Published in final edited form as:

Alcohol Clin Exp Res. 2013 May ; 37(5): 784–793. doi:10.1111/acer.12027.

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

Michelle A. Tanchuck, B.S.^a, Debra K. Cozzoli, Ph.D.^a, Ingrid He^a, Katherine R. Kaufman, Ph.D.^a, Christopher Snelling, B.S.^a, John C. Crabbe, Ph.D.^{a,b}, Gregory P. Mark, Ph.D.^a, and Deborah A. Finn, Ph.D.^{a,b}

^aDepartment of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR 97239

^bPortland Alcohol Research Center, Department of Veterans Affairs Medical Research Portland, OR 97239

Abstract

Background—Allopregnanolone (ALLO) is a potent positive modulator of γ -aminobutyric acid_A receptors (GABA_ARs) that affects ethanol withdrawal. Finasteride (FIN), a 5 α -reductase inhibitor that blocks the formation of ALLO and other GABAergic neurosteroids, alters ethanol sensitivity. Recently, we found that Withdrawal Seizure-Prone mice from the first genetic replicate (WSP-1) exhibited behavioral tolerance to the anticonvulsant effect of intra-hippocampal ALLO during ethanol withdrawal and that intra-hippocampal FIN significantly increased ethanol withdrawal severity. The purpose of the present study was to determine whether neurosteroid manipulations in the substantia nigra reticulata (SNR) and ventral tegmental area (VTA) produced effects during ethanol withdrawal comparable to those seen with intra-hippocampal ALLO and FIN.

Methods—Male WSP-1 mice were surgically implanted with bilateral guide cannulae aimed at the SNR or VTA at two weeks prior to ethanol vapor or air exposure for 72 hrs. Initial studies examined the anticonvulsant effect of a single ALLO infusion (0, 100 or 400 ng/site) at a time corresponding to peak withdrawal in the air- and ethanol-exposed mice. Separate studies examined the effect of four FIN infusions (0 or 10 μ g/site/day) during the development of physical dependence on the expression of ethanol withdrawal.

Results—ALLO infusion exerted a potent anticonvulsant effect in ethanol-naïve mice, but a diminished anticonvulsant effect during ethanol withdrawal. Administration of FIN into the SNR exerted a delayed proconvulsant effect in ethanol-naïve mice, whereas infusion into the VTA increased ethanol withdrawal duration.

Conclusions—Activation of local GABA_ARs in the SNR and VTA via ALLO infusion is sufficient to exert an anticonvulsant effect in naïve mice and to produce behavioral tolerance to the anticonvulsant effect of ALLO infusion during ethanol withdrawal. Thus, ethanol withdrawal reduced sensitivity of GABA_ARs to GABAergic neurosteroids in two neuroanatomical substrates within the basal ganglia in WSP-1 male mice.

Keywords

allopregnanolone; finasteride; GABA; neuroactive steroid

Introduction

The neurosteroid allopregnanolone (ALLO) rapidly potentiates γ -aminobutyric acid_A receptor (GABA_AR) mediated inhibition, and endogenous levels increase during pregnancy and the estrous/menstrual cycle or following stress (reviewed in Finn et al., 2004) to concentrations that potentiate GABAergic inhibition *in vitro* (Belelli and Lambert, 2005). In conjunction with evidence that pharmacological manipulation of local endogenous neurosteroid levels alters GABAergic inhibitory neurotransmission (Belelli and Herd, 2003), it is likely that endogenous levels of ALLO and other GABAergic neurosteroids have physiological relevance via modulation of GABA_AR mediated inhibition (Gunn et al., 2011; Herd et al., 2007). Additionally, ALLO has a pharmacological profile similar to that of ethanol (e.g., anxiolytic, anticonvulsant, sedative), and manipulation of endogenous ALLO levels alters selective effects of ethanol (see reviews by Criswell and Breese, 2005; Gorin-Meyer et al., 2007; Kumar et al., 2009; Morrow et al., 1999). These findings are consistent with the idea that endogenous neurosteroid levels impact ethanol sensitivity.

The interaction of ethanol at GABA_ARs is integral for the development of tolerance to and dependence on ethanol and for the expression of withdrawal-related convulsive activity (e.g., Follés et al., 2006; Grobin et al., 1998; Kumar et al., 2009). Evidence indicates that the transition from acute ethanol intoxication to dependence and withdrawal involves plasticity of GABA_AR properties, including direct and indirect effects of ethanol on GABA_AR function and the synthesis of endogenous GABAergic neurosteroids (Kumar et al., 2009). We hypothesize that the modulatory effect of ALLO on ethanol withdrawal severity reflects a balance between alterations in local concentrations of ALLO at GABA_ARs and the concomitant change in GABA_AR sensitivity to ALLO during ethanol withdrawal (Finn et al., 2004). Decreases in ALLO concentrations following chronic ethanol exposure occur in rodents and humans (Cagetti et al., 2004; Finn et al., 2004; Hill et al., 2005; Romeo et al., 1996; Tanchuck et al., 2009), with an inverse relationship between endogenous ALLO levels and behavioral changes in excitability during ethanol withdrawal in alcoholic patients and in dependent mice with severe withdrawal genotypes (e.g., DBA/2 inbred strain and Withdrawal Seizure-Prone (WSP) selected line). The decrease in GABAergic neurosteroid levels corresponds to an increase in subjective ratings of anxiety and depression during days 4–5 of withdrawal (Hill et al., 2005; Romeo et al., 1996) and an increase in handling-induced convulsions (HICs) at hour 8 of withdrawal in DBA/2 and WSP mice (see Finn et al., 2004), when compared with respective non-withdrawing controls. Collectively, existing evidence suggests that the withdrawal-induced reduction in endogenous ALLO levels in rodents and humans has a physiologically relevant consequence.

Ethanol withdrawal also alters GABA_AR sensitivity to neurosteroids. Sensitivity to the anticonvulsant effect of ALLO and alphaxalone is enhanced in rats (Cagetti et al., 2004; Devaud et al., 1996) and in C57BL/6 mice (Finn et al., 2000) that exhibit mild withdrawal, but is reduced in mouse genotypes with severe withdrawal (DBA/2 and WSP; Finn et al., 2000, 2006b). Work in WSP-1 mice documents that intra-hippocampal ALLO produces a robust anticonvulsant effect in ethanol naïve mice that is absent during ethanol withdrawal, defining the development of behavioral tolerance. This result is mimicked by systemic ALLO administration and suggests mediation by activation of GABA_ARs in the CA1 region of the hippocampus (Gililand-Kaufman et al., 2008). Additionally, intra-CA1 finasteride

(FIN), a 5 α -reductase inhibitor that blocks the anticonvulsant effect of progesterone (e.g., Kokate et al., 1999), increases ethanol withdrawal severity when administered during the development of physical dependence (Gililand-Kaufman et al., 2008). Since 5 α -reductase is the rate-limiting enzyme in the metabolism of progesterone to ALLO (Finn et al., 2006a), the results suggest that selective reduction of GABAergic neurosteroids within the CA1 increases ethanol withdrawal severity.

