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Quantification of Ten Neuroactive Steroids in Plasma in Withdrawal Seizure–Prone and –Resistant Mice During Chronic Ethanol Withdrawal

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Abstract

Rationale—The rapid membrane actions of neuroactive steroids, particularly via an enhancement of γ -aminobutyric acid_A receptors (GABA_ARs), participate in the regulation of central nervous system excitability. Prior evidence suggests an inverse relationship between endogenous GABAergic neuroactive steroid levels and behavioral changes in excitability during ethanol withdrawal.

Objectives—Previously, we found that ethanol withdrawal significantly decreased plasma allopregnanolone (ALLO) levels, a potent GABAergic neuroactive steroid, and decreased GABA_AR sensitivity to ALLO in Withdrawal Seizure–Prone (WSP) but not in Withdrawal Seizure–Resistant (WSR) mice. However, the effect of ethanol withdrawal on levels of other endogenous GABA_AR-active steroids is not known.

Methods—After validation of a gas chromatography-mass spectrometry method for the simultaneous quantification of 10 neuroactive steroids, we analyzed plasma from control male WSP-1 and WSR-1 mice and during ethanol withdrawal.

Results—We quantified levels of 9 neuroactive steroids in WSP-1 and WSR-1 plasma; levels of pregnanolone were not detectable. Basal levels of 5 neuroactive steroids were higher in WSR-1 versus WSP-1 mice. Ethanol withdrawal significantly suppressed 5 neuroactive steroids in WSP-1 and WSR-1 mice, including ALLO.

Conclusions—Due to lower basal levels of some GABA_AR-active steroids in WSP-1 mice, a withdrawal-induced decrease in WSP-1 mice may have a greater physiological consequence than a similar decrease in WSR-1 mice. Because WSP-1 mice also exhibit a reduction in GABA_AR

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sensitivity to neuroactive steroids during withdrawal, it is possible that the combined decrease in neuroactive steroids and GABA_AR sensitivity during ethanol withdrawal in WSP-1 mice represents a neurochemical substrate for severe ethanol withdrawal.

Keywords

allopregnanolone; androstanediol; tetrahydrodeoxycorticosterone; alcohol; gas chromatography; mass spectrometry; DHEA; pregnenolone; male

Introduction

Neuroactive steroids are synthesized in the brain and periphery and have rapid and potent effects on γ -aminobutyric acid_A receptors (GABA_ARs) and other ligand-gated ion channels to alter neuronal activity (Belelli and Lambert, 2005; Finn and Purdy, 2007; Paul and Purdy, 1992; Reddy, 2010). Because the enzymes identified in classic steroidogenic tissues are found in the nervous system (Mellon and Vaudry, 2001), the endogenous concentration of neuroactive steroids in the brain likely reflects a combination of neuroactive compounds produced there *de novo* as well as steroids derived from circulating precursors but metabolized to neuroactive compounds in the brain. The GABA_AR-active neuroactive steroids allopregnanolone (ALLO; 3 α ,5 α -THP), pregnanolone (3 α ,5 β -THP), tetrahydrodeoxycorticosterone (3 α ,5 α -THDOC), and 3 α ,5 α -androstanediol are formed from the 2-step reduction of the parent steroids progesterone (in the case of both THPs), deoxycorticosterone, and testosterone, respectively (Fig. 1). The three most potent pregnane neuroactive steroids characterized to date (ALLO, 3 α ,5 α -THDOC, and pregnanolone) have nanomolar (nM) potencies at GABA_ARs (e.g., Belelli et al., 1990; Purdy et al., 1990; Rupprecht and Holsboer, 1999; Velerio and Burton, 2009). Because endogenous concentrations fluctuate in the 10 – 100 nM range (Barbaccia et al., 2001; Belelli and Lambert, 2005; Finn et al., 2004; Paul and Purdy, 1992), these GABA_AR-mediated actions undoubtedly participate in the regulation of central nervous system (CNS) excitability and have significance in response to physiological conditions such as stress, trauma, and hormonal changes (i.e., pregnancy, puberty, aging, menstrual or estrous cycle changes). Thus, rapid membrane effects of certain specific metabolites of steroid hormones at GABA_ARs provide a mechanism by which these metabolites can influence brain function and behavior, in addition to the classical genomic actions of the parent steroid hormones.

The interaction of alcohol (ethanol) at GABA_ARs is integral for the development of tolerance and withdrawal-related convulsive activity, both of which contribute to the behavioral transition from recreational ethanol use to dependence and addiction (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). Numerous lines of evidence indicate that the transition from acute ethanol intoxication to dependence and the expression of withdrawal involves plasticity of GABA_AR properties with regard to direct and indirect effects of ethanol on GABA_AR function, including the synthesis of endogenous GABA_AR-active neuroactive steroids (see Kumar et al., 2009). In particular, chronic ethanol consumption and the induction of physical dependence reduces ethanol's steroidogenic effect (Boyd et al., 2010), and withdrawal from chronic ethanol exposure decreased ALLO

levels in rodents and humans (Cagetti et al., 2004; Finn et al., 2004; Hill et al., 2005; Romeo et al., 1996; Tanchuck et al., 2009) and reduced sensitivity of the GABA_AR system to ALLO in rodents with severe ethanol withdrawal (e.g., Finn et al., 2000, 2006). And, the decrease in GABA_AR-active neuroactive steroid levels corresponded to an increase in the subjective ratings of anxiety and depression during days 4 – 5 of withdrawal, when compared with control subjects (Hill et al., 2005; Romeo et al., 1996, 2000). Collectively, the data suggest that the withdrawal-induced reduction in endogenous ALLO levels and reduced sensitivity of GABA_ARs to ALLO contributes to increased cellular excitability and associated aversive behavioral effects of ethanol withdrawal.

