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REPLACEMENT WITH GABAERGIC STEROID PRECURSORS RESTORES THE ACUTE ETHANOL WITHDRAWAL PROFILE IN ADX/GDX MICE

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Abstract

The neurosteroid allopregnanolone (ALLO) is a progesterone metabolite that is one of a family of neuroactive steroids (NAS) that are potent positive allosteric modulators of γ -aminobutyric acid_A (GABA_A) receptors. These GABAergic NAS are produced peripherally (in the adrenals and gonads) and centrally in the brain. Peripherally produced NAS modulate some effects of ethanol intoxication (e.g., anxiolytic, antidepressant, and anticonvulsant effects) in rodents. We have found that NAS also may be involved in the rebound neural hyperexcitability following a high ethanol dose. Removal of the adrenals and gonads (ADX/GDX) increased withdrawal severity following 4 g/kg ethanol, as measured by handling-induced convulsions (HICs) in male and female DBA/2J mice. NAS are produced through the metabolism of progesterone (PROG), deoxycorticosterone (DOC), or testosterone, which can be blocked with the administration of finasteride (FIN), a 5 α -reductase enzyme inhibitor. The current investigation was undertaken to clarify the step(s) in the biosynthetic NAS pathway that were sufficient to restore the acute ethanol withdrawal profile in ADX/GDX mice to that seen in intact animals.

Male and female DBA/2J mice underwent ADX/GDX or SHAM surgery. After recovery, separate groups of animals were administered PROG, DOC, PROG+FIN, DOC+FIN, FIN, ALLO, ganaxalone (a synthetic ALLO derivative), corticosterone, or vehicle. Animals were then administered a 4 g/kg ethanol dose and allowed to undergo withdrawal. HICs were measured for 12 hours and again at 24 hours. The results indicate that replacement with PROG and DOC restored the withdrawal profile in ADX/GDX animals to SHAM levels, and that this effect was blocked with co-administration of FIN. Administration of FIN alone increased the withdrawal profile in both SHAM and ADX/GDX males. These findings indicate that the increase in acute withdrawal severity after ADX/GDX may be due to the loss of GABAergic NAS, providing insight into the contribution of endogenous GABAergic NAS to ethanol withdrawal severity.

Keywords

Neurosteroids; alcohol; allopregnanolone; progesterone; deoxycorticosterone; finasteride

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Each year in the United States, almost 5% of the population suffers from alcohol (ethanol; EtOH) abuse or dependence as defined by the DSM IV (Grant et al., 2004). It is estimated that this disease costs the American public millions of dollars a year in lost productivity and healthcare (Mark et al., 2000). While EtOH abuse and dependence are widespread, treatment options are extremely limited (Gardner and Kosten, 2007). One reason for this may be the wide variety of mechanisms through which EtOH can exert its effects. EtOH can have direct effects at receptors, such as acetylcholine, serotonin, γ -aminobutyric acid (GABA), and N-methyl-D-aspartic acid (NMDA) receptors (Chastain, 2006; Davis and de Fiebre, 2006). It can also alter membrane fluidity, enzyme concentrations, as well as several other factors (Busby et al., 1999; Gurtovenko and Anwar, 2009). Additionally, acute and chronic EtOH administration can cause up and down regulation of receptors and receptor subunits (Mhatre and Ticku, 1994; Devaud et al., 1997; Matsumoto et al., 2001), and the relative contribution of any of these factors may change from acute to chronic EtOH exposure. Furthermore, withdrawal from EtOH produces severe rebound neural hyperexcitability, which also may be mediated by numerous mechanisms (Littleton, 1998; Koob, 2003). Thus, in order to provide viable treatment options for alcohol abuse and dependence, it is imperative to understand the etiology of both acute and chronic EtOH intoxication and withdrawal.

Neuroactive steroids (NAS) rapidly alter neuronal excitability through interaction with neurotransmitter-gated ion channels (Paul and Purdy, 1992; Rupprecht, 2003), and many NAS are potent allosteric agonists at the γ -aminobutyric acid_A (GABA_A) receptor (Purdy et al., 1992; Rupprecht, 2003). NAS can be produced in the periphery (mainly the adrenals and the gonads) or *de novo* in the brain (Holzbauer et al., 1985; Mellon and Griffin, 2002b; Mellon and Griffin, 2002a). The production of NAS begins with the translocation of cholesterol across the mitochondrial membrane, which is facilitated by steroidogenic acute regulatory protein or the mitochondrial benzodiazepine receptor (Papadopoulos, 1993; Stocco, 2000). Then, a cytochrome P450 enzyme converts cholesterol into pregnenolone, which is a precursor to several different steroid hormones. Further down the steroidogenic pathway, the two step metabolism of progesterone (PROG), deoxycorticosterone (DOC) and testosterone produces NAS [3 α ,5 α -tetrahydroprogesterone (ALLO), 3 α ,5 α -tetrahydrodeoxycorticosterone (THDOC) and 3 α -androstenediol, respectively] through the enzymes 5 α -reductase and 3 α -hydroxysteroid dehydrogenase (Mellon, 1994; Compagnone and Mellon, 2000). It is possible to modulate the pathways leading to NAS by using enzyme inhibitors such as finasteride (FIN; Rittmaster, 1997) or removing the peripheral sources of NAS [i.e., adrenalectomy; ADX or gonadectomy; GDX (Korneyev et al., 1993)].

