Published in final edited form as:
Neuropharmacology. 2008 February ; 54(2): 365–374.

Decreased Anticonvulsant Efficacy of Allopregnanolone During Ethanol Withdrawal in Female Withdrawal Seizure-Prone vs. Withdrawal Seizure-Resistant Mice

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SUMMARY

The GABAergic neurosteroid allopregnanolone (ALLO) has been repeatedly shown to have an increased anticonvulsant effect during ethanol withdrawal in rats and in C57BL/6J mice. In contrast, the seizure prone DBA/2J inbred strain and the Withdrawal Seizure-Prone (WSP) selected line exhibited decreased sensitivity to ALLO's anticonvulsant effect during ethanol withdrawal, with no change in sensitivity in the Withdrawal Seizure-Resistant (WSR) line. To date, only male mice have been tested. Thus, the present study examined ALLO sensitivity during ethanol withdrawal in female WSP and WSR mice, since females display less severe physical symptoms of withdrawal and have higher circulating ALLO levels than males. Female WSP and WSR mice were exposed to ethanol vapor or air for 72 hr. During peak ethanol withdrawal, separate groups of mice were injected with vehicle or ALLO (0, 3.2, 10, or 17 mg/kg, ip) prior to the timed tail vein infusion of pentylenetetrazol (PTZ). ALLO injection significantly increased the threshold dose for onset to PTZ-induced convulsions, indicating an anticonvulsant effect, in female WSP and WSR mice. During ethanol withdrawal, sensitivity to ALLO's anticonvulsant effect was slightly increased in female WSR mice but was significantly decreased in female WSP mice. This line difference in sensitivity to ALLO during ethanol withdrawal in female mice was similar to that in the male mice. Notably, all seizure prone genotypes tested to date displayed tolerance to the anticonvulsant effect of ALLO during ethanol withdrawal, suggesting that decreased sensitivity of $GABA_A$ receptors to ALLO may contribute to the increased ethanol withdrawal phenotype.

Keywords

alcohol; convulsions; neurosteroid; pentylenetetrazol; selected lines; $GABA_A$ receptors

INTRODUCTION

A large body of evidence indicates that the reduced derivatives of progesterone, deoxycorticosterone, and testosterone are very potent and selective positive modulators of $GABA_A$ receptors in the nanomolar concentration range (reviewed in Belelli & Lambert,

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2005; Paul & Purdy, 1992; Rupprecht & Holsboer, 1999). Numerous reports also suggest that the progesterone derivative allopregnanolone (ALLO, 3α-hydroxy-5α-pregnan-20-one) can modulate sensitivity to some of ethanol's effects (e.g., Criswell and Breese, 2005; Finn et al., 2004a; Grobin et al., 1998; Morrow et al., 1999). Electrophysiologically, ethanol has been shown to have a direct and indirect effect to potentiate $GABA_A$ receptor function, with the indirect effect related to an increase in steroidogenesis (Sanna et al., 2004). This finding is consistent with reports that acute exposure to ethanol by injection or consumption in rodents (Barbaccia et al., 1999; Finn et al., 2004b; Morrow et al., 1999; VanDoren et al., 2000) or consumption in human adolescents (Torres & Ortega, 2003, 2004) significantly increased endogenous ALLO levels to pharmacologically active concentrations. The fact that the 5α-reductase inhibitor finasteride (which decreases endogenous ALLO levels) reduced sensitivity to some, but not all, effects of ethanol (e.g., Dazzi et al., 2002; Gabriel et al., 2004; Hirani et al., 2002, 2005; Khisti et al., 2004; Murphy et al., 2006; VanDoren et al., 2000) suggests that there is a complex interaction between ALLO and ethanol in biological systems.

The effects of chronic ethanol exposure and withdrawal on ALLO levels and on the change in sensitivity of $GABA_A$ receptors to ALLO add an additional layer of complexity to the ALLO/ethanol interaction. Chronic ethanol exposure has been associated with decreased ALLO concentrations in rodents and alcoholic humans (Cagetti et al., 2004; Finn et al., 2004a; Janis et al., 1998; Romeo et al., 1996). Notably, there appeared to be an inverse relationship between endogenous ALLO levels and behavioral changes in excitability during ethanol withdrawal in the alcoholic patients and in rodent genotypes with high withdrawal severity (e.g., DBA/2 inbred strain and Withdrawal Seizure–Prone, WSP, selected line). This observation is consistent with the finding that manipulation of endogenous ALLO levels produced the predicted change in $GABA_A$ receptor mediated inhibition (Belelli & Herd, 2003). However, with regard to ALLO sensitivity, the change in sensitivity to the anticonvulsant effect of alphaxalone or ALLO during ethanol withdrawal was enhanced in rats (Cagetti et al., 2004; Devaud et al., 1996) and C57BL/6 mice that exhibit mild withdrawal (Finn et al., 2000) but was reduced in mouse genotypes with severe withdrawal (DBA/2 and WSP; Finn et al., 2000, 2006). Thus, the results in seizure-prone mouse genotypes during ethanol withdrawal suggest that a reduction in ALLO to levels that could decrease GABAergic inhibition *in vivo*, in conjunction with decreased sensitivity of $GABA_A$ receptors to ALLO, may contribute to their increased withdrawal severity (discussed in Finn et al., 2004a).

Selective breeding offers the opportunity to study genetically correlated traits by comparing the selected lines on responses in addition to the selected phenotype (see Crabbe et al., 1990). The WSP and Withdrawal Seizure–Resistant (WSR) mouse lines have been selectively bred for severe (WSP) or mild (WSR) chronic ethanol withdrawal, measured by handling-induced convulsions (HICs), following 72 hr ethanol vapor exposure. We have recently reported that the anticonvulsant effect of ALLO was decreased during ethanol withdrawal in male WSP mice, whereas sensitivity was unchanged in male WSR mice (Finn et al., 2006). Based on these data, the purpose of the present study was to examine sensitivity to the anticonvulsant effect of ALLO during ethanol withdrawal in female WSP and WSR mice, since female rodents display fewer physical symptoms of withdrawal (Devaud & Chadda, 2001; Gorin-Meyer et al., 2007; Veatch et al., 2007) and have higher circulating plasma ALLO levels than males (Finn et al., 2004b; Morrow et al., 1999; Paul and Purdy, 1992). Sensitivity to the anticonvulsant effect of ALLO during ethanol withdrawal was examined, and circulating hormone levels of progesterone, corticosterone, and estradiol were measured. The range of doses was identical to that used in recent work in male WSP and WSR mice (Finn et al., 2006). We predicted that sensitivity to ALLO would decrease in female WSP and increase in WSR mice during ethanol withdrawal. An increase in knowledge regarding sex and genotype differences in ALLO sensitivity during alcohol withdrawal will aid in our understanding of the human response to alcohol, which also may improve treatments for alcoholism. Portions of this manuscript were

reported at the annual meeting of the Research Society on Alcoholism (Seymour et al., 2006).

