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Sex Differences in Acute Ethanol Withdrawal Severity After Adrenalectomy and Gonadectomy in WSP and WSR Mice

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Abstract

Recent findings suggest that the ability of ethanol (EtOH) to increase the levels of neurosteroids with potent γ -aminobutyric acid (GABA)ergic properties can influence measures of EtOH sensitivity. Earlier studies determined that removal of the adrenals and gonads diminished the steroidogenic effect of EtOH and significantly increased acute EtOH withdrawal severity in two inbred mouse strains that differed in withdrawal severity, suggesting the contribution of anticonvulsant GABAergic steroids to acute withdrawal in intact animals. Thus, the goal of the present studies was to investigate the consequence of steroid removal on acute EtOH withdrawal through excision of the adrenals and gonads, in another genetic animal model of EtOH withdrawal differences, the Withdrawal Seizure-Prone (WSP) and -Resistant (WSR) selected lines. Male and female WSP and WSR mice underwent surgical removal of the adrenals and gonads or no organ removal (SHAM). One to two weeks later, baseline handling-induced convulsions (HICs) were assessed, mice were given a 4 g/kg dose of ethanol, and HICs were measured hourly for 12 hours and then at 24 hours. The combination surgery significantly increased EtOH withdrawal in WSP and WSR female mice, as measured by area under the curve (AUC) and peak HIC scores. AUC was significantly positively correlated with plasma corticosterone levels and significantly negatively correlated with progesterone levels. In contrast, surgical status did not alter withdrawal severity in male WSP and WSR mice. Overall, the increase in acute ethanol withdrawal severity in female WSP and WSR mice following adrenalectomy and gonadectomy corroborate our recent evidence that withdrawal from a high dose of EtOH can be modulated by anticonvulsant steroids produced in the periphery.

Keywords

GABA; progesterone; corticosterone; neurosteroid; convulsions; alcohol

Introduction

Fluctuations in endogenous steroid hormones can affect behaviors through fast-acting membrane receptors (e.g, Purdy et al., 1992; Rupprecht and Holsboer, 1999; Seyle et al., 1942). These steroids have been termed neurosteroids due to their *de novo* synthesis in neural tissue

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and high levels in the brain, and several are potent positive modulators of γ -aminobutyric acid_A (GABA_A) receptors (Belelli and Lambert, 2005; Follesa et al., 2006; Purdy et al., 1992). Neurosteroids enhance GABAergic inhibition, an action mimicked by acute ethanol (EtOH) administration (reviewed in Biggio et al., 2007; Criswell and Breese, 2005; Grobin et al., 1998).

EtOH administration can increase neurosteroid concentrations to biologically and physiologically relevant levels (e.g., Barbaccia et al., 1999; Finn et al., 2004b; VanDoren et al., 2000). This steroidogenic effect of EtOH was able to modulate EtOH's anxiolytic, antidepressant and anticonvulsant effects (Hirani et al., 2002; 2005; VanDoren et al., 2000). Additionally, EtOH's steroidogenic effect was due primarily to synthesis in the periphery, since it was blocked by removal of the peripheral sources of neurosteroid precursors (specifically, the adrenals and gonads; O'Dell et al., 2004). Importantly, EtOH produced both a direct and an indirect effect on GABA_A receptor function, with the indirect effect due to the ability of EtOH to promote brain steroidogenesis via a local action that was independent of the hypothalamic-pituitary-adrenal (HPA) axis (Sanna et al., 2004).

Recent findings suggest that EtOH-induced fluctuations in neurosteroids are seizure protective, since acute EtOH withdrawal severity, measured by handling-induced convulsions (HICs), was significantly increased in adrenalectomized (ADX) and gonadectomized (GDX) mice from two inbred strains that differ in EtOH withdrawal severity (Gililand and Finn, 2007). In DBA/2J (D2) mice, which generally exhibit high acute and chronic EtOH withdrawal (Crabbe et al., 1983; Crabbe, 1998; Roberts et al., 1992), removal of the adrenals significantly increased acute withdrawal severity in males, whereas removal of adrenals and gonads was required to increase withdrawal in female D2 mice when compared with values in intact animals (i.e., SHAM surgery). In C5BL/6J (B6) mice, which exhibit mild EtOH withdrawal (e.g., Crabbe, 1998), only removal of the adrenals was required to increase acute EtOH withdrawal in male B6 mice over values in SHAM animals, but there was no effect of either surgery in female B6 mice (Gililand and Finn, 2007). The inbred strain difference in the effect of ADX and GDX on acute withdrawal severity is consistent with the idea that genetic differences in EtOH withdrawal severity are due in part to modulatory effects of GABAergic neurosteroids (reviewed in Finn et al., 2004a). Moreover, the fact that acute EtOH withdrawal severity was increased in the ADX and GDX animals suggests that an EtOH-induced increase in an anticonvulsant steroid (or steroids) contributes to the withdrawal response in intact animals.

