side from the acute ethanol injection. Seizure latency, the duration of seizure activity and the maximal seizure severity caused by the convulsant were continuously monitored with a cut-off of 5 min (300 sec) for latency and duration because animals that displayed seizures presented with activity well before 5 min post-PTZ injection. Maximal seizure severity was manifested before this cut-off time, with a bimodal distribution for duration: animals presenting with mild seizures or the most severe seizures typically displayed a shorter duration of seizure activity than those presenting with moderate seizure severity. Seizure severity was graded using the scoring system modified from Marsh et al. (1999): Grade 0: no signs of motor seizure activity during the observation period; Grade 1: staring, mouth or facial movements; Grade 2: head nodding or isolated twitches; Grade 3: unilateral/bilateral forelimb clonus; Grade 4: rearing; Grade 5: loss of posture, jumping; Grade 6: clonic/tonic seizures; Grade 7: full tonic seizures; Grade 8: death.

2.5. Data analysis

Each ethanol-treated animal and its paired control were randomly assigned to either the 1 day or 3 days EW time. All studies were the summation of at least two independent experiments, to increase sample size and verify reproducibility of results. Control animals were run with each sex and treatment condition but did not vary between

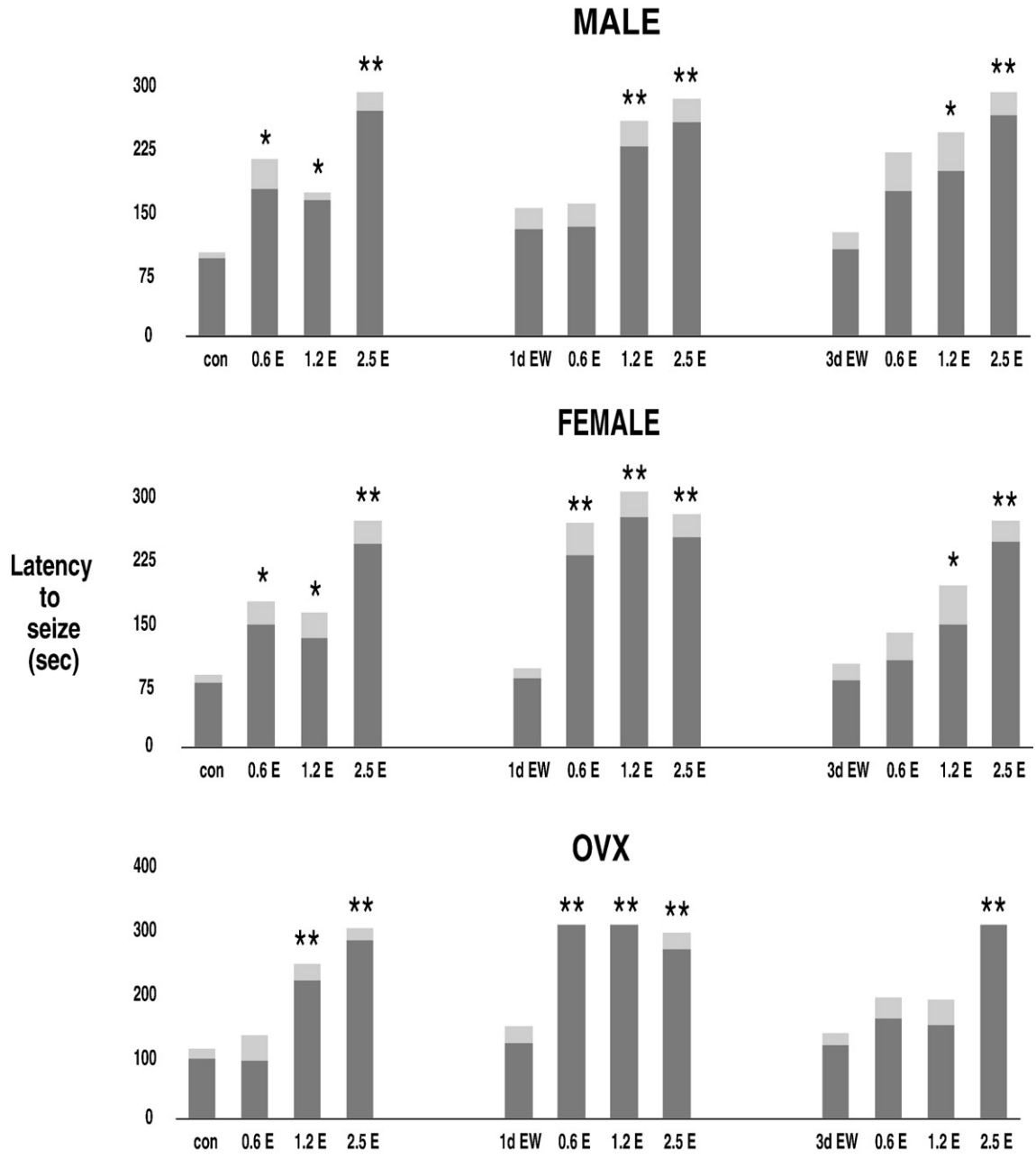

Fig. 2. Effects of acute ethanol (20% v/v) administered at 1 or 3 day EW on seizure latency in male, female and OVX female rats. Con = control (pair-fed control diet) and EW = ethanol withdrawn at either 1 day or 3 days of withdrawal. 0.6 E = the 0.62 g/kg ethanol dose, 1.2 E = the 1.25 g/kg ethanol dose, 2.5 E = the 2.5 g/kg ethanol dose. The con, 1 day EW and 3 days EW conditions received vehicle (0 ethanol) injections. *$P<0.05$ and **$P<0.01$ compared with respective vehicle-treated conditions. Values are expressed as the mean (darker segment of each bar) ± S.E.M (lighter segment of each bar). N = 18–20 animals for control (vehicle-treated) conditions and 7–9 animals for each remaining treatment condition.

groups; therefore control data for each sex condition were pooled for analysis. Data (1 day or 3 days EW) were analyzed by three-way ANOVA (SPSS, Inc., Chicago, IL) comparing diet (6% ethanol v/v or control) by acute ethanol treatment (0, 0.62, 1.25 or 2.5 g/kg) by sex condition (male, female, OVX) for each behavior assessed in the rotorod and PTZ bolus experiments. Each time point of withdrawal was analyzed separately as we were most interested in making the sex×diet×acute ethanol treatment comparisons. Tukey's test was