Two independent genetic replicate lines of Withdrawal Seizure-Prone (WSP) and -Resistant (WSR) mice were selectively bred to have severe (WSP) or mild (WSR) chronic ethanol withdrawal severity after exposure to 72 hr ethanol vapor to induce physical dependence (Crabbe et al., 1985). Studies in our laboratory have used these selected lines to test the hypothesis that genetic differences in ethanol withdrawal severity were due in part to alterations in the GABA_AR-modulatory effects of neuroactive steroids such as ALLO. When we measured ALLO levels with radioimmunoassay (RIA), we found that plasma ALLO levels were significantly decreased in both replicate lines of WSP (but not in WSR) mice that were dependent or undergoing withdrawal, and this corresponded to a decrease in adrenal expression of 5 α -reductase (Tanchuck et al., 2009), one rate-limiting enzyme in ALLO biosynthesis (Fig. 1). Although cortical ALLO and 5 α -reductase activity was decreased to a similar degree in the WSP and WSR lines during withdrawal, hippocampal 5 α -reductase activity and expression was decreased only in dependent WSP mice. Unfortunately, because of the small size of the mouse hippocampus, we were unable to measure hippocampal ALLO levels with RIA. However, previous work in rats showed that withdrawal-induced decreases in expression of 5 α -reductase and 3 α -hydroxysteroid dehydrogenase corresponded to a decrease in hippocampal ALLO levels in rats (Cagetti et al., 2004), suggesting that the chronic ethanol-induced decrease in hippocampal 5 α -reductase activity in WSP mice might produce a corresponding decrease in hippocampal ALLO levels. Collectively, the evidence suggests that during ethanol withdrawal in WSP and WSR mice, there are line specific changes in ALLO levels in plasma and some brain regions, but the effect of ethanol withdrawal on levels of other endogenous GABA_AR-active neuroactive steroids is not known. Considering that steroid synthesis is a dynamic process and that disruptions in one or more steps could impact levels of multiple steroids with positive or negative modulatory effects at GABA_ARs, it is likely that levels of several neuroactive steroids work in concert to coordinate the physiological regulation of CNS excitability.

Recent elegant work (Porcu et al., 2009) utilized gas chromatography-mass spectrometry (GC-MS) to simultaneously quantify levels of 8 neuroactive steroids and the precursor pregnenolone. Here, we have validated the method utilized by Porcu et al. (2009) in our laboratory and achieved good resolution with the simultaneous identification of a panel of 10 neuroactive steroids, which included pregnenolone, dehydroepiandrosterone (DHEA), and the 3 α ,5 α - and 3 α ,5 β -GABA_AR-active metabolites of DHEA, deoxycorticosterone, progesterone, and testosterone. Next, we quantified levels of these 10 neuroactive steroids in plasma from WSP and WSR mice from the first genetic replicate (i.e., WSP-1 and WSR-1;

control and ethanol withdrawal). We predicted that a larger imbalance in ALLO and other GABA_AR-active neuroactive steroids would be evident in WSP-1 versus WSR-1 mice during ethanol withdrawal, which would contribute to a decrease in GABA_AR inhibition and the severe withdrawal phenotype in WSP-1 mice.

Materials and methods

Animals

Drug naïve male WSP and WSR mice from the first pair of replicate lines (WSP-1, WSR-1) were kindly provided by Dr. John C. Crabbe. The animals were bred in the Veterinary Medical Unit at the Veterans Affairs Medical Center (Portland, OR), were housed four per cage with *ad libitum* access to food and water upon weaning, and were acclimated to a 12:12 hr light/dark cycle (lights on at 0600). At the time of testing, mice were from selected generation 26 (filial generation 130) and were 73 – 104 (WSP-1) or 77 – 94 (WSR-1) days old. All procedures complied with the United States Public Health Service Institutes of Health guidelines in accordance with the “Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research” (National Research Council, 2003) and were approved by the local Institutional Animal Care and Use Committee.

Chronic Ethanol Exposure

Separate groups of animals were exposed to ethanol vapor or air for 72 hrs by the Portland Alcohol Research Center, using a standardized method for inducing ethanol dependence (Finn and Crabbe, 1999; Tanchuck et al., 2009; Terdal and Crabbe, 1994). Blood ethanol concentration (BEC) was analyzed by head-space gas chromatography, as previously described (Finn et al., 2007). Animals were euthanized at a time corresponding to peak withdrawal (hrs 6 – 9) after removal from the inhalation chambers. Trunk blood was collected for plasma neuroactive steroid analysis by GC-MS in 100 µl samples. Plasma corticosterone (CORT) levels were measured with a commercially available double antibody RIA kit (MP Biomedicals, Solon, OH) in 5 µl samples, as described recently (Ford et al., 2013).

Chemicals

All extraction and derivatization solvents were chromatography grade from EMD Chemicals (Billerica, MA). Monodeuterated ethanol was obtained from Acros Organics (New Jersey), and heptafluorobutyric acid anhydride was obtained from Fluka (Sigma-Aldrich, St. Louis, MO). Pregnenolone, pregnanolone, 3 α ,5 β -THDOC, and 3 α ,5 β -androstenediol were procured from Steraloids (Newport, RI), and all other native steroid standards were synthesized by Dr. Robert Purdy (Scripps Research Institute, La Jolla, CA). The internal standard (ISTD) that was used in all assays was 17 β -estradiol-2,4,16,16,17-d5 (CDN Isotopes, Pointe-Claire, Quebec, Canada). Stripped human serum (SHS) was purchased from Gemini Bioproducts (West Sacramento, CA).

