Published in final edited form as:

Neuroscience. 2019 January 15; 397: 127–137. doi:10.1016/j.neuroscience.2018.11.045.

Genotype Differences in Sensitivity to the Anticonvulsant Effect of the Synthetic Neurosteroid Ganaxolone During Chronic Ethanol Withdrawal

Michelle A. Nipper, B.S.¹, Jeremiah P. Jensen, B.A.¹, Melinda L. Helms, B.S.¹, Matthew M. Ford, Ph.D.^{1,2}, John C. Crabbe, Ph.D.^{1,3}, David J. Rossi, Ph.D.^{1,4}, Deborah A. Finn, Ph.D.^{1,3}

¹Department of Behavioral Neuroscience, Oregon Health & Science University, Portland OR 97239

²Division of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR

³Portland Alcohol Research Center, VA Portland Health Care System, Portland, OR 97239

⁴Department of Integrative Physiology and Neuroscience, Washington State University, Pullman, WA 99164

Abstract

Sensitivity to anticonvulsant effects of the γ -aminobutyric acid_A receptor-active neurosteroid allopregnanolone (ALLO) during ethanol withdrawal varies across genotypes, with high sensitivity in genotypes with mild withdrawal and low sensitivity in genotypes with high withdrawal. The present studies determined whether the resistance to ALLO during withdrawal in mouse genotypes with high handling-induced convulsions (HICs) during withdrawal could be overcome with use of ganaxolone (GAN), the metabolically stable derivative of ALLO. In separate studies, male and female Withdrawal Seizure-Prone (WSP-1) and DBA/2J (D2) mice were exposed to air (controls) or 72 hour ethanol vapor and then were scored for HICs during withdrawal (hourly for the first 12 hours, then at hours 24 and 25). After the HIC scoring at hours 5 and 9, mice were injected with 10 mg/kg GAN or vehicle. Area under the HIC curve (AUC) for hours 5 – 12 was analyzed. In control WSP-1 mice, GAN significantly reduced AUC by 52% (males) and 63% (females), with effects that were absent or substantially reduced during withdrawal. In contrast, GAN significantly reduced AUC in both control and ethanol-withdrawing male and female D2 mice. AUC was decreased by 81% (males) and 70% (females) in controls and by 35% (males) and 21% (females) during withdrawal. The significant anticonvulsant effect of GAN during withdrawal in D2 but not WSP-1 mice suggests that different mechanisms may contribute to ALLO insensitivity during

Corresponding Author: Michelle A. Nipper, B.S., VAPORHCS Research (R&D-49), 3710 SW U.S. Veterans Hospital Road, Portland, OR 97239, USA, phone: 1-503-220-8262, ext. 5-6600, FAX: 1-503-273-5351, nipper@ohsu.edu.

Author contribution: DAF was responsible for study concept and design, with feedback from JCC, MMF, and DJR. MAN, JPI, MLH, and DAF contributed to the acquisition of the animal data. MAN and DAF analyzed the data and drafted the manuscript. All authors provided critical revision of the manuscript for important intellectual content and approved the final version for publication. The authors have full control of all primary data and agree to allow the journal to review the data if requested.

Declarations of interest: None. The contents do not represent the views of the National Institute of Health, the US Department of Veterans Affairs, or the United States Government.

withdrawal in these two genotypes. Importantly, the results in D2 mice suggest that GAN may be a useful treatment for ethanol withdrawal-induced seizures.

Keywords

Withdrawal Seizure-Prone mice; DBA/2J mice; alcohol; GABA_A receptors; allopregnanolone; sex

Introduction

Alcoholism contributes to, and can exacerbate health problems worldwide, with 15.1 million adults reported as abusing alcohol or alcohol dependent in 2015 (NIAAA, 2017).

Withdrawal is a serious dependence-related medical consequence in up to 71% of hospitalized patients (Saitz, 1998), and it also is one of three genetic factors underlying the criteria for alcohol dependence (Kendler et al., 2012). A hallmark feature of alcohol withdrawal, particularly during the acute phase, is central nervous system (CNS) hyperexcitability, with severe cases culminating in seizures and delirium tremens that can be fatal (peak seizure activity ~ 24 h, peak delirium tremens ~ 72 h; e.g., Heilig et al., 2010; McKeon et al., 2008). Our laboratory uses animal models of withdrawal-induced convulsive activity to examine mechanisms underlying severe withdrawal, due to the similar changes in seizure susceptibility across time in animals and humans (see Heilig et al., 2010).

Additionally, repeated withdrawal episodes further increase withdrawal-induced seizure activity in animals and humans (e.g., Ballenger and Post, 1978; Duka et al., 2004).

Of the numerous neurochemical systems influenced by acute and chronic alcohol (ethanol) administration (e.g., Roberto and Varodayan, 2017; Spanagel, 2009; Tabakoff and Hoffman, 2013), research from many laboratories indicates that the interaction of ethanol at γ -aminobutyric acid_A receptors (GABA_ARs) contributes to ethanol tolerance and dependence as well as withdrawal-induced convulsions (e.g., Devaud et al., 2006; Finn et al., 2010; Follesa et al., 2006; Grobin et al., 1998; Kumar et al., 2009; Liang et al., 2004, 2006, 2009; McKeon et al., 2008). For instance, the plasticity of GABA_ARs following acute and chronic ethanol administration and withdrawal changes in terms of the direct effects of ethanol on GABA_AR function and the indirect effects that involve GABA_AR-active neurosteroid synthesis (reviewed in Kumar et al., 2009). Importantly, GABA_AR-active neurosteroids are potent endogenous allosteric modulators of GABA_AR-mediated inhibition, with *in vitro* and *in vivo* evidence documenting a physiological contribution of fluctuations in neurosteroid levels to CNS excitability (e.g., Belelli and Lambert, 2005; Finn and Purdy, 2007; Porcu et al., 2016; Zorumski et al., 2013).

The progesterone metabolites, allopregnanolone (ALLO; 3 α ,5 α -tetrahydroprogesterone, THP) and pregnanolone (3 α ,5 β -THP), and the deoxycorticosterone (DOC) metabolite 3 α ,5 α -tetrahydroDOC (5 α -THDOC), are the three most potent positive modulators of GABA_ARs identified to date (e.g., Belelli et al., 1990; Carver and Reddy, 2013, 2016; Gee et al., 1988; Morrow et al., 1987; Purdy et al., 1990; Rupprecht and Holsboer, 1999; Veleiro and Burton, 2009). These GABA_AR-active neurosteroids and ethanol share similar behavioral and pharmacological profiles (e.g., anxiolytic, anticonvulsant, sedative/hypnotic;

Finn et al., 2004a; Kumar et al., 2009; Rupprecht and Holsboer, 1999), suggesting that fluctuations in GABA_AR-active neurosteroid levels may influence ethanol sensitivity. Evidence from several laboratories has shown that administration of moderate ethanol doses, either via injection or consumption, produces a significant increase in plasma, cortical, and hippocampal levels of ALLO and 5 α -THDOC in rats, a significant increase in plasma and cortical ALLO levels in mice, with some conflicting results reported in different mouse genotypes, and mixed results in humans (see reviews by Finn et al., 2004a; Kumar et al., 2009; Morrow et al., 2006; Porcu and Morrow, 2014). Use of immunohistochemistry to examine brain regional changes in cellular ALLO levels after ethanol injection revealed that there were divergent brain regional changes in ALLO immunoreactivity and that the ethanol-induced elevations in ALLO levels in some brain regions (e.g., hippocampal CA1, dentate gyrus, bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus but not the medial prefrontal cortex) were independent of peripheral sources such as the adrenals (reviewed in Porcu and Morrow, 2014). In hippocampal minces, ethanol's steroidogenic effect produced a delayed enhancement in GABA_AR-mediated inhibition that corresponded with the increase in ALLO levels (Sanna et al., 2004), consistent with the idea that ethanol-induced elevations in GABA_AR-active neurosteroid levels enhance GABA_AR transmission. With regard to ethanol sensitivity, results from multiple laboratories confirm that ethanol-induced elevations in GABA_AR-active neurosteroid levels influence sensitivity to several of ethanol's behavioral effects (e.g., Finn et al., 2004a; Kumar et al., 2009; Morrow et al., 2006; Porcu and Morrow, 2014), whereas experimental manipulations to decrease neurosteroid levels (e.g., 5 α -reductase inhibitor, adrenalectomy) significantly reduced some of ethanol's effects (Covault et al., 2014; Finn et al., 2004a; Kumar et al., 2009; Morrow et al., 2006; Porcu and Morrow, 2014). Collectively, available evidence confirms that endogenous neurosteroid fluctuations are physiologically relevant and can influence ethanol sensitivity.