METHODS

Animals

Two genetically independent WSP and WSR lines have been bred from the HS/Ibg eight-way cross of inbred strains (Crabbe et al., 1985). Mice were bred in the veterinary medical unit at the Veterans Affairs Medical Center (Portland, OR), and female mice from the first genetic replicate (i.e., WSP-1 and WSR-1) were available for use in the present experiment. Female mice were from selection generation 26 and filial generations 97 and 98, and were 38-62 days old at the time of testing. Mice were housed 2-5 to a cage in Maxi-Miser #1 cages (Thoren Caging Systems, Hazelton, PA) in a temperature-controlled room (21 ± 1 °C) on a 12 hr:12 hr light:dark cycle (lights on at 0600 hours). Mice had *ad libitum* access to food (LabDiet 5001 Rodent Diet, PMI International) and tap water. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the local Institutional Animal Care and Use Committee.

Chronic Ethanol Administration

Female WSP-1 and WSR-1 mice were exposed to 72 hr ethanol vapor or air using a standardized method for inducing physical dependence (Terdal and Crabbe, 1994). Details are provided in Finn and Crabbe (1999) and Finn et al. (2006). Briefly, the ethanol-exposed animals were weighed and injected with a loading dose of ethanol (20% w/v, 1.5 g/kg; Pharmco Products, Brookfield, CT) and pyrazole hydrochloride (pyrazole; 68.1 mg/kg; Sigma Chemical Company, St. Louis, MO), an alcohol dehydrogenase inhibitor used to stabilize blood ethanol concentrations (BECs), prior to ethanol vapor exposure (8-10 mg ethanol/L air) inside inhalation chambers for 72 hrs. Air-exposed animals were treated similarly but received saline (rather than ethanol) and pyrazole injections and were exposed to air for 72 hrs. Each day all mice received an injection of pyrazole. All injections were administered intraperitoneally. Tail blood samples (20 μL) were collected from a subset of the ethanol-exposed mice each day and from all ethanol-exposed mice upon retrieval from the inhalation chambers at 72 hrs, to determine BEC (optimal BEC was 1.5 mg/mL). Tails were nicked for the air-exposed group at 72 hrs, but no blood was taken. Animals were then weighed, placed back into the home cage and taken to a procedure room a minimum of 4 hr prior to the behavioral testing.

BEC Determination

Blood samples (20 μL) were assessed for ethanol content, utilizing a modification of the method originally described by Roach and Creaven (1968). The supernatant, obtained from processing the blood sample (see Finn et al., 2006), was transferred to a crimp top glass vial and analyzed by gas chromatography (Model 6890N, Agilent Technologies, Palo Alto, CA) with flame ionization detection. Seven pairs of ethanol standards (0.25 – 4.0 mg/mL) were used to establish a standard curve.

ALLO Sensitivity

Sensitivity to the anticonvulsant effect of ALLO (purchased from Dr. R. H. Purdy; VA Research Foundation, San Diego, CA) was tested at a time corresponding to peak ethanol withdrawal (5.5 – 9 hrs following removal from the inhalation chambers). Line, dose and treatment groups were counterbalanced across the period of testing.

ALLO was dissolved in a vehicle of 20% w/v 2-hydroxypropyl-β-cyclodextrin (β-cyclodextrin; Cargill, Cedar Rapids, IA) and injected at a volume of 0.01-mL/g body weight. Mice were

injected with vehicle or ALLO (0.0, 3.2, 10, or 17 mg/kg) at 20 min prior to the timed tail vein infusion of pentylenetetrazol (PTZ; Sigma).

The apparatus and procedure for timed tail vein infusion of PTZ have been described in detail (Finn and Crabbe, 1999; Finn et al., 2000, 2006). Briefly, mice were hand-held in a large Plexiglas container, a 27 gauge butterfly needle (Abbott Laboratories, North Chicago, IL) was inserted into a lateral vein, and PTZ was delivered at a rate of 2.5 mg/min (5.0 mg/mL in saline, 0.5 mL/min infusion rate) by one of two syringe pumps (Kd Scientific, Holliston, MA, Model 100; Sage Instruments, Freedom, CA, Model 355). The needle was held in place while one of two trained experimenters recorded the latency of onset to each of four convulsion endpoints that occur in progression and are readily identified (see Table 1). Latencies (sec) were later converted to the threshold dose (mg PTZ/kg body weight) of PTZ to elicit each convulsion endpoint.

Radioimmunoassay (RIA) for Plasma Hormone Determinations

Immediately after each mouse exhibited tonic hindlimb extension (THE), it was decapitated, trunk blood was collected, and plasma was frozen at -80 °C until the RIAs were conducted. For all RIAs, counts per minute were normalized and fit to a least-squares regression equation produced by log-logit transformation of the standards using Prism 4 (GraphPad Software, Inc., San Diego, CA). Mass of samples was calculated by interpolation of the standards.

Although recent work indicates that estrous cycling was abolished in intact female mice during chronic ethanol exposure and early withdrawal (Veatch et al., 2007), we measured plasma progesterone and estradiol levels as an estimate of estrous cycle phase and to confirm that hormone levels were equivalent among ALLO dose groups. Since chronic ethanol exposure and withdrawal can activate the hypothalamic-pituitary-adrenal (HPA) axis (Rivier, 1993), we measured corticosterone levels to determine whether there was a line difference in HPA axis activation during ethanol withdrawal as well as whether ALLO would produce a dose-dependent decrease in this response.

Corticosterone—Plasma (5 μL) was diluted with 100 μL sterile water and samples were immersed in boiling water for 5 min to denature corticosterone-binding globulin. The RIA, which was adapted from a previously described procedure (Keith et al., 1978), used [^{125}I] corticosterone (ICN Pharmaceuticals; Costa Mesa, CA), antiserum (Ventrex; Portland, ME), and standards (10 – 10,000 pg) or samples. The specificity of the assay was very high, with only 4% cross-reactivity to deoxycorticosterone, 1% cross-reactivity to 5β-pregnanedione, and < 0.6% cross-reactivity to other endogenous steroids (Keith et al., 1978).

Estradiol—Using 50 μL of plasma, 17β-estradiol concentrations were determined by using a commercially available kit (ImmuChem Double Antibody 17β-Estradiol [^{125}I] RIA kit; ICN Pharmaceuticals) according to the manufacturer's instructions. Mass of standards ranged from 10 – 3,000 pg/mL. The specificity of the assay was high, with 20% cross-reactivity to estrone, 1.5% cross-reactivity to estriol, and < 1% cross-reactivity to other endogenous steroids (manufacturer's information).