Extraction

Steroids were extracted from plasma using solid phase extraction (SPE) techniques. Plasma (100 µl) was spiked with 1000 pg of estradiol-d5 (ISTD), which was selected for its robust

ionizability in the derivative form and its elution mid-field to the analytes of interest. The extracted sample was then loaded on a Strata-X 33u Polymeric Reversed Phase SPE column (8B-S100-UBJ, 60 mg/3ml, Phenomenex, Torrance, CA), pre-conditioned with 4 ml of dichloromethane, conditioned with 4 ml of acetonitrile, and then equilibrated with 4 ml of deionized water. After loading, the samples were washed with 1 ml of deionized water, and then the SPE columns were dried on a Waters vacuum manifold (WAT200608, Waters, Milford, MA) for 30 min at 12 inHg (inches of mercury, a unit of pressure equal to 0.49 psi) of vacuum. The steroids were then eluted from the SPE columns with 1.5 ml of acetonitrile, and the resulting eluate was dried on an Eppendorf Vacufuge for 90 min (ambient temperature, program 1). The samples were then derivatized or stored in a vacuum desiccator for up to 24 hrs.

Derivatization

Extracted samples were solvated in acetonitrile (450 μ l), and heptafluorobutyric acid anhydride (HFAA, 50 μ l) was added. The reagent mixture was capped and left at ambient temperature for 120 min to allow esterification of 3, 17, and 21 hydroxyl active groups and the resultant formation of perfluoroacyl-steroid species. The mixture was then dried for 90 min using an Eppendorf Vacufuge (ambient temperature, program 1), leaving only dried derivatized extractant. The samples were then re-solvated in acetonitrile (10 μ l) and transferred to a deactivated vial insert for immediate analysis.

Gas Chromatography-Mass Spectrometry

Analysis of samples and calibration curves was performed on an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer detector (Agilent Technologies, Santa Clara, CA). 2 μ l aliquots of samples and calibration curve standards were injected into a 275°C inlet at 12.696 psi. Chromatographic separation was carried out on an HP-5ms capillary column (30 m $\times$ 0.250 mm, 0.25 mm film, Agilent Technologies) at an isobaric flow rate of 0.944 ml/min of helium carrier gas (UHP 5.0 grade, Matheson, Tualatin, OR) with a total run time of 31.41 min. A temperature gradient was employed with an initial oven temp of 75°C for 0.86 min, leading to the first ramp to 210°C (35°C/min) with a hold for 3 min, then a ramp to 235°C (3.25°C/min) with a hold for 11 min, and a final ramp to 310°C (25°C/min) and a hold for 2 min. The transfer line was isothermic at 280°C. Eluates were then analyzed by the mass spectrometer in negative chemical ionization (NCI) using methane (research grade 99.999%, Matheson Tri-gas, La Porte, TX) as the reagent gas at a flow rate of 40%. Both mass spectrometer chemical ionization source and quadrupole mass filter temperatures were set at 150°C. A solvent delay of 14.00 min was established. Selected ion monitoring (SIM) was used, and data were collected for a minimum of two ions per analyte over five retention period groups (Table 1).

Calibration Curves

Calibration curves were formed by spiking 300 μ l of deionized water with 0, 10, 50, 100, 500, 1000, and 3000 pg of all ten analytes of interest. The spiked reference standards were then extracted and derivatized in the identical manner as described above and analyzed via GC-MS. Curves were formed by plotting the ratio of the responses of the analyte divided by the response of the ISTD versus the concentration of the analyte. The response constituted

an integration of the abundance counts collected by the MS detector horn for the target ion from baseline to baseline for each peak representing an analyte of interest. No analytes co-eluted with other analytes or with the ISTD. The calibration curves were then analyzed with linear regression, and unknown plasma samples were interpolated against these regression parameters using their individual response ratios.

Data Analysis

Target ion peaks were integrated in ChemStation and interpolated using Prism5 (GraphPad Software Inc., San Diego, CA). Mean pg/ml of neuroactive steroid per extracted sample was calculated from the interpolated values and statistically analyzed with analysis of variance (ANOVA). Two-way ANOVA assessed line and treatment effects. A similar strategy was used for the analysis of CORT levels. Significant interactions were followed up with one-way ANOVA. Because we predicted line differences in basal neuroactive steroids, significant line effects were examined in the air controls, even in the absence of a significant interaction. Significance was set at a p value of 0.05.

Results

Gas Chromatography and Mass Spectrometry

We were able to simultaneously quantify 10 different neuroactive steroids (see Fig. 1; steroids with an asterisk). We employed SIM during GC-MS analysis to quantify abundances of selected ions per time point. This allowed for both identification of each analyte based on its target ion, qualifier ion(s), and retention times (Table 1) as well as quantification of each analyte by integration of abundances for the target ion at the appropriate retention time. The SIM programming was divided into five SIM groups to limit the maximum of ions tracked per period to six, to ensure ample dwell time per ion and adequate scan rate to obtain approximately 10 – 14 scans per peak. As shown in Figure 2, all analytes were resolved chromatographically with no co-elution of analytes or ISTD.

Detection Limits of the Analytes

Several factors contributed to sensitivity in the assay, such as ionizability of the derivatized analyte, ion-mass selectivity within the quadrupole based on tune parameters, or biased acylation during derivatization due to steric hindrance variability between epimers. However, detection limits were very good for the analytes of interest. The lowest amount in the calibration standard curves that was reliably detected for 3 α ,5 α - and 3 α ,5 β -androstanediol as well as for 3 α ,5 α - and 3 α ,5 β -androsterone was 2 pg per 2 μ l aliquot. DHEA, ALLO, pregnanolone, pregnenolone, and 3 α ,5 β -THDOC were detectable at 10 pg per 2 μ l aliquot, whereas 3 α ,5 α -THDOC was detectable at 20 pg per 2 μ l aliquot. These limits of detection are similar to those previously reported in Porcu et al. (2009). The minimum allowable signal to noise ratio for the GC-MS peaks was three to one, which was the case on occasion for pregnenolone and the THDOCs. However, for all other steroids (even for pregnenolone and the THDOCs under most circumstances), the signal to noise ratio was equal to or greater than five to one.