The current investigation utilizes a genetic animal model of severe ethanol withdrawal (WSP mice) to examine neurosteroid interactions at GABA_ARs during ethanol withdrawal. Notably, animal models of withdrawal convulsive activity are clinically relevant, given that the temporal pattern of seizure susceptibility is similar in animals and humans (see Heilig et al., 2010). Withdrawal-induced seizure activity increases with successive withdrawal episodes in both humans and animals (e.g., Duka et al., 2004), and repeated withdrawals produce deficits in cognitive function and emotional processing (e.g., Stephens and Duka, 2008; Sullivan et al., 2000). Thus, severe withdrawal is a clinically relevant and significant phenotype, and the examination of mechanisms underlying severe withdrawal is crucial for understanding neuroplasticity across the withdrawal continuum from acute withdrawal through severe withdrawal and abstinence (Heilig et al., 2010).

The purpose of the present work was to manipulate GABAergic neurosteroid levels in the basal ganglia of WSP-1 mice and determine the impact on chronic ethanol withdrawal severity. Separate studies gave bilateral microinjections of ALLO or FIN into the substantia nigra reticulata (SNR) or ventral tegmental area (VTA). The SNR is important in the propagation of convulsive activity (e.g., Gale, 1988; Velíšková and Moshé, 2006), and both the SNR and VTA exhibit an activation in c-fos expression during acute (Kozell et al., 2005) and chronic (Chen et al., 2009; Olive et al. 2001) ethanol withdrawal. We predicted that WSP-1 male mice would exhibit behavioral tolerance to the anticonvulsant effect of intra-SNR and intra-VTA ALLO during ethanol withdrawal and that intra-SNR and intra-VTA FIN would enhance ethanol withdrawal severity.

Materials and Methods

Subjects

Drug naïve male WSP-1 mice, selectively bred for severe chronic ethanol withdrawal assessed by HICs following exposure to 72 hr ethanol vapor (Crabbe et al., 1985), were used in the present studies (Selection generation 26, Filial generations 115–118, 70–150 days old). Although there are two genetically independent WSP (WSP-1 and -2) replicate lines available, the present studies focused on WSP-1 mice in order to extend our published findings with CA1 microinjection of ALLO and FIN in male WSP-1 mice (Gililand-Kaufman et al., 2008).

Mice were housed (2 – 4/cage) in the Veterinary Medical Unit of the Portland VA Medical Center (Portland, Oregon) with *ad libitum* access to food and water and were acclimated to a 12:12 hour light dark cycle (lights on at 0600) prior to performing cannulation surgeries. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with NIH guidelines. Efforts were made to reduce the number of animals used and to minimize animal suffering, including the use of post-operative analgesics.

Cannulae Implantation

Two weeks prior to testing, mice were surgically implanted under isoflurane anesthesia with bilateral 26-gauge thin-walled stainless steel guide shafts aimed at the SNR or VTA using previously described procedures (Gililand-Kaufman et al., 2008). The cannulae were

implanted 3.5 mm below the skull surface (A –3.08 mm, L $\pm$ 1.5 mm) for the SNR, and 2.3 mm below the skull surface (A –3.6 mm, L $\pm$ 0.6 mm) for the VTA. Anterior, lateral and ventral coordinates are referenced to bregma, the midsagittal sinus and surface of the level skull (Paxinos and Franklin, 2001). Guide cannulae were kept patent with 28-gauge stainless steel stylets. The tips of the microinjectors extended 1.0 mm beyond the guide shaft to reach the SNR (V –4.5 mm) and 2.0 mm beyond the guide shaft to reach the VTA (V –4.3 mm).

Chronic Ethanol Exposure

All mice were exposed to 72 hrs of ethanol vapor or air by the Portland Alcohol Research Center Dependence Core using a well-documented protocol for the induction of physical dependence (Finn and Crabbe, 1999; Gililand-Kaufman et al., 2008; Metten and Crabbe, 2005). Tail blood samples (20 μ l) were collected from the ethanol exposed mice each day to determine blood ethanol concentration (BEC) by head space chromatography (Finn et al., 2007) and to adjust ethanol vapor concentrations to maintain optimal BEC (1.5 mg/ml).

Microinjections

Mice were gently restrained by hand, the microinjectors were lowered to the target site, and 200 nl of fluid was delivered over sixty sec (Gililand-Kaufman et al, 2008).

Drugs

ALLO was purchased from Dr. Robert Purdy (VA Research Foundation, San Diego, CA) and solubilized using 20% w/v 2-hydroxypropyl- β -cyclodextrin (β -cyclodextrin; Cargill, Incorporated; Minneapolis, MN). Separate groups of mice received a single infusion of ALLO (100 or 400 ng/200 nl/side) or vehicle (VEH, 200 nl/side, 20% β -cyclodextrin). These ALLO doses produce behavioral effects when infused into the VTA (Frye and Rhodes, 2006), CA1 (Gililand-Kaufman et al., 2008), or lateral ventricle of rodents (Ford et al., 2007). FIN (Sterealoids; Newport, RI) was prepared in 20% β -cyclodextrin (10 μ g/200 nl/side), and the dose was based on recent work indicating that local infusion of 10 μ g FIN reduced ALLO levels by 60 – 80% when administered into the VTA or hippocampus (Frye and Vongher, 2001; Rhodes and Frye, 2005a, 2005b). Pentylentetrazol (PTZ; Sigma-Aldrich, St. Louis, MO) was administered via timed tail vein infusion into a lateral tail vein (5 mg/ml in saline; 0.5 ml/min; Finn and Crabbe, 1999; Finn et al., 2006b; Gililand-Kaufman et al., 2008).