Endogenous neurosteroid levels also influence hyperexcitability during ethanol withdrawal. For instance, the ability of adrenalectomy and gonadectomy to decrease endogenous neurosteroid levels and increase withdrawal-induced convulsions was rescued by administration of neurosteroid precursors and metabolism to GABAergic neurosteroids (Kaufman et al., 2010). Several lines of evidence also indicate that ethanol withdrawal produced a decrease in GABA_AR inhibition that was due in part to changes in GABA_AR function, a decrease in ethanol's steroidogenic effect, and a reduction in endogenous ALLO levels (see Finn et al., 2004a; Kumar et al., 2009). The withdrawal-induced decrease in ALLO levels occurred across species (rodents, monkeys, and humans), with reductions reported in plasma and several brain regions such as medial prefrontal cortex, amygdala, dorsolateral striatum, nucleus accumbens core, and ventral tegmental area (VTA; Beattie et al., 2017; Cagetti et al., 2004; Hill et al., 2005; Jensen et al., 2017; Maldonado-Devincci et al., 2014; Romeo et al., 1996; Snelling et al., 2014; Tanchuck et al., 2009). In a small cohort of alcoholic patients, there was an inverse relationship between endogenous ALLO and 5 α -THDOC levels and ratings of anxiety and depression during early withdrawal (days 4 – 5; Romeo et al., 1996), and long-term ethanol consumption was negatively correlated with ALLO immunoreactivity in the lateral and basolateral amygdala in monkeys (Beattie et al., 2017). Several reports also have suggested that brain regional reductions in ALLO levels

during withdrawal may contribute to increased anxiety and hyperexcitability during withdrawal as well as the propensity to increase ethanol consumption during withdrawal (Beattie et al., 2017; Cagetti et al., 2004; Maldonado-Devincci et al., 2014). Taken together, a withdrawal-induced reduction in levels of GABA_AR-active neurosteroids such as ALLO may decrease GABA_AR-mediated inhibition and thereby contribute to withdrawal-induced hyperexcitability and associated symptoms.

In addition to reductions in endogenous neurosteroid levels during ethanol withdrawal, genotype differences in sensitivity to the anticonvulsant effect of GABA_AR-active neurosteroids have been reported (see Finn et al., 2010 and references therein; also Cagetti et al., 2004; Devaud et al., 1995, 1996). To build on existing data in rats, we examined sensitivity to the anticonvulsant effect of ALLO in two different animal models that exhibit marked differences in ethanol withdrawal severity, the Withdrawal Seizure-Prone (WSP) and Withdrawal-Seizure Resistant (WSR) selected lines and the C57BL/6J (B6) and DBA/2J (D2) inbred strains. Both WSP and D2 mice exhibit high ethanol withdrawal severity versus (vs) WSR and B6 mice, respectively, measured by increased handling-induced convulsions (HICs) and anxiety following 72 h of continuous ethanol vapor or chronic intermittent ethanol vapor exposure (Crabbe et al., 1985; Crabbe, 1998; Finn and Crabbe, 1999; Finn et al., 2004a; Gorin et al., 2005; Kosobud and Crabbe, 1986; McCool and Chappell, 2015; Metten and Crabbe, 2005; Metten et al., 2010). Notably, a comparison of results across studies indicated that there was enhanced sensitivity to the anticonvulsant effect of ALLO and alphaxalone in rodents with a low withdrawal convulsive profile (e.g., rats, B6 mouse inbred strain, WSR mouse selected line), when compared to sensitivity in respective controls (Beckley et al., 2008; Cagetti et al., 2004; Devaud et al., 1995, 1996; Finn et al., 2000, 2006). In contrast, resistance to ALLO's anticonvulsant effect during withdrawal was observed in mice with a high withdrawal profile (e.g., D2 mouse inbred strain, WSP mouse selected line), while an anticonvulsant effect was observed in respective controls (Beckley et al., 2008; Finn et al., 2000, 2006). Studies also determined that the above withdrawal-induced behavioral changes in sensitivity to ALLO's anticonvulsant effect were paralleled by shifts in functional sensitivity of GABA_ARs to ALLO during withdrawal: a leftward shift in the ALLO dose-response curve in rats that exhibited enhanced sensitivity to ALLO's anticonvulsant effect (Devaud et al., 1996) and a rightward shift in WSP mice that exhibited resistance to ALLO's anticonvulsant effect (Finn et al., 2006). Finally, the withdrawal-induced resistance to systemic ALLO in WSP mice was mirrored by resistance to ALLO's anticonvulsant effect following bilateral microinjection into three brain regions known to be involved in seizure generation: the hippocampal CA1 region (Gililand-Kaufman et al., 2008) and the substantia nigra reticulata (SNR) or VTA (Tanchuck et al., 2013), whereas there was a significant anticonvulsant effect following ALLO microinjection in the respective controls. Therefore, it is possible that a decrease in endogenous ALLO levels (discussed above) and a reduction in sensitivity of GABA_ARs to ALLO contribute to the greater neural excitability in genotypes that display high withdrawal.

The synthetic neurosteroid ganaxolone (GAN) is a 3 β -methylated analog of ALLO that exhibits a pharmacological profile similar to ALLO, but it has a half-life about 3 – 4 times longer when given systemically because it is not subject to oxidation at C-3 (Carter et al., 1997). This initial report by Carter et al. (1997) documented that GAN exhibited

anticonvulsant activity against chemically-induced convulsions, tonic seizures induced by maximal electroshock, and kindled seizures, consistent with GAN's potent and selective activity as a positive allosteric modulator of GABA_ARs. Subsequent studies confirmed that GAN exerted more potent anticonvulsant activity than ALLO in the majority of diverse rodent seizure models that were examined (see Reddy and Estes, 2016; Reddy and Rogawski, 2012 and references therein; also Carver and Reddy, 2016), although ALLO and GAN were equally effective in a mouse model of treatment-resistant status epilepticus (Zolkowska et al., 2018). These preclinical studies with GAN, in conjunction with the finding that tolerance to GAN's anticonvulsant effect did not develop following repeated administration (Reddy and Rogawski, 2000), renewed interest in the potential therapeutic use of synthetic neurosteroids. For example, GAN is in Phase 2 and Phase 3 clinical trials for the treatment of various forms of epilepsy (see <https://clinicaltrials.gov/>; also reviewed in Reddy and Estes, 2016; Reddy and Rogawski, 2012). Based on the above, we hypothesized that GAN might be more effective in treating ethanol withdrawal-induced convulsions than ALLO.

The purpose of the present studies was to measure sensitivity to the anticonvulsant effect of GAN during ethanol withdrawal in male and female WSP-1 and D2 mice. As discussed above, these two genotypes exhibit resistance to ALLO's anticonvulsant effect during peak (8 h) withdrawal, allowing for the comparison with GAN's anticonvulsant effect during withdrawal in the present set of studies. The results indicate that GAN had a significant anticonvulsant effect in control male and female WSP-1 and D2 mice. During ethanol withdrawal, male and female WSP-1 mice exhibited resistance to GAN's anticonvulsant effect, whereas male and female D2 mice retained sensitivity to GAN's anticonvulsant effect. The results in D2 mice suggest that GAN may be useful for the treatment of ethanol withdrawal-induced seizures.

Experimental Procedures

Animals.

Drug naïve male and female WSP mice from the first genetic replicate (i.e., WSP-1; bred in the Veterinary Medical Unit of the VA Portland Health Care System (VAPORHCS), Portland, OR) and D2 mice (Jackson Laboratory West, Sacramento, CA) were used in the present studies. Each genotype and sex was tested in a separate study to examine sensitivity to GAN's anticonvulsant effect following systemic administration. A total of 26 WSP-1 males, 34 WSP-1 females, 30 D2 males, and 32 D2 females were tested.