Calibration Curves

Analytes in extracted samples were quantified by interpolating integrated abundances against calibration curves formed by reference standards (0, 10, 50, 100, 500, 1000, and 3000 pg), which were run in the concurrent sequence. Each neuroactive steroid had its own calibration curve (Fig. 3) that was formed by plotting the ratio of the response of the analyte divided by the response of the ISTD versus the concentration of the analyte. The r^2 values from linear regression analysis of each calibration curve for the 10 neuroactive steroids ranged from 0.9934 to 0.9999, confirming an excellent linear response for all analytes. A solvent wash of acetonitrile (5 μ l) was conducted every third run during the sequences to limit carryover from previous runs.

Method Validation

Because we modified the GC-MS assay that was utilized by Porcu et al. (2009), we conducted four experiments to verify the accuracy and reproducibility of the current GC-MS procedure. SHS (200 μ l) was spiked with either 30, 300, or 700 pg of the panel of 10 neuroactive steroids, and then extracted, derivatized, and analyzed with GC-MS as if they were plasma samples. The concentrations chosen represented a fairly wide range of the calibration curve, and covered quantities typically found in biological samples. The subsequent abundances were integrated and interpolated against the concurrently run calibration curves, and the resulting interpolated values were plotted against the spiked amount (Fig. 4). Linear regression analysis on the obtained amounts also confirmed the excellent linear response for all neuroactive steroids. The r^2 values ranged from 0.9915 to 0.9996, with the exception of pregnenolone, where r^2 was 0.9832. Notably, the interpolated values of the analytes were statistically similar or identical to the spiked amount, indicating a high level of accuracy in the assay. A non-spiked SHS blank also was analyzed in each experiment, and low levels of 3 α ,5 β -androsterone were detected, leading to a skewing in the obtained amount of that steroid (particularly in the 700 pg spiked sample). The higher obtained levels of 3 α ,5 β -androsterone in the analytical validation is similar to that reported by Porcu et al. (2009).

Intra-assay and Inter-assay Variability

Inter-assay variability was conducted on the validation points from four assays, similar to Porcu et al. (2009). Values ranged from 4.3% – 15.3% for 6 of the steroids, with slightly higher values for 3 α ,5 β -androstenediol (19.4%), pregnenolone (21.0%), 3 α ,5 α -THDOC (23.3%) and 3 α ,5 β -THDOC (21.9%). Intra-assay variability was calculated on the data from the calibration curves depicted in Figure 3. Briefly, the standards were used as unknowns and interpolated values were calculated for each standard. The coefficients of variation were calculated as described by Porcu et al. (2009), and intra-assay variability ranged from 8.5% – 16.5%.

Effect of Chronic Ethanol Withdrawal on Plasma Neuroactive Steroid Levels

We were able to quantify levels of 9 neuroactive steroids in the plasma of WSP-1 and WSR-1 mice following exposure to 72 hr ethanol vapor or air (Fig. 5). Pregnanolone (3 α ,5 β -THP) was not detected, presumably because endogenous levels were below our detection

threshold. For the ethanol-exposed mice, BEC upon removal from the vapor chambers at 72 hrs was 1.598 ± 0.098 mg/ml for WSP-1 mice and 1.591 ± 0.161 mg/ml for WSR-1 mice. Values from the 2-way ANOVAs are provided in Table 2.

There was a main effect of selected line for all steroids examined except pregnenolone and $3\alpha,5\alpha$ -THDOC, with higher levels of neuroactive steroids in WSR-1 versus WSP-1 mice. Post-hoc analysis of the air controls confirmed that basal plasma levels of DHEA ($\uparrow 414\%$, $p < 0.001$; Fig. 5b), $3\alpha,5\alpha$ -androstenediol ($\uparrow 244\%$, $p < 0.001$; Fig. 5d), $3\alpha,5\beta$ -androstenediol ($\uparrow 403\%$, $p < 0.001$; Fig. 5e), $3\alpha,5\alpha$ -androsterone ($\uparrow 319\%$, $p < 0.001$; Fig. 5f), and $3\alpha,5\beta$ -androsterone ($\uparrow 765\%$, $p = 0.01$; Fig. 5g) were significantly higher in WSR-1 versus WSP-1 mice. A trend for higher basal ALLO ($\uparrow 54\%$, $p = 0.06$; Fig. 5c) and $3\alpha,5\beta$ -THDOC ($\uparrow 253\%$, $p = 0.06$; Fig. 5i) in WSR-1 mice also was observed. In contrast, pregnenolone levels (Fig. 5a) were 45% higher in air-exposed WSP-1 versus WSR-1 mice, but this difference was not statistically significant.

Ethanol withdrawal produced a significant suppression in the levels of 5 neuroactive steroids in WSP-1 and WSR-1 mice, respectively (main effect of treatment, see Table 2): DHEA ($\downarrow 47\%$, $\downarrow 61\%$), ALLO ($\downarrow 39\%$ in both lines), $3\alpha,5\alpha$ -androstenediol ($\downarrow 32\%$, $\downarrow 39\%$), $3\alpha,5\beta$ -androstenediol ($\downarrow 78\%$, $\downarrow 70\%$), and $3\alpha,5\alpha$ -androsterone ($\downarrow 56\%$ in both lines) (Fig. 5). There was a trend ($p < 0.07$) for a decrease in $3\alpha,5\beta$ -THDOC levels during withdrawal. The interaction between main effects for DHEA (Fig. 5b), $3\alpha,5\beta$ -androstenediol (Fig. 5e) and $3\alpha,5\alpha$ -androsterone (Fig. 5f) appear to be due to the higher basal neuroactive steroid levels in WSR-1 versus WSP-1 mice (mentioned above) as additional post-hoc tests revealed similar decreases in neuroactive steroid levels during ethanol withdrawal for DHEA, $3\alpha,5\beta$ -androstenediol, and $3\alpha,5\alpha$ -androsterone (also see above). For $3\alpha,5\beta$ -androsterone (Fig. 5g), post-hoc tests indicated that levels were significantly increased (by 96%) during withdrawal in WSP-1 mice and non-significantly decreased (by 53%) during withdrawal in WSR-1 mice. In contrast, pregnenolone (Fig. 5a) was significantly decreased (by 63%) during withdrawal in WSP-1 mice and non-significantly increased (by 72%) during withdrawal in WSR-1 mice. Thus, for both of these neuroactive steroids, pregnenolone and $3\alpha,5\beta$ -androsterone, ethanol withdrawal produced divergent changes in WSP-1 and WSR-1 mice.