Progesterone—Using 100 μL of plasma, progesterone concentrations were determined by using a commercially available kit (Progesterone Double Antibody [^{125}I] RIA kit; MP Biomedicals, formerly ICN Biomedicals, Irvine, CA) according to the manufacturer's instructions. Mass of the standards ranged from 0.2 – 50 ng/mL. The specificity of the assay was high, with 5% cross-reactivity to 20α-progesterone, < 4% cross-reactivity to deoxycorticosterone, and < 1% cross-reactivity to other endogenous steroids (manufacturer's information).

Data Analysis

Analysis of variance (ANOVA) was used to assess line (i.e., WSP vs. WSR), treatment (ethanol vs. air) and steroid (vehicle vs. 3.2, 10 and 17 mg/kg ALLO) effects on the dependent measures BEC, plasma estradiol levels, plasma progesterone levels, plasma corticosterone levels, the threshold dose of PTZ for onset to myoclonic twitch (MC twitch), facial/forelimb clonus (FF clonus), running/bouncing clonus (RB clonus) and THE as well as the % change in threshold dose of PTZ for onset to MC twitch, FF clonus, RB clonus and THE. Since ethanol withdrawal significantly decreased the PTZ threshold dose, these data were transformed to % change in PTZ threshold dose. We have used this strategy to document genotype differences in the change in sensitivity to the anticonvulsant effect of ALLO during ethanol withdrawal, when ethanol withdrawal produced a decrease in PTZ threshold dose in the vehicle-injected animals (Finn et al., 2000, 2006). Therefore, for each line, treatment and steroid group, the % change was calculated for each individual animal as the change from the mean for the respective vehicle-injected group (i.e., air- or ethanol-exposed vehicle group). Data also were analyzed as described above, with age (days) as a covariate (analysis of covariance, ANCOVA).

In the presence of significant interactions among main effects, data for each selected line were analyzed separately. However, based on our *a priori* hypothesis that WSP and WSR mice would be differentially sensitive to ALLO during ethanol withdrawal (from results in male WSP and WSR mice; Finn et al., 2006), simple main effects analyses followed by Tukey's or Dunnett's post hoc tests also were conducted to examine significant treatment and steroid effects within each selected line. Pearson product-moment correlation coefficients were calculated to examine the relationship between progesterone, estradiol and corticosterone levels and each convulsion endpoint. Data from three WSP mice with BEC > 2.6 mg/mL were excluded from the analyses, one for each of the ALLO dose groups. Significance was set at $P \leq 0.05$.

RESULTS

Ethanol exposure produced mean ± SEM BEC that was 1.88 ± 0.06 mg/mL for WSP (n = 37) and 1.36 ± 0.07 mg/mL for WSR (n = 28) mice upon removal from the inhalation chamber. These values were significantly different [$F(1,57) = 34.17$, $P < 0.001$]. However, there was no main effect of ALLO dose, nor was the interaction between line and steroid dose significant. These results suggest that chronic ethanol exposure was comparable in the two genotypes when they were subsequently divided into vehicle or ALLO dose groups.

Basal Seizure Susceptibility to PTZ

Analyses were conducted in the vehicle-injected female WSP and WSR mice to obtain an index of the line and treatment effects on basal sensitivity to PTZ (Figure 1). A decrease in PTZ threshold dose would indicate an increase in sensitivity to PTZ, whereas an increase in PTZ threshold dose would indicate a decrease in sensitivity to PTZ. Since age did not provide a statistically significant adjustment when ANCOVAs were performed, the ANOVA results are reported.

The threshold dose for onset to RB clonus and THE was significantly decreased during ethanol withdrawal [$Fs(1,18) \geq 14.89$, $P \leq 0.001$], confirming that basal sensitivity to PTZ was increased during ethanol withdrawal. RB clonus and THE threshold dose also was significantly lower in WSP versus WSR mice [$Fs(1,18) \geq 16.0$, $P \leq 0.001$], indicating that WSP mice were more sensitive to PTZ with regard to the onset of the two later convulsion endpoints. The trend for an interaction between line and treatment [$F(1,18) = 3.52$, $P < 0.10$] and planned comparisons confirmed that ethanol withdrawal significantly increased sensitivity to PTZ in female WSR mice, measured by the significant decrease in threshold dose for onset to MC

twitch, RB clonus and THE (Figure 1B). Sensitivity to PTZ-induced RB clonus and THE also was increased during ethanol withdrawal in the female WSP mice (Figure 1A).

Sensitivity to ALLO's Anticonvulsant Effect

Sensitivity to the anticonvulsant effect of ALLO was determined as the percent change in PTZ threshold dose. An increase in the percent change PTZ threshold dose would indicate an anticonvulsant effect of ALLO, whereas a decrease would indicate a proconvulsant effect. The effects of ALLO and ethanol treatment on MC twitch were very similar to the effects on FF clonus in both WSP and WSR mice. Thus, only data for MC twitch are depicted in Figure 2 and the corresponding analyses are described. Likewise, the effects of ALLO and ethanol on THE were similar to the effects on RB clonus, so the data and analyses for THE are described and depicted in Figure 3. The results utilizing ANOVA as well as ANCOVA yielded identical findings. Since age did not provide a statistically significant adjustment when ANCOVAs were performed, the ANOVA results are reported.

The percent change in PTZ threshold dose for onset to MC twitch was significantly increased by ALLO in a dose-dependent manner [$F(3,99) = 29.47$, $P < 0.001$]. The significant interaction between line and treatment [$F(1,99) = 12.03$, $P = 0.001$] and trend for an interaction between line and ALLO dose [$F(3,99) = 1.79$, $P = 0.15$] suggested that the anticonvulsant effect of ALLO was differentially altered during ethanol withdrawal in female WSP and WSR mice (Figure 2).

Subsequent analyses in WSP mice confirmed that sensitivity to the anticonvulsant effect of ALLO was significantly reduced during ethanol withdrawal. While the 2-way ANOVA indicated that the percent change in threshold dose for onset to MC twitch was significantly higher in the air- versus ethanol-exposed mice [$F(1,52) = 8.66$, $P = 0.005$] and was significantly increased by ALLO pre-treatment [$F(3,52) = 18.22$, $P < 0.001$], planned comparisons indicated that sensitivity to the 3.2 and 10 mg/kg ALLO doses was significantly decreased ($P \leq 0.05$) and that sensitivity to the 17 mg/kg ALLO dose tended to be decreased ($P = 0.06$) during ethanol withdrawal (Figure 2A). Additional analyses of the dose response data indicated that the 10 and 17 mg/kg ALLO doses produced a significant anticonvulsant effect in the air-exposed WSP mice ($P < 0.01$), whereas only the 17-mg/kg ALLO dose produced a significant anticonvulsant effect ($P < 0.01$) in the ethanol-exposed WSP mice, when compared with values in respective vehicle-injected mice.

