Sex Differences in Steroid Modulation of Ethanol Withdrawal in Male and Female Rats

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ABSTRACT

We investigated the actions of the neuroactive steroid, pregnanolone, and the ovarian steroid, 17β-estradiol, on seizure expression during two time points of ethanol withdrawal (EW). Both steroids can exert rapid, nongenomic actions on the brain that include modulation of seizure activity. Because their basal levels differ in adult males and females and a major symptom of EW is increased seizure risk, we wanted to determine whether these steroids were anticonvulsant during EW. Rats were made ethanol-dependent by administration of 6% ethanol in a nutritionally complete liquid diet for 14 days. After removal of the ethanol-containing diet, EW and paired control rats were tested at 1 or 3 days for seizure responses to pentylenetetrazol. Consistent with previous reports, females seemed to have recovered from EW more quickly than males. We observed significant sex differences in responses to the steroids, primarily at 3 days EW. Pregnanolone afforded protection against seizures with larger effects during EW than in control conditions and greater effects in female than male rats. In contrast, effects of estradiol were mixed. Some responses of ovariectomized female rats were similar to intact females, whereas other responses were more similar to males. Our behavioral findings are consistent with observed EW-induced changes in plasma corticosterone levels, showing persistent elevations in male but not female rats. These results support and extend earlier findings suggesting that although the hormonal milieu influences EW, innate differences in brain structure between the sexes also contribute to sex differences in EW.

Ethanol dependence develops from prolonged intake, is often disruptive to the social, occupational, and physical well being of the individual, and poses significant health and economic burdens. Withdrawal-induced seizures are a significant consequence of ethanol dependence and include risk of injury, even death. Treatment of ethanol withdrawal (EW) remains problematic because of the reduced effectiveness of anticonvulsant drugs due to cross-tolerance with ethanol. The distress and dysphoria of ethanol EW, including enhanced seizure risk, probably contribute to the high risk for relapse.

Several clinical studies have found sex differences in the expression of EW behaviors and ethanol-induced diseases that have important ramifications for treatment (Ashley et al., 1997; Brown et al., 1988; Schenker, 1997; Deshmukh et al., 2003). These differences support a growing body of evidence showing that males and females have significant differences in inherent neurobiology expressed as varying risks for a number of neuronal-based diseases and responses to stressors, including alcohol (Brathen et al., 1999; Blanchard et al., 2001; Figueiredo et al., 2002; Webb et al., 2002). For example, alcoholic women showed a greater dampening of the stress response than male alcoholics (Sinha et al., 1998) and presented with fewer EW symptoms (Deshmukh et al., 2003). While the significance of these differences is beginning to be unraveled, treatment of most diseases, including alcoholism, is not routinely tailored to gender.

Neuroactive steroids are steroid hormone derivatives with rapid nongenomic actions, primarily on the inhibitory GABA system (Paul and Purdy, 1992; Baulieu, 1997). Several of these neuroactive steroids share some of the neurobiological effects and may even contribute to the depressive effects of ethanol (VanDoren et al., 2000). For example, pregnane neuroactive steroids possess significant anxiolytic, sedative/hypnotic, anticonvulsant, and anesthetic effects (Baulieu, 1997). Ethanol acutely enhances these behavioral effects of GABAergic neuroactive steroids, such as ataxia, through actions on the GABA_A receptor (VanDoren et al., 2000; Finn et al., 2004). However, chronic ethanol administration differentially alters levels of the neurosteroid, allopregnanolone, in the plasma and brains of male and female rats (Janis et al., 1998; Finn et al., 2004). Clinical studies have also found significantly reduced plasma neurosteroid levels in alcoholic...
The estrous cycle of intact female animals 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 female rats, although females continued to cycle every 4 to 5 days. After the 2 weeks of liquid diet administration, regular lab chow replaced liquid diet for all animals. Testing was scheduled at 1 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 National Institutes of Health guidelines for the humane care and use of animals and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

Seizure Threshold Determination. Seizures were induced by constant tail vein infusion of PTZ. After gently restraining each animal, a 25-gauge butterfly needle was inserted into a lateral tail vein, taped in place, and the animal was allowed to move while being held lightly by the tip of the tail. PTZ was infused at 1.6 ml/min, and the time to the first myoclonic twitch of the face and/or neck indicated the endpoint of infusion. Seizure thresholds were calculated from the time of infusion (minutes) × dose (5 mg/ml × 1.6 ml/min) of PTZ infused per body weight of the animal and are given as milligrams of PTZ per kilogram body weight. PREG or vehicle (cycloexirin in saline) was administered i.p. 15 min before seizure threshold determinations.

