THE EFFECTS OF NEUROACTIVE STEROIDS ON ACUTE ETHANOL WITHDRAWAL IN MICE

by

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LIST OF ABBREVIATIONS

ADX - adrenalectomy

ALLO - 3α, 5α tetrahydroprogesterone; allopregnanolone

ANOVA - analysis of variance

AUC - area under the curve

B6 – C57BL/6J

BEC - blood ethanol concentration

CNS - central nervous system

CORT - corticosterone

D2 - DBA/2J

DHEA - Dihydroepiandrosterone

DHEAS- Dihydroepiandosterone sulfate

5α-DHP - 5α dihydroprogesterone

DOC - deoxycorticosterone

EtOH - ethanol

FIN - finasteride

$GABA_A$- γ-aminobutyric $acid_A$

GAN - ganaxolone

GDX - gonadectomy

HIC – handling-induced convulsion

HPA - hypothalamic-pituitary-adrenal

5-HT- serotonin

IP - intraperitoneal

KO – knock out

NAS - neuroactive steroid

NMDA - N-methyl-D-aspartate

OVX - ovariectomized

PKA- protein kinase A

PKC- protein kinase C

PROG - progesterone

PS- pregnenolone sulfate
qRT-PCR – quantitative Real-Time reverse transcriptase polymerase chain reaction

RIA - radioimmunoassay

SHAM - sham surgery

StAR - steroidogenic acute regulatory

THDOC - 3α, 5α tetrahydrodeoxycorticosterone

VEH- vehicle

WSP - Withdrawal Seizure-Prone

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ABSTRACT

The alcohol (Ethanol; EtOH) withdrawal syndrome is a hallmark of EtOH dependence, and withdrawal avoidance is one reason that alcoholics relapse. Elucidating the mechanisms underlying EtOH withdrawal may result in improved pharmacotherapy for EtOH dependence. One neural substrate underlying the acute and chronic effects of EtOH is the γ-aminobutyric acid$_A$ ($GABA_A$) receptor system. Neuroactive steroids (NAS) are potent allosteric agonists at $GABA_A$ receptors, and previous work has indicated that a single, acute administration of EtOH increased levels of NAS and that these levels were decreased during EtOH withdrawal. However, the contribution of NAS to the symptoms of EtOH withdrawal is not known.

The main purpose of this dissertation was to elucidate the contributions of endogenous GABAergic NAS to the expression of the acute EtOH withdrawal profile in male and female mice. To accomplish this, the peripheral sources of NAS were removed (through adrenalectomy, ADX; gonadectomy, GDX; and ADX/GDX surgery) in both male and female DBA/2J (D2) and C57BL/6J (B6) mice. Rebound hyperactivity after a 4 g/kg dose of EtOH was measured with handling-induced convulsions (HICs) versus animals with SHAM surgery. I predicted that ADX/GDX surgery would increase the severity of neuronal rebound hyperactivity versus SHAM surgery, indicating that an endogenous anticonvulsant NAS was an important modulator of the neural rebound hyperactivity seen during EtOH withdrawal in intact animals. Acute EtOH withdrawal was increased in male D2 and B6 that had undergone ADX or ADX/GDX surgery and in female D2 mice following ADX/GDX, when compared to respective SHAM mice. In contrast, surgical status did not alter EtOH withdrawal severity in female B6 mice. These

results suggested that progesterone (PROG) or deoxycorticosterone (DOC), but not a testosterone derived NAS, was involved in modulating acute EtOH withdrawal severity.

In order to further examine the contributions of NAS to acute EtOH withdrawal severity, male and female D2 mice underwent ADX/GDX or SHAM surgery. After recovery, separate groups of animals were administered steroids in the NAS biosynthetic pathway or steroids plus the 5α-reductase inhibitor finasteride (FIN). HICs were the index of withdrawal severity after a 4 g/kg dose of EtOH. 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. 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 EtOH withdrawal severity.

A final experiment attempted to elucidate a mechanism for the effect of ADX/GDX and steroid replacement on acute EtOH withdrawal severity. I measured the expression of eight $GABA_A$ receptor subunits and steroidogenic acute regulatory (StAR) protein in the hippocampus of the animals just described using quantitative Real-Time reverse transcriptase polymerase chain reaction. It was found that expression of the $GABA_A$ receptor α1 subunit in male and female ADX/GDX mice was decreased in all groups that had received pretreatments that did not restore the withdrawal profile to that in intact animals. A similar finding was revealed in ADX/GDX female mice when expression of StAR protein mRNA was analyzed. These results suggest that expression levels of the $GABA_A$ receptor α1 subunit and StAR protein may be important in modulating some of the effects seen during acute EtOH withdrawal.

CHAPTER 1. General Introduction

Alcohol Dependence and Withdrawal: Definitions and Clinical Significance

Each year in the United States, 8.5% of the population suffers from alcohol (ethanol; EtOH) abuse or dependence (Grant et al., 2004) as defined by the Diagnostic and Statistical Manual of Mental Disorders-IV (American Psychiatric Association, 2000). The DSM-IV defines both EtOH abuse and dependence as "a maladaptive pattern of substance abuse, leading to clinically significant impairment or distress". One hallmark of EtOH abuse is that EtOH use is continued despite the resultant failures to fulfill obligations, dangerous situations, legal problems and interpersonal problems. The seemingly more serious EtOH dependence is classified by tolerance (defined as a need for greater amounts of EtOH to be consumed to achieve the desired intoxication or for a diminished effect of the same amount of EtOH previously consumed) and withdrawal.

While EtOH withdrawal symptoms can be as mild as increased hand tremor, or as serious as hallucinations and grand mal seizures, symptoms can generally be sorted into three categories. First is that of hyperactivity of the autonomic nervous system (Bayard et al., 2004). These symptoms can be dangerous, since the autonomic nervous system regulates vital functions such as blood pressure, respiration, body temperature and pulse rate, all of which can be abnormal during withdrawal. Second is distortions in sensation and perception, which may include hallucinations and may be dangerous if the patient is in an uncontrolled environment (Grillon et al., 1994). The final category of EtOH withdrawal symptoms is that of hyperexcitability of the central nervous system (CNS). While hyperexcitability of the nervous system can result in minor symptoms

such as agitation and anxiety, it is perhaps the most dangerous withdrawal symptom as it can result in seizures (Becker, 2000; Schuckit et al., 1995).

It is estimated that EtOH abuse and dependence costs the American public millions of dollars a year in lost productivity and healthcare (Mark et al., 2000). Two percent of the individuals diagnosed with an EtOH disorder are admitted to the hospital for treatment necessary to mitigate EtOH use withdrawal related symptoms such as tremors, hallucinations or seizures (Kozak et al., 2002). While treatment for EtOH withdrawal is fairly straight forward with supportive care and benzodiazepine administration, this disease is heavily taxing on already over-worked hospital staff and has an enormous public cost (Holbrook et al., 1999; Mark et al., 2000). Treating EtOH withdrawal must be followed up by continued therapy for the long-term clinical outlook of the patient to improve, and even then over 60% of patients will relapse (Dawson et al., 2007).