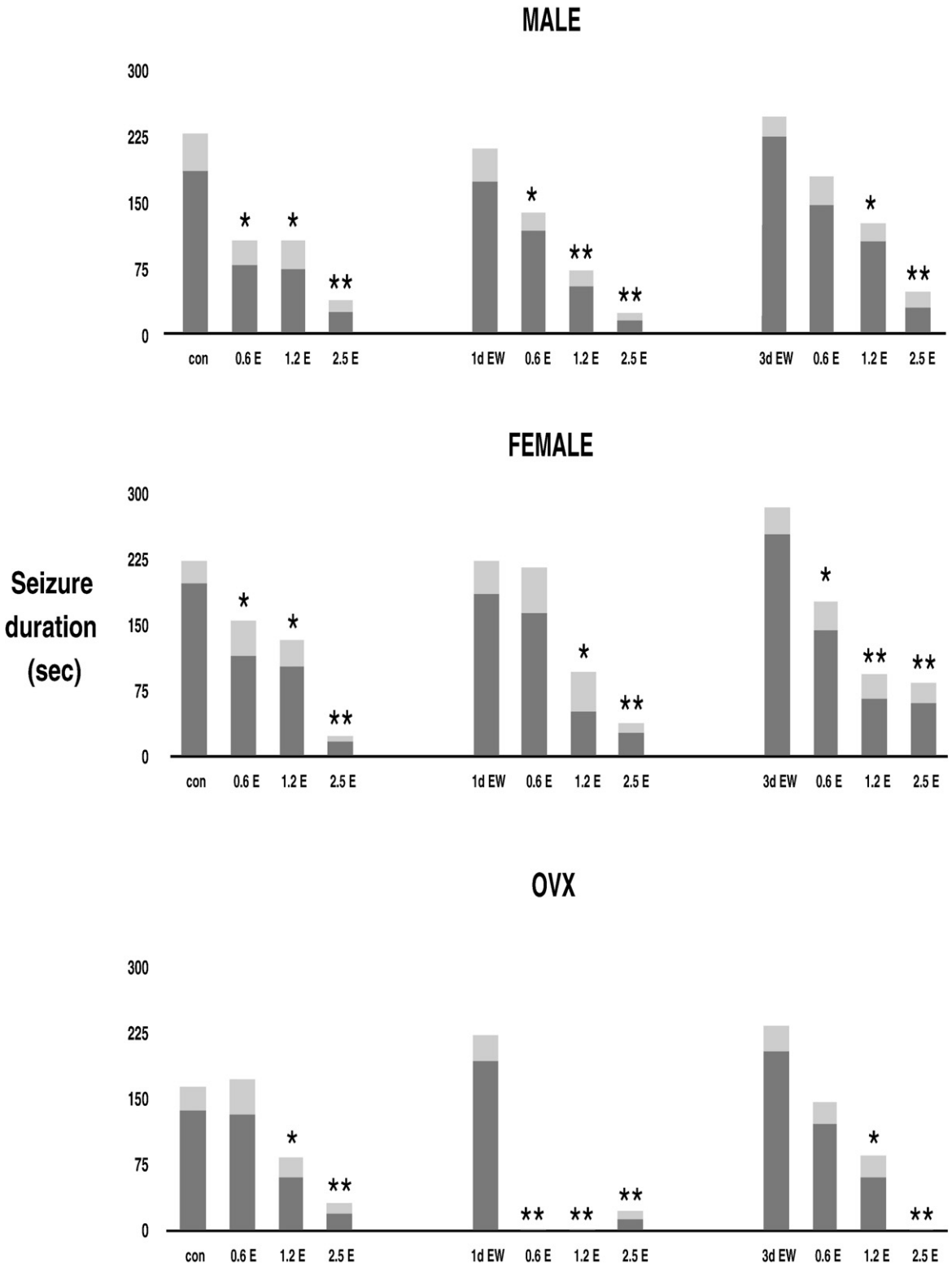

Fig. 3. Effects of acute ethanol (20% v/v) administered at 1 or 3 day EW on seizure duration in male, female and OVX female rats. Con = control (pair-fed control diet) and EW = ethanol withdrawn at either 1 day or 3 days of withdrawal. 0.6 E = the 0.62 g/kg ethanol dose, 1.2 E = the 1.25 g/kg ethanol dose, 2.5 E = the 2.5 g/kg ethanol dose. The con, 1 day EW and 3 days EW conditions received vehicle (0 ethanol) injections. *$P<0.05$ and **$P<0.01$ compared with respective vehicle-treated conditions. Values are expressed as the mean (darker segment of each bar) ± S.E.M (lighter segment of each bar). N = 18–20 animals for control (vehicle-treated) conditions and 7–9 animals for each remaining treatment condition.

used for post hoc analysis. Statistical analysis of amount of ethanol containing diet consumed per day for days 8–14 per sex condition was analyzed by one-way ANOVA, sex × consumption (Prism4, GraphPad, San Diego, CA).

3. Results

The initial set of studies was conducted to determine if we would observe sex differences in ataxia during EW and whether both male and female animals would display the expected tolerance to an acute ethanol challenge at this time. We assessed motor coordination and the effects of the acute ethanol challenge at 1 d or 3 days EW comparing acute ethanol at 0, 1.25 or 2.5 g/kg (Fig. 1). At 1 day EW, there was a significant effect of diet ($F_{1,105}=6.6$, $P<0.01$), and treatment ($F_{2,105}=32.2$, $P<0.001$) on time spent on the rotorod. Control animals were sensitive to the motor-incoordinating effects of the acute ethanol challenge at both doses tested. In addition, there was a sex × diet ($F_{2,105}=8.0$, $P<0.01$) and sex × treatment ($F_{2,105}=4.4$, $P<0.02$) interaction at 1 day EW with females displaying a greater degree of tolerance than males, especially at the 1.25 g/kg dose. The significant