Because we were concerned that small sample sizes may have led us to fail to detect some meaningful differences, we computed effect sizes, and they were consistent with moderate (0.50) to large (0.80) effects (Cohen, 1988). For most of the significant differences observed, the effect size was > 0.80 , and two significant differences had effect sizes of 0.57 and 0.59. The three non-significant trends had effect sizes of 0.56 (ALLO, basal difference), 0.52 ($3\alpha,5\beta$ -THDOC, basal difference) and 0.31 ($3\alpha,5\beta$ -THDOC, treatment). The effect sizes for the non-significant results for $3\alpha,5\alpha$ -THDOC were 0.18 and 0.30. Thus, despite a low group size, the large effect size implies that the power was sufficient to support the significant differences that were observed.

Effect of Chronic Ethanol Withdrawal on Plasma CORT Levels

Mean $\pm$ SEM basal plasma CORT levels were 26.14 ± 2.62 $\mu\text{g/dl}$ for WSP-1 mice ($n=7$) and 26.19 ± 3.71 $\mu\text{g/dl}$ for WSR-1 mice ($n=5$). Ethanol withdrawal produced a similar ($\sim 100\%$) increase in plasma CORT concentrations in WSP-1 (52.50 ± 4.46 $\mu\text{g/dl}$, $n=7$) and WSR-1

(51.51 ± 9.48 $\mu\text{g/dl}$, $n=5$) mice, a finding that is supported by the significant main effect of treatment [$F(1,20) = 24.37$, $p < 0.001$]. There was no significant main effect of selected line [$F(1,20) = 0.008$, $p = 0.93$] or interaction between line and treatment [$F(1,20) = 0.01$, $p = 0.92$]. Effect size for the significant effect of treatment was > 0.80 .

Discussion

The present results indicate that we were able to achieve good resolution with the simultaneous identification of 10 neuroactive steroids, with a slight variation in the GC-MS assay recently published by Porcu et al. (2009). The main differences involved the use of a single ISTD, deuterium-labeled 17β -estradiol, in the present study versus separate deuterium-labeled ISTDs for each neuroactive steroid group (Porcu et al., 2009) as well as a novel extraction methodology incorporating a single solid phase extraction procedure. The retention time for 17β -estradiol was in the mid-range of retention times for all the neuroactive steroid analytes (Table 1), and the peak was easily resolved and separable from the neuroactive steroids with the closest retention times (DHEA and $3\alpha,5\beta$ -androsterone). Importantly, the high resolution and distinct retention times ensured that interference did not produce false estimates of neuroactive steroid levels. The validation of the GC-MS assay in spiked SHS confirmed the high level of accuracy in the current GC-MS assay. Thus, the present findings confirm that we are able to simultaneously identify and quantify 10 neuroactive steroids: the GABA_AR-active reduced metabolites (i.e., $3\alpha,5\alpha$ - and $3\alpha,5\beta$ -) of progesterone, testosterone, deoxycorticosterone, and DHEA as well as the precursor steroids pregnenolone and DHEA.

We recognize that it is not optimal to utilize a single ISTD, based on recommendations that an ideal ISTD should have the same physical and chemical properties as the analyte of interest but also have the ability to be separated by mass, as is found with the use of a deuterated analog of the analyte of interest that differs by 3 – 5 atomic mass units (e.g., Alomary et al., 2001). Initially, we used 5α -pregnane- $3\alpha,21$ -diol- 20 -one- $17,21,21$ - d_3 , 5 -androsten- 3β -ol- 17 -one- $16,16$ - d_2 , 5 -pregnen- 3β -ol- 20 -one- $17,21,21,21$ - d_4 , and 5α -adrostan- 3α -ol- 17 -one- $16,16$ - d_2 as ISTDs in an attempt to be as analogous as possible to the analytes in each of our SIM groupings, as was done by Porcu et al. (2009). Unfortunately, the capabilities of our Mass Spectrometer, which utilizes a single quadrupole mass filter, did not allow for mass resolution between the native species and the deuterated species, even with the $m+4$ deuterated analog. Upon consultation and recommendations by several experts in the GC-MS analysis of steroids, we chose 17β -estradiol- $2,4,16,16,17$ - d_5 as the ISTD for several reasons. First, estradiol has similar structure, derivatization characteristics, and chromatographic tendencies as the analytes of interest. It is a prime candidate for reference to our analytes of interest, as it has similar polarity and heteroatomic substitutions, leading to extraction response consistent to our analytes of interest. It also has two hydroxyl substitutions, allowing for double acylation by the derivatizing agent as is the case with half of our analytes of interest, and it produces excellent ionization in the mass spectrometer source. Further, chromatographically it has retention properties in our method so that it elutes in the middle of our analytes of interest. Most importantly, in using the ratio of response of the deuterated estradiol to each individual analyte of interest, correlation values

of 0.99 or above were achieved for the calibration curves for each analyte. If the response was not analogous, then it is unlikely that we would achieve these correlations.