While EtOH abuse and dependence are widespread, pharmacotherapy treatment options are extremely limited (Gardner and Kosten, 2007). The only treatments that are currently approved by the Federal Food and Drug Administration are disulfiram, naltrexone and acamprosate. Disulfiram prevents the metabolism of acetaldehyde to acetic acid, causing EtOH consumption to result in flushing, nausea and vomiting due to an increase in acetaldehyde levels (Wright and Moore, 1990). While it would be reasonable that disulfiram would cause an aversion to EtOH consumption, early placebo controlled trials did not show an improvement in drinking outcomes over placebo (Fuller et al., 1986). Later studies showed that when patients were extremely motivated to quit and disulfiram was accompanied by with behavioral therapy, disulfiram had minor

efficacy (Chick et al., 1992). Naltrexone is an opioid receptor antagonist that is thought to reduce the rewarding effects of EtOH (O'Malley et al., 1996; Volpicelli et al., 1995). While naltrexone was effective at decreasing relapse over placebo, upwards of 50% of patients still relapsed when exposed to EtOH (Volpicelli et al., 1992). Acamprosate is an N-Methyl-D-asparate (NMDA) receptor modulator that is thought to help control the glutaminergic system that may be overactive in EtOH addicted individuals (Dahchour and De Witte, 1999). Similar to naltrexone, some studies show that acamprosate was effective over placebo at decreasing relapse, even though upwards of 50% of patients still relapsed within 48 weeks and this number increased over time (Sass et al., 1996). Other studies have shown very little efficacy over placebo (Anton et al., 2006).

Despite the enormous cost to society, an effective treatment for EtOH abuse and dependence has been elusive. One reason for this may be the wide variety of mechanisms through which EtOH can exert it effects. EtOH can have direct effects at receptors, such as those responsive to the neurotransmitters acetylcholine (ACh), serotonin (5-HT), GABA, and NMDA (Boone et al., 1997; Breese et al., 2006; Chastain, 2006; Davis and de Fiebre, 2006; Palachick et al., 2008; Strong et al., 1987). There are even further complications among EtOH's direct effects at receptors. For example, at certain nicotinic ACh receptors, specifically those that are insensitive to α-bungarotoxin, applications of EtOH increased ACh-evoked currents. Conversely, nicotinic ACh receptors that are sensitive to α-bungarotoxin show inhibition in response to EtOH application (Aistrup et al., 1999). Again, EtOH has been shown to enhance 5-HT signaling, although this effect is due to one 5-HT receptor subtype, 5-HT3 (Lovinger, 1999), but have an inhibitory effect on NMDA currents (Lovinger et al., 1989).

EtOH can also alter membrane fluidity, enzyme concentrations, as well as several other factors (Busby et al., 1999; Gurtovenko and Anwar, 2009). Like other low-molecular-weight anesthetics, EtOH appears to act through a nonspecific interaction with lipophilic membrane components. EtOH molecules can partition into a lipid membrane, becoming located within the water/lipid interface of the membrane, forming hydrogen bonds with hydrophilic lipid head groups (Holte and Gawrisch, 1997). The presence of EtOH molecules in the membrane has a disordering effect on lipid hydrocarbon chains, increasing the overall fluidity of the membrane and decreasing membrane thickness and rigidity (Ly and Longo, 2004).

Additionally, acute and chronic EtOH administration can cause up and down regulation of receptors and receptor subunits (Devaud et al., 1997; Matsumoto et al., 2001; Mhatre and Ticku, 1994), and the relative contribution of any of these factors may change from acute to chronic EtOH exposure. Furthermore, withdrawal from EtOH produces rebound neural hyperexcitability, which also may be mediated by numerous mechanisms (Koob, 2003; Littleton, 1998). 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

For over half a century, it has been known that sex steroids can influence neuronal excitability (Seyle, 1942). The traditional mechanism of action of steroids involves the steroid binding to its intracellular receptor followed by translocation to the nucleus where the receptor/steroid complex binds to response elements, resulting in the regulation of gene transcription (Beato, 1989; Evans, 1988; Katzenellenbogen and Katzenellenbogen,

1996; McKenna et al., 1999). This is a process that takes place on the order of minutes to hours and requires protein synthesis (McEwen, 1991). It wasn't until 40 years after the work of Seyle, that a mechanism for the fast, depressant effects of steroids was proposed. Specifically, Harrison and Simmonds (1984) showed that alphaxalone (a synthetic steroid) enhanced the stimulus-evoked inhibition at $GABA_A$ receptors. Based on the structure of alphaxalone, Harrison and Simmonds hypothesized that other endogenous steroids might also interact with $GABA_A$ receptors and exert rapid effects on membrane function. Later work established that metabolites of both progesterone (PROG) and deoxycorticosterone (DOC) exerted rapid membrane actions at $GABA_A$ receptors, based on results in electrophysiological, radioligand binding and tracer studies (Barker et al., 1987; Callachan et al., 1987; Gee, 1988; Gee et al., 1987; Harrison et al., 1987). The class of steroids with fast actions at membrane receptors were termed neuroactive steroids (Paul and Purdy, 1992).

Neuroactive steroids (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, 2002a; 2002b). The production of NAS begins with the translocation of cholesterol across the mitochondrial membrane, which is facilitated by steroidogenic acute regulatory (StAR) 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 PROG, DOC, and testosterone produces the NAS 3α,5α-tetrahydroprogesterone (allopregnanalone; ALLO), 3α,5α-tetrahydrodeoxycorticosterone (THDOC) and 3α-androstanediol, respectively, via the

enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase (Compagnone and Mellon, 2000). Steroid biosynthesis leading to the production of NAS can be altered by the use of enzyme inhibitors such as finasteride (FIN; Rittmaster, 1997) or the removal of the peripheral sources of NAS [i.e., adrenalectomy; ADX or gonadectomy; GDX (Korneyev et al., 1993)]. A more detailed diagram of steroid biosynthesis is depicted in Figure 1.1.

A variety of evidence has shown that some NAS can modulate the $GABA_A$ receptor, the 5HT-3 receptor, glycine receptors, glutamate receptors, oxytocin receptors, NMDA receptors and sigma receptors (Finn et al., 2004a; Grazzini et al., 1998; Rupprecht and Holsboer, 1999; Su et al., 1988; Valera et al., 1992; Wu et al., 1990, 1991). However, the 5α-reduced NAS selectively potentiate the action of GABA at the $GABA_A$ receptor at low nanomolar concentrations, which are physiologically relevant, while modulation of other receptors only occurs at higher concentrations ≥10μM (Gee, 1988; Morrow et al., 1987). These 5α-reduced NAS have low affinity at traditional steroid nuclear receptors and are the most potent positive modulators of $GABA_A$ receptors identified to date (Belelli et al., 1990). At micromolar concentrations, NAS can directly gate $GABA_A$ receptors (Puia et al., 1990; Ueno et al., 1997), whereas at nanomolar concentrations NAS steroids generally augment the action of GABA at $GABA_A$ receptors (Belelli and Lambert, 2005; Callachan et al., 1987; Majewska et al., 1986; Shu et al., 2004). While the specifics of the $GABA_A$ receptor will be discussed later in the document, NAS modulation of the $GABA_A$ receptor functions to allow chloride to flux across the membrane, thereby hyperpolarizing the cell and enhancing GABAergic neuronal inhibition.

Behavioral Properties of the 5α-Reduced Neuroactive Steroids

As would be predicted from their profile as potent, positive modulators of $GABA_A$ receptors, 5α-reduced NAS can have wide-spread and considerable behavioral effects in many different species. While NAS can have complicated effects on many behaviors, some of the more noteworthy pharmacological properties include the anxiolytic, sedative, anesthetic, cognitive impairing and anticonvulsant effects (Gasior et al., 1999).