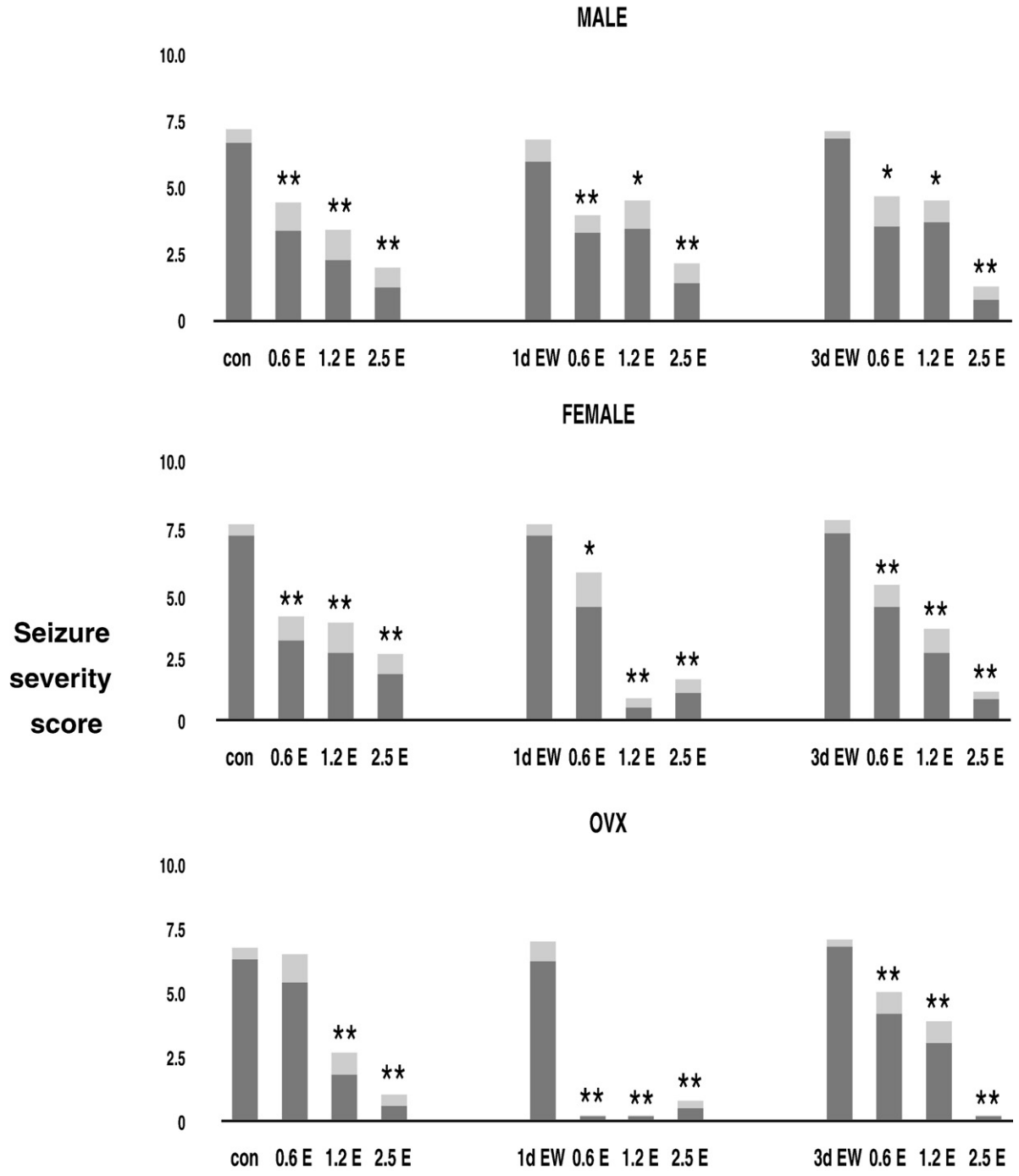

Fig. 4. Effects of acute ethanol (20% v/v) administered at 1 or 3 days EW on seizure severity in male, female and OVX female rats. Con = control (pair-fed control diet) and EW = ethanol withdrawn at either 1 day or 3 days of withdrawal. 0.6 E = the 0.62 g/kg ethanol dose, 1.2 E = the 1.25 g/kg ethanol dose, 2.5 E = the 2.5 g/kg ethanol dose. The con, 1 day EW and 3 days EW conditions received vehicle (0 ethanol) injections. *$P<0.05$ and **$P<0.01$ compared with respective vehicle-treated conditions. Values are expressed as the mean (darker segment of each bar) ± S.E.M (lighter segment of each bar). N = 18–20 animals for control (vehicle-treated) conditions and 7–9 animals for each remaining treatment condition. See Methods section for detailing of scoring measures.

main effect of acute ethanol treatment ($F_{2,118}$=13.0, P<0.001) and diet×treatment interaction ($F_{2,118}$=3.7, P<0.03) persisted through 3 days EW. However, the acute ethanol challenge did not further reduce motor incoordination in either male or female rats at this time. EW alone elicited a significant (P<0.05) decrease in coordination in male, but not female, rats during both time points of EW compared to control measures. EW males displayed a 25% decrease in time spent on the accelerating rotorod at 1 day EW and a 38% decrease at 3 days EW compared to controls. In contrast, EW females were not impaired in the task.