Rationale for Different Experimental Endpoints

We used two strategies to assess sensitivity to ALLO and FIN microinjections for several reasons. First, we had previously shown that the anticonvulsant effect of ALLO against withdrawal-induced HICs was transient (Finn et al., 1995). We subsequently determined that ALLO sensitivity during ethanol withdrawal was more easily quantified when the assessment was limited to a single time point and that the threshold dose of PTZ administered by timed tail vein infusion was a highly sensitive measure of sensitivity at a single time point of withdrawal (Beckley et al., 2008; Finn et al., 2000, 2006b; Gililand-Kaufman et al., 2008). Second, we recently found that the effect of FIN on ethanol withdrawal severity was best quantified by examining the withdrawal time course (Gililand-Kaufman et al., 2008). We also considered the half-life of FIN (4.7 – 7.1 hrs in humans; Finn et al., 2006a) and the fact that we were using FIN to indirectly decrease endogenous GABAergic neurosteroid levels during the development of physical dependence to assess the effect on the expression of withdrawal. Finally, these two strategies were highly successful in our recent comparable work with CA1 microinjections in WSP-1 male mice (Gililand-Kaufman et al., 2008).

Sensitivity to the Anticonvulsant Effect of Intra-SNR/MTA ALLO

At time frames corresponding to peak withdrawal following ethanol vapor exposure (5.5 – 9 hrs after removal from inhalation chambers; Finn and Crabbe, 1999; Metten and Crabbe, 2005), separate groups of air- and ethanol-exposed mice received a single intracranial infusion of ALLO or VEH at 5–15 minutes prior to timed tail vein infusion of PTZ. Microinjections for the different treatment and drug groups (air vs ethanol; VEH vs ALLO) were randomized across the 3.5 hr period of testing during withdrawal (Beckley et al., 2008; Finn et al., 2000, 2006b; Gililand-Kaufman et al., 2008). The latency for onset of each of the four endpoints that characterize PTZ-induced convulsions was recorded in seconds and converted to threshold dose (mg/kg): myoclonic twitch (MC twitch), face and forelimb clonus (FF clonus), running-bouncing clonus (RB clonus), and tonic hindlimb extension (THE). Mice were immediately euthanized upon exhibiting THE. Brains were collected to verify cannulae placements.

The Effect of Intra-SNR/MTA FIN Administered During the Development of Physical Dependence on Convulsion Severity

Separate groups of air- and ethanol-exposed mice received a total of 4 daily infusions of FIN or VEH administered approximately 24 hours prior to, and each day of, exposure to ethanol vapor or air. Prior to the first infusion of FIN or VEH, baseline HIC was measured. Immediately following removal from the inhalation chambers at 72 hrs, HICs were scored hourly for the first 12 hrs and then at 24, 26, 28, and 48 hrs (Gililand-Kaufman et al., 2008; Metten and Crabbe, 2005). Animals were euthanized following the final HIC assessment, and brains were collected for determination of cannulae placement.

Data Analysis

Data from mice with correct probe placements were analyzed. Brains were collected from all animals, flash frozen in isopentane on dry ice, and sectioned at 40 μ m on a cryostat. Brain sections were mounted on superfrost slides, allowed to dry, and photographed for determination of placement hits/misses using a microscope at 1.25 $\times$. Confirmation of cannulae placements was checked by 2 – 3 reviewers, who were blind to treatment conditions.

For the ALLO study, analysis of variance (ANOVA) assessed treatment (ethanol vs air) and drug (ALLO doses vs VEH) effects on BEC and the PTZ threshold dose for onset to MC twitch, FF clonus, RB clonus, and THE. An increase in PTZ threshold dose over VEH indicated an anticonvulsant effect (or decreased sensitivity to PTZ), whereas a relative decrease in PTZ threshold dose would indicate a proconvulsant effect (or increased sensitivity to PTZ). For the FIN study, withdrawal severity was assessed by calculating the area under the withdrawal curve (AUC) and peak withdrawal. AUC was calculated as the summation of HIC scores across the 48 hour period using the trapezoidal method, as previously described (Metten and Crabbe, 2005). ANOVA was used to assess drug (FIN dose vs VEH) effects on BEC. Based on our recent results with intra-CA1 FIN (Gililand-Kaufman et al., 2008), we predicted that FIN infusions would produce different effects on convulsive activity in air- and ethanol-exposed mice. Therefore, separate planned comparisons were conducted on the AUC data in the air- and ethanol-exposed mice (FIN vs VEH). And, since FIN microinjection was found to increase the duration of ethanol withdrawal (Gililand-Kaufman et al., 2008), separate analyses were conducted on the AUC data for hrs 0–24 and hrs 24–48. Data are expressed as the mean $\pm$ SEM. Statistical significance was set at $P = 0.05$.

Results

Distribution of Injector Placements

Microinjections were concentrated in the posterior region of the SNR (Figure 1A) or the VTA (Figure 1B). Of the six experimental passes conducted, the hit rate for successful bilateral infusions was 80.9% for the SNR and 83.6% for the VTA. This resulted in the elimination of 33 animals from the SNR studies and 27 animals from the VTA studies with missed microinjections.

Sensitivity to the Anticonvulsant Effect of ALLO Microinjection

We analyzed all four convulsion endpoints, because evidence indicates that separable and independent anatomical circuits mediate distinct components of convulsive behavior (discussed in Gale, 1988). In general, MC twitch and FF clonus are associated with forebrain neural circuits, with RB clonus and THE depending on hindbrain circuitry. Because early work indicated that GABAergic mechanisms within the midbrain were capable of suppressing both these distinct types of convulsive activity (Gale, 1988), we discuss all four convulsion endpoints.

Each ALLO dose plus VEH controls were tested in a separate experimental pass. Data for the VEH groups did not differ significantly and were collapsed for statistical analysis and presentation.

Intra-SNR ALLO

The mean $\pm$ SEM BEC upon removal from the inhalation chambers at 72 hrs was 1.51 ± 0.05 mg/ml ($n = 32$). There were no significant differences in BEC across ALLO doses.

The threshold dose of PTZ for onset to each convulsant endpoint is depicted in Figure 2. PTZ threshold dose was significantly lower in ethanol- vs air-exposed mice [$F_{(1,54-55)} = 163.16$, $P_s < 0.001$] for all four endpoints, indicative of enhanced sensitivity to PTZ during withdrawal. The significant interaction between treatment and ALLO dose [MC twitch and FF clonus: $F_{(2,55)} = 3.99$, $P_s < 0.05$; RB clonus and THE: $F_{(2,54)} = 7.83$, $P_s = 0.001$] confirmed that sensitivity to the anticonvulsant effect of ALLO differed in the air- vs ethanol-exposed mice.

In the air-exposed animals, ALLO exerted an anticonvulsant effect (Figure 2), measured by the significant increase in PTZ threshold dose for onset to RB clonus and THE [$F_{(2,25)} = 7.61$, $P_s = 0.003$] and trend for increase in PTZ dose for onset to MC twitch and FF clonus [$F_{(2,26)} = 2.90$, $P_s = 0.07$]. Post-hoc tests confirmed that the 100 ng ALLO dose significantly increased PTZ dose for onset to RB clonus and THE by 25–28%.