In animals, NAS administration produces anxiolytic, antidepressant, anticonvulsant and sedative effects (Gasior et al., 1999), consistent with their GABAergic properties. These behavioral effects of NAS are mediated via the GABA_A receptor. Recent evidence indicates that NAS bind in a specific pocket between the α and β subunits, allowing chloride to flux into the cell (Hosie et al., 2009). There are many distinct subunits (Olsen and Sieghart, 2009) and the subunit composition can fluctuate in response to environmental and physiological changes (Smith et al., 2007). While subunit composition of GABA_A receptors may contribute to sensitivity of the receptor to modulation by NAS (Belelli and Lambert, 2005), manipulation of local endogenous GABAergic NAS levels also can alter GABA_A receptor-mediated inhibition (Belelli and Herd, 2003).

Many of the behavioral effects of EtOH intoxication and NAS administration overlap. In fact, research over the past several decades has shown that some of EtOH's behavioral consequences may be modulated by an increase in NAS production (Kumar et al., 2009). Acute EtOH administration increases the production of both plasma and brain concentrations of ALLO and THDOC (Barbaccia et al., 1999; VanDoren et al., 2000; Finn et al., 2004). It has been shown that the increases in these NAS contribute to the delayed actions of EtOH on neuronal inhibition

in medial septal band (VanDoren et al., 2000) and hippocampus (Sanna et al., 2004). That is, EtOH has been shown to have a direct and indirect effect on GABA_A receptor-mediated inhibition, with the indirect effect being due to steroidogenesis (Sanna et al., 2004). Additional research has shown that NAS also contribute to some behavioral effects of EtOH, such as the anxiolytic (Hirani et al., 2005), antidepressant (Hirani et al., 2002), anticonvulsant and sedative/hypnotic effects (VanDoren et al., 2000). Based on these findings, it is likely that NAS may alter sensitivity to, or the duration of, some behavioral effects of EtOH.

Recent work in our lab has also shown that the rebound neuronal hyperexcitability seen during withdrawal from a 4 g/kg acute dose of EtOH may be mediated by peripherally produced NAS (Gililand and Finn, 2007). Male DBA/2J (D2) mice that had their adrenals removed had increased withdrawal severity (as measured by handling induced convulsions; HICs), while female D2 mice had increased withdrawal severity when both their adrenals and gonads were removed. The results of these experiments suggested that an endogenous PROG- or DOC-derived anticonvulsant NAS was an important contributor to the neuronal rebound hyperexcitability seen during withdrawal from an acute, high dose of EtOH in intact animals (i.e., since removal of an anticonvulsant steroid with ADX/GDX would increase withdrawal). Given that removal of the gonads did not alter acute EtOH withdrawal in male mice, we reasoned that testosterone and its GABAergic derivatives may exhibit minimal contributions to the acute EtOH withdrawal profile in intact animals. However, in the current experiments we chose to use animals from both sexes that had both ADX and GDX surgery to insure total removal of the main sources of peripheral NAS production.

In order to further characterize this response, the purpose of the present studies was to identify specific steps along the NAS biosynthetic pathway that were necessary or sufficient to modulate neuronal rebound hyperexcitability following a high dose of EtOH (i.e., acute EtOH withdrawal). In order to fully explore this idea, we felt it was imperative that several arms along the NAS biosynthetic pathway and several steps within each arm were tested in our paradigm. We have chosen to investigate both the PROG and DOC arm of the NAS biosynthetic pathway because of previous data from our laboratory (discussed in the previous paragraph) indicate these two arms may be the involved in EtOH withdrawal. The strategy was two-fold: 1) to administer NAS (ALLO, GAN and CORT) or their precursors (PROG and DOC) and determine the effect on acute EtOH withdrawal in ADX/GDX animals and 2) to determine whether metabolism of NAS precursors was necessary for the modulatory effect on acute EtOH withdrawal in ADX/GDX animals through the use of FIN. We hypothesized that replacing ADX/GDX animals with PROG or DOC, or their 5 α -reduced metabolites would restore acute withdrawal severity back to the levels seen in intact animals and that co-administering FIN would abolish these effects.