Experiments were conducted at Oregon Health & Science University (OHSU), so the WSP-1 mice were transferred from the VAPORHCS to OHSU after being in quarantine for one month prior to transfer. Upon arrival at OHSU, all animals were housed up to four per cage with *ad libitum* access to food and water and were acclimated to a 12:12 hour light dark cycle (lights on at 6:00 am). At the time of testing, the WSP-1 mice were from selected generation 26 [filial generations 111-113] and were 91-135 (males) or 113-153 (females) days old. This age range is similar to our earlier studies that examined sensitivity to ALLO's anticonvulsant effect during ethanol withdrawal following systemic administration (70-100 days old; Finn et al., 2006) and microinjection into the CA1 (~100 days old; Gililand-

Kaufman et al., 2008) or into the SNR or VTA (70-150 days old; Tanchuck et al., 2013) in this mouse line. Male and female D2 mice were received at OHSU from Jackson Labs and were 50-64 days old at the time of testing, consistent with our earlier work examining sensitivity to ALLO's anticonvulsant effect in male D2 mice during ethanol withdrawal (56-63 days old; Finn et al., 2000). So, the older age of the WSP-1 mice was due to both their limited availability and the fact that this genotype underwent quarantine prior to being tested. Regardless, these different age ranges for the WSP-1 and D2 mice are directly comparable to our prior work that measured ALLO sensitivity during ethanol withdrawal. All procedures were compliant with National Institute of Health guidelines and the *Guide for the Care and Use of Laboratory Animals*, 8th ed (National Research Council of the National Academies, 2011), and they were approved by the local Institutional Animal Care and Use Committees at OHSU and VAPORHCS.

Chronic Ethanol Exposure.

Mice were exposed to continuous ethanol vapor or air for 72 h, using a standardized method in the Finn and Crabbe laboratories that involved daily injections of the alcohol dehydrogenase inhibitor pyrazole (68.1 mg/kg; Sigma Chemical Company, St. Louis, MO) to help stabilize blood ethanol concentrations (BECs; see Finn and Crabbe, 1999; Tanchuck et al., 2009; Terdal and Crabbe, 1994 for details). Controls also received daily pyrazole injections. Tail blood samples (20 μ L) were collected from all the ethanol-exposed mice each day and at 72 h to determine BEC during and upon completion of the ethanol vapor exposure, and samples were analyzed by headspace gas chromatography (Finn et al., 2007). Ethanol vapor concentrations were measured hourly via an autosampler, and they were adjusted daily to achieve BECs in the range of 1.5 – 2.0 mg/mL. Briefly, ethanol vapor concentrations started at 7.0 mg ethanol/L air for the males and at 7.3 mg/L for the females. Ethanol vapor concentrations were increased by approximately 0.15 mg/L each day in the male and female WSP-1 mice, whereas they were increased approximately 0.5 mg/L in the male and female D2 mice. At 72 h, all animals were removed from the inhalation chambers, placed back into their home cages, and taken to a procedure room for behavioral testing.

Handling-Induced Convulsions (HICs).

With the exception of the study in male WSP-1 mice, a baseline HIC measurement was taken prior to the ethanol vapor or air exposure. Following removal from the inhalation chambers at 72 h, all mice were scored hourly for HIC for the first 12 h and then again at 24 h and 25 h (only 24 h for male WSP-1). There were two additional HIC measurements at 5.5 h and 9.5 h, which corresponded to 30 min after each injection of GAN or vehicle (VEH). This procedure involved lifting the animal by the tail, gently turning it 180° if necessary, and observing convulsions. HIC scores ranging from 1 to 3 required the gentle turn to elicit a tonic or clonic convulsion; HIC scores ranging from 4 to 6 reflected convulsions that were elicited by merely lifting the mouse by the tail (e.g., Finn and Crabbe, 1999; Kaufman et al., 2010). Mice were euthanized following the final HIC assessment.

Drug Administration.

GAN (synthesized by the late Robert Purdy, PhD) or VEH (20% w/v β -cyclodextrin in water; Cargill, Incorporated; Minneapolis, MN) was administered via intraperitoneal (IP)

injection after the 5 h and 9 h measurement of HICs. GAN was prepared by initially dissolving in a 20% w/v β -cyclodextrin:ethanol solution before being evaporated to dryness and reconstituted in water. The GAN dose administered was 10 mg/kg (IP, 0.01 mL/g body weight), with equivalent volumes of VEH administered to the VEH groups. The 10 mg/kg GAN dose has been well documented to exhibit an anticonvulsant effect in numerous seizure models (e.g., Carver and Reddy, 2016; Reddy and Estes, 2016; Reddy and Rogawski, 2012).

Data Analysis.

Withdrawal severity was assessed by examining the hourly HIC scores and by calculating the area under the withdrawal curve (AUC). AUC was calculated as the summation of HIC scores across the 5 – 12 h period following termination of ethanol exposure, to focus on the time frame of GAN or VEH administration. Similarly, the analysis of hourly HICs focused on the 5 – 12 h period. We also conducted a subsequent analysis of AUC 5 – 7 h and AUC 9 – 12 h to examine the effects at hours surrounding each injection separately. After confirming data normality with the Shapiro-Wilk test, analysis of variance (ANOVA) was used to assess treatment (ethanol vs air) and drug (GAN vs VEH) effects on the dependent variables BEC, baseline HIC, and AUC. Because we were predicting treatment differences in sensitivity to GAN, only planned comparisons were conducted on hourly HIC data. Each genotype and sex was tested in a separate study, so data initially were analyzed in independent tests. To explore sex differences, planned comparisons were conducted on the AUC 5 – 12 h data. Data are expressed as the mean $\pm$ SEM. Statistical significance was set at $p = 0.05$.

Results

Reduced efficacy of the anticonvulsant effect of systemic GAN during ethanol withdrawal in WSP-1 male and female mice.

In the male WSP-1 mice, mean $\pm$ SEM BECs across the 72 h of ethanol vapor exposure were 1.97 ± 0.10 mg/mL ($n=8$) and 1.85 ± 0.11 mg/mL ($n=6$) for the mice that subsequently received GAN or VEH injections, respectively. BECs did not differ between these two drug groups. Figure 1 depicts the pattern of hourly HICs and AUC across the 5 – 12 h period following removal from the inhalation chambers at 72 h and following two IP injections of GAN, which were administered 4 h apart. AUC 5 – 12 h was significantly increased during ethanol withdrawal compared to air-exposed controls [$F(1,22)=166.44$, $p<0.001$] and was significantly decreased by GAN injections [$F(1,22)=56.26$, $p<0.001$]. The significant interaction between treatment and drug [$F(1,22)=23.20$, $p<0.001$] reflected the different sensitivity to GAN's anticonvulsant effect in the ethanol- vs air-exposed mice. Post-hoc tests confirmed that AUC 5 – 12 h with GAN treatment was significantly decreased by 52% in the air-exposed mice ($p<0.001$), but it was not significantly reduced during ethanol withdrawal ($\downarrow 8\%$, $p=0.10$; Figure 1B). Similar results were observed when the analyses were conducted on AUC 5 – 7 h and AUC 9 – 12 h (not shown), suggesting that there were comparable effects of GAN at hours surrounding each injection separately. Planned comparisons of hourly HICs in the air-exposed mice indicated that GAN produced a significant reduction in HICs at 5.5 h, 6 h, 9.5 h, 10 h, and 11 h vs VEH-treated mice (Figure 1A). In the ethanol-exposed mice, no planned comparisons were significant.

Ethanol exposure was comparable in the female WSP-1 mice that were subsequently injected with GAN or VEH. Mean $\pm$ SEM BECs across the 72 h were 1.83 ± 0.04 mg/mL (n=9) and 1.85 ± 0.03 mg/mL (n=11) for the GAN- and VEH-treated mice, respectively. BECs did not differ between these two drug groups. Baseline HICs were measured in this study, and they were lower in the mice that were subsequently exposed to air vs the mice subsequently exposed to ethanol vapor [$F(1,30)=4.65$, $p<0.05$]. However, baseline HICs did not differ in the GAN vs VEH subgroups, nor was there a significant interaction between treatment and drug on baseline HIC, indicating that the difference in baseline HICs across treatment groups did not influence the subsequent response to GAN or VEH. Figure 2 depicts the pattern of HIC and AUC results across the 5 – 12 h time frame in female WSP-1 mice following the two injections of GAN or VEH. AUC 5 – 12 h was increased significantly during ethanol withdrawal [$F(1,30)=94.30$, $p<0.001$] and was significantly decreased by GAN injection [$F(1,30)=9.37$, $p<0.01$]. The interaction between treatment and drug was not significant, suggesting that GAN similarly influenced AUC 5 – 12 h in the ethanol- and air-exposed mice. However, GAN decreased AUC 5 – 12 h by 63% in the air-exposed mice and only by 13% during ethanol withdrawal (Figure 2B). Similar results were obtained with the analysis of AUC 5 – 7 h and AUC 9 – 12 h (not shown). Planned comparisons across hours 5 – 12 in the air-exposed mice indicated that GAN significantly decreased HICs at 5.5 h, 6 h, 7 h, 9.5 h, and 10 h (Figure 2A). In the ethanol-exposed mice, there was a significant reduction in HICs in the GAN- vs VEH-treated mice at 5.5 h, 6 h, 7 h, and 10 h.