Seizure Induction by Bolus Injection of PTZ. Animals were injected i.p. with PREG, estradiol, or vehicle 15 min before a bolus injection of PTZ (40 mg/kg), administered on the opposite side from the pretreatment. This dose was chosen after preliminary studies showed that it reliably induced major signs of seizure in our laboratory rat model but without causing death in most animals. The time to first signs of a seizure (seizure latency), the duration of seizure activity, and the maximal seizure severity caused by the convulsant were continuously monitored for 30 min. Seizure severity was graded using the scoring system modified from Marsh et al. (1989): grade 0, no signs of motor seizure activity during the 30-min 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; and grade 8, death.

Corticosterone Radioimmunoassay. In a separate set of experiments, animals were sacrificed at either of the two time points of EW for collection of plasma and assay of corticosterone. Trunk blood was collected in tubes containing ethylenediamine tetraacetic acid. All tubes were centrifuged at low speed, and supernatant was collected. Plasma samples were stored frozen at −70°C until time of assay. Corticosterone assays were conducted by radioimmunoassay in the laboratory of Dr. Deborah Finn (Oregon Health and Sciences University, Portland, OR). The RIA used 125I-corticosterone from ICN Pharmaceuticals and antisera from Ventrex (Portland, ME). The specificity of the assay is very high, with only 4% cross-reactivity to deoxycorticosterone.

Plasma (5 µl) was diluted with 100 µl of sterile water. Samples were immersed in boiling water for 5 min to denature corticosterone-binding globulin. Counts per minute were normalized and fit to a least squares regression equation produced by log-logit transforma-
tion of the standards. Mass of samples was calculated by interpolation of the standards. The detectable range of the assay was from 0.1 to 400 μg of corticosterone per 100 ml of plasma. Intra- and interassay coefficients of variation were <10%.

Data Analysis. Each ethanol-treated animal and its paired control were randomly assigned to either the 1- or 3-day test time and was tested once only. Data from the different time points (1 and 3 days EW) were analyzed separately using two-way analysis of variance comparing treatment (PREG, estradiol, or vehicle) by ethanol or control diet. For our analyses, we used GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA). We used two-way analysis of variance because of our previous hypothesis that we would find sex differences in recovery from withdrawal (Devaud and Chadda, 2001; Devaud and Alele, 2004). We used Tukey’s post hoc test to further analyze significant main or interactive effects.

Results

The previously observed sex differences in seizure threshold measures to assess recovery from EW were again seen in this study, with males and female rats having significantly lowered seizure threshold (increased seizure susceptibility) at 1 day EW that persisted through 3 days EW in male, but not female, rats (Fig. 1). Acute administration of PREG (4 mg/kg) significantly increased PTZ seizure thresholds in control male and female rats with no observable sedative or ataxic effects. There was a significant main effect of PREG ($F_{1,31} = 19.25, p < 0.001$) on seizure thresholds at 1 day EW. At 3 days EW, there was a significant main effect of PREG treatment ($F_{1,30} = 12.98, p < 0.01$). However, seizure threshold values of EW female rats were similar to basal levels at 3 days EW, and the anticonvulsant effect of PREG was reduced in these animals compared with 1 day EW. These findings agreed with determinations of corticosterone levels. Both EW male and female rats showed significantly elevated corticosterone levels ($F_{1,15} = 9.16, p = 0.0085$) at 1 day compared with controls (Fig. 2). However, at 3 days EW, corticosterone levels remained significantly elevated in male, but not female, rats.