To further examine genetic differences in the contribution of peripheral sources of GABAergic neurosteroids during acute EtOH withdrawal, we investigated lines of mice that were selectively bred for either severe (Withdrawal Seizure-Prone; WSP) or minimal (Withdrawal Seizure-Resistant; WSR) chronic EtOH withdrawal HICs (Crabbe et al., 1985). WSP mice exhibit greater than a 10-fold more severe withdrawal than WSR mice following 72 hours of equivalent EtOH vapor exposure (Crabbe et al., 1985), as well as following administration of a single high dose of EtOH (i.e., acute withdrawal; Crabbe et al., 1991). The WSP and WSR lines also differ during chronic EtOH withdrawal in endogenous levels of, and sensitivity to, the GABAergic 5 α -reduced progesterone metabolite allopregnanolone (ALLO; Beckley et al., 2008; Finn et al., 2004a, 2006). Specifically, endogenous ALLO levels and sensitivity to ALLO were significantly reduced in WSP versus WSR mice during EtOH withdrawal (Beckley et al., 2008; Finn et al., 2004a, 2006). Based on these data and the recent findings in B6 and D2 mice (Gililand and Finn, 2007), the purpose of the present study was to determine whether removal of the gonads and adrenals would differentially alter acute EtOH withdrawal severity in the WSP and WSR selected lines. We hypothesized that removal of the peripheral sources of neurosteroids would increase acute withdrawal severity in both selected lines, but that the effect would be more prominent in the WSP line. This would suggest that GABAergic compounds produced in the adrenals and gonads play a crucial role in seizure protection during withdrawal

from acute EtOH exposure, and that differences in genotype as well as steroid manipulation contribute to EtOH withdrawal severity.

Methods

Subjects

The WSP/WSR selection has produced two independent pairs of selected lines with similar characteristics, both of which were generated initially from HS/Ibg mice (Crabbe et al., 1985). The WSP and WSR mice used in this study were from the replicate-1 line (WSP-1 and WSR-1). At the time of testing, mice were from selected generation 26 [filial generations 108–110]. Sexually mature, 8–12 week old drug naïve, male and female WSP and WSR mice were bred in the Veterinary Medical Unit at the Veterans Affairs Medical Center (Portland, OR). Upon weaning, mice were separated by sex and line and housed 2–4 per polycarbonate cage. All mice were allowed *ad libitum* access to rodent chow (Labdiet 5001 rodent diet; PMI International, Richmond, IN) and water and were maintained on a 12 hour light/dark cycle (6am–6pm), at $21 \pm 2^\circ\text{C}$. All rodent cages were changed once per week.

Procedure

Since our recent work determined that a combination of ADX/GDX produced the greatest effect on acute EtOH withdrawal severity, with no effect in GDX-only groups and minimal effects in the ADX-only groups (Gililand and Finn, 2007), mice were randomly assigned to one of two surgery groups: ADX/GDX combination or no organ removal (SHAM). Following surgery, mice were given a period of 7–14 days to recover and to ensure that all endogenous steroids were depleted (Khisti et al., 2003). After this recovery period, baseline HIC scores were determined, and mice were then given a 4 g/kg intraperitoneal (IP) injection of ethanol (20% v/v in saline). HICs were recorded hourly for 12 consecutive hours starting immediately after EtOH injection (hour 0) and then again at the 24-hour time point. Following the last HIC score, mice were decapitated, trunk blood was collected for subsequent analysis of steroid levels by radioimmunoassay (RIA), and proper organ removal was confirmed. Due to the large number of animals that were tested, the experiments were conducted in separate passes for each genotype and sex. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted by the U.S. National Institutes of Health and were approved by the local Institutional Animal Care and Use Committee.