The 5α-reduced NAS steroids have been shown to be anxiolytic in several animal models and may be a promising treatment in humans with anxiety disorders (Longone et al., 2008). Early work in rats found that administration of PROG was anxiolytic in the elevated plus-maze paradigm and that co-administering FIN (and thereby preventing the metabolism to ALLO) abolished this effect (Bitran et al., 1999; Bitran et al., 1991; Bitran et al., 1993; Bitran et al., 1995). An anxiolytic effect in the elevated plus maze also was shown in mice following administration of several different 5α-reduced NAS, including ALLO and THDOC (Finn et al., 1997; Rodgers and Johnson, 1998), and these results were replicated in several other models of rodent anxiety-like behavior such as the light/dark box, open-field, the staircase test and lick suppression test (Crawley et al., 1986; Wieland et al., 1995; Wieland et al., 1991). In humans, patients with panic disorder had significantly greater concentrations of plasma ALLO levels when compared with control patients (Strohle et al., 2002). Additionally, patients with panic disorders exhibited a decrease in ALLO levels following a chemically induced panic attack, while normal controls did not (Strohle et al., 2003). These finding suggest that manipulations in

endogenous GABAergic NAS could alter anxiety-related behaviors in rodents and humans.

Many GABAergic compounds have sedative properties and the NAS are no exception. Early work showed that 5 and 10 mg/kg administration of THDOC in rats caused potent dose-dependent sleep-inducing properties and increased non-REM sleep (Mendelson et al., 1987). ALLO administration can induce anesthesia within minutes, and it is more potent than benzodiazepines and barbiturates (Norberg et al., 1999). In humans, administration of PROG was sedative in men, and further research indicated that ALLO administration was sedative in both men and women (Schulz et al., 1996; van Broekhoven et al., 2007). NAS may be a viable treatment option for insomnia, as their administration in rats shortens sleep latency (Edgar et al., 1997) but does not seem to cause tolerance or rebound insomnia (Damianisch et al., 2001), frequent side effects of other GABAergic sleep-aids.

Again, as would be expected from their GABAergic profile, NAS can cause cognitive impairment. In the mouse, ALLO administration caused deficits in both the Y-maze and the Morris water maze, tasks that measure the animal's spatial memory (Johansson et al., 2002; Ladurelle et al., 2000). A single administration of PROG in young women caused them to have memory deficits during a face recall task when compared to women administered vehicle (van Wingen et al., 2007). PROG administration also caused cognitive impairment in healthy women, and this impairment was positivity correlated with circulating levels of ALLO (Freeman et al., 1993).

The 5α-reduced NAS are powerful anticonvulsants, as would be predicted by their GABAergic profile. Inhibition of the NMDA receptor would also confer anticonvulsant properties. Some of the more potent anticonvulsant steroids are the reduced metabolites of PROG. It was first shown in 1942 that PROG had anticonvulsant properties (Seyle, 1942). Recent studies determined that PROG's anticonvulsant properties were due to its metabolism to 5α-dihydroprogesterone (5α-DHP) and ALLO (Frye et al., 2002). 5α-DHP has been shown to be seizure protective in very low doses and at time points as soon as 15 minutes post-injection (Lonsdale and Burnham, 2003).

The previous examples are just a few of the behaviors that can be affected through administration of 5α-reduced NAS that positively modulate the $GABA_A$ receptor. The NAS that negatively modulate the $GABA_A$ receptor, such as pregnenolone sulfate (PS) and dihydroepiandrosterone sulfate (DHEAS), generally have the opposite effects on behavior as the positive GABAergic modulators (Majewska and Schwartz, 1987; Reddy and Kulkarni, 1998). While an entire book could be written on the behavioral affects of NAS (and indeed has!), the behavior that it was important to focus on for the current project was that of seizure susceptibility.

Steroids and Seizure Susceptibility

The actions of steroids on seizure susceptibility are varied. While the GABAergic effects of NAS would warrant the assumption that most NAS are anticonvulsant, this is actually not the case. The truth is that it depends greatly on the steroid being administered and the pathway through which it is metabolized. Two steroids that have been found to be consistently anticonvulsant are PROG and DOC. PROG administration

in humans have been shown to be anticonvulsant in catamenial epilepsy (Backstrom et al., 1984; Herzog, 1995), but studies in animals has shown that in order for PROG to be anticonvulsant, it must be metabolized to its 5α-reduced derivative ALLO (Frye et al., 2002; Frye and Scalise, 2000; Kokate et al., 1999a). The 5α-reduced NAS have been shown to be anticonvulsant in several models (Belelli et al., 1989; Belelli et al., 1990; Kokate et al., 1994). Much like PROG, DOC has long been known to be anticonvulsant when administered to humans (Aird and Gordan, 1951), and these results have been replicated in animal models (Edwards et al., 2002a; 2002b, 2005; Perez-Cruz et al., 2007). The mechanism for DOC's anticonvulsant activity has also been shown to require 5α –reduction (Perez-Cruz et al., 2006; Perez-Cruz et al., 2007; Reddy and Rogawski, 2002).

The majority of data indicate that corticosterone (CORT) administration is proconvulsant. Acute doses of EtOH increased CORT levels in both DBA/2 (D2) and C57BL/6 (B6) mice, which are two inbred strains with well-documented differences in EtOH withdrawal severity (Crabbe, 1998; Crabbe et al., 1983; Roberts et al., 1992). However, the CORT response in the seizure prone D2 animals was significantly higher at 60 minutes post-injection than in B6 mice (Roberts et al., 1992). Administration of high levels of CORT (100 mg/day) to rats accelerated the presentation of tonic-clonic seizures in animals undergoing kindling epileptogenesis (Karst et al., 1999). CORT administration has also been shown to increase convulsions due to acute withdrawal from several drugs, including EtOH, in mice (Roberts et al., 1994). While CORT may be proconvulsant, it has been suggested that DOC, the precursor to CORT, may be protective against

convulsions, along with other 5α-reduced GABAergic DOC metabolites (Reddy and Rogawski, 2002).

Data indicate that testosterone can be both anti- and proconvulsant, depending on its metabolism. Testosterone replacement in GDX animals increased seizure susceptibility in rats administered kainic acid, indicating that testosterone can be proconvulsant (Mejias-Aponte et al., 2002). However, studies examining testosterone metabolism have shown that when testosterone was aromatized into 17β-estradiol, it was proconvulsant. In contrast, when testosterone was reduced at the A-ring by 3α-HSD and 5α-reductase into 5α-dihydrotestosterone and 3α-androstanediol, it was anticonvulsant (Frye and Reed, 1998; Reddy, 2004a). When the formation of estrogen was inhibited following testosterone administration, both rats and mice were protected against picrotoxin (a $GABA_A$ receptor channel blocker) induced seizures, but not against kainic acid induced seizures (Reddy, 2004b; 2004c). These data indicate that testosterone's reduced metabolites are anticonvulsant via a $GABA_A$ receptor-mediated pathway.