The remaining experiments used the second method of delivery of the convulsant agent to evaluate EW and steroid interactions in male and female rats. Furthermore, to better
delineate the role of hormonal milieu, a third sex group was included (OVX female rats). At 1 day EW, there were no significant differences in seizure latency across groups (control, EW males and OVX; see Fig. 3, top panel). At this time, there was a significant main effect of PREG on seizure latency ($F_{1,63} = 11.14, p < 0.01$). Compared with vehicle-treated EW males, seizure latency increased by 82% in male, by 21% in intact female, and by 142% in OVX female rats after acute PREG administration. There was a significant effect of treatment on seizure duration in OVX rats ($F_{1,42} = 9.54, p < 0.05$). PREG administration reduced seizure duration in EW OVX females by 62% compared with EW OVX females treated with vehicle.

As shown in Fig. 4, at 3 days EW, there was a significant main effect of PREG ($F_{1,80} = 31.46, p < 0.001$), on seizure latency. PREG increased seizure latency by 113% in EW males compared with EW males treated with vehicle, by 55% in EW females compared with vehicle-treated EW females, and by 74% in EW OVX females compared with vehicle-treated EW OVX females. Similar to findings at 1 day EW, PREG treatment did not significantly alter seizure duration in control or EW males. In contrast, PREG-induced decreases occurred in seizure duration in intact ($F_{1,82} = 11.33, p < 0.05$) and OVX ($F_{1,42} = 10.2, p < 0.05$) females. At 3 days EW, seizure duration decreased by 67% in EW intact females after acute PREG administration compared with EW vehicle-treated females; seizure duration decreased by 82% in EW OVX females pretreated with PREG, compared with EW OVX females treated with vehicle.

Seizure severity increased across all sex conditions (male, female, and OVX) during EW at both 1 and 3 days. Significant main effects of PREG ($F_{1,67} = 16.92, p < 0.001$) were seen at 1 day EW (Fig. 3, bottom panel). The decrease in seizure severity in EW males administered PREG was 46% compared with EW males treated with vehicle; PREG also decreased seizure severity by 46% in EW intact females compared with EW intact females treated with vehicle, and by 66% in EW OVX females compared with EW OVX females treated with vehicle. At 3 days EW (Fig. 4, bottom panel), there was a significant main effect of PREG ($F_{1,89} = 54.84, p < 0.001$) on seizure severity across treatments for each sex. At this time, acute administration of PREG decreased seizure severity by 49% in EW males compared with vehicle-treated EW males, by 44% in EW intact females compared with vehicle-treated EW intact females, and by 61% in EW OVX females compared with vehicle-treated EW OVX females.

In addition to characterizing different aspects of seizure expression, we determined levels of survival after seizure induction following the bolus injection of PTZ. For control conditions, 16% males, 24% intact females, and 13% of OVX females died from PTZ-induced seizures. In contrast, no control males or intact females treated with PREG died, and only 1 of 15 OVX females (7%) died (Table 1). At 1 day EW, seizure severity remained submaximal, and none of the PREG-treated EW animals died. At 3 days EW, 10% males, 47% intact females, and 62% OVX females died. Acute administration of PREG prevented mortality for all EW animals.

A second set of experiments studied the same PTZ seizure assessments but tested for interactions with the steroid, 17β-estradiol. Similar to the previous study, there was a decreased seizure latency at 1 day EW in males ($F_{1,46} = 8.51, p = 0.005$) compared with controls (Fig. 5), a 44% decrease. At this time, there was a significant main effect of estradiol ($F_{1,46} = 8.51, p = 0.005$) on seizure latency; estradiol-treated males in EW showed a 27% reduction in seizure latency compared with vehicle-treated EW males. In addition, at 1 day EW, estradiol treatment of EW OVX females increased latency to seize 2-fold (205%), compared with EW OVX females treated with vehicle. Seizure duration significantly increased by 120% in EW males ($F_{1,41} = 8.57, p < 0.05$) as shown in Fig. 5, middle panel. Estradiol pretreatment of EW males decreased seizure duration by 35% compared with EW males treated with vehicle. Estradiol pretreatment of EW intact females increased seizure duration by 22% compared with EW intact females treated with vehicle. There was a significant main effect of estradiol ($F_{1,44} = 11.71, p < 0.05$) on seizure severity (Fig. 5, bottom panel) at 1 day EW. At 1 day EW, estradiol treatment decreased seizure severity by 46% in EW OVX females compared with EW OVX females treated with vehicle, but severity was similar for EW estra-
diol- and vehicle-treated male rats; likewise, severity was similar for EW estradiol- and vehicle-treated intact female rats.