Surgery

Anesthesia was induced with 5% isoflurane and maintained throughout surgery at 2%. To begin the ADX/GDX, a dorsal midline incision was made in the skin. The incision was shifted to either side to expose the muscle wall lateral of the midline. To remove the adrenal gland, an incision was made in the muscle wall just behind the last rib on either side to expose the anterior pole of the kidney and the adrenal gland. To separate the adrenal gland from the surrounding tissue, it was grasped with tweezers and gently pulled through the incision in the muscle wall. In female mice, the ovaries were removed through the same incision, but it was made slightly larger to facilitate removal of both the ovary and the oviduct. Female mice received 4–0 chromic gut sutures in the muscle wall incision, and all midline incisions (both male and female) were closed with metal clips. To remove the testicles, a cranial pubic incision was made to enter the peritoneal cavity. The testes, epididymis and surrounding fat were separated from the body with a cauterizing gun, and the incision was closed with tissue adhesive (Vetbond, St. Paul MN). Following surgery, all animals were allowed to recover on a heating pad and were given a daily 3 mg/kg subcutaneous injection of Keterolac (Sigma-Aldrich, St. Louis, MO) for three days following surgery. All mice were given unlimited access to 0.9% saline to maintain sodium balance (Beers and Barkow, 2005; Baxter, Deerfield, IL), as well as water and chow. Daily weights were recorded to ensure that animals recovered to pre-surgery

fitness. When mice exhibited signs of dehydration or weight loss, daily supplemental fluids were administered (0.9% NaCl, 1 ml) subcutaneously until stable weight was reached.

HIC Scoring

In order to quantify withdrawal severity, HICs were measured on a scale of 0–7. This mild convulsion can be induced by gently lifting the mouse by the tail and turning it 180°. A score of 0 indicates no convulsion, scores of 1–3 indicate tonic or clonic convulsions elicited by the turn, scores of 4–6 indicate convulsions elicited only by lifting the mouse by the tail, and a score of 7 indicates spontaneous convulsions observed in the home cage (Crabbe et al., 1991b). All recorded scores were in the range of 0–6.

RIA

Following the HIC scoring at 24 hours, trunk bloods were collected. Plasma was separated and stored at –80 °C until the RIA was conducted. Corticosterone (CORT) and progesterone (PROG) levels were analyzed by RIA. Due to the amount of plasma that could be obtained from one animal (which would allow us to conduct 2 steroid assays in most animals), we reasoned that CORT and PROG levels would be the most informative steroids to be examined.

CORT—Plasma concentrations of CORT were determined using a commercially available kit (Corticosterone Double Antibody [¹²⁵I] RIA kit; MP Biomedicals), and used 5 µl of plasma. Concentration of the standards ranged from 25 to 1000 ng/mL. The assay had a very high specificity, with 0.3% cross-reactivity to deoxycorticosterone and less than 0.1% cross-reactivity to other endogenous steroids. Across three assays, the inter-assay coefficient of variability was 9%, and the average intra-assay coefficient of variability was 6.4%, consistent with values reported by the manufacturer.

PROG—Plasma concentrations of PROG were determined using a commercially available kit (Progesterone Double Antibody [¹²⁵I] RIA kit; MP Biomedicals), and used 100 µl of plasma. Concentration of the standards ranged from 0.2 to 50 ng/mL. The assay had a high specificity, with 5% cross-reactivity to 20α-progesterone, less than 4% cross-reactivity to deoxycorticosterone, and less than 1% cross-reactivity to other endogenous steroids. One assay was used to obtain all values, and the intra-assay coefficient of variation was 8.9%.

Data Analysis

Each data point is expressed as the mean ± SEM. All animals included in the ADX/GDX groups had confirmed organ removal, and all animals in SHAM groups had organs intact. To analyze hourly HIC score, a multivariate ANOVA using time as a repeated measure factor and sex, genotype and surgery as between subject factors was performed. When significant interactions were observed, data were separated by sex and genotype and a two-way ANOVA (surgery by time) was performed. As an overall index of withdrawal severity, area under the curve (AUC) was calculated from hour 0 using the trapezoidal method. To calculate peak withdrawal score, the highest HIC score and the two HIC scored in the hours before and after it were averaged. There were no significant differences in baseline HIC scores between sexes in either genotype. To determine the significance of AUC, peak HIC, plasma CORT and PROG levels, a three-way ANOVA (surgery by sex by genotype) was performed. In the event of significant interactions, data were separated by sex and genotype and *t*-tests on surgical condition were performed. Correlations also were calculated between AUC and plasma CORT and PROG. Significance was set at $P \leq 0.05$. Any data points that were two or more standard deviations from the mean were considered outliers and excluded from hormone data analysis.

Results

The time course for the change in HICs following administration of a high EtOH dose is shown in Figure 1 for all groups. Details of the analysis of the hourly HIC data are not presented, as similar results were found with the analysis of AUC. As depicted in Figure 1, hourly HIC scores were significantly higher in WSP versus WSR mice and were significantly elevated in ADX/GDX versus SHAM animals in WSP females (hrs 2, 8–24), WSP males (hr 1, 11 & 12), and WSR females (hr 7, 8 & 10).