In contrast, sensitivity to the anticonvulsant effect of ALLO was markedly reduced during withdrawal (Figure 2). While ALLO infusion significantly altered the PTZ dose for onset to MC twitch and FF clonus [$F_{(2,29)} = 3.85$, $P_s < 0.05$], post-hoc tests revealed that the 400 ng dose significantly increased PTZ dose by 14–18%. ALLO did not significantly increase the PTZ dose for onset to RB clonus or THE.

Intra-VTA ALLO

The mean $\pm$ SEM BEC was 1.47 ± 0.10 mg/ml upon removal from the inhalation chambers at 72 hrs ($n=35$), but there were significant differences in BEC across ALLO doses [$F_{(2,32)} = 6.63$, $P < 0.01$]. Mean BEC in the animals that were infused with 400 ng ALLO (1.96 mg/ml) was significantly higher than in animals infused with VEH (1.37 mg/ml; $P < 0.02$) or 100 ng ALLO (1.17 mg/ml; $P < 0.01$). Because we found that withdrawal severity in WSP

mice was not markedly different following ethanol vapor exposures that yielded BECs ranging from 1.0 – 2.0 mg/ml (Finn and Crabbe, 1999; Terdal and Crabbe, 1994), we do not believe that differences in BEC across dose groups confounded the present results.

The threshold dose of PTZ for onset to each convulsion endpoint is depicted in Figure 3. PTZ dose was lower in ethanol- versus air-exposed mice [$F(1,79-81) = 14.92$, $P < 0.001$] for all four endpoints, indicative of enhanced sensitivity to PTZ during withdrawal. The significant interaction between treatment and ALLO dose for RB clonus and THE [$F(2,79) = 3.35$, $P < 0.05$] confirmed that the anticonvulsant effect of ALLO differed in the air- vs ethanol-exposed mice for these two endpoints.

In the air-exposed mice, ALLO exerted a significant anticonvulsant effect, measured by the significant increase in PTZ dose for onset to RB clonus and THE [$F(2, 48) = 4.31$, $P < 0.02$] (Figure 3C–D). Post-hoc tests revealed that the 400 ng ALLO dose significantly increased PTZ dose by 15–20%. In contrast, mice were insensitive to the anticonvulsant effect of ALLO during ethanol withdrawal.

Effect of FIN Microinjection on Convulsion Severity

One dose of FIN was tested, with two experimental passes for the VEH controls. Data for the VEH groups did not differ and were collapsed for statistical analysis and presentation.

Hourly HIC scores are shown in Figures 4A and 5A for illustrative purposes. AUC was used as the index of withdrawal severity. Statistical analyses of hourly HICs yielded similar results to the analysis of AUC and for simplicity are not shown.

Intra-SNR FIN

The mean $\pm$ SEM BEC upon removal from the inhalation chambers at 72 hrs was 1.73 ± 0.17 mg/ml ($n=39$), and there were no significant group differences.

Baseline HIC scores did not differ between groups that subsequently were assigned to the FIN vs VEH treatments or air vs ethanol exposure (Figure 4A). HIC scores were higher in the ethanol- vs air-exposed mice starting at 3–4 hr of withdrawal. Analysis of AUC 0–24 indicated that FIN did not alter AUC in the air- or ethanol-exposed mice (Figure 4B), but the analysis of AUC 24–48 indicated that FIN infusion significantly increased AUC only in the air-exposed mice [$F(1,38) = 5.21$, $P < 0.03$] (Figure 4C). Examination of peak withdrawal (hours 6–10 in this study) revealed that FIN significantly increased HICs when averaged across hrs 6–10 [$F(1,38) = 6.98$, $P = 0.01$] or when hrs 6–9 were analyzed separately (Figure 4A). These results suggest that the 10 μ g FIN dose enhanced convulsive activity in the air-exposed mice.

Intra-VTA FIN

The mean $\pm$ SEM BEC upon removal from the inhalation chambers at 72 hrs was 1.53 ± 0.09 mg/ml ($n = 25$), and there were no significant group differences.

Baseline HIC scores did not differ between groups that subsequently were assigned to the VEH vs FIN groups or air vs ethanol exposure (Figure 5A). HIC scores were higher in the ethanol- vs air-exposed mice starting at 3–4 hr of withdrawal. While FIN did not increase AUC 0–24 in the air- or ethanol-exposed mice (Figure 5B), FIN selectively increased AUC 24–48 only in the ethanol-exposed mice [$F(1,21) = 4.21$, $P = 0.05$]. For this study, peak withdrawal occurred during hrs 8–11, and FIN pretreatment tended to increase the average HIC during peak withdrawal in the ethanol-exposed mice [$F(1,23) = 3.90$, $P = 0.06$] (Figure 5A). Collectively, these results suggest that FIN increased ethanol withdrawal duration.

Discussion

Published data from our laboratory in genetic animal models of severe ethanol withdrawal found that male DBA/2J and male and female WSP-1 and WSP-2 mice exhibited behavioral tolerance to ALLO's anticonvulsant effect during ethanol withdrawal (Beckley et al., 2008; Finn et al., 2000, 2006b) and that the behavioral tolerance seen during withdrawal in male WSP-1 and WSP-2 mice corresponded to a right-ward shift in the dose-response efficacy of ALLO to potentiate GABA-stimulated chloride flux in forebrain microsacs (Finn et al., 2006b). Thus, the withdrawal-induced reduction in GABA_AR sensitivity to ALLO and in sensitivity to ALLO's anticonvulsant effect was a consistent finding across three genetic animal models of severe withdrawal and across male and female mice. Here, and in our earlier studies, we report brain regional differences in sensitivity to the anticonvulsant effect of ALLO microinjection in air-exposed mice (CA1 SNR>VTA). We also found that dependent WSP-1 mice exhibit behavioral tolerance to the anticonvulsant effect of ALLO microinjection into all three brain regions (Gililand-Kaufman et al., 2008; present results). Collectively, these results provide strong evidence for the sufficiency of the CA1 and SNR in mediating both the anticonvulsant effect of ALLO in ethanol-naïve mice and the behavioral tolerance to ALLO's anticonvulsant effect during ethanol withdrawal. There is weaker evidence for the involvement of the VTA. Based on consistent findings following systemic ALLO injection, we anticipate that future microinjection studies in a second genetic animal model of high withdrawal (i.e., WSP-2 or DBA/2J mice) and in female mice of these genotypes would confirm the current microinjection results in male WSP-1 mice and thereby increase the generalizability and impact of our results.