While mentioned briefly above as proconvulsant, it is important to note that estrogen's convulsant actions depend upon several variables, notably treatment duration, dose and mode of administration (Veliskova, 2007). In ovariectomized (OVX) animals, chronic replacement of estrogen was seizure protective against picrotoxin induced seizures (Schwartz-Giblin et al., 1989). However, in a similar paradigm, estrogen replaced OVX animals took fewer daily amygdala stimulations to develop seizures and developed more intense seizures to repeated pentylenetetrazol injections (Buterbaugh,

1987), suggesting that estrogen enhanced kindling. Thus, estrogen can exhibit proconvulsant and anticonvulsant properties.

The sulfated NAS, such as PS, pregnanolone sulfate and epipregnanolone sulfate can bidirectionally modulate NMDA receptors, with PS being a robust positive modulator of NMDA, while pregnanolone sulfate and epipregnanalone sulfate are negative modulators of NMDA (Irwin et al., 1994; Park-Chung et al., 1994; Wu et al., 1991). When administered intracerebroventriculy, PS is a robust proconvulsant as measured by sensitivity to PTZ (Kokate et al., 1999b) . The effects of pregnanolone sulfate and epipregnanalone sulfate on seizure susceptibility have not been investigated, but their negative modulation of NMDA would indicate that they would have anticonvulsant effects.

Recent research has also focused on the anticonvulsant activity of DHEA. As shown in Figure 1.1, DHEA can be metabolized into several different compounds. Its metabolism to 3α,5α androsterone renders it into an anticonvulsant NAS when administered to mice in several models of seizure susceptibility, and it has been shown that the anticonvulsant effect was due to its actions at the $GABA_A$ receptor (Kaminski et al., 2006; Rafal et al., 2005). However, when DHEA was transformed by sulfotranferase into DHEA sulfate, it became pro-convulsant (Park-Chung et al., 1999). In humans, a case study of a woman who was previously seizure free showed that she developed seizures one month after starting daily DHEA treatment (Galia et al., 2009).

In addition to steroid hormones and their metabolites, the adrenal glands are a significant source of epinephrine, which also affects seizure susceptibility. Injecting

systemic epinephrine retarded the development of kindling-induced seizures in rats (Welsh and Gold, 1986), and this effect was modulated through the vagus nerve (Krahl et al., 2000). While epinephrine is produced in the adrenal medulla in response to stress, it is important to note that following ADX, epinephrine can still be produced by other organs (Ricordi et al., 1988) as well as in the brain (Santibañez et al., 2005).

Ethanol and Neuroactive Steroids

As discussed above, NAS-induced potentiation of the action of GABA at $GABA_A$ receptors can cause anticonvulsant, anxiolytic, sedative, anesthetic, ataxic and cognitive impairing properties (Eser et al., 2006; Gasior et al., 1999; Rupprecht, 2003), much like the effects of EtOH (Breese et al., 2006; Finn et al., 2004a; Gasior et al., 1999; Khisti et al., 2003; Paul and Purdy, 1992). It has been shown that the increases in these NAS contributed 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).While the exact mechanism of EtOH-induced potentiation of $GABA_A$ receptor function remains controversial, it is thought that sensitivity to some of the effects of EtOH may be due to potentiation of GABA at $GABA_A$ receptors (Grobin et al., 1998).

Recent work has found that acute EtOH intoxication caused a rise in GABAergic neuroactive steroids in both plasma and brain (Barbaccia et al., 1999; Finn et al., 2004b; Schuckit et al., 1987; VanDoren et al., 2000) and that the effect of EtOH on steroidogenesis produced an indirect effect on $GABA_A$ receptor function (Sanna et al.,

2004). However, replicating this effect in the human population has not been determined. Some studies showed that adolescents admitted to the emergency room for high levels of EtOH intoxication had increased NAS levels compared to adolescents admitted for non-EtOH related injuries (Torres and Ortega, 2003, 2004). In a more controlled study, adults with a history of social drinking that were administered moderate doses of EtOH in a laboratory setting did not show increases in plasma ALLO levels (Holdstock et al., 2006). The rise seen in animals in response to EtOH is mostly attributable to activation of the hypothalamic-pituitary-adrenal (HPA) axis, as ADX/GDX surgery abolishes the rise in NAS seen in response to EtOH injections (Khisti et al., 2003; O'Dell et al., 2004; Porcu et al., 2004). However, the brain can continue to make NAS, despite ADX (Follesa et al., 2006). Thus, it is possible that an interaction of EtOH and GABAergic NAS at $GABA_A$ receptors could influence sensitivity to some of the behavioral effects of EtOH.

Consistent with this idea, blocking the formation of ALLO with the 5α-reductase inhibitor FIN decreased the anticonvulsant properties of an acute dose of EtOH at 40 minutes, but not at 10 minutes, post EtOH injection (VanDoren et al., 2000). Likewise, removal of the peripheral sources of neurosteroids eliminated the EtOH-induced increase in ALLO levels (O'Dell et al., 2004) and altered several behavioral aspects of EtOH intoxication such as anxiety, depression and loss of righting reflex (Hirani et al., 2002; Hirani et al., 2005; Khisti et al., 2003). ALLO levels are reduced during withdrawal in both humans and animals when compared to non-withdrawn controls (Cagetti et al., 2004; Hill et al., 2005; Janis et al., 1998; Romeo et al., 1996; Tanchuck et al., 2009). Collectively, these findings suggest that removal of the peripheral sources of GABAergic neuroactive steroids may alter behaviors associated with acute EtOH administration.

Importantly, they also suggest that NAS may be modulating EtOH withdrawal-related behaviors. The work in this dissertation will investigate the contributions of NAS to the neural rebound hyperexcitability seen during withdrawal from an acute dose of EtOH.

Animal Models of Ethanol Withdrawal-Related Convulsive Behavior

As outlined above, one of the major complications in EtOH dependence is the appearance of a withdrawal syndrome upon cessation of EtOH consumption. Many EtOH-related deaths stem from withdrawal, and many alcoholics continue drinking in order to avoid withdrawal. Investigating the underlying etiology of withdrawal could yield important information on EtOH dependence. However, it is difficult to investigate the underlying causes of EtOH withdrawal in humans, as withdrawal in humans can be variable in both incidence and intensity. The variability seen may be related to the fact that no two alcoholics consume EtOH in the same way and that the amount of alcohol consumed, duration of use and other co-morbid disorders may affect EtOH withdrawal severity (Saitz, 1998). In order to investigate EtOH withdrawal, the use of animal models is imperative, but careful selection of the model must be exercised.

It is possible to investigate EtOH withdrawal with *in vitro* models. In these models, either cells (Hu and Ticku, 1997) or brain slices (Bailey et al., 1998) are exposed to EtOH, and it is then removed. Electrophysiological, biochemical or molecular strategies can then be used to examine mechanisms underlying the neuroadaptation occurring during chronic EtOH exposure and withdrawal. While *in vitro* models are very useful, it is important to remember that cells and slices are devoid of the complex interactions that are found in the whole animal.

A variety of *in vivo* models of EtOH withdrawal have been developed. *In vivo* models have two components. The first is that the animal must be exposed to EtOH (and withdrawn) in some manner, while the second must measure the withdrawal severity in some way. Although it would seem that the most desirable strategy to expose an animal to EtOH would be to allow the animal to willingly consume EtOH, this poses several problems. First of all, many animals will not consume large amounts of EtOH voluntarily and second, and this does not allow the experimenter to control the amount or timing of EtOH consumption. For these reasons, many animal models employ forced EtOH procedures. The most common models employ either inhalation of alcohol, a liquid diet containing alcohol, EtOH vapor exposure, or injections or infusions of EtOH. The goal of these procedures is to achieve stable EtOH levels over time (Finn and Crabbe, 1997).