We quantified the effect of surgical status on the severity of EtOH withdrawal by analyzing AUC. There was a significant effect of both surgery (ADX/GDX > SHAM) [$F(1,138) = 11.660$, $P = 0.001$] and genotype (WSP > WSR) [$F(1,138) = 694.420$, $P < 0.001$], but not sex (Figure 2). The analysis also revealed an interaction between surgery and sex [$F(1,138) = 6.161$, $P = 0.014$] and surgery and genotype [$F(1,138) = 5.470$, $P = 0.021$]. Further tests indicated that AUC was significantly altered by surgical status in only WSP females (Figure 2D) [$t(30) = 4.275$, $P < 0.001$] and WSR females (Figure 2B) [$t(27) = 2.301$, $P < 0.05$], with AUC in ADX/GDX animals elevated over the respective SHAM groups.

To determine whether surgical status altered the magnitude of withdrawal severity, peak withdrawal was analyzed. Following the initial injection of EtOH, the mice are sedated for several hours. As the EtOH is metabolized (Gililand and Finn, 2007; Prediger et al., 2006), mice undergo symptoms of acute withdrawal, indicated by an elevation in HIC scores above baseline. Peak HIC scores are usually recorded approximately 4–7 hours following EtOH administration. Analysis of peak HIC score revealed that there were significant main effects of sex (male > female) [$F(1,138) = 74.419$, $P < 0.001$] and genotype (WSP > WSR) [$F(1,138) = 26.904$, $P < 0.001$], but no effect of surgery (Table 1). There was a trend for an interaction of surgery and sex [$F(1,138) = 3.661$, $P = 0.058$] and a significant interaction between sex and genotype [$F(1,138) = 59.413$, $P < 0.001$]. Subsequent analyses revealed that ADX/GDX produced a very slight (i.e., possibly not physiologically relevant), but significant increase in peak HIC score versus SHAM in WSR female mice [$t(27) = 2.574$, $P < 0.05$].

To gain a better understanding of the role of peripheral steroids in acute withdrawal, plasma CORT and PROG levels were measured. It should be noted that hormone data presented represent a subset of the total number of animals per group, due to rejection of statistical outliers that were more than two standard deviations from the mean ($n_{\text{PROG}} = 4$; $n_{\text{CORT}} = 1$) and insufficient plasma to conduct all hormone assays. Main effect analysis for ANOVA indicated that plasma PROG levels (Figure 3) were significantly higher in SHAM versus ADX/GDX animals [$F(1,134) = 30.899$, $P < 0.001$] and in female versus male animals [$F(1,134) = 9.507$, $P = 0.002$]. It should be noted that PROG levels were not significantly higher in WSR SHAM versus ADX/GDX animals. There were significant interactions of surgery and sex, surgery and genotype, and sex and genotype [$F(1,134) > 3.986$, $P < 0.05$] and a three-way interaction between surgery, sex and genotype [$F(1,134) = 8.518$, $P = 0.004$]. Additional analyses revealed a significant effect of surgery in WSP males (Figure 3C) [$t(51) = 2.595$, $P = 0.015$] and females (Figure 3D) [$t(29) = 6.330$, $P < 0.001$], and in WSR females [$t(28) = 3.151$, $P = 0.004$], with PROG levels in SHAM animals elevated over values in ADX/GDX animals in all but WSR male mice. Within SHAM animals, plasma PROG levels in WSR female mice were significantly elevated over values in males [$t(29) = 2.907$, $P = 0.007$]. Plasma CORT levels (Figure 4) also were significantly altered by surgery (SHAM > ADX/GDX) [$F(1,137) = 47.233$, $P < 0.001$], sex (male > female) [$F(1,137) = 8.119$, $P = 0.005$], and genotype (WSP > WSR) [$F(1,137) = 42.235$, $P < 0.001$], with a significant interaction between surgery and genotype [$F(1,137) = 21.947$, $P < 0.001$]. Subsequent analyses indicated that there was a significant effect of surgery in WSP males (Figure 4C) [$t(52) = 5.213$, $P < 0.001$] and females (Figure 4D)

[$t(30) = 5.834$, $P < 0.001$], and WSR females (Figure 4B) [$t(27) = 2.030$, $P = 0.052$], with CORT levels in SHAM animals elevated over values in ADX/GDX animals in all cases.