The brain regional differences in sensitivity to ALLO's anticonvulsant effect in the SNR vs VTA are likely due to their distinct impact on output of the basal ganglia (Figure 6), to brain regional differences in GABA_AR plasticity following chronic ethanol exposure, or both. The SNR is one of the two major output nuclei of the basal ganglia, with projections to superior colliculus (SC; tectum), brainstem nuclei (e.g., pedunculopontine nucleus, reticular formation) and thalamus and from the thalamus to the cortex (e.g., Gale, 1988; Yin and Knowlton, 2006). Considering the role of the projection from SNR to SC in the anticonvulsant action of intra-SNR ALLO microinjection, an activation of GABA_ARs in the SNR would suppress nigroreticular GABA activity and produce a net disinhibition of target neurons in the SC (Gale, 1988). The posterior region of the VTA projects to the substantia nigra compacta (Ferreira et al. 2008), which connects the striatum to the output nuclei of the basal ganglia through an indirect pathway via the subthalamic nucleus (Figure 6). Because our microinjections were concentrated in the posterior VTA, it is likely that activation of GABA_ARs in the posterior VTA affected basal ganglia output through this indirect pathway. Thus, one potential explanation for the greater sensitivity to ALLO's anticonvulsant effect in the SNR than the VTA may be the direct vs indirect effects, respectively, on GABA_AR mediated output of the basal ganglia.

Several factors can influence neurosteroid sensitivity of GABA_ARs, such as GABA_AR subunit composition, phosphorylation state of the GABA_AR, and local neurosteroid synthesis and metabolism (reviewed in Herd et al., 2007). Another potential explanation is that the ability of neurosteroids to increase GABA release (Herd et al., 2007) may differ in the SNR and VTA, which could affect GABA_AR sensitivity and/or desensitization. That is, receptor desensitization or internalization could explain the lack of sensitivity to the 400 ng dose in the SNR. Differences between SNR and VTA could be investigated in future studies.

The current results demonstrate that a history of chronic ethanol exposure reduces sensitivity to the anticonvulsant effect of ALLO microinjections within the SNR and VTA. Because chronic ethanol exposure and withdrawal produces many adaptations in GABA_AR subunit

expression and function (reviewed in Kumar et al., 2009), alterations in GABA_AR composition following chronic ethanol exposure may contribute to the withdrawal-induced reduction in ALLO sensitivity. In support of this theory, preliminary results in WSP-1 male mice indicate that ethanol withdrawal significantly increased midbrain (e.g., SNR and VTA) expression of the $\alpha 4$ subunit by 25% and decreased midbrain expression of the $\alpha 1$ and $\alpha 5$ subunits by 30 and 38%, respectively (Tanchuck et al., 2011). These preliminary findings are similar to reports of changes in GABA_AR subunits in other animal models of ethanol dependence and withdrawal (reviewed in Kumar et al., 2009). For example, a withdrawal-induced increase in hippocampal $\alpha 4$ expression was associated with decreased sensitivity to the hypnotic effect of the synthetic neurosteroid alphaxalone in rats (Cagetti et al., 2003). This is compatible with the idea that the withdrawal-induced increase in midbrain $\alpha 4$ expression in WSP mice (Tanchuck et al., 2011) was associated with behavioral tolerance to the anticonvulsant effect of ALLO during ethanol withdrawal. Interestingly, mice with a point mutation in the $\alpha 1$ gene do not experience the anticonvulsant effect of diazepam against PTZ convulsions (Rudolph et al., 1999), supporting the possibility that a withdrawal-induced decrease in expression of the $\alpha 1$ subunit could be an important neuroadaptation for reducing the efficacy of anticonvulsants such as ALLO. Regardless, additional studies are necessary to determine whether the reduction in GABA_AR sensitivity to ALLO's anticonvulsant effect during ethanol withdrawal in WSP mice (Beckley et al., 2008; Finn et al., 2006b; Gililand-Kaufman et al., 2008; present findings) is consistent with selective GABA_AR subunit changes.

Studies with FIN utilized a different endpoint for assessing the effects of this manipulation of endogenous neurosteroid levels on basal convulsive activity and ethanol withdrawal severity, namely the assessment of HICs over the 48 hr time course of withdrawal. The present findings revealed interesting brain regional differences, in that intra-SNR FIN produced a delayed increase in basal convulsive activity and intra-VTA FIN produced a delayed increase in ethanol withdrawal severity. While the mechanism for the brain regional differences is not known, one possibility is variations in local ALLO metabolism within these regions. Metabolic variation across subregions of the hippocampus (CA1 vs dentate gyrus) in rats revealed a divergence in neurosteroid tone that significantly impacted GABAergic inhibition (Belelli and Herd, 2003). Although speculative, it is possible that FIN produced a similar reduction in endogenous ALLO biosynthesis in the SNR and VTA, but that the subsequent metabolism of ALLO was differentially altered in air- and ethanol-exposed WSP-1 mice in the SNR and VTA. The net result of an increase in ALLO metabolism would be a decrease in GABAergic neurosteroid tone, hence decreased GABAergic inhibition that could have increased convulsive activity.

The change in convulsive activity in ethanol naïve mice following FIN infusion was less pronounced than that seen with ALLO infusion. An explanation for this difference could be secondary to the manner in which the local neurosteroid environment was manipulated, if, for example infusion of FIN decreases local basal endogenous ALLO levels, whereas infusion of ALLO increases ALLO to supra-physiological concentrations. Alternately, or in addition, the relative importance of GABA_AR mediated inhibition in the neural pathways that mediate HIC vs PTZ-induced convulsions may differ.

Although ALLO levels were not measured in the present studies, we believe that it is unlikely that the brain regional differences were due to a differential effectiveness of FIN to alter ALLO biosynthesis. Recent work has found that ethanol withdrawal did not significantly decrease midbrain 5 α -reductase levels in WSP mice, using both qRT-PCR (Tanchuck et al., 2009) and in situ hybridization (Roselli et al., 2011) techniques, suggesting that FIN would be equally effective at inhibiting 5 α -reductase activity in air- and ethanol-exposed mice in both brain regions. Also, other studies show that infusions of 10 μ g FIN into

the VTA or hippocampus reduced ALLO levels by 60–80% (Frye and Vongher, 2001; Rhodes and Frye, 2005a, 2005b). Thus, we presume that FIN produced a similar decrease in ALLO biosynthesis in SNR and VTA. Another consideration is that the FIN-induced inhibition of 5 α -reductase could alter levels of other 5 α -reduced steroids or could shift local steroid metabolism to favor the production of steroids with proconvulsant effects, such as corticosterone or estrogen. This possibility will be examined in future studies, as we are currently optimizing a procedure for the simultaneous quantification of nine neurosteroids and their precursor pregnenolone in dissected tissues using gas chromatography–mass spectrometry as recently described (Porcu et al., 2009).