Experimental Procedures

Subjects

Drug naïve DBA/2J (D2) male and female mice were purchased from Jackson West Laboratories (Davis, CA) and were 8-12 weeks old at the time of experiment. Animals were group housed (4/cage, separated by sex) and were allowed free access to rodent chow (Labdiet 5001 rodent diet; PMI International, Richmond, IN) and water. Mice were maintained on a 12-hour (6 am to 6 pm) light/dark cycle in polycarbonate cages (Thorens, Hazleton, PA) in a room kept at 21 $\pm$ 2° C with humidity control. Mice were allowed to acclimate to the facility for at least 1 week before any experimental manipulations were undertaken. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and were approved by the local Institutional Animal Care and Use Committee.

Procedure

All animals were assigned to one of two groups: ADX/GDX surgeries (in which both the adrenals and the gonads were removed; surgery detailed below) or SHAM surgery (in which no organs were removed). After the appropriate surgery was performed, animals were allowed to recover for 7-14 days. Once recovered, animals were assigned to one of nine treatment groups (outlined below in drug section and in Table 1). Due to the large number of animals, the experiments were completed in nine passes, one with each drug treatment and several control animals. Baseline HIC scores were assessed and then the appropriate treatment was administered. Following drug treatment, all animals were administered a 4 g/kg intraperitoneal (IP) dose of EtOH (Aaper alcohol and chemical company, Shelbyville, KY; 20% v/v in saline). Animals were monitored for HICs for 12 hours following EtOH injection and again 24 hours later. Upon completion of HIC scoring, animals were decapitated and dissected to confirm organ removal.

A separate group of animals was utilized to explore the timing of FIN treatment (see Drugs and Treatment section, below). Male and female D2 mice underwent SHAM surgery and were allowed to recover for one week. Animals received either an injection of 20% cyclodextrin (VEH), or 50 mg/kg finasteride (FIN) in 20% cyclodextrin (both IP; 0.01 ml/gm). Animals then received a second injection of 50 mg/kg finasteride directly followed by a 4 g/kg dose of IP EtOH. Following treatment, all animals were returned to their home cage. Fifteen minutes, 2 and 8 hours following EtOH injection, subsets of animals were placed in a clean cage, transported to another room, and decapitated. Brains were collected on ice and immediately frozen at -80°C until ALLO levels were measured with radioimmunoassay (RIA).

Surgery

Surgeries were performed as previously described (Gililand & Finn, 2007). Briefly, anesthesia was induced with 5% isoflurane (Abbott Laboratories, North Chicago, IL) and maintained at 2% throughout surgery. A dorsal midline incision was made on all animals. This incision was used to access the lateral muscle walls. Incisions were made in the muscle walls, the adrenal glands were extracted, and the incision was closed with surgical steel wound clips. In female animals, this incision was also used to extract the ovaries; this caused the muscle wall incision to be larger and it was closed with 4-0 chromic gut suture material (Davis & Geck, Danbury, CT). In male animals, the peritoneal cavity was entered via a small cranial pubic incision from which the testes were removed. This incision was closed with tissue adhesive. ADX and GDX were performed during the same surgical session. Animals in the SHAM surgery group received all incisions, but no organs were removed. After surgery, all animals were administered a subcutaneous dose of 3 mg/kg keterolac (Sigma-Aldrich, St Louis, MO) for 2 days and had unlimited access to 0.9% NaCl (in order to maintain sodium balance (Beers and Berkow, 2005); Baxter, Deerfield, IL), water and chow for the remainder of the experiment. The weight of all animals was monitored.

Drugs and Treatments

Animals were administered one of nine treatment paradigms: vehicle (VEH), progesterone (PROG), finasteride (FIN), allopregnanolone (ALLO), deoxycorticosterone (DOC), corticosterone (CORT), ganaxalone (GAN), PROG+FIN, and DOC+FIN. All drugs were dissolved in VEH (20% w/v β -cyclodextrin in 0.9% saline; Cerestar USA, Hammond, IN) in a concentration to facilitate dosing at 0.01 ml/gram body weight and were administered IP. VEH, PROG (5 mg/kg; Sigma; St. Louis, MO), ALLO (10 mg/kg; Purchase from Dr. Robert Purdy, San Diego, CA), DOC (50 mg/kg; Sigma), CORT (20 mg/kg; Sigma) and GAN (10 mg/kg; Purchased from Dr. Robert Purdy) were administered 30 minutes prior to EtOH administration. FIN (50 mg/kg; Steraloids, Newport, RI) was administered twice, 24 hours before and immediately prior to EtOH administration. Groups that received two treatments

(PROG+FIN and DOC+FIN) received the two doses of FIN, as described, in addition to steroid administration 30 minutes prior to EtOH administration. Doses were chosen from the literature in order to mimic the increases in steroid levels seen after acute EtOH administration. See Table 1 for a summary of treatment groups.