To examine the relationship between acute withdrawal severity and plasma PROG and CORT levels, Pearson correlations were calculated. Correlations were performed in order to quantify variance in hormone levels and to point to how a change in hormone levels due to experimental manipulation may have contributed to the outcome variable—withdrawal severity. Since the ability of ADX/GDX to increase acute withdrawal severity was influenced by sex rather than genotype, the correlations were conducted for each sex and collapsed across both surgery groups. In female mice, AUC was significantly positively correlated with plasma CORT [$r(59) = 0.352$, $P < 0.01$] and significantly negatively correlated with plasma PROG [$r(57) = -0.273$, $P < 0.02$]. These findings suggest that acute ethanol withdrawal was higher in animals with high CORT levels and/or low PROG levels. In the male mice, AUC also was significantly positively correlated with plasma CORT [$r(82) = 0.250$, $P < 0.05$], but it was not correlated with plasma PROG [$r(81) = 0.027$].

We did observe that the survival rate following ADX/GDX surgery in male WSP mice (~69%) was much lower than in all other ADX/GDX groups (>90%). Despite extensive preventative measures (daily subcutaneous fluid replacement and nesting materials given), the survival rate did not improve across several passes of the WSP male group. Consultation by the in-house veterinarian confirmed that sick mice were showing signs of dehydration, and necropsy on select mice revealed no other visible signs of illness.

Discussion

Since it was first postulated that steroids produced peripherally (rather than in the brain) were important in regulating convulsions (e.g., Pericic et al., 1999), there has not been much direct evidence for their importance in EtOH withdrawal-related phenotypes. Based on recent findings in B6 and D2 mice (Gililand and Finn, 2007), the purpose of the present experiments was to pursue further the idea that acute EtOH withdrawal is worsened in animals that have been depleted of neurosteroid precursors. Notably, the present finding that ADX/GDX significantly increased acute EtOH withdrawal severity in female WSP and WSR mice provides additional evidence that gonadal and adrenal steroids contribute to the withdrawal profile in intact animals (Gililand and Finn, 2007; O'Dell et al., 2004). Taken in conjunction with previous work indicating that certain GABAergic neurosteroids are anticonvulsant during chronic EtOH withdrawal (e.g., Alele and Devaud, 2007; Cagetti et al., 2004; Devaud et al., 1996; Finn et al., 2000, 2006), the evidence suggests that peripherally-derived endogenous anticonvulsant steroids can play an important role in modulating the severity of acute EtOH withdrawal severity.

We chose to use an acute EtOH withdrawal model as it is thought to provide information on neuronal hyperexcitability and adaptation and is considered by some to model “hangover” in humans (Prediger et al., 2006). Depending on the mouse genotype, total clearance time for a 4 g/kg dose of EtOH has been reported to range from 4.2 – 8.5 hours in intact male mice, with the appearance of increased anxiety-like behavior or HIC occurring after the high dose of EtOH had been eliminated (Prediger et al., 2006; Gililand and Finn, 2007). In the present study, the time to peak withdrawal ranged from 4.6 – 6.3 hours (see Table 1), consistent with the reported clearance time for this high dose of EtOH. Since the initial suppression in HICs following EtOH injection was followed by an exacerbation of HIC scores, these changes in convulsive behavior are thought to reflect a state of rebound central nervous system hyperexcitability during acute withdrawal (Crabbe et al., 1991a). Additionally, the initial suppression in HIC scores did not differ in WSP and WSR mice, consistent with early work indicating that the lines did not differ in loss of righting reflex (Crabbe and Kosobud, 1986). Given the strong

genetic correlation between acute and chronic EtOH withdrawal severity in inbred strains (Metten and Crabbe, 2005) and in the WSP and WSR selected lines (Crabbe et al., 1991a), examination of hyperexcitability following withdrawal from a single high EtOH dose may provide insight regarding neuroadaptation following chronic EtOH withdrawal.