In conclusion, the present and previous results confirm the reduced sensitivity of GABA_ARs to ALLO during ethanol withdrawal in brain regions important in the withdrawal seizure circuit in WSP-1 male mice. Future mechanistic studies examining the altered pharmacology of synaptic and extrasynaptic GABA_ARs in animal models of severe withdrawal will provide important information that will aid in our understanding of mechanisms fundamental to alcohol withdrawal and will help in the development of new strategies for the treatment of alcohol dependence.

Acknowledgments

This research was supported by USPHS grants AA12439 (DAF) and AA13519 (JCC) from the National Institute on Alcohol Abuse and Alcoholism and by grants from the Department of Veterans Affairs (DAF, JCC). Additionally, this material is the result of work supported with resources and the use of facilities at the Portland VA Medical Center (DAF, JCC). We thank the Portland Alcohol Research Center Dependence Core (funded by AA10760 to JCC) for their assistance with the induction of physical dependence.

References

- Beckley EH, Fretwell AM, Tanchuck MA, Gililand KR, Crabbe JC, Finn DA. Decreased anticonvulsant efficacy of allopregnanolone during ethanol withdrawal in female Withdrawal Seizure-Prone vs. Withdrawal Seizure-Resistant mice. *Neuropharmacology*. 2008; 54:365–374. [PubMed: 18045626]
- Belelli D, Herd MB. The contraceptive agent Provera enhances GABA_A receptor-mediated inhibitory neurotransmission in the rat hippocampus: Evidence for endogenous neurosteroids? *J Neurosci*. 2003; 23:10013–10020. [PubMed: 14602815]
- Belelli D, Lambert JJ. Neurosteroids: Endogenous regulators of the GABA_A receptor. *Nature Rev Neurosci*. 2005; 6:565–575. [PubMed: 15959466]
- Cagetti E, Liang J, Spigelman I, Olsen RW. Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of GABA_A receptors. *Mol Pharmacol*. 2003; 63:53–64. [PubMed: 12488536]
- Cagetti E, Pinna G, Guidotti A, Baicy K, Olsen RW. Chronic intermittent ethanol (CIE) administration in rats decreases levels of neurosteroids in hippocampus, accompanied by altered behavioral responses to neurosteroids and memory function. *Neuropharmacology*. 2004; 46:570–579. [PubMed: 14975681]
- Chen G, Reilly MT, Kozell LB, Hitzemann R, Buck KJ. Differential activation of limbic circuitry associated with chronic ethanol withdrawal in DBA/2J and C57BL/6J mice. *Alcohol*. 2009; 43:411–420. [PubMed: 19801271]
- Crabbe JC, Kosobud A, Young ER, Tam BR, McSwigan JD. Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus*. *Behav Genet*. 1985; 15:521–536. [PubMed: 4096679]
- Criswell HE, Breese GR. A conceptualization of integrated actions of ethanol contributing to its GABA_Amimetic profile: A commentary. *Neuropsychopharmacology*. 2005; 30:1407–1425. [PubMed: 15856077]

- Devaud LL, Purdy RH, Finn DA, Morrow AL. Sensitization of γ -aminobutyric acid_A receptors to neuroactive steroids in rats during ethanol withdrawal. *J Pharmacol Exp Ther*. 1996; 278:510–517. [PubMed: 8768698]
- Duka T, Gentry J, Malcolm R, Ripley TL, Borlikova G, Stephens DN, Veatch LM, Becker HC, Crews FT. Consequences of multiple withdrawals from alcohol. *Alcohol Clin Exp Res*. 2004; 28:233–246. [PubMed: 15112931]
- Ferreira JGP, Del-Fava F, Hasue RH, Shammah-Lagnado SJ. Organization of ventral tegmental area projections to the ventral tegmental area-nigral complex in the rat. *Neuroscience*. 2008; 153:196–213. [PubMed: 18358616]
- Finn DA, Crabbe JC. Chronic ethanol differentially alters susceptibility to chemically-induced convulsions in Withdrawal Seizure-Prone and -Resistant mice. *J Pharmacol Exp Ther*. 1999; 288:782–790. [PubMed: 9918589]
- Finn DA, Beadles-Bohling AS, Beckley EH, Ford MM, Gililand KR, Gorin-Meyer RE, Wiren KM. A new look at the 5 α -reductase inhibitor finasteride. *CNS Drug Rev*. 2006a; 12:53–76. [PubMed: 16834758]
- Finn DA, Douglass AD, Tanchuck MA, Long SL, Crabbe JC. Selected line difference in sensitivity to a GABAergic neurosteroid during ethanol withdrawal. *Genes Brain Behav*. 2006b; 5:53–63. [PubMed: 16436189]
- Finn DA, Ford MM, Wiren KW, Roselli CE, Crabbe JC. The role of pregnane neurosteroids in ethanol withdrawal: Behavioral genetic approaches. *Pharmacol Ther*. 2004; 101:91–112. [PubMed: 14761701]
- Finn DA, Gallaher EJ, Crabbe JC. Differential change in neuroactive steroid sensitivity during ethanol withdrawal. *J Pharmacol Exp Ther*. 2000; 292:394–405. [PubMed: 10604976]
- Finn DA, Roberts AJ, Crabbe JC. Neuroactive steroid sensitivity in Withdrawal Seizure-Prone and -Resistant mice. *Alcohol Clin Exp Res*. 1995; 19:410–415. [PubMed: 7625575]
- Finn DA, Snelling C, Fretwell AM, Tanchuck MA, Underwood L, Cole M, Crabbe JC, Roberts AJ. Increased drinking during withdrawal from intermittent ethanol exposure is blocked by the CRF receptor antagonist D-Phe-CRF(12–41). *Alcohol Clin Exp Res*. 2007; 31:939–949. [PubMed: 17403068]
- Follesa P, Biggio F, Talani G, Murru L, Serra M, Sanna E, Biggio G. Neurosteroids, GABA_A receptors, and ethanol dependence. *Psychopharmacology*. 2006; 186:267–280. [PubMed: 16163526]
- Ford MM, Mark GP, Nickel JD, Phillips TJ, Finn DA. Allopregnanolone influences the consummatory processes that govern ethanol drinking in C57BL/6J mice. *Behav Brain Res*. 2007; 179:265–272. [PubMed: 17376546]
- Frye CA, Rhodes ME. Infusions of 5 α -pregnan-3 α -ol-20-one (3 α ,5 α -THP) to the ventral tegmental area, but not the substantia nigra, enhance exploratory, anti-anxiety, social and sexual behaviors and concomitantly increase 3 α ,5 α -THP concentrations in the hippocampus, diencephalon and cortex of ovariectomised oestrogen-primed rats. *J Neuroendocrinol*. 2006; 18:960–975. [PubMed: 17076771]
- Frye CA, Vongher JM. Ventral tegmental area infusions of inhibitors of the biosynthesis and metabolism of 3 α ,5 α -THP attenuate lordosis of hormone-primed and behavioral oestrous rats and hamsters. *J Neuroendocrinol*. 2001; 13:1076–1086. [PubMed: 11722704]
- Gale K. Progression and generalization of seizure discharge: Anatomical and neurochemical substrates. *Epilepsia*. 1988; 29(Suppl 2):S15–S34. [PubMed: 2844521]
- Gililand-Kaufman KR, Tanchuck MA, Ford MM, Crabbe JC, Beadles-Bohling AS, Snelling C, Mark GP, Finn DA. The neurosteroid environment in the hippocampus exerts bi-directional effects on seizure susceptibility in mice. *Brain Res*. 2008; 1243:113–123. [PubMed: 18840414]
- Gorin-Meyer RE, Wiren KM, Tanchuck MA, Long SL, Yoneyama N, Finn DA. Sex differences in the effect of finasteride on acute ethanol withdrawal severity in C57BL/6J and DBA/2J mice. *Neuroscience*. 2007; 146:1302–1315. [PubMed: 17428611]
- Grobin AC, Matthews DB, Devaud LL, Morrow AL. The role of GABA_A receptors in the acute and chronic effects of ethanol. *Psychopharmacology*. 1998; 139:2–19. [PubMed: 9768538]