Handling-Induced Convulsion (HIC) scoring

HICs were scored according to a previously published scale (Crabbe et al., 1991). The HIC scores range from 0 to 7; a score of 0 indicates no convulsions, a score including 1 to 3 indicates tonic or clonic convulsions obtained by a gentle turn, a score including 4 to 6 indicates convulsions elicited by only lifting the mouse by the tail, and a score of 7 indicates a spontaneous convulsion. At each HIC measurement, the animal was briefly observed in the home cage for spontaneous convulsions (which would indicate a score of 7). If spontaneous seizures were not observed, the animal was lifted by the tail. If a seizure was elicited by lifting the animal alone, the HIC was scored as a 4 to 6, depending upon the severity of the convulsion (4 indicates a tonic convulsion, 5 indicates a tonic clonic convulsion, and 6 indicates a severe tonic clonic convulsion) and the animal was returned to its home cage. If no seizure was elicited from lifting the animal, the animal was gently turned 180° by the tail. Seizures elicited by this turn were scored as 1 to 3, depending upon the severity (1 indicates a facial grimace, 2 indicates a tonic convulsion, and 3 indicates a tonic clonic convulsion). If no seizure was observed after one 180° spin, the HIC was scored as a zero and the animal was returned to the home cage.

Allopregnanolone extraction and Radioimmunoassay (RIA)

ALLO was extracted from whole brain according to the methods of Janis et al. (1998). Briefly, whole brain was digested with 385 µl of 0.3N NaOH and then 100 µl [³H] ALLO (200 counts per minute (CPM)/100 µl in EtOH) was added to monitor extraction efficiency (65 Ci/mmol; New England Nuclear, Boston, MA). The samples were each extracted three times with 10% (v/v) heptane and then sonicated for 1 minute, followed by 2 minutes of centrifuging at 1000 g. The supernatants from these 3 extractions were collected, combined and applied to solid silica phase columns (Honeywell, Burdick and Jackson, Muskegon, MI), which were then washed with heptanes. ALLO was eluted under gravity with 25% (v/v) acetone in pentane and then dried under nitrogen. These samples were frozen until the RIA could be performed.

The RIA for ALLO was conducted according to the methods described in detail by Finn and Gee (1994) and Finn et al (2004). Assays used used [³H] ALLO (10,000 cpm in 100 µl l in RIA buffer) and a polyclonal antiserum that was a generous gift from Dr. Kelvin Gee (University of California, Irvine, CA) and had minimal cross reactivity. Results were normalized and fit to a least square regression equation produced by log-logit transformation of the standards (0.0195 to 20 ng). The mass of the samples was calculated by interpolation of the standards and were corrected for extraction efficiency. The minimum detectable limit in the assay was 25 pg and all samples were run in the same assay.

Data Analysis

Data are expressed as the mean ± SEM. Analyses were conducted in SHAM and ADX/GDX animals with verified organ removal. Withdrawal severity was quantified by calculating the area under the curve (AUC) for each animal (hours 0 to 24), using the trapezoidal method, as previously described (Crabbe et al., 1983; Metten and Crabbe, 2005). Hourly HIC scores were analyzed by a multifactorial ANOVA with time as a within subjects factor and sex (2 levels), surgery (2 levels), FIN treatment (2 levels) and steroid treatment (6 levels) as between subjects factors. When appropriate (due to significant interactions), data were separated by sex and surgery and analyzed with a two-way ANOVA (surgery by time) with repeated measures and a Greenhouse–Geiser correction. Each time point was analyzed separately when appropriate using a one-way ANOVA. AUC was analyzed with a multifactorial ANOVA (sex by surgery

by treatment) and then followed by a one-way ANOVA analyzing each sex and surgery separately. Baseline HIC scores were analyzed with a multifactorial ANOVA and if necessary, a one-way ANOVA due to interactions. Several apriori hypotheses were tested with one-way ANOVAs. Significance was set at $p < 0.05$.

Results

A multifactorial repeated measures ANOVA was used in order to determine if FIN administration had suppressed ALLO levels during the withdrawal period. Time was a within subjects factor, while sex and drug administration were between subject factors. There were significant effects of sex [$F(1,107)=12.99$, $p < 0.001$; female > male] and drug administration [$F(1,107)=49.27$, $p < 0.001$; VEH > FIN], and no other interactions. These results confirmed that FIN pretreatment had suppressed ALLO production during the test phase (see Figure 1).