In contrast, analyses in female WSR mice suggested that sensitivity to the anticonvulsant effect of ALLO was slightly increased during ethanol withdrawal. The percent change in threshold dose for onset to MC twitch was significantly increased by ALLO pre-treatment [$F(3,47) = 12.36$, $P < 0.001$], with planned comparisons confirming that sensitivity to the 10 mg/kg ALLO dose tended to be increased ($P < 0.10$) during ethanol withdrawal (Figure 2B). Analyses of the ALLO dose response data confirmed that the 17-mg/kg ALLO dose was anticonvulsant in the air-exposed WSR mice ($P < 0.01$), whereas both the 10 and 17 mg/kg ALLO doses were anticonvulsant in the ethanol-exposed WSR mice ($P < 0.05$).

Analyses conducted on the percent change in threshold dose for onset to THE, the terminal convulsion endpoint, indicated that there was a significant line difference [$F(1,97) = 11.75$, $P = 0.001$] (WSP > WSR) and a significant increase in the percent change THE threshold dose following ALLO pre-treatment [$F(3,97) = 34.285$, $P < 0.001$]. The significant interaction between line and steroid [$F(3,97) = 7.57$, $P < 0.001$] and trend for an interaction between line and treatment [$F(1,97) = 2.59$, $P = 0.11$] suggested that the anticonvulsant effect of ALLO was differentially altered during ethanol withdrawal in female WSP and WSR mice (Figure 3).

Subsequent analyses in WSP mice indicated that the percent change in threshold dose for onset to THE was significantly increased by ALLO pre-treatment [$F(3,50) = 22.71$, $P < 0.001$]. Planned comparisons indicated that sensitivity to the 3.2 mg/kg ALLO dose tended to be decreased ($P < 0.10$) during ethanol withdrawal (Figure 3A). Analysis of the ALLO dose response data indicated that the 10 and 17 mg/kg ALLO doses produced a significant anticonvulsant effect in the air-exposed WSP mice ($P < 0.01$), whereas only the 17-mg/kg ALLO dose produced a significant anticonvulsant effect in the ethanol-exposed WSP mice ($P < 0.01$), when compared with values in respective vehicle-injected mice.

In contrast, analyses in female WSR mice suggested that sensitivity to the anticonvulsant effect of ALLO was increased during ethanol withdrawal. The percent change in threshold dose for onset to THE was significantly increased by ALLO pre-treatment [$F(3,47) = 15.91$, $P < 0.001$] and was significantly increased in ethanol- versus air-exposed WSR mice [$F(1,47) = 4.54$, $P < 0.05$]. Planned comparisons confirmed that sensitivity to the 3.2 mg/kg ALLO dose was significantly increased ($P < 0.05$) during ethanol withdrawal (Figure 3B). However, analysis of the ALLO dose response data indicated that both the 10 and 17 mg/kg ALLO doses were anticonvulsant in the air-exposed ($P < 0.01$) and ethanol-exposed WSR mice ($P < 0.05$).

Plasma Hormone Levels

Mean hormone concentrations are shown in Table 2. Age provided a statistically significant adjustment of estradiol [$F(1,94) = 7.79$, $P < 0.01$] and progesterone levels [$F(1,65) = 5.75$, $P < 0.05$], with a trend for an adjustment of corticosterone levels [$F(1,85) = 3.08$, $P < 0.09$]. Importantly, the pattern of the results was unaffected by the elimination of this covariate (i.e., ANCOVA did not significantly alter the main effects or interactions for any of the three hormones over results with ANOVA). Based on the recommendations of Tabachnick and Fidell (2007), the results of ANOVAs without age covariates are reported to facilitate comparisons across analyses and to eliminate uninformative covariates.

Corticosterone levels were measured to get an index of activation of the HPA axis. Corticosterone levels were significantly increased during ethanol withdrawal [$F(1,86) = 22.55$, $P < 0.001$], tended to be decreased by ALLO pre-treatment [$F(3,86) = 2.36$, $P < 0.10$], and tended to be higher in WSR than in WSP mice [$F(1,86) = 3.05$, $P < 0.10$]. There were no significant interactions between main effects.

Progesterone levels were measured to obtain an index of estrous cycle phase as well as HPA axis activation. Progesterone levels were significantly higher in WSR versus WSP mice [$F(1,66) = 37.24$, $P < 0.001$] and in animals that had been pre-treated with ALLO [$F(3,66) = 9.455$, $P < 0.001$]. However, the significant interaction between line and steroid [$F(3,66) = 2.795$, $P < 0.05$] and trend for an interaction between line and treatment [$F(1,66) = 3.675$, $P = 0.06$] suggested that the line difference in progesterone levels varied across treatment and ALLO dose. Subsequent analyses determined that the main effect of ALLO dose on progesterone levels in WSP [$F(3,38) = 6.99$, $P = 0.001$] and WSR [$F(3,28) = 4.875$, $P < 0.01$] mice was due to the significantly higher levels in the animals pre-treated with the 17 mg/kg ALLO dose. Progesterone levels in the WSR females also tended to be higher in the air- versus ethanol-exposed mice [$F(1,28) = 2.74$, $P < 0.11$].

Estradiol levels also were measured to obtain an index of estrous cycle phase. As with progesterone levels, estradiol concentrations were significantly higher in WSR than in WSP mice [$F(1,95) = 13.49$, $P < 0.001$]. Estradiol levels were significantly higher during ethanol withdrawal [$F(1,95) = 6.835$, $P = 0.01$], and there was a significant interaction between line and treatment [$F(1,95) = 4.53$, $P < 0.05$]. Subsequent analyses confirmed that the significant interaction was due to the higher estradiol levels during ethanol withdrawal only in the WSR mice [$F(1,43) = 5.88$, $P < 0.05$].

Pearson product-moment correlation analyses were performed between plasma concentrations of each hormone and the PTZ threshold dose for onset to each convulsion endpoint from the individual data in all the air- and ethanol-exposed WSP and WSR mice. Plasma corticosterone levels were significantly, negatively correlated with the PTZ threshold dose for onset to MC twitch, FF clonus, and RB clonus (range of $r = -0.20$ to -0.28, all $p < .05$). Estradiol levels were significantly, negatively correlated with the threshold dose for onset to MC twitch ($r = -0.23$, $p < .05$). Progesterone levels were significantly, positively correlated with PTZ threshold dose for onset to RB clonus and THE (both $r \geq 0.28$, $p < .05$). Although multiple significant correlations were detected, the goodness of fit (r^2 ranged from 4.2% to 9.1%) indicated that only 4 – 9% of the variation in convulsion endpoints could be accounted for by the variation in hormone levels.