We next conducted a set of experiments to determine if the observed responses to the incoordinating effects of an acute ethanol challenge during EW would generalize to a second behavioral measure, that of seizure induction by the GABAergic chemoconvulsant, pentylenetetrazol (PTZ). Ovariectomized female (OVX) rats were included in these experiments to see if we could distinguish activational versus organizational influences of ovarian steroids on any observed sex differences in responses. We employed a single, bolus injection of PTZ to quantify the increased seizure risk associated with EW across several seizure parameters. In general, the acute ethanol challenge caused a pronounced decrease in PTZ-induced seizure activity for both control and EW treatment conditions with limited expression of tolerance to its anticonvulsant actions during EW.

There was a significant main effect of ethanol treatment on seizure latency in 1 day EW animals ($F_{3,222}$=49.6, P<0.001) as shown in Fig. 2. At 1 day EW, administration of either 1.25 or 2.5 g/kg ethanol significantly increased seizure latency (P<0.001) in EW male, female, and OVX rats. At 3 days EW, the overall main effect of the acute ethanol challenge against PTZ-induced seizures remained significant ($F_{3,225}$=48.6, P<0.001); however responses to ethanol appeared somewhat attenuated compared to the 1 day EW time point. The lowest dose of ethanol used (0.62 g/kg) still significantly increased seizure latency (P<0.04). Administration of 1.25 or 2.5 g/kg ethanol still significantly increased seizure latency across all sex conditions (P<0.03–0.01).