- Gunn BG, Brown AR, Lambert JJ, Belelli D. Neurosteroids and GABA_A receptor interactions: a focus on stress. *Front Neurosci.* 2011; 5:1–20. (article 131) [Epub Dec 5]. [PubMed: 21390287]
- Heilig M, Egli M, Crabbe JC, Becker HC. Acute withdrawal, protracted abstinence and negative affect in alcoholism: are they linked? *Addiction Biol.* 2010; 15:169–184.
- Herd MB, Belelli D, Lambert JJ. Neurosteroid modulation of synaptic and extrasynaptic GABA_A receptors. *Pharmacol Ther.* 2007; 116:20–34. [PubMed: 17531325]
- Hill M, Popov P, Havlíková H, Lyudmila K, Vrbíková J, Kancheva R, Pouzar V, erný I, Stárka L. Altered profiles of serum neuroactive steroids in premenopausal women treated for alcohol addiction. *Steroids.* 2005; 70:515–524. [PubMed: 15894035]
- Kokate TG, Banks MK, Magee T, Yamaguchi S, Rogawski MA. Finasteride, a 5 α -reductase inhibitor blocks the anticonvulsant activity of progesterone in mice. *J Pharmacol Exp Ther.* 1999; 288:679–684. [PubMed: 9918575]
- Kozell LB, Hitzemann RJ, Buck KJ. Acute alcohol withdrawal is associated with c-fos expression in the basal ganglia and associated circuitry: C57BL/6J and DBA/2J inbred mouse strain analysis. *Alcohol Clin Exp Res.* 2005; 29:1939–1948. [PubMed: 16340450]
- Kumar S, Porcu P, Werner DF, Matthews DB, Diaz-Granados JL, Hefland RS, Morrow AL. The role of GABA_A receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology.* 2009; 205:529–564. [PubMed: 19455309]
- Metten P, Crabbe JC. Alcohol withdrawal severity in inbred mouse (*Mus musculus*) strains. *Behav Neurosci.* 2005; 119:911–925. [PubMed: 16187819]
- Morrow AL, Janis GC, VanDoren MJ, Matthews DB, Samson HH, Janak PH, Grant KA. Neurosteroids mediate pharmacological effects of ethanol: a new mechanism of ethanol action? *Alcohol Clin Exp Res.* 1999; 23:1933–1940. [PubMed: 10630613]
- Olive MF, Mehmert KK, Nannini MA, Camarini R, Messing RO, Hodge CW. Reduced ethanol withdrawal severity and altered withdrawal-induced c-fos expression in various brain regions of mice lacking protein kinase C-epsilon. *Neuroscience.* 2001; 103:171–179. [PubMed: 11311798]
- Paxinos, G.; Franklin, K. *The Mouse Brain in Stereotaxic Coordinates*. 2nd ed.. San Diego: Academic Press; 2001.
- Porcu P, O'Buckley TK, Alward SE, Marx CE, Shampine LJ, Girdler SS, Morrow AL. Simultaneous quantification of GABAergic 3 α ,5 α /3 α ,5 β neuroactive steroids in human and rat serum. *Steroids.* 2009; 74:463–473. [PubMed: 19171160]
- Rhodes ME, Frye CA. Actions at GABA_A receptors in the hippocampus may mediate some antiseizure effects of progestins. *Epilepsy Behav.* 2005a; 6:320–327. [PubMed: 15820338]
- Rhodes ME, Frye CA. Attenuating 5 α -pregnane-3 α -ol-20-one formation in the hippocampus of female rats increases pentylenetetrazole-induced seizures. *Epilepsy Behav.* 2005b; 6:140–146. [PubMed: 15710296]
- Romeo E, Brancati A, De Lorenzo A, Fucci P, Furnari C, Pompili E, Sasso GF, Spalletta G, Troisi A, Pasini A. Marked decrease of plasma neuroactive steroids during alcohol withdrawal. *Clin Neuropharmacology.* 1996; 19:366–369.
- Roselli CE, Finn TJ, Ronnekleiv-Kelly SM, Tanchuck MA, Kaufman KR, Finn DA. Localization of brain 5 α -reductase messenger RNA in mice selectively bred for high chronic alcohol withdrawal severity. *Alcohol.* 2011; 45:763–772. [PubMed: 21917407]
- Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy J-M, Martin JR, Bluethmann H, Möhler H. Benzodiazepine actions mediated by specific γ -aminobutyric acid_A receptor subtypes. *Nature.* 1999; 401:796–800. [PubMed: 10548105]
- Stephens DN, Duka T. Cognitive and emotional consequences of binge drinking: role of amygdala and prefrontal cortex. *Phil Trans R Soc B.* 2008; 363:3169–3179. [PubMed: 18640918]
- Sullivan EV, Rosenbloom MJ, Pfefferbaum A. Pattern of motor and cognitive deficits in detoxified alcoholic men. *Alcohol Clin Exp Res.* 2000; 24:611–621. [PubMed: 10832902]
- Tanchuck MA, Long SL, Ford MM, Hashimoto J, Crabbe JC, Roselli CE, Wren KM, Finn DA. 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.* 2009; 33:2077–2087. [PubMed: 19740134]