DISCUSSION

WSP and WSR mice were selectively bred for high (WSP) or low (WSR) chronic ethanol withdrawal severity, as assessed using HICs. The present findings continue our efforts to test the hypothesis that the line difference in ethanol withdrawal severity is mediated, in part, by changes in the modulatory effects of the neurosteroid ALLO on $GABA_A$ receptor function. Recent findings in male mice indicate that the anticonvulsant efficacy of ALLO (versus limbic convulsion endpoints) was significantly decreased during ethanol withdrawal in WSP mice, whereas sensitivity was unchanged in similarly treated WSR mice (Finn et al., 2006). The present findings support the same line differences, and importantly, show that the change in sensitivity is more robust in female mice.

The age range of the female mice (38 – 62 days) suggests that mice were tested during late adolescence and early adulthood. Early work in 100 mice suggests that first stage of estrus occurred over a range of 28 – 49 days of age, with a median at the 35th day (Engle and Rosasco, 1927). The fact that 74% of the mice exhibited normal estrous cycles by day 38, with 89% exhibiting normal cycles by day 42, suggests that the majority of mice in the present study were sexually mature when tested. Additionally, age did not provide a significant adjustment for any of the PTZ seizure endpoints or percent change scores, nor did it change the pattern of any results, when age was included as a covariate. Thus, the age range of the animals did not significantly alter the present findings.

The reduced efficacy during withdrawal was observed following all ALLO doses (3.2 – 17 mg/kg) in female WSP mice against limbic convulsion endpoints, whereas male WSP mice exhibited reduced efficacy to only the 17-mg/kg ALLO dose, when measured by the percent change in threshold dose for onset to MC twitch (Finn et al., 2006). A bigger difference between the present findings and those in male mice is that female WSP mice also exhibited reduced sensitivity to the anticonvulsant effect of ALLO against the brainstem convulsion endpoints. Specifically, the percent change in threshold dose for onset to THE was reduced following the 3.2 and 10 mg/kg ALLO doses in female WSP mice, but unchanged in male WSP mice (Finn et al., 2006). This suggests that the plasticity of $GABA_A$ receptors in limbic versus hindbrain convulsion circuits may be differentially altered during ethanol withdrawal in male versus female WSP mice. Regardless, since the reduced sensitivity to ALLO's anticonvulsant effect in the male WSP mice was accompanied by a rightward shift in the ability of ALLO to potentiate GABA-stimulated chloride uptake (Finn et al., 2006), we presume that functional sensitivity of $GABA_A$ receptors to ALLO also is decreased in female WSP mice during ethanol withdrawal.

In contrast to the results in WSP mice, sensitivity to the anticonvulsant effect of ALLO was either enhanced or unchanged in female WSR mice during ethanol withdrawal, depending on the dose and convulsion endpoint. Specifically, sensitivity to the anticonvulsant effect of the

10 mg/kg ALLO dose was enhanced versus the limbic convulsion endpoints, whereas sensitivity to the 3.2 mg/kg ALLO dose was enhanced versus the brainstem convulsion endpoints. Sensitivity to the other ALLO doses for each convulsion endpoint was unchanged during ethanol withdrawal, consistent with recent results in male WSR mice (Finn et al., 2006). However, the enhanced sensitivity to the anticonvulsant effect of ALLO during ethanol withdrawal at selected doses is consistent with previous findings in male C57BL/6 mice (Finn et al., 2000) and in male and female rats in which sensitivity to the anticonvulsant effect of GABAergic steroids such as ALLO, pregnanolone and alphaxalone was enhanced during ethanol withdrawal (e.g., Alele and Devaud, 2007; Cagetti et al., 2004; Devaud et al., 1996). Taken in conjunction with the findings that functional sensitivity of $GABA_A$ receptors to ALLO was unchanged in male WSR mice (Finn et al., 2006) but was enhanced in male (Devaud et al., 1996) and female rats (Alele and Devaud, 2007) during ethanol withdrawal, it is possible that functional sensitivity of $GABA_A$ receptors to ALLO might be enhanced during ethanol withdrawal in female WSR mice. Additional studies are necessary to confirm this assumption.

The ALLO dose range examined (3.2 – 17 mg/kg) is identical to that used in previous studies in male mice, where we identified genetic differences in the change in sensitivity to ALLO during ethanol withdrawal in seizure prone versus seizure resistant genotypes (Finn et al., 2000, 2006). We reasoned that use of the same dose range would allow us to make comparisons across studies. While our earlier work documented that the 17-mg/kg dose of ALLO decreased rotarod performance and forelimb grip strength in air-exposed mice (Finn et al., 1997, 2000), mice were tolerant to the ataxic and muscle relaxant effects of ALLO during ethanol withdrawal (Finn et al., 2000). Importantly, the potential ataxic or sedative effects of the 17-mg/kg dose of ALLO did not confound our assessment of ALLO's anticonvulsant effect, as PTZ was infused until each animal exhibited each of the four readily identifiable convulsion endpoints.

ALLO's efficacy as an anticonvulsant in the air- and ethanol-exposed mice was fairly dose-dependent, exhibiting a graded dose-response function for the increase in threshold dose of PTZ for onset to MC twitch and THE (that may not have been readily apparent due to the scaling of the y-axes in Figures 2 and 3). Although ALLO levels were not measured in the present study, earlier work has documented that administration of the 10 and 17 mg/kg doses of ALLO increased plasma levels to approximately 200 ng/ml and 300 ng/ml, respectively, in both air- and ethanol-exposed mice (Finn et al., 2000). Since ALLO concentrations below 10 μM are selective for activity at $GABA_A$ receptors (discussed in Finn et al., 2004a;Rupprecht and Holsboer, 1999), the present findings most likely represent actions at $GABA_A$ receptors, rather than a contribution from effects at serotonin type 3 ($5\text{-}HT_3$), nicotinic or sigma receptors.

Ethanol withdrawal significantly increased basal sensitivity to PTZ in female WSP and WSR mice, measured by the significant decrease in the threshold dose for onset to RB clonus and THE. This finding is similar to that reported earlier in male WSP and WSR mice (Finn and Crabbe, 1999) and male rats (Kokka et al., 1993) during ethanol withdrawal. Increased sensitivity to (+)bicuculline also has been reported in male and female rats (e.g., Devaud et al., 1995, 1996) during ethanol withdrawal. However, subtle statistical differences in basal sensitivity to PTZ during ethanol withdrawal were apparent in the comparison of data in female and male mice from the WSR and WSP lines. In particular, ethanol withdrawal significantly decreased the threshold dose of PTZ to elicit RB seizures and THE in male WSP, female WSP, and female WSR mice, while it produced a non-significant decreased in male WSR mice. Regardless, the overall pattern of altered PTZ sensitivity across convulsion endpoints in both male and female WSR (and WSP) mice indicates that both sexes experienced increased seizure susceptibility during ethanol withdrawal, as evidenced by the significant main effects of air versus ethanol vapor exposure. Taken in conjunction with the sex difference in basal PTZ sensitivity (i.e., PTZ threshold dose in air controls was higher in female than in male WSP and WSR mice), it is likely that the greater shift in seizure risk during ethanol withdrawal in the

female WSR mice was due to their higher basal PTZ threshold dose in the air control treatment. Overall, these results are consistent with previous work and indicate that the increase in basal sensitivity of $GABA_A$ receptors to antagonist ligands during ethanol withdrawal (reviewed in Morrow, 1995) appears to generalize across sexes and genotypes. However, the time course for these changes differs in male and female rats (Alele & Devaud, 2007; Devaud and Chadda, 2001), with male rats exhibiting increased sensitivity to (+)bicuculline for a longer duration during ethanol withdrawal than female rats (3 days versus 1 day, respectively).