GC-MS analysis was conducted on plasma from WSP-1 and WSR-1 male mice exposed to air (controls) or 72 hr ethanol vapor and euthanized at a time corresponding to peak withdrawal in WSP mice. The goal was to extend our previous analysis of selected line differences in ALLO levels during ethanol withdrawal, which relied on a RIA-based method. Our earlier study (Tanchuck et al., 2009) found that plasma ALLO levels were decreased by 34% during withdrawal in WSP but not in WSR mice from both genetic replicates. This finding also corresponded to a downregulation of adrenal 5 α -reductase during ethanol withdrawal in WSP mice. However, in the present study, we observed a 39% decrease in plasma ALLO levels during withdrawal in both WSP-1 and WSR-1 mice. It is possible that the exclusive use of replicate-1 animals in the present study, or the different methods of analysis (i.e., GC-MS versus RIA), account for this discrepancy. Yet, we did not observe replicate-1 versus replicate-2 differences in the Tanchuck et al. (2009) study, making a replicate line explanation unlikely. Regardless, a withdrawal-induced suppression in ALLO levels during ethanol withdrawal is consistent with prior evidence in humans and rodents (e.g., Cagetti et al., 2004; Finn et al., 2004; Hill et al., 2005; Romeo et al., 1996, 2000; Tanchuck et al., 2009). Notably, studies in rodents also determined that ethanol withdrawal did not significantly alter levels of the precursor progesterone (Cagetti et al., 2004; Finn et al., 2004), while expression of 5 α -reductase and 3 α -hydrosteroid dehydrogenase (3 α -HSD) was suppressed (e.g., Cagetti et al., 2004). Collectively, these results suggest that an ethanol withdrawal-induced decrease in ALLO levels is not due to a concomitant decrease in progesterone, and that it likely involves an effect on 5 α -reductase and 3 α -HSD, the two biosynthetic enzymes downstream of progesterone that are important for ALLO synthesis (see Fig. 1).

When considering the pattern of the results across the GABA_AR-active neuroactive steroids examined, there was a significant suppression in the levels of 5 neuroactive steroids (including ALLO) during ethanol withdrawal in WSP-1 and WSR-1 mice. The GABA_AR-active testosterone metabolites, 3 α ,5 α - and 3 α ,5 β -androstenediol, were decreased by 32 or 39% (3 α ,5 α -) and by 78 or 70% (3 α ,5 β -) during ethanol withdrawal in WSP-1 and WSR-1 mice, respectively. Recent work indicated that serum testosterone was significantly decreased at the 8 hr withdrawal time point in male WSP and WSR mice (Forquer et al., 2011), suggesting that ethanol withdrawal produced a similar suppression in the biosynthesis of testosterone and its GABA_AR-active metabolites in male WSP and WSR mice in the present study. This finding is consistent with the reported decrease in plasma testosterone levels in male alcoholics (e.g., Castilla-Garcia et al., 1987; Iturriaga et al., 1999; Krüger et al., 2006; Maneesh et al., 2006; Martinez-Riera et al., 1995; Ruusa et al., 1997; Sierksma et al., 2004; Ylikahri et al., 1980). Ethanol withdrawal also produced a similar decrease (56%) in levels of the GABAergic DHEA metabolite 3 α ,5 α -androsterone in WSP-1 and WSR-1 mice, which corresponded to a withdrawal-induced suppression in levels of the parent steroid DHEA ($\downarrow$ 47% in WSP, $\downarrow$ 61% in WSR). While we did not measure plasma levels of the sulfated form of DHEA (i.e., DHEAS), the present results with DHEA are consistent with a reported decrease in DHEAS in a small cohort of alcoholic patients during the early withdrawal phase (Ozsoy and Esel, 2008). Overall, findings to date suggest that a consistent

suppression in the DHEA and testosterone steroid pathways occurs during ethanol withdrawal in male WSP-1 and WSR-1 mice.

It is interesting that plasma pregnenolone was suppressed by 63% during ethanol withdrawal only in WSP-1 but not in WSR-1 mice. This finding would be consistent with an overall suppression of steroid synthesis during withdrawal in WSP-1 mice, given that pregnenolone is the precursor of all the steroids examined (Fig. 1), but it also would suggest that ethanol withdrawal was altering steroid synthesis at a step downstream from pregnenolone in WSR-1 mice. However, we have found previously that ethanol withdrawal did not decrease plasma progesterone levels in WSP or WSR mice (Finn et al., 2004) and plasma CORT levels were significantly increased during withdrawal in both WSP and WSR mice in the present and prior work (Tanchuck et al., 2009). Taken in conjunction with non-significant changes in the GABA_AR-active deoxycorticosterone metabolites, 3 α ,5 α - and 3 α ,5 β -THDOC, it is likely that ethanol withdrawal is exerting independent effects at several steps in the steroidogenic pathway rather than an overall suppression in steroid synthesis.

While plasma ALLO and related GABA_AR-active neuroactive steroids were significantly decreased during ethanol withdrawal in both WSP-1 and WSR-1 male mice, absolute values of the neuroactive steroids during ethanol withdrawal also should be considered, as a decrease below the physiologically relevant range would no longer potentiate GABA_AR function. Since basal neuroactive steroid levels were lower in WSP-1 versus WSR-1 mice for 5 of the steroids examined, a withdrawal-induced decrease in WSP-1 mice may have a greater physiological consequence versus a similar decrease in WSR-1 mice (e.g., 3 α ,5 α - and 3 α ,5 β -androstane diols). Additionally, based on evidence that brain and peripheral neuroactive steroid synthesis can be regulated independently, it is possible that the withdrawal-induced changes in plasma neuroactive steroid levels in the present study do not reflect local changes in discrete brain regions. Consistent with this idea, hippocampal 5 α -reductase enzyme activity and gene expression was decreased only in dependent WSP mice from both genetic replicates (Tanchuck et al., 2009), suggesting that there may be brain regional and selected line differences in the effect of ethanol withdrawal on neuroactive steroid levels.