Exploratory data analysis revealed that the hourly data set violated assumptions of sphericity, and therefore, subsequent analysis on all hourly data employed a Greenhouse-Gieser correction. A multifactorial ANOVA revealed that there were significant main effects of time [$F(7.64, 3771.98)=1178.22$, $p < 0.0001$], surgery [$F(1,494)=23.88$, $p < 0.0001$] and treatment [$F(8,494)=10.72$, $p < 0.0001$] but no main effect of sex on hourly HIC scores. This analysis also revealed that all interactions were significant, including a four-way interaction between time, sex, surgery and drug treatment [$F(61.085, 3771.93)=2.312$, $p < 0.0001$]. Due to these highly significant interactions, further analysis on the hourly HIC scores was conducted separately on groups divided by sex and surgery. Data in male mice are depicted in Figure 2, while data in female mice are depicted in Figure 3.

A repeated measure ANOVA using time as a within subjects factor and drug treatment in ADX/GDX operated animals revealed a main effect of time [$F(7.15, 793.32)=302.06$, $p < 0.0001$], a main effect of drug treatment [$F(8,111)=17.42$, $p < 0.0001$] and a significant interaction [$F(57.18, 793.18)=7.26$, $p < 0.0001$]. A Dunnett's post-hoc (comparing all male ADX/GDX treatment groups to the male ADX/GDX, VEH treated group) revealed that PROG and DOC administration lowered hourly HIC scores and that DOC+FIN, GAN and CORT administration increased hourly HIC scores. There was no effect of PROG+FIN, ALLO, or FIN administration on hourly HIC scores in male ADX/GDX animals (see Figure 2A and 2B for representative hourly HIC scores).

A repeated measure ANOVA using time as a within subjects factor and drug treatment as a between subjects factor in the male SHAM operated animals revealed a main effect of time [$F(7.327, 981.79)=321.634$, $p < 0.0001$], a main effect drug treatment [$F(8,134)=7.543$, $p < 0.0001$] and a significant interaction [$F(58.61, 981.79)=4.582$, $P < 0.001$]. A Dunnett's post-hoc test (comparing all male SHAM treatment groups to the male SHAM, VEH treated group) revealed that animals treated with PROG, DOC, GAN, CORT and FIN had increased hourly withdrawal (see Figure 2C and 2D for representative hourly HIC scores).

A similar strategy was used in the analysis of HIC scores in female mice. Hourly HIC scores in female ADX/GDX animals were significantly influenced by time [$F(7.07, 841.55)=342.30$, $p < 0.0001$] and drug treatment [$F(8,119)=8.57$, $p < 0.0001$], with a significant interaction between time and treatment [$F(56.58, 841.55)=5.33$, $p < 0.0001$]. A Dunnett's post-hoc test (comparing all female ADX/GDX drug treatments to the female ADX/GDX, VEH treated group) revealed that when compared to VEH animals, PROG, DOC and ALLO administration significantly decreased hourly HICs (See Figure 3A and 3B for representative hourly withdrawal curves).

Analysis in female SHAM operated animals revealed a main effect of time [$F(6.29, 817.10)=249.50$, $p < 0.0001$], no main effect of drug treatment, and a significant interaction [$F(50.28,$

817.10)=3.29, $p<0.0001$]. Due to the lack of a main effect of drug treatment, no post-hoc tests were performed (See Figure 3C and 3D for representative hourly HIC curves).

In order to determine whether surgery or the FIN pre-treatment affected baseline HIC scores, a multivariate ANOVA determined the effects of sex, surgery and treatment on baseline HIC scores. This analysis showed a main effects of sex [male>female; $F(1,530)=4.98$, $p=0.026$] and drug treatment [$F(8,530)=8.83$, $p<0.0001$] and no interactions. A post hoc Dunnett's test comparing each drug group to VEH treated mice, showed that FIN pre-treatment significantly increased baseline withdrawal scores from 1.32 to 2.11 or 1.77 in the FIN or PROG+FIN groups (regardless of sex), respectively.

In order to analyze the effects of surgical status, sex and drug treatment on total withdrawal severity, the area under the curve (AUC) was calculated. A three way ANOVA on AUC revealed main effects of sex [male>female; $F(1,530)=3.856$, $p=0.05$], surgery [ADX/GDX>SHAM; $F(1,530)=20.17$, $p<0.0001$] and drug treatment [$F(8,530)=6.14$, $p<0.001$]. There were significant interactions between sex and surgery, surgery and drug treatment, and a trend toward a three way interaction between sex, surgery and drug treatment (Figure 4). Due to these interactions and to keep the data analysis consistent with that conducted on the hourly data, the effect of drug treatment on AUC was analyzed for each sex and surgery.

A one way ANOVA evaluating the effect of drug treatment on AUC in ADX/GDX males found that drug treatment significantly altered AUC [$F(8,119)=11.73$, $p<0.0001$] (Figure 4A). Administration of PROG and DOC significantly decreased AUC, but the administration of FIN significantly increased AUC in these animals (Figure 4A). In SHAM males, there was a significant effect of drug treatment [$F(8,142)=3.25$, $p=0.002$]. Post-hoc tests comparing each drug group to VEH indicated that PROG, DOC and FIN all significantly increased AUC. Additionally, there was a trend for CORT administration to increase AUC ($p=0.08$; Figure 4B). A one-way ANOVA comparing the SHAM VEH treated males to ADX/GDX PROG and DOC treated males revealed a strong trend toward these groups being different from each other [$F(2,64)=3.03$, $p=0.056$].