As shown in Fig. 3, at 1 day EW there was a significant main effect of ethanol treatment ($F_{3,222}$=45.0, P<0.001) on seizure duration with the acute ethanol challenge providing a dose-dependent reduction in seizure time. Injection of 0.62 g/kg ethanol caused a 35% decrease in seizure duration in EW males (P<0.05) and a 100% decrease in EW OVX females (P<0.001), with a smaller effect in EW females (P<0.01). Both the mid-range dose of 1.25 g/kg ethanol (P<0.001) and the 2.5 g/kg dose (P<0.001) caused an even greater reduction in seizure duration across all three sex conditions during this early time point of EW. The significant main effect of EW and acute ethanol challenge persisted to 3 days EW ($F_{3,225}$=42.2, P<0.01). At this time, all EW animals remained sensitive to the anticonvulsant effects of the acute ethanol challenge on seizure duration by displaying significant decreases in duration that varied from 35–50% across the three sex conditions.

For seizure severity measurements (Fig. 4), a significant main effect of the acute ethanol challenge was observed across all treatment conditions for the 1 day EW analysis ($F_{3,222}$=92.6, P<0.001). In addition a significant sex×treatment interaction was observed ($F_{4,222}$=29.2, P<0.01). At 3 days EW, a significant main effect of ethanol treatment was again observed across all conditions, although it was somewhat attenuated compared to the 1 day EW response ($F_{3,225}$=152.2, P<0.001). Moreover, there was a seizure severity×sex interaction ($F_{2,219}$=6.3, P<0.02) with OVX showing the greatest sensitivity to the acute ethanol challenge at 1 day EW.

In this set of studies, PTZ-induced seizure mortality ranged from 32–43% for males, 47–62% for females, and 31–45% for OVX across control and EW conditions (Table 1). There was an overall main effect at 1 day EW ($F_{3,245}$=23.7, P<0.001) and 3 days EW ($F_{3,248}$=29.9, P<0.0001) only for acute ethanol treatments, with no sex or diet interactions. Each dose of acute ethanol significantly reduced mortality for all sex and diet conditions compared to vehicle-only treated controls at both times of EW (P<0.001), with no significant diet×treatment or sex×diet interactions. Interestingly, we also observed some basal differences in PTZ-seizure induction that appeared to be modulated by the hormonal environment. Pair-fed control female animals were generally more sensitive to seizure induction by PTZ than were either males or OVX animals, displaying a nearly 40% increase in percent mortality (at 47%) compared to control males or OVX (34% and 31%, respectively).

Table 1
Mortality from PTZ bolus administration

	Male	Female	OVX
Control	11/32 (34%)	18/38 (47%)	11/36 (31%)
+0.62 g/kg EtOH	1/12 (8%)**	0/12 (0%)**	2/12 (17%)**
+1.25 g/kg EtOH	0/7 (0%)**	0/6 (0%)**	0/5 (0%)**
+2.5 g/kg EtOH	0/19 (0%)**	0/20 (0%)**	0/18 (0%)**
1 day EW	6/19 (32%)	12/20 (60%)	7/18 (39%)
+0.62 g/kg EtOH	0/7 (0%)**	1/6 (17%)**	0/6 (0%)**
+1.25 g/kg EtOH	0/6 (0%)**	0/5 (0%)**	0/6 (0%)**
+2.5 g/kg EtOH	0/10 (0%)**	0/9 (0%)**	0/9 (0%)**
3 days EW	10/23 (43%)	13/21 (62%)	9/20 (45%)
+0.62 g/kg EtOH	0/7 (0%)**	0/7 (0%)**	0/6 (0%)**
+1.25 g/kg EtOH	0/6 (0%)**	0/6 (0%)**	0/7 (0%)**
+2.5 g/kg EtOH	0/10 (0%)**	0/11 (0%)**	0/9 (0%)**

Data is presented as number of animals that died/ number of animals tested, with % mortality in parentheses. Ethanol (EtOH), at 20% v/v in normal saline, was injected 15 min prior to the PTZ bolus injection. Analysis found a significant reduction in mortality following the acute ethanol treatment across all doses for each sex and diet condition. **P<0.001 compared to vehicle-treated controls.

One confound for this experiment was selection of the optimal dose of PTZ to reliably cause moderate convulsions in all animals. Identifying the optimal dose was a challenge as sensitivity to the PTZ seizure induction was expected to vary among the treatment groups, especially for control versus EW. Other investigators have used 40–70 mg/kg PTZ, depending on the type of experiment they were conducting and the measures of interest. We ran parametric studies using several doses of PTZ when we initiated our PTZ bolus studies, with 40 mg/kg reliably producing seizures but not a high level of mortality across the various treatment conditions (see Alele and Devaud, 2007). As we prepared to conduct the current set of experiments, we noted an apparent increase in sensitivity to PTZ-induced mortality, shifting upwards from approximately 20% to 30%; therefore we reduced the dose of PTZ to 36 mg/kg. However, we still observed a greater degree of mortality than in the earlier set of studies, which suggests an overall shift in sensitivity of the rat strain. The end result for these experiments was that we had a higher level of mortality than desired, but also noted a greater level of sensitivity than expected to the anticonvulsant effect of the acute ethanol challenge.

