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 depressant effects of ethanol (VanDoren et al., 2000). For example, progesterone neuroactive steroids possess significant anxiolytic, sedative/hypnotic, anticonvulsant, and anesthetic effects (Baulieu, 1997). Ethanol acutely enhances some 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

ABBRVIATIONS: EW, ethanol withdrawal; OVX, ovariectomy/ovariectomized; PTZ, pentylenetetrazol; PREG, pregnanolone.
patients during EW (Romeo et al., 1996), which probably contributes to the withdrawal syndrome (Devaud et al., 1996; Cagetti et al., 2004).

A second steroid, 17β-estradiol, is the most abundant and potent estrogen in the female mammalian body. Evidence suggests that estradiol also possesses neuroactive properties and is produced by the brain in small quantities (Steckelbroeck et al., 2001a,b). Although earlier studies showed 17β-estradiol to be excitatory with proconvulsant properties (Reibel et al., 2000), more recent studies have reported its anticonvulsant actions. 17β-Estradiol enhanced the anticonvulsant actions of progesterone (Frye and Rhodes, 2005), lowered EW severity scores, reduced cerebellar neuronal damage in ovarioectomized (OVX) rats (Rewal et al., 2003), and protected against EW-induced lipid peroxidation in the cerebellum and hippocampus of OVX rats (Jung et al., 2004).

In the present study, we hypothesized that sex-selective differences in severity of, and recovery from, EW result, at least in part, from differential actions of steroid hormones among males and females. We measured EW at 1 and 3 days because we had previously found significant sex differences in measures of GABAergic systems at these time points (Devaud and Alele, 2004; Alele and Devaud, 2005). In addition, we observed significant sex differences in seizure susceptibility at 3 days of EW (Devaud and Chadda, 2001) and wanted to better characterize this difference in seizure risk as well as determine the pharmacological effects of steroids on EW seizure parameters. We chose two methods of seizure induction to compare and contrast responses. Seizure threshold determinations monitor the very first signs of a seizure and can be precisely quantified and compared. The i.p. bolus method of administration allows for additional comparisons of seizure responses with a standard dose of the convulsant. Therefore, our overall intent was to assess steroid modulation of seizure behaviors by two methods of seizure induction in male, intact female, and OVX rats during EW using the chemoconvulsant pentylenetetrazol (PTZ). We wanted to determine whether these differences extended to responses to two steroids with therapeutic potential. We included OVX female rats to determine the influence of the underlying hormonal milieu on EW and responses to the two steroids.

Materials and Methods

Animals. Male and female rats (Simonsen Labs, Gilroy, CA) approximately 50 days old at the start of experiments were made ethanol-dependent by administration of 6% ethanol in a nutritionally complete liquid diet (ICN Biochemicals, Costa Mesa, CA) for 14 days. Before liquid diet administration, OVXs were performed on half of the female rats. Ovarioectomies were performed under ketamine/xylazine anesthesia; two lateral incisions were made in the abdomen directly above the ovaries, and the ovaries were ligated and excised. The abdominal incisions were closed with stainless steel wound clips, and the animals were allowed at least 4 days to recover from surgery before initiation of the experiment. Control animals were administered the same liquid diet but with dextrose substituted isocalorically for the ethanol to ensure equivalent caloric intake. The amount of liquid diet consumed was recorded daily. Animals were habituated 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. (1999): 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 tetracetic 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 intersay 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 \( \sim 44\% \) reduction in latency to seize 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>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>OVX</th>
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<tr>
<td>Control</td>
<td>4/25 (16%)</td>
<td>6/25 (24%)</td>
</tr>
<tr>
<td>Control + PREG</td>
<td>0/28 (0%)</td>
<td>0/28 (0%)</td>
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At 3 days EW

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<th>Female</th>
<th>OVX</th>
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<tr>
<td>EW</td>
<td>2/19 (10.5%)</td>
<td>9/19 (47.4%)</td>
</tr>
<tr>
<td>EW + PREG</td>
<td>0/21 (0%)</td>
<td>0/20 (0%)</td>
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Fig. 4. Effects of PREG at 3 days EW on seizure expression measured by latency to seizures (top), duration of seizures (middle), and mean severity score (bottom). PREG-pretreated EW males showed a significantly greater delay to have seizures than pair-fed controls. However, EW females showed a reduced response to PREG, which suggests tolerance rather than sensitization to PREG. EW had no effect on seizure latency in either male or female rats at 3 days of EW, but sex differences in the actions of PREG were seen. *, p < 0.05 compared with pair-fed controls from two independent experiments; **, p < 0.05 comparing effect of PREG with vehicle on EW. Values are the mean ± S.E.M.; n = 6 to 13 animals per treatment condition across two independent experiments.

TABLE 1
Proportions of death in animals treated with vehicle or PREG following bolus administration of PTZ

Data are presented as number of animals that died/number of animals tested and percentages in parentheses. PREG was injected at a dose of 4 mg/kg i.p., 20 min prior to the PTZ bolus injection. PTZ was injected at a dose of 40 mg/kg.
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<sub>A</sub> 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

Fig. 5. Effects of estradiol (E2) pretreatment on seizure responses at 1-day EW. The top panel shows latency to seizures, the middle panel shows seizure duration, and the bottom panel shows mean severity scores. a, p < 0.05 comparing main effect of ethanol and control treatments from two independent experiments; **, p < 0.05 comparing effect of PREG with vehicle on EW. Values are the mean ± S.E.M.; n = 7 to 8 animals per treatment condition across two independent experiments.
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$_\text{A}$ 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-

TABLE 2
Proportions of deaths in animals treated with vehicle or 17$\beta$-estradiol following bolus administration of PTZ
Data are presented as number of animals that died/number of animals tested and percentages in parentheses. Estradiol was injected at a dose of 20 $\mu$g/kg i.p. 20 min prior to the PTZ bolus injection. PTZ was injected at a dose of 40 mg/kg.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1/16 (6.25%)</td>
<td>3/16 (18.75%)</td>
<td>4/16 (25%)</td>
</tr>
<tr>
<td>Control + E2</td>
<td>1/16 (6.25%)</td>
<td>4/16 (25%)</td>
<td>8/16 (50%)</td>
</tr>
<tr>
<td>At 1 day EW</td>
<td>2/7 (28.57%)</td>
<td>4/8 (50%)</td>
<td>3/8 (37.5%)</td>
</tr>
<tr>
<td>EW + E2</td>
<td>3/7 (42.86%)</td>
<td>4/8 (50%)</td>
<td>3/8 (37.5%)</td>
</tr>
<tr>
<td>At 3 days EW</td>
<td>5/8 (62.5%)</td>
<td>2/8 (25%)</td>
<td>1/8 (12.5%)</td>
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E2, 17$\beta$-estradiol.

Fig. 6. Effects of estradiol (E2) pre-treatment on seizure responses at 3 days EW. The top panel shows latency to seizures, the middle panel shows seizure duration, and the bottom panel shows mean severity scores. *, $p < 0.05$ compared with pair-fed controls from two independent experiments. Values are the mean ± S.E.M.; $n = 8$ to 9 animals per treatment condition across two independent experiments.
nings 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 Schwatzkroin, 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 be 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 be-

References


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Estradiol and progesterone have also been shown to modulate seizure activity. These steroids may modulate seizure activity during EW and may modify responses to existing therapies for EW in alcoholic men and women.


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