Planned comparisons also were conducted on AUC 5 – 12 h for each treatment and drug condition to evaluate potential sex differences in withdrawal severity and in response to GAN. In the air-exposed mice, AUC 5 – 12 h was significantly lower in female vs male WSP-1 mice injected with VEH [$F(1,12)=5.82$, $p<0.05$] and GAN [$F(1,10)=12.71$, $p=0.005$]. During ethanol withdrawal, there was a statistical trend for AUC 5 – 12 h to be lower in female vs male mice injected with GAN [$F(1,15)=3.89$, $p<0.07$], but AUC 5 – 12 h did not differ between the sexes in the mice injected with VEH.

Anticonvulsant effect of systemic GAN in air- and ethanol-exposed D2 male and female mice.

Ethanol exposure was comparable in the GAN- and VEH-treated male D2 mice, as BECs did not differ between these two drug groups. Mean $\pm$ SEM BECs were 1.83 ± 0.05 mg/mL (n=8) and 1.74 ± 0.13 mg/mL (n=7) across the 72 h of ethanol vapor exposure for the mice that were subsequently injected with GAN and VEH, respectively. Additionally, baseline HICs did not differ in the male D2 mice that were later exposed to air- or ethanol-vapor or that were injected with GAN or VEH. As depicted in Figure 3, GAN significantly decreased hourly HICs and AUC across the 5 – 12 h time frame that encompassed the two injections of GAN or VEH in both the air controls and the mice undergoing withdrawal. Analysis of AUC 5 – 12 h revealed that there was a main effect of treatment [$F(1,26)=47.08$, $p<0.001$] and drug [$F(1,26)=37.98$, $p<0.001$], but no significant interaction between main effects. GAN suppressed AUC 5 – 12 h by 81% in the air-exposed mice and by 35% during ethanol withdrawal (Figure 3B). The analysis of AUC 5 – 7 h and AUC 9 – 12 h yielded similar results. Planned comparisons indicated that GAN significantly decreased HIC scores at 5.5h,

6h, 7h, 8h, 9h, 11 h, and 12 h in the air-exposed controls (Figure 3A). During ethanol withdrawal, GAN significantly decreased HIC scores at 5.5 h, 6h, 7h, 9.5h, 10h, and 11h.

In the female D2 mice, mean $\pm$ SEM BECs across the 72 h of ethanol vapor exposure were 1.83 ± 0.08 mg/mL (n=8) and 1.89 ± 0.03 mg/mL (n=8) for the GAN- and VEH-treated mice, respectively, indicating that ethanol exposure was comparable in these two groups. Baseline HICs also did not differ in the mice that were later exposed to air- or ethanol-vapor or that were injected with GAN or VEH. Figure 4 depicts the pattern of hourly HICs and AUC across the 5 – 12 h period following removal from the inhalation chambers at 72 h and following two injections of GAN. AUC 5 – 12 h was increased significantly during ethanol withdrawal compared to the air-controls [$F(1,28)=99.47$, $p<0.001$] and was significantly decreased by GAN injection [$F(1,28)=4.86$, $p<0.05$]. However, the interaction between treatment and drug was not significant, suggesting that GAN similarly influenced HIC and AUC in the ethanol- and air-exposed mice. GAN produced decreases in AUC 5 – 12 h of 70% and 21% in the air controls and the mice undergoing withdrawal, respectively (Figure 4B). The overall analysis of AUC 5 – 7 h and AUC 9 – 12 h yielded similar results to that of AUC 5 – 12 h. Planned comparisons in the air-exposed mice indicated there was a significant decrease in HICs in the GAN- vs VEH-treated mice at 6 h. This restriction of significance to a single time point is likely due to a floor effect created by the already low HICs following air-exposure in these mice. During ethanol withdrawal, GAN significantly reduced HICs at 5.5 h, 6 h, 9.5 h, 10 h, 11 h, and 12 h (Figure 4A).

Planned comparisons to evaluate sex differences in AUC 5 – 12 h confirmed the lower basal convulsive activity in the air-exposed female vs male D2 mice injected with VEH [$F(1,14)=11.13$, $p=0.005$]. However, the air-exposed mice did not differ in AUC 5 – 12 h following injection with GAN. During ethanol withdrawal, male and female D2 mice did not differ in AUC 5 – 12 h following injection with VEH or GAN.

Discussion

The present studies utilized male and female mice from two genotypes that display a high ethanol withdrawal phenotype, and they measured sensitivity to the anticonvulsant effect of GAN under control conditions and during ethanol withdrawal. Consistent with the potent anticonvulsant effect of GAN in diverse rodent models of epilepsy (see Reddy and Estes, 2016; Reddy and Rogawski, 2012 and references therein; also Carver and Reddy, 2016; Zolkowska et al., 2018), GAN administration produced a marked and significant anticonvulsant effect in control mice of both sexes and genotypes. However, there was no anticonvulsant effect of GAN during withdrawal in male WSP-1 mice, and GAN had extremely low efficacy during withdrawal in female WSP-1 mice, indicating that male and female WSP-1 mice were resistant to the anticonvulsant effect of GAN during withdrawal. In male and female D2 mice, GAN exerted a marked and significant anticonvulsant effect during withdrawal, but the duration of effect appeared to be shorter than in the controls. Our earlier work determined that mice from these two genotypes were resistant to the anticonvulsant effect of ALLO during ethanol withdrawal (Beckley et al., 2008; Finn et al., 2000, 2006), measured by a right-ward shift in the dose response for ALLO's anticonvulsant effect. Thus, use of a metabolically stable derivative of ALLO was able to overcome the

chronic ethanol and withdrawal-induced resistance to an endogenous GABA_AR-active neurosteroid (ALLO) that was observed previously in one of the two genotypes tested.

Both ALLO and GAN are very potent positive allosteric agonists at synaptic and extrasynaptic GABA_ARs (e.g., Belelli and Lambert, 2005; Finn and Purdy, 2007; Reddy and Estes, 2016), with low concentrations (i.e., nanomolar) potentiating GABA_AR-mediated currents, and high concentrations (i.e., micromolar) directly activating GABA_ARs. Additionally, endogenous neurosteroids such as ALLO are subject to oxidation at the C-3 position; this oxidation decreases bioavailability due to the back-conversion of ALLO to 5 α -dihydroprogesterone (5 α -DHP), which is a steroid with genomic effects at progesterone receptors but no activity at GABA_ARs (Rupprecht et al, 1993). Isoforms of the enzyme 3 α -hydroxysteroid dehydrogenase (3 α -HSD) are responsible for the conversion of 5 α -DHP to ALLO (and vice versa; Li et al., 1997), so the relative activity of the respective 3 α -HSD isoforms can influence the concentration of endogenous (or exogenously applied) neurosteroids that GABA_ARs are exposed to, and thereby play a crucial role in shaping inhibitory neurotransmission, as was observed in the dentate gyrus (Belelli and Herd, 2003). Notably, GAN overcomes the limitations of the lower bioavailability of endogenous neurosteroids by preventing the oxidation of the 3 α -hydroxyl group (Carter et al., 1997). A recent pharmacokinetic study indicated that peak brain concentrations of GAN following injection were significantly greater than brain ALLO levels, and that brain levels of both neurosteroids were approximately 3-fold greater than levels achieved in plasma (Zolkowska et al., 2018). And, while volume of distribution was similar for the two neurosteroids, the clearance of ALLO from the central vs peripheral component was more than 50% greater than the clearance of GAN, consistent with the extended half-life for GAN (Zolkowska et al., 2018). This higher bioavailability of GAN than ALLO likely contributed to the more potent anticonvulsant activity of GAN vs ALLO in many rodent seizure models (see Reddy and Estes, 2016; Reddy and Rogawski, 2012 and references therein; also Carver and Reddy, 2016). Therefore, we reasoned that GAN would exert an anticonvulsant effect during ethanol withdrawal unless the prior resistance to ALLO's anticonvulsant effect that was observed during withdrawal was due solely to a withdrawal-induced decrease in GABA_AR sensitivity to neurosteroids.