The differences in sensitivity to the acute ethanol challenge across sex conditions was of particular interest in light of the differences in ethanol consumption, with females and OVX drinking larger quantities of ethanol (g/kg) than males for this study as well as a series of previous studies (Fig. 5). Across the set of studies conducted during the past 2 years (including the experiments presented herein), males drank an average of 11.0±0.2 g/kg ethanol during the final week of each study whereas females drank 12.8±0.2 g/kg and OVX drank 13.3±0.2 g/kg during this same time. This was a significantly greater ($F_{2,39}$=20.3, P<0.01) ethanol consumption for both groups of females compared to males, with no differences in levels of drinking between the two female groups. That females consume more ethanol g/kg body weight concurs with our earlier studies and reports from other investigators who have compared ethanol consumption between male and female rats (Almeida et al., 1998; Devaud et al., 1999, 2000; Lancaster and Spiegel, 1992). However, this is the first report on

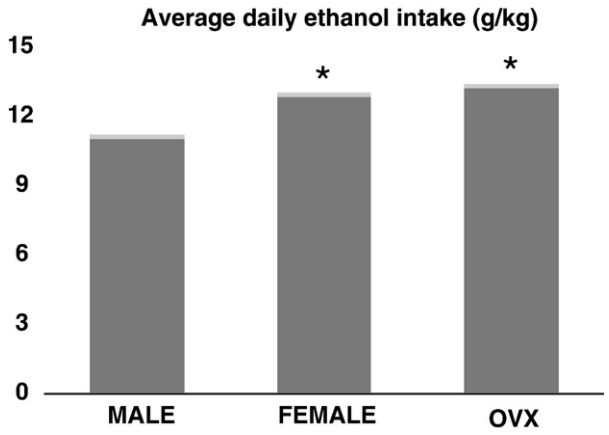

Fig. 5. Average g/kg ethanol consumed per day by males, females or OVX rats for the final 7 days of ethanol diet administration from 15 independent studies conducted in the past 2 years, including the studies being presented in this paper. Values are expressed as the mean (darker segment of each bar)±S.E.M (lighter segment of each bar). Both sets of females drank significantly ($P<0.01$) more ethanol g/kg than males.

comparisons of intake in ovariectomized female rats, and it was interesting to note that OVX females tend to drink quantities more similar to intact females than males even though their body weight tended to fall between males and females.

4. Discussion

Investigation of behavioral responses to an acute ethanol challenge during ethanol withdrawal was the major focus of this study. Additionally, we wanted to assess influences of hormonal status on these responses. These studies were designed to expand and extend earlier findings focusing on the role of GABAergic transmission in ethanol dependence and withdrawal, and how ovarian steroids may modulate responses at $GABA_A$ receptors. The key finding to this study was that EW animals generally displayed tolerance to the motor-incoordinating actions of the acute ethanol challenge whereas they remained quite sensitive to its anticonvulsant effects at this same time. This is consistent with studies that have shown a rapid development of tolerance to the ataxic effects of ethanol (Bell et al., 2000; Radcliffe et al., 2006). It also supports additional reports showing a divergence in sensitivity to $GABA_A$ receptor modulators depending on the response measured (Cagetti et al., 2003, 2004). In the present set of experiments, tolerance was more marked in females than in males compared to same sex controls, leading us to question whether mechanisms underlying coordination include some manner of modulation by sex steroids. This idea is supported by the recent evidence that repeated administration of either of two steroids (progesterone or dehydroepiandrosterone) differentially altered EW behaviors, such as ataxia, in male rats (Sharma et al., 2007). Interestingly, we observed a sex difference in coordination between EW male and female rats, with EW males, but not EW females, showing impairment. Underlying control of motor coordination involves several brain regions that share partial overlap with areas involved in seizure expression, such as the thalamus, motor cortex and cerebellum. While it is possible that chronic ethanol-induced adaptations in $GABA_A$ receptors play a role in this sex difference in motor coordination during EW between males and females, it is more likely that multiple neurotransmitter systems involved in fine motor control are involved in this response and are likely modulated by the hormonal condition. Additionally, there appeared to be a small sex difference in basal coordination between males and females, with ethanol naïve males remaining on the rotorod approximately 12% longer than ethanol naïve females. As we control for age rather than weight in our studies, male rats generally weigh about 30–50 g more than females at the time of testing. It is possible that the difference in size influences basal responses on the accelerating rotorod, and underscores why we make our comparisons within each sex condition.