In order to measure EtOH withdrawal severity, several animal models have been developed. Regardless of the method by which EtOH is administered, withdrawal can be measured through a variety of behaviors. The enhanced autonomic activation can be measured via dysregulation in body temperature (Ritzmann and Tabakoff, 1976). Distortions in sensation and perception have been modeled using auditory or tactile stimuli. In general, enhanced responsiveness to these stimuli was observed during withdrawal, which was thought to model the human condition (Chester et al., 2004; Rassnick et al., 1992). The hyperexcitability of the CNS has been most successfully modeled by measuring enhanced susceptibility to convulsions. Susceptibility to convulsions can be measured by the occurrence of spontaneous tonic or clonic convulsions or of handling induced convulsions (HICs), elicited by sound stimuli,

exposure to mild electric stimuli or the administration of chemical substances. Measuring convulsive behavior is easy to identify and quantify, which makes this an excellent model to study one aspect of withdrawal (Becker, 2000).

The model that will be most discussed in this dissertation is that of acute EtOH withdrawal severity measured by HICs. 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). The hyperexcitability can be visualized through the measurement of HICs, which has been shown to be a reliable measure of withdrawal severity (Goldstein, 1972a; Goldstein, 1972b). This model involves picking the animal up by the tail, and gently spinning it 180 degrees and rating small, discrete convulsions on a previous established scale (Crabbe et al., 1991). This is a useful model as the animals recover from this non-invasive measurement almost instantly, and it allows for within subject measurements over the entire withdrawal period. Acute EtOH withdrawal severity measured by HICs provides a high through-put and reliable model. This model has face validity since convulsions, as discussed above, are part of the human EtOH withdrawal behavior. The model also has predictive validity, as benzodiazepine administration decreases HIC scores during withdrawal (Crabbe et al., 1993) and as discussed earlier, are used to treat withdrawal-induced convulsions in humans.

Notably, acute (single dose) and chronic intermittent withdrawal procedures have been reported to produce 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 Role of the Hippocampus

The hippocampus is part of the limbic system that plays an important role in many biological functions, including memory and spatial mapping. The hippocampus also is important in seizure propagation, as it is one of the first regions to exhibit epileptic seizure discharge (Gale, 1992). The circuitry of seizures is complicated and involves many brain area, however the limbic pathway (including the hippocampus) is important during the seizures seen with EtOH withdrawal (Gale, 1988). C-fos studies reveal that the hippocampus is activated during withdrawal from both acute and chronic EtOH withdrawal (Chen et al., 2009; Kozell et al., 2005; Morgan et al., 1992).

Relevant to GABAergic NAS, several studies documented that the brain regional rank order of potency for ALLO to potentiate $GABA_A$ receptor function was: hippocampus>cortex=amygdala (Finn and Gee, 1993; Gee et al., 1988; Wilson and Biscardi, 1997). During NAS withdrawal, the GABAergic kinetics of cells in the hippocampus were altered, with the time constant for decay of $GABA_A$-gated currents reduced six-fold, indicating that NAS withdrawal decreased total $GABA_A$ receptor-mediated inhibition in the hippocampus (Smith et al., 1998b). Collectively, these results document that the hippocampus is an important area for both NAS and EtOH withdrawal.

As mentioned above, the GABAergic NAS are very potent in the hippocampus. ALLO administration in the hippocampus produced an inhibition of pyramidal cell

population spikes following stimulation of the CA1 area (Tokunaga et al., 2003). Additionally, the hippocampus has been shown to be an important region for ALLO's anticonvulsant effects (Gililland-Kaufman et al., 2008; Martin-Garcia and Pallares, 2005b).

Several recent studies have revealed that NAS may be exerting part of their effects on EtOH behaviors through the CA1 region of the hippocampus (Silvers et al., 2003). Acute EtOH administration produced a reduction of c-fos in the hippocampus, but not in the striatum of rats (Ryabinin et al., 1997). In addition, EtOH inhibited the activity of pyramidal neurons and pre-treatment with the 5α-reductase inhibitor FIN (which would inhibit the formation of ALLO and other GABAergic NAS) attenuated this inhibitory effect of EtOH (Tokunaga et al., 2003). Data from our lab has recently shown that administering FIN directly to the hippocampus during the development of physical dependence augmented the expression of chronic EtOH withdrawal (Gililland-Kaufman et al., 2008). Since acute EtOH administration has been shown to increase ALLO levels in both the hippocampus and the frontal cortex (Barbaccia et al., 1999; O'Dell et al., 2004), it is not known whether changes in specific NAS are modulating acute EtOH withdrawal severity via the hippocampus or frontal cortex.

The $GABA_A$ Receptor

The $GABA_A$ receptor is an ionophore comprised of five subunits that when activated, allows chloride to flux into the cell. The $GABA_A$ receptor is the most widely distributed receptor in the brain, with 20-30% of all synapses within the mammalian brain thought to be GABAergic (Young and Chu, 1990). There are 19 known different subunit

proteins, including α1-α6, β1-β3, γ1-γ3, δ, ε, θ, ρ1- ρ2 and π, but these 19 subunits only have only been documented to assemble into a few dozen receptor conformations (Whiting et al., 1999). The most common subunit arrangement is α1β2γ2, while α2β3γ2 and α2βxγ2 are the next most ubiquitous arrangements (McKernan and Whiting, 1996). Each conformation of $GABA_A$ receptors has a specific anatomical distribution (Pirker et al., 2000) and specific pharmacological and physiological properties (Hevers and Luddens, 1998).

One of the more interesting recent developments has been the characterization of phasic vs. tonic activation of $GABA_A$ receptors. Phasic activation of the receptors involves the release of several thousand GABA molecules into the synaptic cleft (Mody et al., 1994), which activates receptors clustered close to the release site. This exposure causes the $GABA_A$ receptors to open and chloride to flow into the cell. These receptors are being exposed to high concentrations of GABA for a short amount of time, as the GABA diffuses away from the synapse in a time frame around 100μs (Mozrzymas, 2004). This short exposure means that even though the concentration of GABA in the synapse is high, not all receptors will become activated, as the binding rate of GABA to the $GABA_A$ receptor is slow (Jones et al., 1998). This form of receptor activation allows swift and exact transmission of a pre- to post-synaptic signal (Farrant and Nusser, 2005). It appears as though expression of the γ2 subunit may be necessary for phasic $GABA_A$ receptors, as it facilitates interactions with scaffolding proteins and clusters the receptor into the synaptic cleft (Leil et al., 2004; Schweizer et al., 2003). Overall, it has been shown that $GABA_A$ receptors made of α1-3,5βxγ2 subunits are responsible for phasic GABA currents.

Tonic GABAergic currents result from GABA spillage outside of the synaptic cleft and activation of $GABA_A$ receptors that are located on the soma, dendrite or axon of the neuron (Kullmann et al., 2005). The receptors in these areas encounter low GABA concentrations [estimated at 10 nanomolar to one micromolar (Kennedy et al., 2002; Lerma et al., 1986; Tossman et al., 1986)], but for a much more extended period of time. These receptors are predominantly comprised of α4-6βxδ subunits, are responsive to low levels of GABA, and are slow to desensitize (Nusser et al., 1998; Saxena and Macdonald, 1994; Serwanski et al., 2006). Inhibition via tonic $GABA_A$ receptors is hypothesized to increase the input conductance of a cell, rendering the cell less responsive to modulation by input neurons.