The present study was conducted in intact female mice, since recent findings indicated that ovariectomy altered the sex difference in ethanol withdrawal-related responses (Alele & Devaud, 2007). Although estrous cycles were not assessed in the current experiment, hormone levels suggest that females may have been in the late diestrus or early proestrus phase of the estrous cycle, based on the high estradiol and low progesterone levels. A recent study utilizing multiple phases of ethanol exposure and withdrawal in C3H/Hecr mice reported that intermittent ethanol exposure interrupted estrous cycles, resulting in vaginal cell samples consistent with diestrus (Veatch et al., 2007). Since estradiol and progesterone levels were sufficiently variable to suggest that estrous cycles may not have been suppressed, it is possible that procedural or genetic differences might explain the variation between studies in estrous-cycle suppression during ethanol exposure. Another possibility is that the hormone measurements were affected by the seizure induction procedure, since blood was collected after PTZ infusion. Nonetheless, plasma estradiol or progesterone levels were not consistently correlated with all convulsion endpoints, and the extremely low r^2 values indicated that the variation in convulsion endpoints could not be explained by the variation in hormone levels. Thus, it is unlikely that any potential estrous cycle-related differences in treatment groups contributed to the present findings.

Although corticosterone levels were measured upon the termination of PTZ-induced convulsions, values were significantly higher in the ethanol- versus air-exposed mice. Thus, ethanol withdrawal produced a comparable increase in corticosterone concentrations in the female WSP and WSR mice. This finding is consistent with earlier work indicating that chronic ethanol exposure and withdrawal activates the HPA axis in male and female rats (e.g., Rivier, 1993) and in male WSP and WSR mice (Finn, unpublished). The negative correlation between plasma corticosterone levels and PTZ threshold dose for three of the four convulsion endpoints suggests that increases in corticosterone levels were associated with decreased PTZ doses to elicit a convulsion (i.e., increase PTZ sensitivity). This finding could be related to the reported proconvulsant effect of corticosteroids (reviewed in Roberts and Keith, 1995). However, pre-treatment with ALLO decreased plasma corticosterone levels in female WSP mice (in both air- and ethanol-exposed), with variable results in WSR mice (no effect in air-exposed, decrease following 3.2 and 10 mg/kg ALLO in ethanol-exposed). This finding is consistent with an earlier parallel study that was conducted in male C57BL/6 and DBA/2 mice (Finn et al., 2000). The ability of ALLO pre-treatment to decrease plasma corticosterone levels may be due to the anxiolytic effect of this steroid (e.g., Akwa et al., 1999; Bitran et al., 1991, 1999; Finn et al., 1997) and/or the ability of ALLO to decrease the stress-induced increase in HPA axis activation (Patchev et al., 1996).

The WSR and WSP selected lines can be used to examine genetically correlated traits (Crabbe et al., 1990). In male mice (Finn et al., 2006) tolerance to ALLO's anticonvulsant effect and reduced sensitivity of $GABA_A$ receptors to ALLO was observed in both replicate lines of WSP mice during ethanol withdrawal, providing strong evidence that the change in sensitivity to ALLO during ethanol withdrawal is a correlated response to selection. The present findings indicate that similar results were found in female mice, although only one of the two replicate lines was tested in the current experiment. Collectively, the results in male and female WSP and WSR mice are consistent with the hypothesis that selection for sensitivity to ethanol

withdrawal severity is genetically associated with tolerance to the anticonvulsant effect of ALLO during ethanol withdrawal. These findings suggest that genes related to the sensitivity of $GABA_A$ receptors to ALLO may play an important role in genetic susceptibility to ethanol withdrawal-induced convulsions.

In both male and female WSP and WSR mice, the most consistent line difference in the change in sensitivity to ALLO during withdrawal was observed versus the limbic convulsion endpoints. While microinjection of ALLO or pregnanolone into the hippocampus, amygdala and lateral septum can produce anticonvulsant (Finn et al., 2005; Martin-Garcia and Pallares, 2005a) and anxiolytic (Akwa et al., 1999; Bitran et al., 1999, Martin-Garcia and Pallares, 2005b) effects, preliminary data indicates that male WSP mice exhibit tolerance to the anticonvulsant effect of intra-hippocampal ALLO during ethanol withdrawal (Finn et al., 2005). Thus, the hippocampus may be one of several brain regions in the limbic neuroanatomical circuit underlying PTZ-induced convulsions whereby line differences in $GABA_A$ receptors exist. Certainly, chronic ethanol exposure and withdrawal significantly alters the expression and peptide levels of several $GABA_A$ receptor subunits in the hippocampus of male rats ($\downarrow \alpha 1$, $\uparrow \alpha 4$, $\uparrow \gamma 2$, $\downarrow \delta$; Cagetti et al., 2003; Grobin et al., 2000; Matthews et al., 1998), but the relevance of these changes in expression to $GABA_A$ receptor subunit assembly or trafficking, composition of synaptic versus extra-synaptic receptors, or phosphorylation state of $GABA_A$ receptors (see Kumar et al., 2004; Liang et al., 2004) to the present findings is not known. Additional factors influencing $GABA_A$ receptor function are the rapid diffusion of hippocampal receptors from extra-synaptic to synaptic domains (Thomas et al., 2005) and $GABA_A$ receptor associated proteins (Chen and Olsen, 2007). Taken in conjunction with the finding that the effects of ALLO on GABAergic transmission in the amydgala may depend on neural network activity (Wang et al., 2007), it is possible that multiple mechanisms affect $GABA_A$ receptor sensitivity to ALLO within a coordinated limbic convulsion circuit and thereby contribute to the tolerance to the anticonvulsant effect of ALLO in WSP mice during ethanol withdrawal.

In conclusion, the present findings and previous work indicate that cross-tolerance to ALLO is a correlated response to selection in male and female WSP and WSR mice. In other words, the results suggest that some of the genes that confer reduced sensitivity of $GABA_A$ receptors to ALLO during ethanol withdrawal may impart increased severity of ethanol withdrawal. Taken in conjunction with the finding that other seizure-prone genotypes, such as the DBA/2 inbred strain (Finn et al., 2000) also exhibit cross-tolerance to ALLO during ethanol withdrawal, an understanding of the GABAergic neural differences that underlie alcohol withdrawal severity may lead to improved therapies for the treatment of alcohol dependence and withdrawal.