In ADX/GDX female mice, a one way ANOVA revealed a significant effect of drug treatment [$F(8,127)=6.86$, $p<0.0001$]. A post-hoc comparison of each treatment group to the VEH group revealed that treatment with PROG or DOC significantly ($p<0.05$) decreased AUC (Figure 4C), and that there was a trend for ALLO administration to decrease AUC compared to VEH ($p=0.07$). The same analysis in SHAM female animals revealed no effect of drug treatment on AUC [Figure 4D; $F(8,138)=0.86$, $p=0.56$]. A one-way ANOVA comparing female SHAM VEH treated mice to ADX/GDX PROG and DOC treated mice revealed that these groups were not different from each other [$F(2,64)=0.277$, $p=0.759$].

Discussion

Previous work in our lab has shown that removing the main peripheral sources of PROG- and DOC-derived NAS increased acute EtOH withdrawal severity in D2 male and female mice (Gililand and Finn, 2007). These studies replicated these results as animals that had undergone ADX/GDX surgery had a more severe withdrawal profile than animals that had undergone a SHAM surgery. Further, the lack of effect of GDX on withdrawal severity in D2 male mice suggested that testosterone-derived NAS had no effect on acute EtOH withdrawal. This model of EtOH withdrawal utilizes a single, acute injection of a sedative dose of EtOH. The initial depressant effect produced by administration of the high dose of EtOH is followed by rebound hyperexcitability as the EtOH is metabolized (i.e., at approximately 4-8 hr post-injection). Notably, acute (single dose) and chronic intermittent withdrawal procedures produced similar changes in GABA_A receptor composition and function, with differences in the persistence of

the changes in GABA_A receptor plasticity (Liang et al., 2007). Thus, the examination of neuroadaptation following withdrawal from a single high dose of EtOH should provide insight regarding neuroadaptation following chronic EtOH withdrawal.

The current experiments replicated the findings of enhanced acute EtOH withdrawal in ADX/GDX animals (Gililand and Finn, 2007). Importantly, the present results expanded on our previous work by attempting to elucidate the steps in the NAS biosynthetic pathway that were necessary or sufficient to restore the withdrawal profile to levels seen in intact animals. It had previously been shown that in certain conditions, PROG and DOC can exert an anticonvulsant effect, and that metabolism to their GABAergic NAS derivatives was important for their anticonvulsant properties (Frye et al., 2002; Reddy and Rogawski, 2002). Consistent with these data, our results indicate that in male and female D2 animals that have undergone surgery to remove the main peripheral sources of NAS, the NAS precursors PROG and DOC were sufficient to restore the acute EtOH withdrawal profile to that of intact animals. Furthermore, the ability of PROG and DOC to decrease withdrawal in ADX/GDX mice was abolished by inhibiting the 5 α -reduction of PROG and DOC, indicating that the GABAergic NAS metabolites of PROG and DOC were mostly likely responsible for the restoration of EtOH withdrawal severity to levels in intact animals. These results agree with and add to previous data that has shown that some of EtOH's effects (such as anticonvulsant, anxiolytic and antidepressant effects) are also modulated by 5 α -reduced NAS.

While these results indicate that 5 α -reduced anticonvulsant NAS modulate the rebound neuronal hyperexcitability seen during acute EtOH withdrawal, they also suggest that withdrawal severity in ADX/GDX animals was not modulated by glucocorticoids. Replacing ADX/GDX animals with 20 mg/kg of CORT before EtOH withdrawal did not restore the withdrawal profile to that in intact animals. In fact, this drug regimen tended to increase withdrawal severity in male SHAM animals. These data are consistent with previous results showing that CORT administration significantly increased withdrawal from an acute dose of EtOH in intact male mice (Roberts et al., 1992). While the current data only tended toward an increase, the previous experiments were performed in an animal model that was selectively bred for high EtOH withdrawal severity (Withdrawal Seizure-Prone, WSP mice) and that was shown to be very sensitive to manipulations in steroid levels. Additionally, as we were trying to mimic the increase in CORT seen directly following the administration of EtOH, our dosing regimen was slightly different than that in the Roberts et al. (1992) study.