In the second set of experiments, we assayed the effects of an acute ethanol challenge on seizure risk using the $GABA_A$ receptor channel blocker, pentylenetetrazol (Huang et al., 2001). We saw a consistent and high degree of sensitivity, rather than tolerance, to the anticonvulsant actions of ethanol through the first 3 days of EW. The sensitivity to acute ethanol exposure was most pronounced when assessing seizure severity and PTZ-induced mortality. This sensitivity was in marked contrast to the clear cross tolerance to the anticonvulsant action of a $GABA_A$ receptor positive modulator, the benzodiazepine, diazepam, noted in an earlier study (Devaud et al., 1996).

Seizure activity was induced by a bolus injection of the convulsant, allowing us assess several seizure parameters, encompassing a series of events that involves multiple brain areas, including cortical, limbic and thalamic regions, as recently detailed by in vivo imaging studies of seizure activity following a PTZ infusion (Brevard et al., 2006). These data showed that the more severe the seizure, the more extensive the involvement of seizure circuitry. Evidence supports the intrinsic involvement of GABAergic transmission in seizure activity (see Gale, 1992; MacDonald and Barker, 1978). The present set of experiments found some distinction between various components of seizure pathways during EW. For example, there was no significant effect of EW on latency. In contrast, duration of seizures and % mortality tended to increase with EW compared to ethanol naïve controls, and was overlaid by some sex differences in extent of response. These data were similar to our previous studies where we noted the most prominent anticonvulsant effects of the GABAergic neuroactive steroid, pregnanolone on PTZ-induced seizure duration and severity (Alele and Devaud, 2007). Taken together, these findings suggest new avenues for development of non-benzodiazepine pharmacotherapies that will be effective in reducing the risk for harm from prolonged seizures that can be a serious consequence of alcohol withdrawal.

Chronic ethanol-induced, regionally-selective alterations in $GABA_A$ receptor subunit assembly is one likely mechanism underlying the increased seizure risk as well as sensitivity to the anticonvulsant effects of an acute ethanol challenge during EW. Earlier studies showed region-specific alterations in gene expression for various subunits of $GABA_A$ receptors elicited by the development of ethanol dependence (Devaud et al., 1995b; Mahmoudi et al., 1997; Matthews et al., 1998), with a number of these alterations varying between male and female rats (Devaud et al., 1998, 1999; Devaud and Alele, 2004). Of particular interest was the consistent increase in $GABA_A$ receptors α4 subunit levels observed in both male and female ethanol dependent animals (Devaud and Alele, 2004; Devaud et al., 1998). One study uncovered a clear relationship between elevations in this subunit and the increased seizure risk of EW (Devaud and Alele, 2004). Furthermore, this increase was prevented by treatment with the benzodiazepine receptor antagonist, flumazenil, and correlated with reduction in EW $GABA_A$ receptor functional changes (Biggio et al., 2007).

Trafficking of receptors between locations (i.e. synaptic versus extrasynaptic) appears to be an important regulatory mechanism that is driven, in part, by receptor subunit composition (Kullmann et al., 2005; Luscher and Keller, 2004; Thomas et al., 2005). $GABA_A$ receptor isoforms containing the α4 subunit appear to be expressed predominantly at extrasynaptic sites and are involved in GABA-mediated tonic inhibition. Relevant to these studies, investigations have shown that α4 and delta subunit-containing $GABA_A$ receptors are quite sensitive to low doses of ethanol (Olsen et al., 2007; Smith et al., 2007; Wallner et al., 2006) and may be stimulated to translocate between extrasynaptic and synaptic sites following various stimuli, including exposure to ethanol (Liang et al., 2006). To better determine the precise role of α4 subunit-containing $GABA_A$ receptors, an α4 knockout mouse model was developed and tested for behavioral and neuronal responses to an acute ethanol application. Deletion of

this subunit resulted in alterations in $GABA_A$ receptor synaptic currents and compensatory changes in levels of several other receptor subunits, such as the γ2 (Liang et al., 2008). Interestingly, behavioral assessment of responses to ethanol found normal acute responses across a number of measures, including anxiety, temperature regulation and hypnosis. However, these knockout animals displayed a significantly enhanced sensitivity to PTZ-induced seizures (Chandra et al., 2008). Therefore, the elevated seizure risk observed during EW could be caused, at least in part, by the increased expression of α4 subunit-containing $GABA_A$ receptors and is in concert with evidence for a link between prevalence of α4 subunit-containing $GABA_A$ receptors and seizure risk (Lagrange et al., 2007; Raol et al., 2006; Sierra-Paredes and Sierra-Marcuno, 2007). These findings, however, do not rule out the likelihood that chronic ethanol-induced adaptations in additional neurotransmitter systems also play a role in EW seizure susceptibility. For example, a recent report by Le Strat and coworkers found evidence for an association between an enhanced withdrawal seizure phenotype and expression of a particular variant of the dopamine transporter in alcoholic patients (Le Strat et al., 2008).