In male WSP-1 mice, the insensitivity to GAN when administered systemically during withdrawal is similar to our earlier report of insensitivity to ALLO's anticonvulsant effect during ethanol withdrawal in male WSP mice from both genetic replicates (WSP-1 and WSP-2, which were not studied here) when administered systemically (Finn et al., 2006) or via bilateral microinjection into the hippocampal CA1, VTA, or SNR (Gililand-Kaufman et al., 2008; Tanchuck et al., 2013) in male WSP-1 mice. The combined results suggest that ethanol withdrawal produces a decrease in functional sensitivity of GABA_ARs to neurosteroids in male WSP mice. Consistent with this interpretation, we found that ethanol withdrawal produced a rightward shift in the sensitivity of GABA_ARs to ALLO in a biochemical assay conducted in forebrain microsacs from male mice of both genetic replicates (Finn et al., 2006). Also, in our unpublished electrophysiological studies of hippocampal dentate gyrus granule cell (GC) GABA_AR currents from male WSP-1 mice, we found that ethanol withdrawal decreased presynaptic GABA release by a greater degree than GAN could increase GABA release (Mohr, Richardson, Finn, and Rossi, unpublished). The

presynaptic enhancement of vesicular GABA release by GAN in GCs is consistent with reports that physiologically relevant ALLO levels exert effects on presynaptic GABA_ARs to increase GABA release (e.g., Park et al., 2011; also reviewed in Herd et al., 2007), but our electrophysiological study suggests that it may not be enough to dampen hippocampal hyperexcitability during ethanol withdrawal in WSP-1 mice. Collectively, the withdrawal-induced reduction in GABA release and reduction in sensitivity of GABA_ARs to neurosteroids may explain the insensitivity to the anticonvulsant effect of ALLO and GAN during withdrawal in male WSP-1 mice.

Fewer studies have been conducted in female than in male WSP-1 mice. Our prior study determined that female WSP-1 mice exhibited resistance to the anticonvulsant effect of ALLO during ethanol withdrawal (Beckley et al., 2008), similar to what we had observed in male WSP mice (Finn et al., 2006). In the present studies, we also observed similar anticonvulsant effects of GAN in controls and during ethanol withdrawal in the male and female WSP-1 mice, based on the % decrease in AUC 5 – 12 h (compare Figures 1B and 2B). Despite some statistical differences between the sexes, there were very similar reductions in efficacy of GAN during withdrawal (only a ↓ 8% in AUC in males; ↓ 13% in females), which we interpret as a minimally efficacious anticonvulsant effect of GAN during ethanol withdrawal and indicative of reduced sensitivity in the male and female WSP-1 mice. Taken in conjunction with the insensitivity to ALLO's anticonvulsant effect during withdrawal in males and females and with the mechanistic studies showing reduced GABA_AR function during withdrawal in males, we presume that functional sensitivity of GABA_ARs to ALLO and GAN also is decreased in female WSP-1 mice during withdrawal. Unfortunately, the WSP replicate lines are now available only as cryopreserved embryos, so mechanistic studies in female WSP-1 mice are not immediately possible.

In male and female D2 mice, GAN exerted a significant anticonvulsant effect during ethanol withdrawal, although the AUC 5 – 12 h results suggest that efficacy was reduced when compared to the controls. One interpretation of a decrease in efficacy is that some resistance had occurred to the GAN dose that was administered. However, the reduction in efficacy appeared to be due in part to a robust anticonvulsant effect of GAN during withdrawal, but of a shorter duration than in the controls, according to the pattern of hourly HICs results (see Figures 3A and 4A). Importantly, the significant anticonvulsant effect of GAN following both injections in D2 mice is consistent with the report that tolerance to GAN's anticonvulsant effect did not develop following repeated administration (Reddy and Rogawski, 2000). In contrast, early work determined that male D2 mice were resistant to the anticonvulsant effect of ALLO during ethanol withdrawal (females not tested; Finn et al., 2000). Plasma ALLO levels did not differ in the ethanol- vs air-exposed mice following injection of the ALLO doses (3.2, 10 or 17 mg/kg; Finn et al., 2000), indicating that a withdrawal-induced change in ALLO bioavailability or metabolism did not contribute to the resistance to ALLO's anticonvulsant effect during ethanol withdrawal. Mechanistic studies have not been conducted in this genotype, so the potential contribution of a withdrawal-induced change in GABA_AR function to the present findings is not known. Collectively, the significant anticonvulsant effect of GAN during withdrawal suggests that withdrawal did not produce a marked decrease in GABA_AR sensitivity to neurosteroids in D2 mice.

The present studies were conducted in intact female mice. Although hormone levels or stage of estrous cycle were not assessed in the current study, our prior work that measured progesterone and estradiol levels after 72 h ethanol vapor or air exposure suggested that female WSP-1 mice were in the late diestrus phase of the estrous cycle during withdrawal and following air exposure (Beckley et al., 2008). A subsequent study in WSP females determined that 72 h chronic ethanol vapor exposure disrupted normal cycling so that females remained in diestrus during withdrawal, but controls continued to cycle (Forquer et al., 2011). Similarly, chronic intermittent ethanol vapor exposure or consumption of an ethanol containing liquid diet abolished estrous cycling in female C3H/HeCr mice and Sprague-Dawley rats, respectively, with female rodents remaining in diestrus during withdrawal and 1 – 2 weeks of abstinence (Rettori et al., 1987; Veatch et al., 2007). As a result, we presume that female mice in the present studies were in diestrus during withdrawal, but that the control females continued to cycle normally. Notably, convulsive activity in the control females was lower than in control males (compare Figures 1A vs 2A and 3A vs 4A), which may be due in part to the reported higher endogenous levels of GABA_AR-active neurosteroids such as ALLO in female vs male rodents (e.g., Finn et al., 2004b; reviewed in Finn and Purdy, 2007; Paul and Purdy, 1992). Consistent with this idea, preliminary results suggest that cortical and hippocampal ALLO levels in control WSP-1 females (JP Jensen, MA Nipper, and DA Finn, unpublished) were higher than levels in control WSP-1 males (Jensen et al., 2017), but comparable studies in female D2 mice have not been conducted. Nonetheless, higher endogenous neurosteroid levels in female vs male mice may contribute to the lower convulsive activity in female vs male air controls, but the similar GAN results between the male and female mice suggest that it is unlikely that potential estrous cycle-related differences in treatment groups (i.e., air vs ethanol) contributed to the present findings related to changes in GAN sensitivity.

In conclusion, several lines of evidence indicate that the synthetic neurosteroid GAN is a promising compound for the treatment of ethanol withdrawal-related convulsions. First, GAN exerted a significant anticonvulsant effect during withdrawal in D2 mice. The different results between D2 and WSP-1 mice suggests that distinct GABA_AR mechanisms influence high withdrawal, which would be consistent with the many other differences that exist between these genotypes. Directional selection pressure was used to maximize withdrawal convulsive activity in the WSP selected line, making WSP mice a true genetic animal model of high withdrawal, whereas high withdrawal is one of many characteristics of D2 mice that arose fortuitously during inbreeding and make them different from other inbred strains. Additionally, D2 mice were sensitive to the ability of steroid replacement and metabolism to GABA_AR-active neurosteroids to rescue high acute ethanol withdrawal that was exacerbated by adrenalectomy and gonadectomy (Kaufman et al., 2010), suggesting that the insensitivity of WSP mice to neurosteroids during withdrawal is unique to that genotype. Second, rodents that display mild withdrawal (e.g., B6 mice and rats) exhibit enhanced sensitivity to the anticonvulsant effect of ALLO during withdrawal (Cagetti et al., 2004; Devaud et al., 1995, 1996; Finn et al., 2000), which corresponded to a leftward shift in functional sensitivity of GABA_ARs to ALLO in rats (Devaud et al., 1996). Although GAN has not been tested in these animals, the results with ALLO suggest that GAN will exert a highly efficacious and potent anticonvulsant effect during withdrawal in rodents that exhibit mild or moderate

withdrawal. Finally, GAN is being investigated in many advanced clinical trials for the treatment of various forms of epilepsy, with promising results (see Reddy and Estes, 2016; Reddy and Rogawski, 2012 and references therein). Because seizure activity can propagate across neural circuits, neurosteroids may disrupt seizure activity by augmenting tonic inhibition within the network path, in addition to potentiating phasic inhibition. Thus, it is likely that the positive modulatory effect of neurosteroids on tonic inhibition mediated by extrasynaptic GABA_ARs containing δ subunits (Carver and Reddy, 2016) can exert a significant influence on network excitability and seizure activity, including during withdrawal.