An unexpected finding of these experiments was that in male SHAM animals (but not any other group), administration of PROG and DOC *increased* withdrawal severity from an acute dose of EtOH. Co-administration with FIN eliminated this effect, suggesting that the proconvulsant effect of PROG and DOC also might be due to the 5 α -reduced metabolites of these steroids. Although speculative, it is possible that administering PROG and DOC to intact animals increased ALLO and THDOC to levels that were additive to EtOH with respect to rebound GABAergic hyperexcitability following the decline in concentrations of these NAS during withdrawal. In fact, it has been shown that administering high doses of ALLO (75 mg/kg) to the WSP line of mice increased HIC's over a 25 hour period (Reilly et al., 2000), consistent with an acute withdrawal response from high supra-physiological ALLO levels. While it is unlikely that the animals in our study are reaching the very high ALLO levels seen in the study by Reilly et al. (2000), it may be that intact male D2 mice are sensitive to NAS withdrawal under particular conditions of acute EtOH withdrawal, perhaps due to changes in GABA_A plasticity (e.g., receptor density, distribution or subunit composition). Further experiments exploring these effects must be undertaken (including dose response curves) in order to make any firm conclusions from these data.

Also opposite to our initial prediction was the finding that replacing ADX/GDX animals with ALLO or GAN did not restore the withdrawal profile to that of intact animals. The ALLO results are most likely due to the fact that ALLO is rapidly metabolized. The current experiments administered ALLO only once in order to try and mimic the increases seen after EtOH administration (Barbaccia et al., 1999; Morrow et al., 1999; VanDoren et al., 2000; Finn et al., 2004). However, the single administration of ALLO would not model the endogenous NAS tone that would be present in intact animals. Nonetheless, to try and mitigate the fast half-life of ALLO, we administered GAN, a 3 β -methyl-substituted analog of ALLO that has been reported to have a longer half-life while still being an allosteric agonist at GABA_A receptors (Carter et al., 1997). However, this treatment also did not restore the acute withdrawal profile in ADX/GDX animals. It may be that the dosage of GAN that was used (10 mg/kg) was not high enough to increase brain concentrations to achieve a level of GABAergic inhibition that would be required to restore the acute withdrawal profile in the ADX/GDX animals. Alternately, it could be that a different time point for administration should have been chosen in order for GAN to alter GABAergic inhibition in the brain. Due to these results, THDOC was not tested in our model. THDOC also has a fast half life of less than 20 minutes (Reddy, 2003), so we reasoned that using this drug would have the same potential confounds as with ALLO administration.

While the current experiment was designed to try and imitate the rise in NAS seen in intact animals in response to an acute dose of EtOH, it is possible that being deprived of NAS and their precursors for one to two weeks prior to the experiment changed GABA_A subunit composition in the ADX/GDX mice. In fact, it has been shown that two days after ADX/GDX in female rats, expression of the GABA_A receptor α_1 , α_2 , and γ_2 subunits increased, while β_2 subunit expression decreased in the hippocampus (Orchinik et al., 1994). Changes such as these could be influencing the animals' sensitivity to steroid replacement, and in fact steroid replacement may also be changing subunit expression. In ADX/GDX female rats that received a single dose of PROG and were killed five hours later, there was a decrease in α_1 and α_2 subunit mRNA, but a further increase in γ_2 mRNA (Weiland and Orchinik, 1995). It should be noted that these animals also had estrogen replacement, so it is unknown if PROG alone elicited these effects. We plan to investigate the effects of our dosing regimens on GABA_A subunits in the hippocampus in a future experiment.

It is interesting that both PROG and DOC administration were sufficient to restore the withdrawal profile in ADX/GDX male and female mice to that seen in intact animals. Both PROG and DOC can be metabolized through a two step process that yields NAS. Both of the 5 α -reduced NAS of PROG and DOC have been shown to be positive allosteric agonists of the GABA_A receptor, although ALLO was more potent than THDOC (Morrow et al., 1987; Belelli et al., 1990). Nonetheless, the present findings suggest that both ALLO and THDOC could be sufficient to restore acute withdrawal severity to that seen in intact animals if correctly dosed. It is not known whether administration of both NAS precursors (i.e., PROG + DOC) could further reduce withdrawal severity.

Overall, removing the peripheral sources of NAS during EtOH withdrawal increases the severity of the withdrawal. Restoring either the PROG or DOC arm of the biosynthetic NAS pathway can restore the withdrawal profile to that seen in SHAM operated animals. The current results indicate that the increase in NAS steroids seen following an acute dose of EtOH functions to modulate neural rebound hyperexcitability and helps maintain homeostasis of the GABAergic system. Furthermore, they show that peripheral sources of the 5 α -reduced NAS are important for modulating the rebound neural hyperexcitability seen during withdrawal from an acute dose of EtOH. While these findings provide another important step forward in understanding how EtOH withdrawal modulates brain excitability, they are by no means conclusive data. Follow-up experiments further exploring the mechanisms behind these results

must be undertaken in order to draw more conclusions. An understanding of the protective contribution of endogenous GABAergic NAS to EtOH withdrawal severity may lead to new treatment strategies for EtOH abuse and dependence in the future.