Another interesting finding was that basal levels of 5 neuroactive steroids were significantly higher in WSR-1 versus WSP-1. This finding is consistent with the recent report of higher serum neuroactive steroid levels in C57BL/6J versus DBA/2J male mice (Porcu et al., 2010), another genetic animal model of chronic ethanol withdrawal severity (i.e., mild withdrawal in C57BL/6J mice; severe withdrawal in DBA/2J mice). Because brain neuroactive steroid concentrations reflect a combination of *de novo* synthesis as well as peripheral sources, it is possible that greater fluctuations in circulating neuroactive steroids in WSR-1 and C57BL/6J mice result in higher brain concentrations than in WSP-1 and DBA/2J mice. Taken in conjunction with evidence that GABAergic neuroactive steroids modify the function of GABA_A receptors *in vivo* and participate in the physiological control of CNS excitability (e.g., Belelli and Lambert, 2005), the selected line and inbred strain differences in basal GABA_AR-active neuroactive steroid levels may have a physiologically significant consequence on GABA_AR inhibition.

We also noticed that the basal plasma neuroactive steroid levels in the present study were significantly higher overall than in the study by Porcu et al. (2010) in C57BL/6J and DBA/2J mice. And, the plasma ALLO levels in the present study were significantly higher than those in our earlier study (Tanchuck et al., 2009; if we converted the pg/ml ALLO concentrations to ng/ml). We suspected that the air-exposure in the present study produced a greater activation of the hypothalamic-pituitary-adrenal (HPA) axis than in our prior work, which was confirmed by our analysis of plasma CORT concentrations. In the present study, basal CORT levels were ~ 26 µg/dl; these values are over 5-fold higher than in our prior work (< 5 µg/dl for both WSP and WSR mice; Tanchuck et al., 2009) and are associated with a moderate degree of stress. Ethanol withdrawal did produce a further activation of the HPA axis in the present study, measured by the 100% increase in plasma CORT concentrations, but this increase was much lower than what we observed in our prior work (~ 6-fold increase in both WSP and WSR mice; Tanchuck et al., 2009). We do not know why exposure to 72 hr air in the present study produced a greater activation of the HPA axis than in prior work as the methods and procedures were very similar, but this difference could explain the higher basal neuroactive steroid levels that were detected in the present study.

In conclusion, use of GC-MS to quantify GABAergic neuroactive steroids in male WSP-1 and WSR-1 mice indicated that there was a similar pattern of changes in 5 GABA_AR-active neuroactive steroids during peak withdrawal. However, basal levels of 5 neuroactive steroids were lower in WSP-1 versus WSR-1 mice, so a withdrawal-induced decrease in WSP-1 mice may have a greater physiological consequence versus a similar decrease in WSR-1 mice (e.g., 3α,5α- and 3α,5β-androstanediols). Additionally, it should be considered that ethanol withdrawal renders WSP mice (but not WSR) from both genetic replicates tolerant to the anticonvulsant effects of exogenous ALLO administration (e.g., Finn et al., 2006; Gililland-Kaufman et al., 2008; Tanchuck et al., 2013). This behavioral tolerance corresponded to a rightward shift in the ability of ALLO to potentiate GABA-stimulated chloride flux in forebrain microsacs (Finn et al., 2006), consistent with a reduction in GABA_AR sensitivity to GABA_AR-active neuroactive steroids during ethanol withdrawal in WSP mice. And, recent preliminary results confirm a reduced sensitivity of extrasynaptic GABA_ARs to the GABA_AR agonist THIP (gaboxadol) during ethanol withdrawal in male WSP-1 mice, measured by voltage-clamp recordings of tonic GABA_AR current responses in hippocampal dentate gyrus granule cells (Rossi and Finn, unpublished). Collectively, a withdrawal-induced decrease in GABA_AR sensitivity to GABAergic neurosteroids and a concomitant decrease in endogenous GABAergic neurosteroid levels may represent a neurochemical substrate for enhanced susceptibility to ethanol withdrawal severity.

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Fig. 1. Biosynthesis of select neuroactive steroids

Steroids that were simultaneously quantified in the GC-MS assay are indicated with a BOLD asterisk. Note that the potent positive modulators of GABA_A receptors (THP, THDOC, androstenediol) are formed by the 2-step reduction of the parent steroids, progesterone, deoxycorticosterone, and testosterone, respectively. Also depicted are the sulfated derivatives of pregnenolone and DHEA (dehydroepiandrosterone), which are negative modulators of GABA_A receptors. [DHDOC = dihydrodeoxycorticosterone; THDOC = tetrahydrodeoxycorticosterone; DHP = dihydroprogesterone; THP = tetrahydroprogesterone; DHT = dihydrotestosterone; HSD = hydroxysteroid dehydrogenase]

Abundance

Fig. 2. Total Ion Chromatogram (TIC) of a panel of 9 neuroactive steroids and pregnenolone exhibiting full resolution to baseline

TIC was obtained for a reference standard mixture (1000 pg of each steroid) using selected ion monitoring (SIM) in negative chemical ionization mode. There was high response and good resolution for all analytes of interest; x-axis depicts range of retention times (min) for elution of the neurosteroid groups (also see Table 1). [Testosterone derivatives = 3α,5α-A-Diol & 3α,5β-A-Diol (androstane diols); Androstenedione derivatives = 3α,5α-A & 3α,5β-A (hydroxyandrostane-17-ones); Progesterone derivatives = 3α,5α-THP (allopregnanolone) & 3α,5β-THP (pregnanolone); Deoxycorticosterone derivatives = 3α,5α-THDOC & 3α,5β-THDOC (tetrahydrodeoxycorticosterones)] See Figure 1 for parent steroids.