At 3 days EW, although latency to seize was not significantly altered by either EW or estradiol administration, in male, female, and OVX control or EW rats (Fig. 6), there was ~44% reduction in latency to seizure in EW males compared with control males. Estradiol pretreatment reduced seizure duration by approximately 42% in EW males compared with EW males treated with vehicle. EW significantly increased seizure severity in EW male rats by 78% ($F_{1,43} = 4.61, p < 0.05$), and estradiol pretreatment reduced seizure severity by 36% in EW males compared with EW males treated with vehicle.

In this set of experiments, PTZ-induced seizures caused death in 6% of control males, 19% of control females, and 25% of control OVX animals (Table 2). In marked contrast, at 3 days EW, no PTZ-induced deaths occurred in EW male and OVX females treated with estradiol, and only one intact female (12.5%) died at this time. Similar to findings with PREG, mortality was lowered in animals treated with estradiol at 3 days EW compared with vehicle-treated animals.

**Discussion**

Our present findings provide further support to earlier observations of significant differences in behavioral measures of EW in male and female rats. We previously noted that male rats still show signs of EW (reduced seizure thresholds) at 3 days, whereas females have recovered to baseline (control) levels by this time (Devaud and Chadda, 2001; Devaud et al., 2003). Here, we employed an additional...
method of seizure induction and tested for proconvulsant or anticonvulsant effects of two steroids. Because seizure induction involves a series of processes (initiation, propagation, generalization, and termination) that are probably influenced by the hormonal milieu (Foldvary-Schaefer et al., 2004), we used the two methods to compare and contrast several parameters of seizure risk relevant to EW seizures in an animal model operating under a differing hormonal milieu. The rationale for this approach is the clinical finding that alcoholic women show fewer withdrawal symptoms compared with men, even when alcohol consumption is equivalent (Deshmukh et al., 2003).

At 1 day EW, we again observed a consistent and reliable increase in PTZ seizure susceptibility (decreased seizure

### TABLE 1

Proportions of death in animals treated with vehicle or PREG following bolus administration of PTZ

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4/25 (16%)</td>
<td>6/25 (24%)</td>
<td>2/15 (13.3%)</td>
</tr>
<tr>
<td>Control + PREG</td>
<td>0/28 (0%)</td>
<td>0/28 (0%)</td>
<td>1/15 (6.7%)</td>
</tr>
<tr>
<td>At 1 day EW</td>
<td>1/8 (12.5%)</td>
<td>1/8 (12.5%)</td>
<td>1/8 (12.5%)</td>
</tr>
<tr>
<td>EW + PREG</td>
<td>0/10 (0%)</td>
<td>0/8 (0%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>At 3 days EW EW</td>
<td>2/19 (10.5%)</td>
<td>9/19 (47.4%)</td>
<td>5/8 (62.5%)</td>
</tr>
<tr>
<td>EW + PREG</td>
<td>0/21 (0%)</td>
<td>0/20 (0%)</td>
<td>0/8 (0%)</td>
</tr>
</tbody>
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threshold), a hallmark of EW. Acute administration of PREG protected both control and EW animals against first signs of a seizure. The anticonvulsant actions of PREG were enhanced in EW animals compared with controls, consistent with earlier reports of sensitization to the anticonvulsant actions of its neuroactive steroid analog, allopregnanolone, during EW (Devaud et al., 1995; Finn et al., 1995). However, at 3 days EW, the anticonvulsant effect of pregnanolone was reduced in EW females compared with controls, whereas male rats still showed sensitization (and the persistent reduction in seizure threshold).