The combined removal of both gonads and adrenals significantly increased EtOH withdrawal severity in female, but not in male, WSP and WSR mice, as measured by hourly HIC scores, AUC, and peak HIC scores. This result suggests that sex, rather than genotype, was a more important predictor of the impact of peripheral steroid removal on acute EtOH withdrawal severity. Notably, the present findings are consistent with recent expression profiling results in WSP and WSR mice during the early phase of chronic EtOH withdrawal (i.e., 8 hours following termination of EtOH vapor exposure; Hashimoto and Wiren, 2008). Specifically, of the total EtOH-regulated genes that were identified, cluster analysis revealed that the transcriptional response correlated with sex rather than the selected withdrawal phenotype. The distinct signaling pathways and targeted classes of genes that were altered by EtOH suggested enhanced neurotoxicity in the female mice (Hashimoto and Wiren, 2008). However, during protracted withdrawal (i.e., 21 days following termination of EtOH vapor exposure), the pattern of EtOH-regulated genes correlated with genotype rather than sex (Hashimoto et al., 2008). In conjunction with the present findings, it is possible that a distinct neuradaptive response occurs in female versus male WSP and WSR mice during acute EtOH withdrawal and the early phase of chronic EtOH withdrawal. Put another way, both our data and the Hashimoto and Wiren data from early withdrawal do not suggest pleiotropic influences of genes associated with withdrawal HIC severity. However, the strongest evidence for this would require findings in both WSP-1 vs WSR-1 and WSP-2 vs WSR-2 lines of mice. While we studied only one pair of replicate lines, Hashimoto and Wiren (2008) tested both genetic replicates and reported data collapsed across replicates.

While the effect of ADX/GDX in WSP and WSR mice differs from the results in B6 and D2 mice (Gililand and Finn, 2007), the different pattern of the present versus previous results may be due to inherent differences in the two genetic models examined. That is, selected line differences are due to changes in the allelic frequencies for genes important for the selection phenotype (from an initial genetically heterogeneous population comprised of alleles from 8 inbred strains) whereas inbred strain differences are attributed to the allelic differences encapsulated by chance with each strain (> 20 generations of brother-sister matings eliminated all genetic variability within an inbred strain so that all members are essentially a clone of all others). Since the B6 and D2 inbred strains were only 2 of the 8 strains that comprised the genetically heterogeneous foundation population for the generation of the WSP and WSR selected lines, it is likely that there will be similarities as well as differences in the genes contributing to acute and chronic EtOH withdrawal severity in the WSP and WSR selected lines versus the B6 and D2 inbred strains.

Though analysis of AUC and peak HIC scores can provide insight as to overall differences in acute EtOH withdrawal between ADX/GDX and SHAM groups, examination of the HIC scores each hour can give hints regarding the manner in which withdrawal was changing (i.e., increase in magnitude or duration of withdrawal, precipitation in onset of withdrawal). ADX/GDX produced a slight increase in the magnitude of withdrawal in female WSR mice (↑ peak withdrawal, Table 1; ↑ HIC scores, Figure 1B), whereas ADX/GDX increased the duration of withdrawal in female WSP mice (no change in peak withdrawal, but sustained ↑ HIC scores, Figure 1D), when compared with the withdrawal profile in respective SHAM animals. These data suggest that removal of peripherally derived steroids increased withdrawal severity following a single high dose of EtOH in both female WSP and WSR mice, but that there were slight differences in the manner by which withdrawal was increased.

In contrast, there was no effect of surgical status in the male WSR mice (Figure 1A), consistent with the lack of effect of ADX/GDX on acute withdrawal severity in these animals. In the WSP mice, hourly HIC scores only were elevated in the ADX/GDX versus SHAM animals at hrs 1, 11 and 12 (Figure 1C), suggesting that there was a transient increase in the duration of withdrawal in male WSP mice. These results in male WSP mice were somewhat surprising, given that our recent work suggested that chronic EtOH withdrawal rendered male WSP mice much more sensitive than naïve mice to the proconvulsant effect of pharmacologically decreasing endogenous ALLO levels (Gililand-Kaufman et al., 2008) and that chronic withdrawal produced a persistent decrease in endogenous ALLO levels in conjunction with a decreased sensitivity to an exogenous ALLO challenge in WSP versus WSR mice (Finn et al., 2004a, 2006). While these results suggest that WSP mice would be more sensitive to the removal of peripherally derived steroids (including ALLO) on acute EtOH withdrawal severity, it is possible that the ADX/GDX surgery represented a greater physiological challenge to WSP mice that negatively impacted their survival rate (see Results). Although speculative, it is possible that the WSP male mice that survived the ADX/GDX surgery were physiologically less sensitive to steroid manipulations, which may have contributed to the lack of effect of surgical status on acute withdrawal severity. However, more research is needed to determine the degree of hormone sensitivity in WSP male mice.

Another possible explanation for the present findings is that there were sex differences in the effect of ADX/GDX on ethanol metabolism. While we did not examine this possibility in the present study, we recently determined that there were no sex or strain differences in the effect of ADX/GDX on ethanol metabolism, when compared with values in respective SHAM animals (Gililand and Finn, 2007). Additionally, ADX/GDX did not produce a significant change in the time to achieve peak withdrawal in the present study (Table 1), which might imply a difference in EtOH metabolism. These findings suggest that the increase in acute EtOH withdrawal following ADX/GDX in female WSP and WSR mice was not due to an indirect effect of surgical status on EtOH metabolism.