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Abbreviations used

ALLO	allopregnanolone
PROG	progesterone
DOC	deoxycorticosterone
NAS	neuroactive steroid
HIC	handling-induced convulsion
EtOH	ethanol
GABA	γ -aminobutyric acid
NMDA	N-methyl-D-aspartic acid
ADX	adrenalectomy
GDX	gonadectomy
FIN	finasteride
CORT	corticosterone
GAN	ganaxalone
THDOC	3 α ,5 α -tetrahydrodeoxycorticosterone
D2	DBA/2J
AUC	area under the curve
WSP	Withdrawal Seizure-Prone

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Figure 1.

Two doses of 50 mg/kg finasteride (FIN) reliably decreased allopregnanolone (ALLO) production over an 8 hour period. Separate groups of male and female DBA/2J animals were dosed with either 50 mg/kg FIN or 20% cyclodextrin at 24 hours before and immediately prior a 4 g/kg dose of EtOH. Different groups of animals were euthanized at 15 minutes, 2 hours and 8 hours following the EtOH injection. FIN administration significantly decreased ALLO production during the entire 8 hour period when compared to VEH treated animals. Bars represent the mean $\pm$ the SEM for 8-10 animals/group. * $p < 0.05$.

Figure 2.

The effects of steroid replacement on acute ethanol (EtOH) withdrawal severity in male DBA/2J mice, measured by hourly handling-induced convulsions (HICs). Overall, in ADX/GDX males (panels A and B), replacement with progesterone (PROG) or deoxycorticosterone (DOC) decreased withdrawal severity. Co-administration of FIN blocked this effect. In SHAM males (panels C and D), administration of PROG or DOC increased withdrawal severity, and co-administration of FIN blocked this effect. Points represent the mean $\pm$ the SEM for 9-16 for treatment groups and 36-40 for VEH treated groups that were collapsed across passes.

A. ADX/GDX Females

B. ADX/GDX Females

C. SHAM Females

D. SHAM Females

Figure 3.

The effects of steroid replacement on acute EtOH withdrawal severity in female DBA/2J mice, measured by hourly HICs. Overall, in ADX/GDX females (panels A and B), replacement with PROG or DOC decreased withdrawal severity. Co-administration of FIN blocked this effect. In SHAM females (panels C and D), there was no effect of any drug treatment on acute withdrawal severity. Points represent the mean $\pm$ the SEM for 12-16 for treatment groups and 28-40 for VEH treated groups that were collapsed across passes.

Figure 4.

The effect of steroid replacement on acute EtOH withdrawal severity in DBA/2J mice, measured by AUC. In ADX/GDX males (panel A), administration of PROG and DOC decreased AUC, co-administration with FIN abolished this effect, and administration of FIN alone increased AUC. In SHAM males (panel B), PROG and DOC administration increased AUC, while co-administration with FIN abolished this effect. FIN administration alone significantly increased AUC, and corticosterone (CORT) administration tended to increase AUC. In ADX/GDX females (panel C), administration of PROG and DOC significantly decreased AUC, and administration of ALLO tended toward a decrease. Co-administration of FIN abolished these effects. In SHAM females (panel D), there were no effects of any drug administration on AUC. Bars represent the mean $\pm$ the SEM for the animals depicted in Figures 2 and 3. + $p < 0.10$, * $p < 0.05$ vs. respective VEH treated group.

Table 1

Summary of Treatment Regimens

Treatment	Dosage	Reference for dosage
20% Cyclodextrin (VEH)	0.01 ml/gm of body weight	N/A
Progesterone (PROG)	5 mg/kg, 30 min prior to EtOH	Hirani et al., 2005
PROG+FIN	PROG: 5 mg/kg, 30 min prior to EtOH FIN: 50 mg/kg $\times$ 2, 24 hours before and with EtOH	Hirani et al., 2005; Gorin et al., 2005 and current experiments
Deoxycorticosterone (DOC)	50 mg/kg, 30 min prior to EtOH	Reddy and Rogawski, 2002
DOC + FIN	DOC: 50 mg/kg, 30 min prior to EtOH FIN: 50 mg/kg $\times$ 2, 24 hours before and with EtOH	Reddy and Rogawski, 2002; Gorin et al., 2005 and current experiments
Finasteride (FIN)	50 mg/kg $\times$ 2, 24 hours before and with EtOH	Gorin et al., 2005 and current experiment
Corticosterone (CORT)	20 mg/kg, 30 min prior to EtOH	Roberts et al., 1994
Allopregnanolone (ALLO)	10 mg/kg, 30 min prior to EtOH	Beckley et al., 2008; Finn et al., 1997
Ganaxolone (GAN)	10 mg/kg, 30 min prior to EtOH	Carter et al., 1997