Acknowledgements

This research was supported by USPHS grants RO1 AA012439 (DAF and DJR, MPIs) and P60 AA10760 (JCC, CoI) from the National Institute on Alcohol Abuse and Alcoholism and by VA Merit Review grant BX000313 from the US Department of Veterans Affairs (JCC). The WSP-1 line was supported by R24 AA020245 and VA Merit Review grant BX000313 (JCC), but this line has now been cryopreserved. This material also is the result of work supported with resources and the use of facilities at the VA Portland Health Care System (DAF, JCC).

References

- Ballenger JC, Post RM (1978) Kindling as a model for alcohol withdrawal syndromes. *Br J Psychiatry* 133:1–14. [PubMed: 352467]
- Beattie MC, Maldonado-Devicci AM, Porcu P, O'Buckley TK, Daunais JB, Grant KA, Morrow AL (2017) Voluntary ethanol consumption reduces GABAergic neuroactive steroid (3 α ,5 α)3-hydroxypregnan-20-one (3 α ,5 α -THP) in the amygdala of the cynomolgus monkey. *Addiction Biol* 22:318–330.
- Beckley EH, Fretwell AM, Tanchuck MA, Gililand KR, Crabbe JC, Finn DA (2008) Decreased anticonvulsant efficacy of allopregnanolone during ethanol withdrawal in female Withdrawal Seizure-Prone vs. Withdrawal Seizure-Resistant mice. *Neuropharmacology* 54:365–374. [PubMed: 18045626]
- Belelli D, Herd M (2003). The contraceptive agent Provera enhances GABA_A receptor-mediated inhibitory neurotransmission in the rat hippocampus: Evidence for endogenous neurosteroids? *J Neurosci* 23:10013–10020. [PubMed: 14602815]
- Belelli D, Lambert JJ (2005) Neurosteroids: Endogenous regulators of the GABA_A receptor. *Nature Rev Neurosci* 6:565–575. [PubMed: 15959466]
- Belelli D, Lan NC, Gee KW (1990) Anticonvulsant steroids and the GABA/benzodiazepine receptor-chloride ionophore complex. *Neurosci Biobehav Rev* 14:315–322. [PubMed: 2172879]
- Cagetti E, Pinna G, Guidotti A, Baisey K, Olsen RW (2004) Chronic intermittent ethanol (CIE) administration in rats decreases levels of neurosteroids in hippocampus, accompanied by altered behavioral responses to neurosteroids and memory function. *Neuropharmacology* 46:570–579. [PubMed: 14975681]
- Carter RB, Wood PL, Wieland S, Hawkinson JE, Belelli D, Lambert JJ, White HS, Wolf HH, Mirsadeghi S, Tahir SH, Bolger MB, Lan NC, Gee KW (1997) Characterization of the anticonvulsant properties of ganaxolone (CCD 1042; 3 α -hydroxy-3 β -methyl-5 α -pregnan-20-one), a selective, high-affinity, steroid modulator of the γ -aminobutyric acid_A receptor. *J Pharmacol Exp Ther* 280:1284–1295. [PubMed: 9067315]
- Carver CM, Reddy DS (2013) Neurosteroid interactions with synaptic and extrasynaptic GABA_A receptors: regulation of subunit plasticity, phasic and tonic inhibition, and neuronal network excitability. *Psychopharmacology* 230:151–188. [PubMed: 24071826]
- Carver CM, Reddy DS (2016) Neurosteroid structure-activity relationships for functional activation of extrasynaptic δ GABA_A receptors. *J Pharmacol Exp Ther* 357:188–204. [PubMed: 26857959]

- Covault J, Pond T, Feinn R, Arias AJ, Oncken C, Kranzler HR (2014) Dutasteride reduces alcohol's sedative effects in men in a human laboratory setting and reduces drinking in the natural environment. *Psychopharmacology* 231:3609–3618. [PubMed: 24557088]
- Crabbe JC (1998) Provisional mapping of quantitative trait loci for chronic ethanol withdrawal severity in BXD recombinant inbred mice. *J Pharmacol Exp Ther* 286:263–271. [PubMed: 9655868]
- Crabbe JC, Kosobud A, Young ER, Tam BR, McSwigan JD (1985) Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus*. *Behav Genet* 15:521–536. [PubMed: 4096679]
- Devaud LL, Purdy RH, Morrow AL (1995) The neurosteroid, 3 α -hydroxy-5 α -pregnan-20-one, protects against bicuculline-induced seizures during ethanol withdrawal in rats. *Alcohol Clin Exp Res* 19:350–355. [PubMed: 7625568]
- Devaud LL, Purdy RH, Finn DA, Morrow AL (1996) Sensitization of γ -aminobutyric acid_A receptors to neuroactive steroids in rats during ethanol withdrawal. *J Pharmacol Exp Ther* 278:510–517. [PubMed: 8768698]
- Devaud LL, Risinger FO, Selvage D (2006) Impact of the hormonal milieu on the neurobiology of alcohol dependence and withdrawal. *J Gen Psychol* 133:337–356. [PubMed: 17128955]
- Duka T, Gentry J, Malcolm R, Ripley TL, Borlikova G, Stephens DN, Veatch LM, Becker HC, Crews FT (2004) Consequences of multiple withdrawals from alcohol. *Alcohol Clin Exp Res* 28:233–246. [PubMed: 15112931]
- Finn DA, Crabbe JC (1999) Chronic ethanol differentially alters susceptibility to chemically-induced convulsions in Withdrawal Seizure-Prone and -Resistant mice. *J Pharmacol Exp Ther* 288:782–790. [PubMed: 9918589]
- Finn DA, Purdy RH (2007) Neuroactive steroids in anxiety and stress In: Sibley DR, Hanin I, Kuhar M, Skolnick P (eds) *Handbook of Contemporary Neuropharmacology, vol 2* John Wiley & Sons, Inc, New Jersey, pp 133–176.
- Finn DA, Gallaher EJ, Crabbe JC (2000) Differential change in neuroactive steroid sensitivity during ethanol withdrawal. *J Pharmacol Exp Ther* 292:394–405. [PubMed: 10604976]
- Finn DA, Ford MM, Wiren KM, Roselli CE, Crabbe JC (2004a) The role of pregnane neurosteroids in ethanol withdrawal: behavioral genetic approaches. *Pharmacol Ther* 101:91–112. [PubMed: 14761701]
- Finn DA, Sinnott RS, Ford MM, Long SL, Tanchuck MA, Phillips TJ (2004b) Sex differences in the effect of ethanol injection and consumption on brain allopregnanolone levels in C57BL/6 mice. *Neuroscience* 123:813–819. [PubMed: 14751275]
- Finn DA, Douglass AD, Beadles-Bohling AS, Tanchuck MA, Long SL, Crabbe JC (2006) Selected line difference in sensitivity to a GABAergic neurosteroid during ethanol withdrawal. *Genes Brain Behav* 5:53–63. [PubMed: 16436189]
- Finn DA, Snelling C, Fretwell AM, Tanchuck MA, Underwood L, Cole M, Crabbe JC, Roberts AJ (2007) Increased drinking during withdrawal from intermittent ethanol exposure is blocked by the CRF receptor antagonist D-Phe-CRF(12–41). *Alcohol Clin Exp Res* 31:939–949. [PubMed: 17403068]
- Finn DA, Beckley EH, Kaufman KR, Ford MM (2010) Manipulation of GABAergic steroids: Sex differences in the effects on alcohol drinking- and withdrawal-related behaviors. *Horm Behav* 57:12–22. [PubMed: 19615369]
- Follesa P, Biggio F, Talani G, Murru L, Serra M, Sanna E, Biggio G (2006) Neurosteroids, GABA_A receptors, and ethanol dependence. *Psychopharmacology* 186:267–280. [PubMed: 16163526]
- Forquer MR, Hashimoto JG, Roberts ML, Wiren KM (2011) Elevated testosterone in females reveals a robust sex difference in altered androgen levels during chronic alcohol withdrawal. *Alcohol* 45:161–171. [PubMed: 20843636]
- Gee KW, Bolger MB, Brinton RE, Coirini H, McEwen BS (1988) Steroid modulation of the chloride ionophore in rat brain: Structure-activity requirements, regional dependence and mechanism of action. *J Pharmacol Exp Ther* 246:803–812. [PubMed: 2841455]
- Gililand-Kaufman KR, Tanchuck MA, Ford MM, Crabbe JC, Beadles-Bohling AS, Snelling C, Mark GP, Finn DA (2008) The neurosteroid environment in the hippocampus exerts bi-directional effects on seizure susceptibility in mice. *Brain Res* 1243:113–123. [PubMed: 18840414]