The mechanism(s) for the overall sex, but not genotype, differences in the effects of surgical status on withdrawal severity following a single high dose of EtOH are not known. With the proviso that ADX/GDX is removing all peripheral steroids, we measured 2 adrenal and/or gonadal steroids with documented proconvulsant or anticonvulsant properties (CORT, PROG), to determine the relative contribution of these steroids to the acute EtOH withdrawal response (i.e., by comparing the change in steroid levels with the change in acute withdrawal severity in ADX/GDX versus SHAM animals). It has been known for decades that PROG has anticonvulsant properties (Seyle, 1942), and that this anticonvulsant effect primarily was due to its 5 α -reduction to ALLO (Frye et al., 2002; Kokate et al., 1999). Additionally, the anticonvulsant action of ALLO and other GABAergic steroids during EtOH withdrawal has been well-documented (e.g., Alele and Devaud, 2007; Cagetti et al., 2004; Devaud et al., 1996; Finn et al., 2000, 2006). Although we were unable to measure testosterone levels in the present study, it has been shown to exhibit proconvulsant and anticonvulsant properties, depending on its metabolism (discussed in Reddy, 2004b). When testosterone was aromatized into 17 β -estradiol, it was proconvulsant (Reddy, 2004b). However, when testosterone was reduced to 5 α -dihydrotestosterone and 3 α -androstenediol, it was anticonvulsant (Frye and Reed, 1998; Reddy, 2004a). Finally, the excitatory versus inhibitory effects of CORT are mediated by mineralocorticoid (MR) and glucocorticoid (GR) receptors in the brain, with predominant MR activation increasing excitatory hippocampal output (discussed in De Kloet et al., 1998). Relevant to the present study, activation of MR exerted a proconvulsant effect against pentylenetetrazol-induced convulsions in WSP mice (Roberts et al., 1993), and acute EtOH withdrawal severity was significantly increased by acute and chronic administration of CORT in WSP mice (Roberts et al., 1991, 1994). However, even though the proconvulsant effect of exogenous administration of CORT is fairly well-established, it should be noted that

deoxycorticosterone (DOC, precursor to CORT) has been reported to have anticonvulsant properties due to its 5 α -reduction to GABAergic metabolites (Reddy and Rogawski, 2002).

Based on the above, we reasoned that ADX/GDX would significantly decrease PROG and CORT levels, and that the change in acute withdrawal severity in ADX/GDX versus SHAM animals would point to the relative contribution of these steroids to the withdrawal profile. That is, an increase in acute withdrawal severity in the ADX/GDX animals would suggest that an anticonvulsant steroid (such as PROG or DOC) was important for maintaining the withdrawal profile in intact animals, whereas a decrease in acute withdrawal severity in ADX/GDX animals would suggest the opposite (i.e., a proconvulsant steroid was important, such as CORT). The results indicated that PROG and CORT levels were decreased in the ADX/GDX WSP mice by 72% and 71% in males and by 70% and 81% in females, respectively. However, in the WSR mice, PROG and CORT levels were decreased in the ADX/GDX animals by 31% and 25% in males and by 96% and 83% in females, respectively. Even with the proviso that the steroid levels were measured following a high dose of EtOH and scoring for withdrawal over a 24 hour period, WSR males did not exhibit the marked decrease in steroid levels following ADX/GDX that was apparent in WSR females as well as in WSP males and females. Though a study by Croft and colleagues (Croft et al., 2008) indicates that 24 hours after acute ethanol administration is a sufficient time for both blood and brain CORT concentrations to return to baseline levels in intact male TO strain mice, it is still possible that in WSR male mice, the 24 hour time point may not be sufficient for levels to return to baseline. Therefore, future studies may want to examine the 48 hour time point. Since organ removal was confirmed in all animals, these results in WSR mice, in conjunction with the lack of effect of ADX/GDX on acute withdrawal severity, are consistent with previous work indicating that WSR mice are more resistant to steroid manipulations (Finn et al., 2004a; Roberts et al., 1991).

The fact that all ADX/GDX female groups tested experienced a significant increase in HICs over values in SHAM animals suggests that endogenous anticonvulsant steroids are important modulators of the behavioral effects of acute EtOH withdrawal and that the major source of these steroids is the adrenals (and gonads) rather than the brain. Since withdrawal was unaffected by ADX/GDX in the male mice, it is unlikely that testosterone or its metabolites were contributing to the present findings. Thus, the results in female mice are consistent with the idea that removal of PROG (or DOC) and their respective 5 α -reduced GABAergic metabolites contributed to the increase in acute withdrawal severity in the ADX/GDX animals. However, the relative involvement of a single or multiple GABAergic steroids to the present findings is unclear.