The influence of hormonal status on alterations in seizure risk as well as sensitivity to drugs during EW is not surprising, as ovarian steroids and/or their neuroactive derivatives (which are positive $GABA_A$ receptor modulators) have been shown to be generally protective against seizure induction (Finn and Gee, 1994; Foldvary-Schaefer et al., 2004; Maguire et al., 2005; Standley et al., 1995; Wilson, 1992). However, there are intriguing findings on effects of several GABAergic neuractive steroids on EW symptoms that vary by experimental paradigm and the neuroactive steroids studied. In general, most laboratories have observed increased anticonvulsant effects of the progesterone-derivatives, allopregnanolone and pregnanolone on EW seizure risk (Alele and Devaud, 2007; Becker et al., 1998; Devaud et al., 1995a, 1996; Finn et al., 2000). However, this response did not generalize to all EW behaviors or all models studied. For example, WSP and WSR mice displayed divergent responses to the anticonvulsant actions of allopregnanolone during EW with WSR mice maintaining sensitivity whereas WSP mice showed attenuation of this steroid's anticonvulsant effects (Finn et al., 2006). A follow-up study noted that female WSP mice showing even greater cross tolerance than males (Beckley et al., 2008). Cross tolerance to the hypnotic effects of the anesthetic neurosteroid, alphaxalone was noted during EW (Cagetti et al., 2003). In contrast, increased sensitivity to its anticonvulsant and anxiolytic effects was observed using their chronic, intermittent ethanol exposure paradigm (Cagetti et al., 2004). A recent study by Knapp and coworkers that used acute administration of alphaxalone during an early ethanol withdrawal period found enhanced anxiety-like behaviors during subsequent withdrawals (Knapp et al., 2005). Finally, WSP mice displayed withdrawal signs (increased handling-induced convulsions) following administration of allopregnanolone, similar to responses seen after ethanol exposure (Reilly et al., 2000), supporting overlap in actions of ethanol and neurosteroids at $GABA_A$ receptors. Therefore, while various neuroactive steroids have the potential to be beneficial in reducing some EW symptoms, these findings show that responses may vary by symptom as well as by patterns or extent of ethanol consumption. These data also support the clinical evidence that hormonal status influences seizure risk during alcohol withdrawal, with both organizational as well as activational effects of ovarian steroids likely affording some protection for women (Deshmukh et al., 2003).

We did note that both sets of females drank more ethanol g/kg than males, achieving higher blood and brain levels as it has previously been shown via microdialysis that brain levels of ethanol correlate with amount ingested for both male and female rats (Crippens et al., 1999). This would lead us to expect to see more severe withdrawal signs in the females compared to males, but this was not observed. This finding is consistent with an earlier study showing decreased sensitivity of EW female rats compared to EW males to bicuculline-induced seizures when adjusting for amount of ethanol consumed (Devaud et al. 1995a). It is also similar to when responses were compared between intact and OVX females (Devaud et al., 2000).

Additional studies have highlighted interactions between ethanol and sex condition on EW responses. Sex differences in EW seizure severity in two mouse lines were observed when animals were pretreated with finasteride, an inhibitor of 5-alpha reductase, resulting in reduced formation of the neuroactive steroids allopregnanolone and pregnanolone from progesterone (Gorin-Meyer et al., 2007). Pair-fed control females did display an increased sensitivity to seizure induction with PTZ compared to males or OVX rats. This finding agreed with earlier studies suggestive of inherent differences in seizure sensitivity between males and females (Kokka et al., 1992), and infers some role for the hormonal milieu in modulating basal sensitivity to at least one parameter of seizure activity.

In summary, these findings provide further evidence for distinctive modulation of EW behaviors by GABAergic drugs. The divergence in sensitivity to an acute ethanol challenge during EW across behavioral measures highlights the complex modulation of ethanol-targeted neurotransmitter systems by both chronic and acute ethanol exposure. These studies also provided additional evidence for differences in some, but not all, EW behaviors between males and females, which includes contributions from brain sexual dimorphism. The lack of tolerance to the anticonvulsant effect of the acute ethanol challenge during EW is similar to what was noted for the neuroactive steroids, allopregnanolone, alphaxalone and pregnanolone, in contrast to the significant cross-tolerance seen to the anticonvulsant actions of diazepam during EW (Devaud et al., 1996). This is a key finding, as it provides promise for the development of pharmacotherapies that retain effectiveness against the potential for life threatening seizures during EW. Finally, the complexity of the influence of hormonal status, involving both activational and organizational effects, on responses to ethanol has not yet been well explored, but could be found to have important implications for treatment of alcoholic men and women.

Acknowledgments

The expert technical assistance of Emily Baergen, Mike Fisher, Praweg Koirala and Jacob VanVleck is greatly appreciated. We are also indebted to Fred Risinger, Department of Biomedical and Pharmaceutical Sciences, ISU, for his assistance with statistical analysis of the data. This research was supported by AA011877 and RR016454.

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