ACKNOWLEDGEMENTS

We thank the Portland Alcohol Dependence Core, particularly Dr. Pamela Metten, Michelle Sorensen, Lauren Brown, and Crissy Cotman, for their assistance with the induction of physical dependence and gas chromatography. This work was supported by National Institutes of Health grants AA10760 (J.C.C.) and AA12439 (D.A.F.) from the National Institute on Alcohol Abuse and Alcoholism (NIAAA), and Merit Review grants (J.C.C., D.A.F.) from the Department of Veterans Affairs. Additional support was provided by training grant T32-AA07468 from NIAAA (E.H.B., K.R.G.) and by the Nancy and Dodd Fischer Scholarship/Portland ARCS Foundation (E.H.B.).

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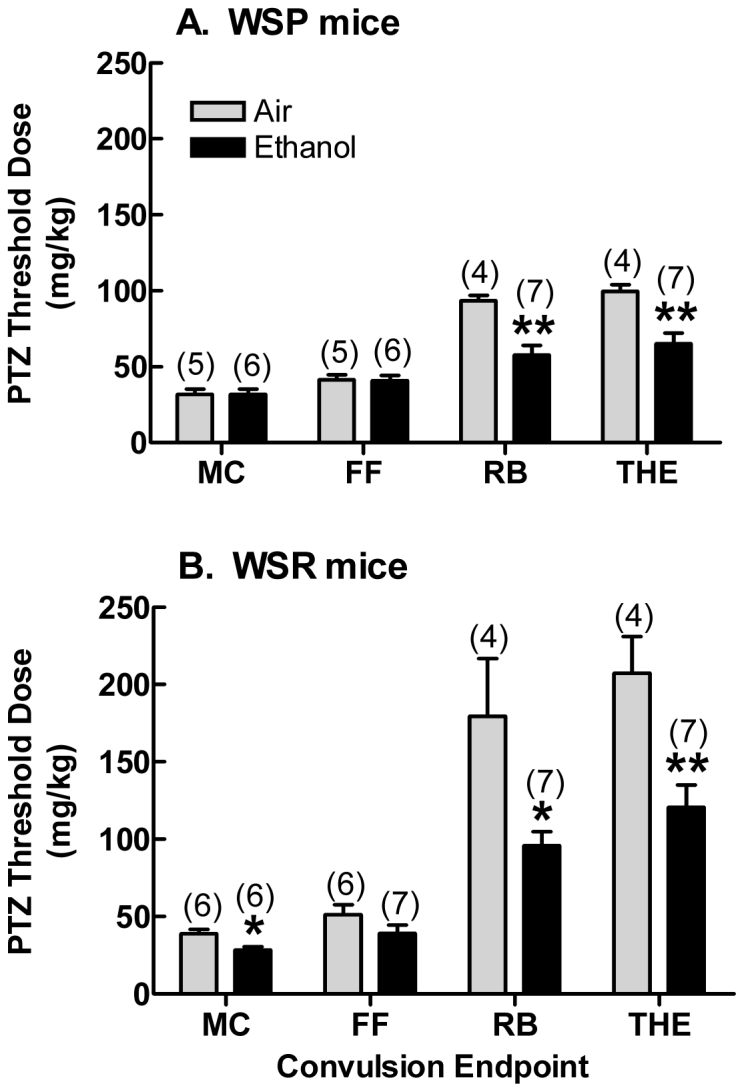

FIGURE 1. Basal sensitivity to pentylenetetrazol (PTZ) in ethanol- and air-exposed (A) WSP and (B) WSR female mice

PTZ was administered at 20 min post-injection of vehicle. Values represent the mean (± SEM) for the number of animals in parentheses and for the 4 convulsion endpoints that characterize PTZ-induced convulsions: MC (myoclonic twitch), FF (facial/forelimb clonus), RB (running/bouncing clonus), and THE (tonic hindlimb extension). *$P < 0.05$, **$P < 0.01$ vs. respective air-exposed mice

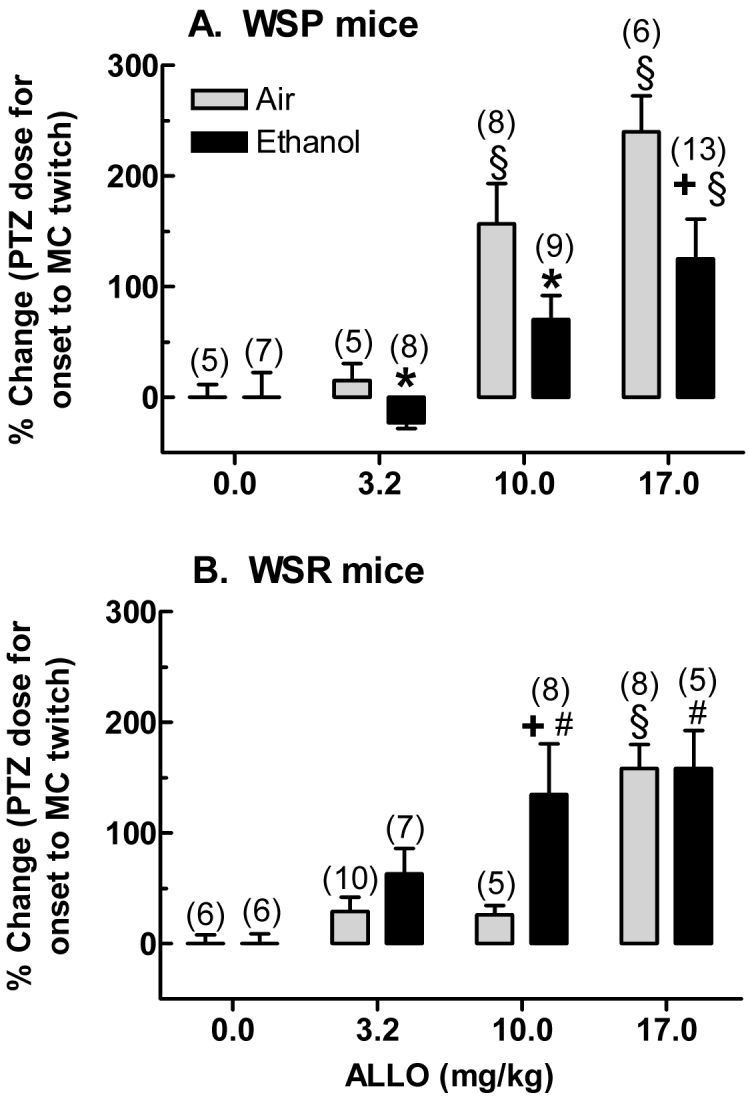

FIGURE 2. Differential change in sensitivity to the anticonvulsant effect of ALLO, measured by the percent change in PTZ threshold dose for onset to MC twitch, in (A) WSP and (B) WSR female mice

PTZ was administered at 20 min post-injection of ALLO or vehicle. Values represent the mean (± SEM) for the number of animals in parentheses.