As mentioned above, each conformation of $GABA_A$ receptor has specific pharmacological and physiological properties, which makes the changes in subunit composition an important factor when studying the interaction of $GABA_A$ receptors and substances like EtOH and NAS. $GABA_A$ receptors containing α1 and β2 subunits are sensitive to modulation by NAS, while receptors containing the α4 subunits are less sensitive to modulation by NAS (Brussaard et al., 1999; Rick et al., 1998; Smith et al., 1998a; Zhu et al., 1996). Administration of and withdrawal from steroids and NAS has also been shown to modulate $GABA_A$ receptor subunit expression. Withdrawal from PROG increased α4 subunit mRNA expression, along with an increase in δ subunit expression (Biggio et al., 2009; Smith et al., 1998b; Sundstrom-Poromaa et al., 2002). Withdrawal of steroids through ADX increased expression of the α1, α2 and γ2 subunits in the hippocampus, but decreased expression of the β2 subunit (Orchinik et al., 1994).

While there has been much debate about the ability of EtOH to directly potentiate the $GABA_A$ receptor (Breese et al., 2006), recent evidence shows that a receptor comprised of α4,α6,β3,δ subunits as being highly sensitive to modulation by low concentrations of EtOH (Wallner et al., 2006), although these results are debatable (Borghese et al., 2006). It is important to note that if these receptors are sensitive to low levels of EtOH, they would be an extra-synaptic, tonic $GABA_A$ receptor.

It has been well documented that exposure to EtOH can significantly change expression of several $GABA_A$ subunits. Exposure to chronic EtOH significantly decreased expression of α1 and α2 mRNA in the cerebral cortex (Montpied et al., 1991; Morrow et al., 1990). In the hippocampus, expression of the α1 subunit was decreased, there was no change in expression of the α2 subunit, and α4 subunit expression was increased (Matthews et al., 1998). It has been theorized that these changes are responsible for the increased seizure severity that is seen during withdrawal, as the increase in α4 subunit expression and decrease in α1 subunit expression persisted during withdrawal (along with increase in β2, β3 and γ1), and α4 containing receptors were insensitive to modulation by benzodiazepines (Devaud et al., 1997).

Hypothesis and Goals of the Dissertation

The evidence presented above makes it clear that there are contributions of the $GABA_A$ receptor-modulatory NAS on EtOH behaviors and that $GABA_A$ receptor subunit composition, notably in the hippocampus, may be important to these effects. The goal of this dissertation was to elucidate the effects of NAS during the neuronal rebound hyperexcitability seen during acute EtOH withdrawal and to elucidate the effects of

$GABA_A$ receptor subunits during these events. As discussed above, we chose to do this in a model of acute EtOH withdrawal in mice. The overall hypothesis of the proposed work was that endogenous GABAergic NAS contributed to the expression of the acute EtOH withdrawal profile in male and female mice.

The first experiment (Chapter 2) involved removing the peripheral sources of NAS (through ADX, GDX and ADX/GDX surgery) in both male and female D2 and B6 mice and assessing the severity of neural rebound hyperactivity measured by HICs versus animals with SHAM surgery. I reasoned that peripheral sources of steroids, the adrenals and gonads, were important during withdrawal from a single acute EtOH exposure, and that the direction of change in acute withdrawal severity would provide insight into the relative contribution of pro- versus anti-convulsant steroids to acute EtOH withdrawal severity. That is, an increase in EtOH withdrawal severity following removal of the adrenals and gonads would suggest that endogenous anticonvulsant steroids were protective against HICs. In contrast, a decrease in acute EtOH withdrawal severity following removal of the adrenals and gonads would suggest that endogenous proconvulsant steroids were increasing HICs in intact animals. I predicted that we would see an increased severity of neuronal rebound hyperactivity in animals that had undergone ADX/GDX surgery, indicating that an anticonvulsant NAS was important to modulate the neural rebound hyperactivity seen during EtOH withdrawal. Since these initial studies were characterizing the effect of organ removal on acute withdrawal severity, I examined two inbred strains that differ in acute and chronic EtOH withdrawal severity [i.e. D2>B6; (Crabbe, 1998; Crabbe et al., 1983; Roberts et al., 1992)] and in sensitivity to the anticonvulsant effect of ALLO [i.e. B6>D2 (Finn et al., 2000; Finn et

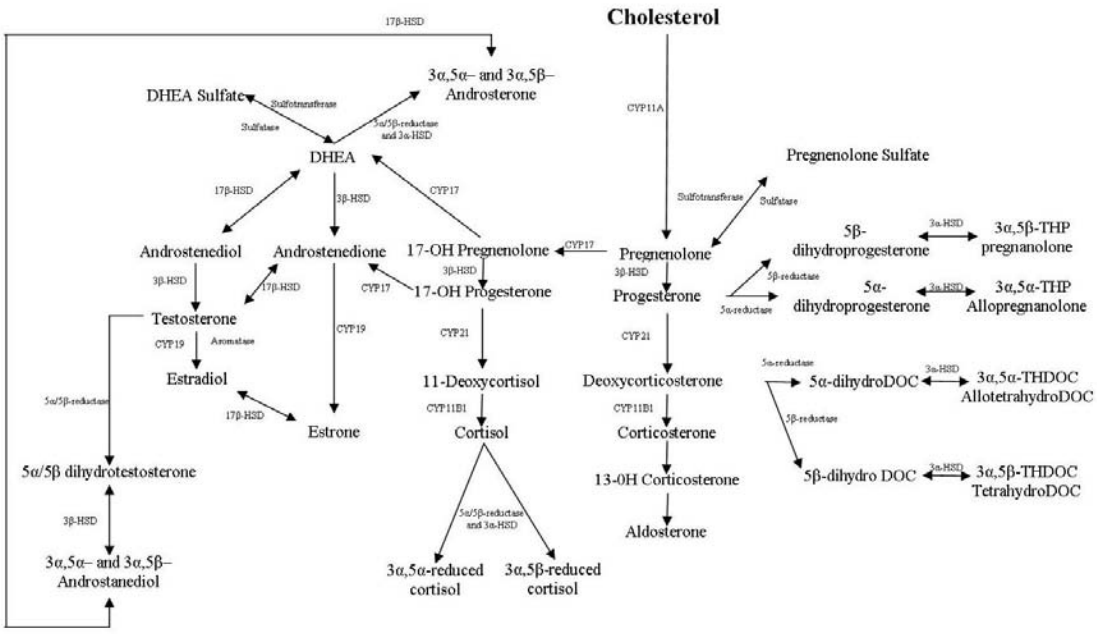
al., 1997)]. Male and female mice were tested since acute EtOH withdrawal is lower in female versus male B6 and D2 mice (Gorin-Meyer et al., 2007), and endogenous ALLO levels are higher in female versus male rodents (Finn et al., 2004a; Paul and Purdy, 1992)