- Gorin RE, Crabbe JC, Tanchuck MA, Long SL, Finn DA (2005) Effects of finasteride on chronic and acute ethanol withdrawal severity in the WSP and WSR selected lines. *Alcohol Clin Exp Res* 29:939–948. [PubMed: 15976519]
- Grobin AC, Matthews DB, Devaud LL, Morrow AL (1998) The role of GABA_A receptors in the acute and chronic effects of ethanol. *Psychopharmacology* 139:2–19. [PubMed: 9768538]
- Heilig M, Egli M, Crabbe JC, Becker HC (2010) Acute withdrawal, protracted abstinence and negative affect in alcoholism: are they linked? *Addiction Biol* 15:169–184.
- Herd MB, Belelli D, Lambert JJ (2007) Neurosteroid modulation of synaptic and extrasynaptic GABA_A receptors. *Pharmacol Ther* 116:20–34. [PubMed: 17531325]
- Hill M, Popov P, Havlíková H, Kancheva L, Vrbíková J, Kancheva R, Pouzar V, erný I, Stárka L (2005) Altered profiles of serum neuroactive steroids in premenopausal women treated for alcohol addiction. *Steroid* 70:515–524.
- Jensen JP, Nipper MA, Helms ML, Ford MM, Crabbe JC, Rossi DJ, Finn DA (2017) Ethanol withdrawal-induced dysregulation of neurosteroid levels in plasma, cortex, and hippocampus in genetic animal models of high and low withdrawal. *Psychopharmacology* 234:2793–2811. [PubMed: 28664280]
- Kaufman KR, Tanchuck MA, Strong MN, Finn DA (2010) Replacement with GABAergic steroid precursors restores the acute ethanol withdrawal profile in ADX/GDX mice. *Neuroscience* 166:5–14. [PubMed: 20004707]
- Kendler KS, Aggen SH, Prescott CA, Crabbe J, Neale MC (2012) Evidence for multiple genetic factors underlying the DSM-IV criteria for alcohol dependence. *Mol Psychiatry* 17:1306–1315. [PubMed: 22105626]
- Kosobud A, Crabbe JC (1986) Ethanol withdrawal in mic bred to be genetically prone or resistant to ethanol withdrawal seizures. *J Pharmacol Exp Ther* 238:170–177. [PubMed: 3723396]
- Kumar S, Porcu P, Werner DF, Matthews DB, Diaz-Granados JL, Helfand RS, Morrow AL (2009) The role of GABA_A receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology* 205:529–564. [PubMed: 19455309]
- Li X, Bertics PJ, Karavolas HJ (1997) Regional distribution of cytosolic and particulate 5 α -dihydroprogesterone 3 α -hydroxysteroid oxidoreductases in female rat brain. *J Steroid Biochem Mol Biol* 60:311–318. [PubMed: 9219922]
- Liang J, Cagetti E, Olsen RW, Spigelman I (2004) Altered pharmacology of synaptic and extrasynaptic GABA_A receptors on CA1 hippocampal neurons is consistent with subunit changes in a model of alcohol withdrawal and dependence. *J Pharmacol Exp Ther* 310:1234–1245. [PubMed: 15126642]
- Liang J, Zhang N, Cagetti E, Houser CR, Olsen RW, Spigelman I (2006) Chronic intermittent ethanol-induced switch of ethanol actions from extrasynaptic to synaptic hippocampal GABA_A receptors. *J Neurosci* 26:1749–1758. [PubMed: 16467523]
- Liang J, Spigelman I, Olsen RW (2009) Tolerance to sedative/hypnotic actions of GABAergic drugs correlates with tolerance to potentiation of extrasynaptic tonic currents of alcohol-dependent rats. *J Neurophysiol* 102:224–233. [PubMed: 19420124]
- Maldonado-Devincci AM, Cook JB, O'Buckley TK, Morrow DH, McKinley RE, Lopez MF, Becker HC, Morrow AL (2014) Chronic intermittent ethanol exposure and withdrawal alters (3 α ,5 α)-3-hydroxy-pregnan-20-one immunostaining in cortical and limbic brain regions of C57BL/6J mice. *Alcohol Clin Exp Res* 38:2561–2571. [PubMed: 25293837]
- McCool BA, Chappell AM (2015) Chronic intermittent ethanol inhalation increases ethanol self-administration in both C57BL/6J and DBA/2J mice. *Alcohol* 49:111–120. [PubMed: 25659650]
- McKeon A, Frye MA, Delanty N (2008) The alcohol withdrawal syndrome. *J Neurol Neurosurg Psychiatry* 79:854–862. [PubMed: 17986499]
- Metten P, Crabbe JC (2005) Alcohol withdrawal severity in inbred (*Mus musculus*) strains. *Behav Neurosci* 119:911–925. [PubMed: 16187819]
- Metten P, Sorensen ML, Cameron AJ, Yu C-H, Crabbe JC (2010) Withdrawal severity after chronic intermittent ethanol in inbred mouse strains. *Alcohol Clin Exp Res* 34:1552–1564. [PubMed: 20586758]

- Morrow AL, Suzdak PD, Paul SM (1987) Steroid hormone metabolites potentiate GABA receptor-mediated chloride ion flux with nanomolar potency. *Eur J Pharmacol* 142:483–485. [PubMed: 2828079]
- Morrow AL, Porcu P, Boyd KN, Grant KA (2006) Hypothalamic-pituitary-adrenal axis modulation of GABAergic neuroactive steroids influences ethanol sensitivity and drinking behavior. *Dialogues Clin Neurosci* 8:463–477. [PubMed: 17290803]
- National Institute on Alcohol Abuse and Alcoholism (2017) Alcohol Facts and Statistics. <https://pubs.niaaa.nih.gov/publications/AlcoholFacts&Stats/AlcoholFacts&Stats.htm>, downloaded 11/27/2017.
- National Research Council of the National Academies (2011) Guide for the Care and Use of Laboratory Animals, Eighth Edition. The National Academies Press: Washington, DC.
- Park H-M, Choi I-S, Nakamura M, Cho J-H, Lee M-G, Jang I-S (2011) Multiple effects of allopregnanolone on GABAergic responses in single hippocampal CA3 pyramidal neurons. *Eur J Pharmacol* 652:46–54. [PubMed: 21118679]
- Paul SM, Purdy RH (1992) Neuroactive steroids. *FASEB J* 6:2311–2322. [PubMed: 1347506]
- Porcu P, Morrow AL (2014) Divergent neuroactive steroid responses to stress and ethanol in rat and mouse strains: relevance for human studies. *Psychopharmacology* 231:3257–3272. [PubMed: 24770626]
- Porcu P, Barron AM, Frye CA, Walf AA, Yang S-Y, He X-Y, Morrow AL, Panzica GC, Melcangi RC (2016) Neurosteroidogenesis today: Novel targets for neuroactive steroid synthesis and action and their relevance for translational research. *J Neuroendocrinology* 28:12351. [PubMed: 26681259]
- Purdy RH, Morrow AL, Blinn JR, Paul SM (1990) Synthesis, metabolism, and pharmacological activity of 3 α -hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptoneurosomes. *J Med Chem* 33:1572–1581. [PubMed: 2160534]
- Reddy DS, Estes WA (2016) Clinical potential of neurosteroids for CNS disorders. *Trends Pharmacol Sci* 37:543–561. [PubMed: 27156439]
- Reddy DS, Rogawski MA (2000) Chronic treatment with the neuroactive steroid ganaxolone in the rat induces anticonvulsant tolerance to diazepam but not to itself. *J Pharmacol Exp Ther* 295:1241–1248. [PubMed: 11082461]
- Reddy DS, Rogawski MA (2012) Neurosteroids – Endogenous regulators of seizure susceptibility and role in the treatment of epilepsy In: Jasper's Basic Mechanisms of the Epilepsies [Internet], 4th edition (Noebels JL, Avoli M, Rogawski MA et al., eds). Bethesda (MD): National Center for Biotechnology Information (US).
- Rettori V, Skelley CW, McCann SM, Dees WL (1987) Detrimental effects of short-term ethanol exposure on reproductive function in the female rat. *Biol Reprod* 37:1089–1096. [PubMed: 2832006]
- Roberto M, Varodayan FP (2017) Synaptic targets: Chronic alcohol actions. *Neuropharmacology* 122:85–99. [PubMed: 28108359]
- Romeo E, Brancati A, De Lorenzo A, Fucci P, Furnari C, Pompili E, Sasso GF, Spalletta G, Troisi A, Pasini A (1996) Marked decrease of plasma neuroactive steroids during alcohol withdrawal. *Clin Neuropharmacol* 19:366–369. [PubMed: 8829001]
- Rupprecht R, Holsboer F (1999) Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci* 22:410–416. [PubMed: 10441302]
- Rupprecht R, Reul JMHM, Trapp T, van Steensel B, Wetzel C, Damm K, Ziegglänsberger W, Holsboer F (1993) Progesterone receptor-mediated effects of neuroactive steroids. *Neuron* 11:523–530. [PubMed: 8398145]
- Saitz R (1998) Introduction to alcohol withdrawal. *Alcohol Health Res World* 22:5–12. [PubMed: 15706727]
- Sanna E, Talani G, Busonero F, Pisu MG, Purdy RH, Serra M, Biggio G (2004) Brain steroidogenesis mediates ethanol modulation of GABAA receptor activity in rat hippocampus. *J Neurosci* 24:6521–6530. [PubMed: 15269263]
- Snelling C, Tanchuck-Nipper MA, Ford MM, Jensen JP, Cozzoli DK, Ramaker MJ, Helms M, Crabbe JC, Rossi DJ, Finn DA (2014) Quantification of ten neuroactive steroids in plasma in Withdrawal