$^{+}P < 0.10$, $^{*}P < 0.05$ vs. respective air-exposed mice

$^{\#}P < 0.05$, $^{\S}P < 0.01$ vs. respective vehicle-injected mice

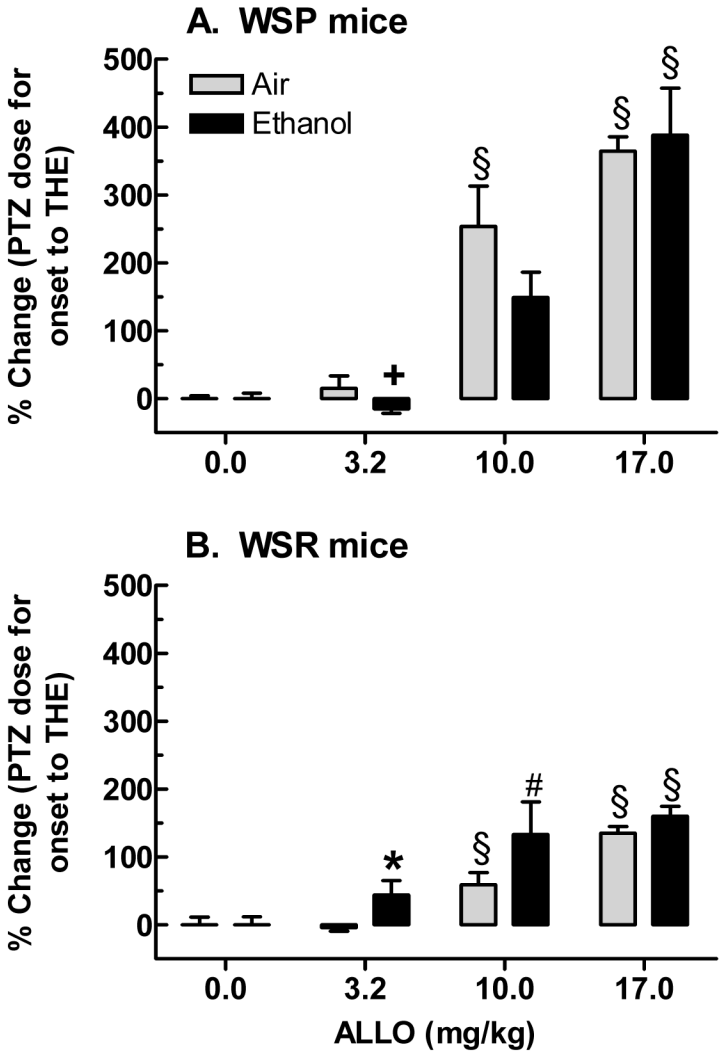

FIGURE 3. Differential change in sensitivity to the anticonvulsant effect of ALLO, measured by the percent change in PTZ threshold dose for onset to THE, in (A) WSP and (B) WSR female mice

PTZ was administered at 20 min post-injection of ALLO or vehicle. Values represent the mean (± SEM) for the number of animals depicted in Figure 2.

$^{+}P < 0.10$, $^{*}P < 0.05$ vs. respective air-exposed mice

$^{\#}P < 0.05$, $^{\S}P < 0.01$ vs. respective vehicle-injected mice

Table 1

PTZ Convulsion Endpoints

Progression of Convulsion Endpoints	Description
myoclonic (MC) twitch	sudden involuntary muscle jerk
facial/forelimb (FF) clonus	rapid writhing of the head and neck with forelimb clonus
running/bouncing (RB) clonus	whole-body clonus with jumping and running movements
tonic hindlimb extension (THE)	whole-body rigidity with caudal extension of all limbs

As discussed in detail by Gale (1988), there are two qualitatively distinct components to the PTZ convulsion endpoints that are mediated by independent anatomical circuits. MC twitch and FF clonus are associated with forebrain circuits, whereas RB clonus and THE are associated with hindbrain circuits. Since genetic susceptibility to these two distinct convulsion types also may be distinct in mice (Kosobud and Crabbe, 1990), interpretations of the present studies will discuss MC twitch and FF clonus as similar types of convulsions and will similarly group results for RB clonus and THE.

Table 2

Hormone Concentrations

Line	Treatment	ALLO	Progesterone		Estradiol		Corticosterone	
		mg/kg	ng/mL	*n*	pg/mL	*n*	µg/dL	*n*
WSP-1	Air	0	1.0 ± 0.6	2	22.1 ± 2.6	5	38.9 ± 7.2	3
		3.2	0.4 ± 0.2	3	25.9 ± 8.4	5	24.0 ± 8.4	4
		10	1.0 ± 0.3	3	17.7 ± 4.9	7	29.1 ± 7.4	7
		17	1.9 ± 0.4	4	15.3 ± 2.4	6	21.0 ± 7.2	5
	Ethanol	0	0.7 ± 0.2	7	21.9 ± 3.9	8	69.0 ± 5.7	7
		3.2	0.7 ± 0.2	6	21.5 ± 4.4	8	59.2 ± 10.9	6
		10	1.7 ± 0.3	8	25.9 ± 4.0	9	54.9 ± 5.7	9
		17	1.8 ± 0.2	13	19.0 ± 2.8	12	43.5 ± 9.6	12
WSR-1	Air	0	2.6 ± 1.8	4	34.6 ± 8.9	5	41.7 ± 6.4	6
		3.2	4.0 ± 1.7	3	30.5 ± 8.7	8	40.2 ± 10.1	7
		10	3.8 ± 0.4	2	12.1 ± 6.6	3	42.2 ± 9.6	4
		17	5.4 ± 2.3	3	27.0 ± 8.9	7	39.6 ± 10.7	8
	Ethanol	0	1.2 ± 0.2	6	30.8 ± 3.7	7	86.9 ± 11.0	6
		3.2	1.5 ± 0.1	6	40.0 ± 10.7	7	35.5 ± 10.1	7
		10	2.3 ± 0.3	7	46.5 ± 10.2	8	50.9 ± 11.8	7
		17	5.9 ± 1.1	5	57.9 ± 14.2	6	75.4 ± 15.3	4

Plasma was collected from all animals immediately following the terminal convulsion endpoint, and hormone levels were measured by radioimmunoassay (RIA). Values are the mean ± SEM for the number of animals listed. Due to the amount of plasma required for both the estradiol and progesterone RIAs, we were unable to obtain estradiol and progesterone levels for a small number of animals.