The second set of experiments (Chapter 3) was designed to identify specific steps along the NAS biosynthetic pathway that were necessary or sufficient to modulate the neuronal rebound hyperexcitability following a high dose of EtOH (i.e., acute EtOH withdrawal). In order to fully explore this idea, I felt it was imperative that several arms along the NAS biosynthetic pathway and several steps within each arm were tested in our paradigm. I chose to investigate both the PROG and DOC arm of the NAS biosynthetic pathway because of data obtained in Chapter 2 indicated these two arms may be the most important ones that were involved in EtOH withdrawal (i.e., GDX alone in male B6 and D2 mice did not alter withdrawal, suggesting minimal contribution of testosterone). The strategy was two-fold: 1) to administer NAS (ALLO, ganaxolone 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. I hypothesized that replacing ADX/GDX animals with doses of PROG or DOC meant to emulate the rises seen in these NAS in response to EtOH administration, or their 5α-reduced metabolites, would restore acute withdrawal severity back to the levels seen in intact (i.e. SHAM) animals and that co-administering FIN would abolish these effects. Parallel studies were conducted in SHAM animals to determine the effects of these steroid manipulations in intact animals. Since the results of Chapter 2 indicated that ADX/GDX produced a significant increase in acute EtOH

withdrawal in both male and female D2 mice (but only in male B6 mice), only the D2 strain was examined in these studies.

The purpose of the third set of experiments (Chapter 4) was also two-fold. First, I wanted to see if the expression of specific $GABA_A$ receptor subunits or of StAR protein changed in a manner that corresponded to the behavioral results presented in Chapter 3. Specifically, I hypothesized that animals that had undergone ADX/GDX surgery and had steroid treatments that restored their behavior to intact levels, would display subunit conformations similar to those seen in intact animals. Second, I hoped that these experiments would shed light on basal differences in expression in vehicle treated animals with regard to the effects of sex and surgical status on acute EtOH withdrawal severity. In order to accomplish these goals, the hippocampi from animals that had previously participated in our behavioral acute withdrawal paradigm (Chapter 3) were assayed for mRNA expression of $GABA_A$ receptor subunits ($\alpha1$, $\alpha2$, $\alpha4$, $\alpha5$, $\beta1$, $\beta2$, δ, $\gamma2$) and StAR protein using quantitative, Real-Time reverse transcriptase polymerase chain reaction (qRT-PCR). These subunits were chosen based on their involvement in sensitivity to either NAS or EtOH and their involvement in synaptic vs. extrasynaptic $GABA_A$ receptor mediated transmission. StAR protein was chosen based on its involvement as a rate limiting step in NAS production.

Figure 1.1. Biosynthesis of Neuroactive steroids



The biosynthetic pathway for the neurosteroids that are potent positive modulators of $GABA_A$ receptors is represented. The sulfated derivatives of pregnenolone and DHEA, which are negative modulators of $GABA_A$ receptors, are also depicted. ↑ indicates a unidirectional reaction; ↔ indicates a bi-directional reaction. Adapted from Morrow 2007.

Table 1. Handling-induced convulsion (HIC) scoring

HIC Score	Description (adapted from Crabbe et al., 1991)
0	No convulsion after 180 degree spin.
1	Facial grimace after 180 degree spin.
2	Tonic convulsion after 180 degree spin.
3	Tonic-clonic convulsion after 180 degree spin.
4	Tonic convulsion after being lifted by the tail.
5	Tonic-clonic convulsion after being lifted by the tail.
6	Severe tonic-clonic convulsion after being lifted by the tail. May continue after mouse is released.
7	Spontaneous tonic-clonic convulsions or tonic-clonic convulsions in response to minor environmental stimuli such as movement of the lid of the cage.

CHAPTER 2: THE IMPACT OF GONADECTOMY AND ADRENALECTOMY ON ACUTE WITHDRAWAL SEVERITY IN MALE AND FEMALE C57BL/6J AND DBA/2J MICE FOLLOWING A SINGLE HIGH DOSE OF ETHANOL.

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ABSTRACT

Background: Steroid hormones can influence neuronal excitability and subsequent seizure susceptibility through genomic and non-genomic mechanisms. For example, there are pro-convulsant steroids such as estradiol and corticosterone (CORT) and anticonvulsant steroids such as testosterone, progesterone and their γ-aminobutyric acid (GABA) -ergic metabolites. Recent findings indicated that a single, acute administration of ethanol increased levels of GABAergic steroids and that the source of this increase was peripheral organs such as the adrenals and gonads. Thus, the purpose of the present study was to determine the impact of removal of the adrenals and/or gonads on withdrawal severity following a single high dose of ethanol in two genotypes that differ in ethanol withdrawal severity. *Method:* Male and female C57BL/6J (B6) and DBA/2J (D2) mice were either left intact (SHAM), adrenalectomized (ADX), gonadectomized (GDX) or underwent ADX/GDX surgery. Seven days following surgery, baseline handling-induced convulsions (HICs) were measured prior to administration of a 4-g/kg dose of ethanol. HICs were assessed following the ethanol injection, then hourly for 12 hours and at 24 hours. A separate group of mice were used to measure the impact of surgical status on ethanol metabolism at 30, 60, 120 and 240 minutes after a single 4-g/kg dose of ethanol. *Results:* ADX and ADX/GDX treatments in male B6 and D2 mice increased ethanol withdrawal severity following a single dose of ethanol, measured by area under the withdrawal curve and peak HIC scores. Acute ethanol withdrawal also was increased in female D2 mice that had undergone ADX/GDX. In contrast, surgical status did not alter ethanol withdrawal severity in female B6 mice. Surgical status had only minor effects on ethanol metabolism. *Conclusions:* Removal of peripherally-derived

steroids with anticonvulsant properties significantly increased HIC scores during acute ethanol withdrawal following a single dose of ethanol in male and female D2 mice and in male B6 mice. These increases were not due to changes in ethanol metabolism.

Key Words: Alcohol, $GABA_A$ receptors, Convulsions, Neuroactive steroids, Steroid hormones.

Introduction

For over half a decade, it has been known that sex steroids can influence neuronal excitability (Seyle, 1942). More specifically, it has been shown that the hormones progesterone (PROG), deoxycorticosterone (DOC) and testosterone and some of their metabolites are protective against several types of seizures (Belelli et al., 1989; Reddy, 2004b), whereas estrogens and CORT are pro-convulsant (Reddy, 2004c; Roberts et al., 1992). While steroids are traditionally thought of as affecting gene transcription through nuclear binding to hormone response elements, the rapid steroid effect on neuronal excitability has been shown to be mediated through interactions with membrane bound receptors (Paul and Purdy, 1992).

The metabolism of DOC, testosterone and PROG with 5α-reductase and 3α-hydroxysteroid dehydrogenase yields steroids that have actions at membrane receptors and that have been termed neuroactive steroids (NAS; Rupprecht and Holsboer, 1999). The three major sites of neurosteroid production are the gonads (Mellon and Griffin, 2002b), the adrenals (Holzbauer et al., 1985) and the brain (Baulieu, 1998; Mellon and Griffin, 2002a). For example, a variety of evidence has shown that some NAS, such as the PROG derivative allopregnanolone (ALLO), are potent positive modulators of the $GABA_A$ receptor at low nanomolar concentrations (Gee, 1988; Morrow et al., 1987). Additionally, NAS have low affinity at traditional steroid nuclear receptors (Belelli et al., 1990).