- Seizure-Prone and -Resistant mice during chronic ethanol withdrawal. *Psychopharmacology* 231:3401–3414. [PubMed: 24871700]
- Spanagel R (2009) Alcoholism: A systems approach from molecular physiology to addictive behavior. *Physiol Rev* 89:649–705. [PubMed: 19342616]
- Tabakoff B, Hoffman PL (2013) The neurobiology of alcohol consumption and alcoholism: An integrated history. *Pharmacol Biochem Behav* 113:20–37. [PubMed: 24141171]
- Tanchuck MA, Long SL, Ford MM, Hashimoto J, Crabbe JC, Roselli CE, Wren KM, Finn DA (2009) Selected line difference in the effects of ethanol dependence and withdrawal on allopregnanolone levels and 5 α -reductase enzyme activity and expression. *Alcohol Clin Exp Res* 33:2077–2087. [PubMed: 19740134]
- Tanchuck MA, Cozzoli DK, He I, Kaufman KR, Snelling C, Crabbe JC, Mark GP, Finn DA (2013) Local changes in neurosteroid levels in the substantia nigra reticulata and the ventral tegmental area alter chronic ethanol withdrawal severity in male Withdrawal Seizure-Prone mice. *Alcohol Clin Exp Res* 37:784–793. [PubMed: 23278716]
- Terdal ES, Crabbe JC (1994) Indexing withdrawal in mice: matching genotypes for exposure in studies using ethanol vapor inhalation. *Alcohol Clin Exp Res* 18:542–547. [PubMed: 7943652]
- Veatch LM, Wright TM, Randall CL (2007) Only male mice show sensitization of handling-induced convulsions across repeated ethanol withdrawal cycles. *Alcohol Clin Exp Res* 31:477–485. [PubMed: 17295733]
- Veleiro AS, Burton G (2009) Structure-activity relationships of neuroactive steroids acting on the GABA_A receptor. *Curr Med Chem* 16:455–472. [PubMed: 19199916]
- Zolkowska D, Wu C-Y, Rogawski MA (2018) Intramuscular allopregnanolone and ganaxolone in a mouse model of treatment-resistant status epilepticus *Epilepsia* 2 17 [Epub ahead of print].
- Zorumski CF, Paul SM, Izumi Y, Covey DF, Mennerick S (2013) Neurosteroids, stress and depression: Potential therapeutic opportunities. *Neurosci Biobehav Rev* 37:109–122. [PubMed: 23085210]

Figure 1. Insensitivity to the anticonvulsant effect of ganaxolone (GAN) during ethanol (EtOH) withdrawal in male WSP-1 mice, measured by hourly handling-induced convulsions (HICs; panel A) and area under the withdrawal curve (AUC, panel B).

Mice were exposed to 72 h EtOH vapor or air, and HICs were measured over the time course of withdrawal. Immediately following the HIC measurement at 5 h and 9 h, mice received an injection of 10 mg/kg GAN or equivalent volume of vehicle (VEH; see arrows on figure). Hourly HIC scores (**panel A**) and AUC (**panel B**) were significantly higher in the EtOH vs air exposed mice ($p < 0.001$), and the significant interaction between GAN and EtOH treatment ($p < 0.001$) indicated that the anticonvulsant effect of GAN was significantly reduced during withdrawal. Values are the mean $\pm$ SEM for the number of animals in parentheses. In cases where the SEM is not visible, the value is contained within the symbol for the mean. **Panels A and B:** $^+p = 0.10$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs respective VEH treatment, planned comparisons (**panel A**) or post-hoc tests (**panel B**).

Figure 2. Minimal anticonvulsant effect of ganaxolone (GAN) during ethanol (EtOH) withdrawal in female WSP-1 mice, measured by hourly handling-induced convulsions (HICs; panel A) and area under the withdrawal curve (AUC, panel B).

Mice were treated as described in Figure 1. Hourly HICs (**panel A**) and AUC (**panel B**) were increased significantly during EtOH withdrawal ($p < 0.001$) and were decreased significantly by GAN injection ($p < 0.01$). The interaction between GAN and EtOH treatment was not significant, suggesting a similar anticonvulsant effect of GAN in the air- and EtOH-exposed mice. However, there was a marked reduction in efficacy during withdrawal. Depicted are the mean $\pm$ SEM for the number of mice in parentheses. In cases where the SEM is not visible, the value is contained within the symbol for the mean. Arrows indicate times when the mice were administered GAN or vehicle (VEH). **Panel A:** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs respective VEH treatment, planned comparisons. **Panel B:** ** $p < 0.01$ vs VEH, main effect of GAN.

Figure 3. Significant anticonvulsant effect of ganaxolone (GAN) in control male DBA/2J mice and during ethanol (EtOH) withdrawal, measured by hourly handling-induced convulsions (HICs; panel A) and area under the withdrawal curve (AUC, panel B). Mice were treated as described in Figure 1. Hourly HICs (**panel A**) and AUC (**panel B**) were increased significantly during EtOH withdrawal ($p < 0.001$) and were decreased significantly by GAN injection ($p < 0.001$). The lack of an interaction between GAN and EtOH treatment suggested that GAN exerted a similar anticonvulsant effect in the air- and EtOH-exposed mice, and the slight reduction in efficacy appeared due to a more rapid return to values in vehicle (VEH)-treated mice during EtOH withdrawal. Mice received 2 injections of GAN or VEH, separated by 4 h (see arrows). Values are the mean $\pm$ SEM for the number of mice in parentheses. In cases where the SEM is not visible, the value is contained within the symbol for the mean. **Panel A:** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs respective VEH (air) or GAN (EtOH) treatment, planned comparisons. **Panel B:** *** $p < 0.001$ vs VEH, main effect of GAN.

Figure 4. Despite low convulsive activity in control animals, there was a significant anticonvulsant effect of ganaxolone (GAN) in control female DBA/2J mice and during ethanol (EtOH) withdrawal, measured by hourly handling-induced convulsions (HICs; panel A) and area under the withdrawal curve (AUC, panel B).

Mice were treated as described in Figure 1. Hourly HIC (**panel A**) and AUC (**panel B**) were increased significantly during EtOH withdrawal ($p < 0.001$) and were decreased significantly by GAN injection ($p < 0.05$). The lack of an interaction between GAN and EtOH treatment suggested that GAN exerted a similar anticonvulsant effect in the air- and EtOH-exposed mice. Even with low convulsive activity in the controls, GAN suppressed HIC scores across time. During EtOH withdrawal, GAN had a pronounced anticonvulsant effect, and the more rapid return to values in vehicle (VEH)-treated mice likely contributed to the reduction in efficacy. Mice received 2 injections of GAN or VEH, separated by 4 h (see arrows). Values are the mean $\pm$ SEM for the number of mice in parentheses. In cases where the SEM is not visible, the value is contained within the symbol for the mean. **Panel A:** * $p < 0.05$, *** $p < 0.001$ vs respective VEH (air) or GAN (EtOH) treatment, planned comparisons. **Panel B:** * $p < 0.05$ vs VEH, main effect of GAN.