These data showed that enhancement of the anticonvulsant action of PREG was associated with the increased seizure risk of EW. When animals had recovered from withdrawal, cross-tolerance to this GABA\(_A\) receptor modulator became evident in intact female rats. The enhanced anticonvulsant action and the cross-tolerance to a GABAergic modulator on recovery from EW are in contrast to the tolerance and cross-tolerance observed in other GABAergic agents, such as ethanol and diazepam, during EW. Tolerance and cross-tolerance to therapeutic GABAergic agents have made effective treatment of EW symptoms problematic in alcoholic patients because of the need for increasing drug doses to
suppress symptoms. The enhanced anticonvulsant action of PREG that coincides with increased seizure risk and the cross-tolerance to GABAergic agents on recovery add further support to the novelty of actions of GABAergic neuroactive steroids; it also supports their potential as effective treatment of EW symptoms during early EW. Therefore, dynamic adaptations in GABA_{A} receptors occur across withdrawal recovery.

Stress has a prominent role in EW. It is difficult to separate the contributions of stress from other effects of EW. Clinical evidence supports a greater increase in stress-induced craving in alcohol-dependent individuals during early abstinence (Breese et al.; 2005), with an increased likelihood for relapse among dependent individuals confronted with stressful situations. In the present study, elevations in plasma corticosterone levels at 1 and 3 days EW followed the same pattern as EW-induced increases in seizure responses. The increases in plasma corticosterone levels verify the stressful nature of EW and substantiate the evidence that female rats recover from this stressor more quickly than male rats. This finding was in agreement with earlier find-
ings of a greater stress response during early withdrawal in males than in females (Janis et al., 1998). Clinical findings have also shown that women with a family history of alcoholism and women with a history of anxiety disorders have lower stress responses to the effects of alcohol compared with men (Sinha et al., 1998), signifying that gender differences are present in response to the stress of ethanol and that this animal model provides findings representative of the human condition.

17β-Estradiol is an important ovarian steroid that has been shown to display either proconvulsant or anticonvulsant effects, depending on the context (Woolley and Schwartzkroin, 1998; Jung et al., 2002; Rewal et al., 2003; Wise, 2003). Estradiol is presumably protective to the brain under different conditions, such as concentration, than those that enhance neuronal excitability (Reibel et al., 2000). We next assayed the effects of acute administration of a low dose of this steroid on seizure latency, duration, and severity in control and EW rats. In general, the effects of an acute administration of 17β-estradiol on seizure expression were more variable than that seen for progesterone. Estradiol protected against mortality during EW but exacerbated other seizure parameters differentially in male, female, and OVX rats. This highlights the need to aware of the impact of context on responses, i.e., the context dependence of results. Whether an animal experiences cycling levels of ovarian steroids, differences in levels of testosterone, or the absence of steroids seems to influence acute effects of estradiol on seizure induction. In our treatment paradigm, actions of estradiol were assessed quickly, making it unlikely that effects resulted from the genomic actions of the steroid. These findings suggest that estradiol-like steroids should be investigated further for their potential in treatment of EW and may prove to be especially beneficial in postmenopausal women.

The increased susceptibility to seizures represents the heightened state of excitation in the brains of recovering alcoholics and probably contributes to the exigent nature of EW and its problematic treatment. The dual responses (protective versus excitatory) observed for estradiol between the sexes and hormonal conditions suggest inherent differences in pathways modulated by ovarian steroids. Our data suggest that these pathways may elicit different responses in basal seizure risk parameters because intact females showed a greater overall sensitivity to PTZ seizures (most marked in measuring severity by bolus injection), a finding in agreement with earlier reports (Kokka et al., 1992). By extension, the heightened seizure responses observed during EW may also involve differential hormonal modulation of seizure circuitry between males and females (men and women).

The neuroprotective effect of PREG and low-dose 17β-estradiol on seizure activity in acute EW has important implications for treatment of EW-induced seizures. A greater understanding of the role of hormonally mediated sex differences and of the role of hormonal milieu in EW behaviors in men and women may provide additional insights into how current therapies of EW can be tailored based on gender. Given that approximately 20% of the population chronically abuses alcohol or becomes dependent on it (Alcohol and Health, 2000), it is important to continue studying potential alternative therapies such as the steroids pregnanolone and estradiol. These steroids may modulate seizure activity during EW and may modify responses to existing therapies for EW in alcoholic men and women.

References


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