- Tanchuck MA, Strong MN, Snelling C, Hashimoto JG, Wiren KM, Finn DA. Selection for ethanol withdrawal severity alters the expression of select GABA-A receptor subunits in controls and during withdrawal. *Alcohol Clin Exp Res.* 2011; 35:190A.
- Terdal ES, Crabbe JC. Indexing withdrawal in mice: matching genotypes for exposure in studies using ethanol vapor inhalation. *Alcohol Clin Exp Res.* 1994; 18:542–547. [PubMed: 7943652]
- Velíšková J, Moshé SL. Update on the role of substantia nigra pars reticulata in the regulation of seizures. *Curr Rev Basic Sci.* 2006; 6:83–87.
- Yin HH, Knowlton BJ. The role of the basal ganglia in habit formation. *Nature Rev Neurosci.* 2006; 7:464–476. [PubMed: 16715055]

A. SNR

B. VTA

Figure 1. Schematic showing concentration of microinjection placements within the (A) posterior SNR and (B) posterior VTA

The dark squares indicate the range of confirmed hits in one experimental pass, which is representative of the range of hits for all experimental passes.

(SNR)

Figure 2. Decreased sensitivity to the anticonvulsant effect of intra-SNR ALLO during ethanol withdrawal, measured by the PTZ threshold dose for onset to (A) MC Twitch, (B) FF Clonus, (C) RB Clonus, and (D) THE

During withdrawal from 72 hr exposure to ethanol vapor or air, separate groups of animals received bilateral infusions of ALLO (100 or 400 ng/side) or VEH approximately 5–15 minutes prior to the timed tail vein infusion of PTZ. In air-exposed mice, ALLO exerted a significant anticonvulsant effect. The 100 ng ALLO dose significantly increased PTZ threshold dose by 25–28%. In contrast, mice exhibited behavioral tolerance to the anticonvulsant effect of ALLO infusion during ethanol withdrawal. The 400 ng ALLO dose significantly increased PTZ dose for onset to MC twitch and FF clonus by 14–18%, without altering PTZ dose for onset to RB clonus and THE. Values are the mean $\pm$ SEM for the number of animals in parentheses. $^+P = 0.065$; $*P < 0.05$, $**P < 0.01$ versus respective VEH group.

(VTA)

Figure 3. Ethanol withdrawal decreased sensitivity to the anticonvulsant effect of intra-VTA ALLO, measured by the PTZ threshold dose for onset to (A) MC Twitch, (B) FF Clonus, (C) RB Clonus, and (D) THE

During withdrawal from 72 hr exposure to ethanol vapor or air, separate groups of animals received bilateral infusions of ALLO (100 or 400 ng/side) or VEH approximately 5–15 minutes prior to the timed tail vein infusion of PTZ. ALLO (400 ng dose) exerted a significant anticonvulsant effect in the air-exposed animals, measured by the 15–20% increase in PTZ threshold dose for onset to RB clonus and THE. Importantly, this effect was absent during ethanol withdrawal. Values are the mean $\pm$ SEM for the number of animals in parentheses. * $P < 0.05$, ** $P < 0.01$ versus respective VEH group.

(SNR)

Figure 4. Intra-SNR FIN alters convulsive behavior only in air-exposed mice, measured by (A) hourly HIC and peak withdrawal, (B) AUC 0–24, and (C) AUC 24–48

Mean $\pm$ SEM baseline HIC scores prior to any experimental manipulation were 2.17 ± 0.14 . Animals received 4 daily bilateral infusions of FIN (10 μ g/side) or VEH at 24 hr before, and each day of the exposure to 72 hr ethanol vapor or air. Animals were scored for HIC hourly for 12 hr and then at 24, 26, 28, and 48 hr. Subsequently, AUC was calculated. FIN significantly increased AUC 24–48 and the average HIC over the peak withdrawal time frame (hrs 6–10) only in the air-exposed mice, without altering ethanol withdrawal severity. Values represent the mean $\pm$ SEM for the number of animals in parentheses (Panel B). * $P < 0.05$ versus respective VEH.

(VTA)

Figure 5. Increase in ethanol withdrawal severity following FIN microinjections into the VTA, measured by (A) hourly HIC and peak withdrawal, (B) AUC 0–24, and (C) AUC 24–48 Mean $\pm$ SEM baseline HIC scores prior to any experimental manipulation were 2.23 ± 0.16 . Mice were treated as described in the legend to Figure 4. AUC was calculated and is depicted as the mean $\pm$ SEM for the number of animals in parentheses (Panels B & C; missing data point between hrs 26 – 48 for 1 animal in each group). FIN significantly increased AUC 24–48 and tended to increase the average HIC over the peak withdrawal time frame (hrs 8–11) in the ethanol-exposed mice, indicative of an enhancement of the duration of withdrawal. $^+P = 0.06$; $*P < 0.05$ versus respective VEH.

Figure 6. Schematic of basal ganglia circuitry pertinent to convulsive activity

GABAergic (solid lines) and glutamatergic (dashed lines) projections are highlighted in this simplified depiction of basal ganglia circuitry. In the normal brain, the striatum receives an excitatory projection from the motor and somatosensory cortex. The striatal neurons communicate with the substantia nigra reticulata (SNR) and internal segment of the globus pallidus (GPi) through a direct inhibitory pathway or through a multi-synaptic indirect pathway [via the external segment of the globus pallidus (GPe) and the subthalamic nucleus (STN)]. The posterior region of the ventral tegmental area (VTA) projects to the substantia nigra compacta (SNC), which connects the striatum to the output nuclei of the basal ganglia through the indirect pathway. The GPi and SNR are the major output nuclei of the basal ganglia, and they project to the thalamus, superior colliculus (SC) and brainstem nuclei (e.g., pedunculopontine nucleus, reticular formation). Of the various outputs from the SNR, the GABAergic projections to thalamus, reticular formation and/or SC most likely influence propagation of convulsive activity.