Fig. 3. Calibration curves for each neuroactive steroid standard

Reference standards (0, 10, 50, 100, 500, 1000 and 3000 pg) were plotted against the ratio of the responses of the analyte over the response of the internal standard per sample (2 μl). Data are the mean $\pm$ SEM of three experiments for each neuroactive steroid, which are depicted in two separate panels. The top panel contains the 3α,5α- and 3α,5β-androstenediols and the 3α,5α- and 3α,5β-androsterones; the bottom panel contains DHEA, the 3α,5α- and 3α,5β-THPs, pregnenolone, and the 3α,5α- and 3α,5β-THDOCs.

Fig. 4. Validation of assay by determination of spiked serum samples

Stripped human serum (SHS, 200 μ l) was spiked with internal standard and three selected concentrations (30, 300 and 700 pg/sample) of each neuroactive steroid and extracted in the same manner as the reference standards. The resultant interpolated values (obtained amount $\blacktriangle$ with dashed line, from a standard curve of each analyte) are plotted along with the value for the amount added to the sample (spiked amount $\square$ with solid line). The r^2 values from the linear regression on the obtained amounts ($\blacktriangle$) ranged from 0.9915 to 0.9996 for all

analytes except pregnenolone, where $r^2 = 0.9832$. Data are the mean $\pm$ SEM of four experiments per analyte. Note the different range in y-axis for 3 α ,5 β -androsterone.

Fig. 5. Effect of chronic ethanol withdrawal on plasma neuroactive steroid levels in male WSP-1 and WSR-1 mice

Mice were exposed to 72 hr ethanol vapor or air and euthanized at a time corresponding to peak withdrawal in WSP-1 mice (6 – 9 hrs after removal from the inhalation chambers). Basal neuroactive steroid levels (air controls) were significantly higher in WSR-1 versus WSP-1 mice for 5 of the 9 steroids depicted, and ethanol withdrawal significantly decreased neuroactive steroid levels in 5 of the 9 steroids examined. The mean $\pm$ SEM neuroactive steroid levels are shown in pg/ml; note the different ranges in the y-axes. Group size for WSP-1 mice was 5 (air) and 7 (ethanol), except for 3 α ,5 α -androstanediol, where n=6

(ethanol). Groups size for WSR-1 mice was 5 (air) and 4 (ethanol). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus respective air control (main effect of treatment for panels B – F; post-hoc test for panels A & G); ** $p < 0.01$, *** $p < 0.001$ versus respective WSP-1 (post-hoc test)

Table 1

Group, retention time, target ion, and qualifier ion for each analyte in assay.

Analyte	Retention Time (min)	Target Ion (m/z)	Qualifier Ion (m/z)
Group 1			
3 α ,5 α -Androstenediol	14.41	659.85	679.60
3 α ,5 β -Androstenediol	14.82	659.85	679.60
3 α ,5 α -Androsterone	15.22	463.35	483.10
3 α ,5 β -Androsterone	15.61	443.70	483.10
Group 2			
Estradiol-d5 (ISTD)	16.35	429.80	469.20
DHEA	17.22	462.75	442.15
Group 3			
3 α ,5 α -THP	18.91	471.50	491.05
3 α ,5 β -THP	19.16	471.50	491.05
Group 4			
Pregnenolone	20.47	489.15	469.90
Group 5			
3 α ,5 α -THDOC	23.70	488.10	470.60
3 α ,5 β -THDOC	24.06	488.10	470.60

Each neuroactive steroid was detected by selected ion monitoring (SIM) tracking of a target ion and qualifier ion specific for that analyte and further confirmed by retention time. Five SIM groups were established to limit the number of ions being tracked per group and to allow for longer dwell times per ion without loss of data. ISTD = internal standard; m/z = mass to charge

Table 2

ANOVA Results for Plasma Neuroactive Steroids

Steroid	Main Effect: Selected Line	Main Effect: Treatment	Interaction
Pregnenolone	$F(1,17) = 0.32, p = 0.58$	$F(1,17) = 0.22, p = 0.646$	$F(1,17) = 6.09, p < 0.05$
DHEA	$F(1,17) = 59.86, p < 0.001$; WSR > WSP	$F(1,17) = 25.20, p < 0.001$; air > ethanol	$F(1,17) = 13.92, p = 0.002$
ALLO (3 α ,5 α -THP)	$F(1,17) = 8.12, p = 0.01$; WSR > WSP	$F(1,17) = 10.42, p = 0.005$; air > ethanol	$F(1,17) = 0.50, p = 0.49$
3 α ,5 α -androstanediol	$F(1,16) = 35.43, p < 0.001$; WSR > WSP	$F(1,16) = 6.72, p < 0.05$; air > ethanol	$F(1,16) = 2.59, p = 0.127$
3 α ,5 β -androstanediol	$F(1,17) = 70.39, p < 0.001$; WSR > WSP	$F(1,17) = 46.24, p < 0.001$; air > ethanol	$F(1,17) = 18.75, p < 0.001$
3 α ,5 α -androsterone	$F(1,17) = 85.34, p < 0.001$; WSR > WSP	$F(1,17) = 34.45, p < 0.001$; air > ethanol	$F(1,17) = 13.02, p = 0.002$
3 α ,5 β -androsterone	$F(1,17) = 15.75, p = 0.001$; WSR > WSP	$F(1,17) = 2.20, p = 0.156$	$F(1,17) = 5.15, p < 0.05$
3 α ,5 α -THDOC	$F(1,17) = 1.58, p = 0.226$	$F(1,17) = 0.004, p = 0.95$	$F(1,17) = 0.89, p = 0.358$
3 α ,5 β -THDOC	$F(1,17) = 4.86, p < 0.05$; WSR > WSP	$F(1,17) = 3.75, p < 0.07$; air > ethanol	$F(1,17) = 2.55, p = 0.129$

Results for the 2-way ANOVAs are shown for the 9 neuroactive steroids (and in the same order) that are depicted in Figure 5.