The present findings revealed that PROG levels were decreased significantly by ADX/GDX in all but male WSR mice, and that they were higher in female than in male WSR, but not WSP SHAM mice. Based on the low PROG levels in SHAM female mice, it is likely that the mice were in estrus, though estrous cyclicity was not monitored due to the short time frame of the experiment and the robust manipulation of the animals involved. Although we were unable to measure ALLO levels in the present studies, it is likely that the significant reduction in PROG levels in the ADX/GDX mice would correspond to a marked decrease in ALLO levels. Additionally, there is evidence that female mice have higher circulating ALLO levels than male mice (Finn et al., 2004b). Removing the main peripheral sources of PROG would produce a concomitant reduction in the synthesis of ALLO and other GABAergic steroids, which could contribute to the increase in HICs during acute withdrawal in the ADX/GDX female mice. Consistent with this idea, plasma PROG levels were significantly negatively correlated with acute withdrawal-related AUC in the female mice. Thus, the increase in acute EtOH withdrawal severity in the female ADX/GDX mice is consistent with the idea that endogenous anticonvulsant steroids (such as ALLO) may contribute to seizure protection during EtOH withdrawal.

Our previous work provides strong evidence that reduced sensitivity to the anticonvulsant effect of ALLO (Beckley et al., 2008; Finn et al., 2006) and to the ability of ALLO to potentiate GABA_A receptor function during chronic EtOH withdrawal (Finn et al., 2006) represents a correlated response to selection in male and female WSP and WSR mice (discussed in Crabbe et al., 1990). That is, some of the genes influencing chronic EtOH withdrawal severity also affect sensitivity to the anticonvulsant effect of ALLO, which is a metabolite of PROG, during EtOH withdrawal. Since the line difference in ALLO sensitivity during EtOH withdrawal was even greater in the female mice, it is possible that the hormonal environment in female WSP mice is such that changes in endogenous anticonvulsant steroid levels can have a more pronounced effect on GABA_A receptor function (and acute and chronic EtOH withdrawal severity) than in WSP males, similar to what we observed in the present study.

In conclusion, the present findings have further corroborated the evidence that withdrawal from an acute high EtOH dose can be modulated by anticonvulsant steroids produced in the periphery. Based on these findings, it can be postulated that these anticonvulsant steroids contribute to the neuroadaptation and neuroexcitability produced by high doses of EtOH, with sex and genotype differences in the magnitude of the effect. Given that acute and chronic EtOH withdrawal may be under the control of a common group of genes (Crabbe et al., 1991a; Metten and Crabbe, 2005), the examination of acute withdrawal-related hyperexcitability can provide insight regarding neuroadaptation following chronic EtOH withdrawal. Thus, the finding that certain peripherally-derived GABAergic anticonvulsant steroids can modulate the severity of alcohol withdrawal provides information that can be important for the treatment of alcohol dependence as well as for understanding mechanism(s) contributing to the development of physical dependence and the expression of withdrawal.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1

Peak withdrawal parameters following a single high dose of EtOH

	Sex	Surgery	Peak HIC score	Peak HIC hour
<i>WSP-1</i>				
	M	ADX/GDX	4.17 ± 0.17 (n=23)	6.04 ± 0.23 (n=23)
		SHAM	4.13 ± 0.13 (n=32)	6.26 ± 0.31 (n=32)
	F	ADX/GDX	4.07 ± 0.22 (n=17)	5.35 ± 0.34 (n=17)
		SHAM	3.68 ± 0.29 (n=15)	6.00 ± 0.28 (n=15)
<i>WSR-1</i>				
	M	ADX/GDX	0.66 ± 0.07 (n=15)	4.64 ± 0.27 (n=15)
		SHAM	0.55 ± 0.05 (n=15)	4.73 ± 0.38 (n=15)
	F	ADX/GDX	0.63 ± 0.10 (n=13)*	6.00 ± 0.77 (n=13)
		SHAM	0.39 ± 0.04 (n=16)	5.18 ± 0.71 (n=16)

Peak withdrawal scores (i.e. an average of the highest HIC score, the previous hour's score and the following hour's score) and peak hour were calculated for male and female WSP and WSR mice. All values represent mean ± SEM for the animals depicted in Figure 1.

* $P \leq 0.05$ versus respective SHAM.