NAS-induced potentiation of the action of GABA at $GABA_A$ receptors can cause anticonvulsant, anxiolytic, sedative, ataxic and cognitive impairing properties (EtOH;

Gasior et al., 1999), much like the effects of ethanol (Gasior et al., 1999; Khisti et al., 2003; Paul and Purdy, 1992), much like the effects of ethanol (EtOH). While the exact mechanism of EtOH-induced potentiation of $GABA_A$ receptor function remains controversial, it is widely accepted that some of the effects of EtOH may be due to potentiation of GABA at $GABA_A$ receptors (Grobin et al., 1998). Recent work has found that acute EtOH intoxication causes a rise in GABAergic neuroactive steroids in both plasma and brain (Barbaccia et al., 1999; Finn et al., 2004b; Schuckit et al., 1987; VanDoren et al., 2000) and that the effect of EtOH on steroidogenesis produced an indirect effect on $GABA_A$ receptor function (Sanna et al., 2004). Thus, it is possible that an interaction of EtOH and GABAergic neuroactive steroids at $GABA_A$ receptors could influence sensitivity to some of the behavioral effects of EtOH. Consistent with this idea, blocking the formation of ALLO with the 5α-reductase inhibitor finasteride (FIN) decreased the anticonvulsant properties of an acute dose of EtOH at 40 minutes, but not at 10 minutes, post EtOH injection (VanDoren et al., 2000). Likewise, removal of the peripheral sources of neurosteroids eliminated the EtOH-induced increase in ALLO levels (O'Dell et al., 2004) and altered several behavioral aspects of EtOH intoxication such as anxiety, depression and loss of righting reflex (Hirani et al., 2002; Hirani et al., 2005; Khisti et al., 2003). Collectively, these finding suggest that removal of the peripheral sources of GABAergic neuroactive steroids may alter behaviors associated with acute EtOH withdrawal following a single dose of EtOH.

As discussed in the General Introduction (Chapter 1), hormones and their metabolites can have either pro- or anticonvulsant properties, and these steroids may be important for some of the behavioral effects of EtOH, such as seizure susceptibility.

Whereas these steroids can be produced both peripherally and de novo in the brain, little attention has been paid to which source of steroid hormones is important in modulating specific effects of EtOH. The few studies that have examined these effects have shown an interesting relationship between peripherally derived steroids and sensitivity to EtOH withdrawal-induced seizures. Adrenalectomized (ADX) mice that were exposed to 14 days of chronic EtOH treatment showed a marked decrease in audiogenic-induced seizures, but these seizures were reinstated with administration of glucocorticoids (Sze et al., 1974). In a similar fashion, it was again shown that ADX was seizure protective in rats against harmine (a beta-carboline tremorogenic agent)-induced seizures after withdrawal from three weeks of exposure to vaporized EtOH (Lamblin et al., 1996). However, these investigations were undertaken in chronic EtOH-exposed animals, a procedure that can drastically affect receptor sensitivity (Follesa et al., 2006) and the steroid profile from acute exposure (Finn et al., 2004b; Romeo et al., 1996), leaving one unable to extend the conclusions from these studies to similar undertakings in an acute EtOH withdrawal model (i.e., withdrawal from a single high dose of EtOH).

The current experiments investigated the role of peripherally derived steroids on acute EtOH withdrawal severity following a single dose of EtOH, measured by HICs. We hypothesized that peripheral sources of steroids, the adrenals and gonads, are important during withdrawal from a single EtOH exposure, and that the direction of change in acute withdrawal severity would provide insight into the relative contribution of pro- versus anti-convulsant steroids to acute EtOH withdrawal severity. That is, an increase in EtOH withdrawal severity following removal of the adrenals and gonads would suggest that endogenous anticonvulsant steroids were protective against HICs. In

contrast, a decrease in acute EtOH withdrawal severity following removal of the adrenals and gonads would suggest that endogenous proconvulsant steroids were increasing HICs. These effects were investigated in C57BL/6 (B6) and DBA/2J (D2) male and female mice, based on the several lines of evidence. First, it is well established that acute and chronic EtOH withdrawal (Crabbe, 1998; Crabbe et al., 1983; Roberts et al., 1992) is significantly greater in D2 than B6 mice. Additionally, sensitivity to the anticonvulsant effect of ALLO is significantly greater in EtOH-naïve B6 vs. D2 mice (Finn et al., 1997) and sensitivity to the anticonvulsant effect of ALLO is enhanced in B6 and reduced in D2 mice during chronic EtOH withdrawal (Finn et al., 2000). Finally, endogenous ALLO levels are higher in female than in male mice (Finn et al., 2004b) and acute EtOH withdrawal is lower in female than in male B6 and D2 mice (Gorin-Meyer et al., 2007). An additional consideration was that ADX and GDX have been reported to have varying effects on EtOH metabolism (Becker et al., 1985; Budec et al., 2002; Mezey et al., 1980; Powis et al., 1977; Wallis et al., 1984). Thus, a final study examined the effect of ADX and GDX on acute EtOH metabolism in order to account for changes in metabolism on acute EtOH withdrawal severity.

Materials and Methods

Subjects

Eight to 12 week old drug naïve, sexually mature, male and female B6 and D2 mice were obtained from Jackson West Laboratories (Davis, CA). Mice were separated by sex and strain; group housed four to a cage, and were allowed free access to rodent

chow (Labdiet 5001 rodent diet, PMI international) and water. Mice were maintained on a 12 hr (6 am to 6 pm) light/dark cycle in polycarbonate cages (Thorens) in a room kept at 21 ± 2°C with humidity control. Mice were allowed to acclimate to the facility for at least one week before any experimental manipulations were undertaken.

Procedure

Male and female B6 and D2 mice were randomly assigned to one of four groups: adrenalectomy (ADX), gonadectomy (GDX), both surgeries (ADX/GDX), or no organs removed (SHAM), and had the appropriate surgery performed (details below). Seven to 14 days after surgery, baseline handling-induced convulsions (HICs) were assessed in the mice. This time period allowed the animals to recover from the surgery and clear all endogenous steroids (Khisti et al., 2003). The mice were then given an intraperitoneal (IP) injection of 4 g/kg of EtOH (Aaper alcohol and chemical company, Shelbyville, KT; 20% v/v in saline). HIC severity was monitored hourly for twelve hours following injection and again at 24 hours. Following HIC scoring, animals were decapitated and dissected to confirm organ removal. Due to the large number of animals, several experiments were conducted; animals from each sex and genotype were tested at the same time. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted by the U.S. National Institutes of Health and were approved by the local Institutional Animal Care and Use Committee.

Surgery

All surgeries were adapted from "The Biology and Medicine of Rabbits and Rodents" (Harkness and Wagner, 1995). Anesthesia was induced with 5% isoflurane (Abbott Laboratories, North Chicago, IL) and maintained at 2% throughout surgery. Briefly, to remove the adrenal glands, a dorsal midline incision was made. This incision was shifted to either side to expose the areas lateral of the midline. Two more incisions were made to the muscle wall just behind the last rib on both sides in order to expose the anterior pole of the kidney and the adrenal gland. The adrenal gland was removed by separating the gland from the surrounding tissue with tweezers and then gently pulling the gland through the flank incision. 4-O chromic gut sutures (Davis & Geck, Danbury, CT) were used to close the incisions in the muscle walls, and metal clips were used to close the flank incision. To remove the testicles, the peritoneal cavity was entered via a small cranial pubic incision. The testicular vessels were severed using a cauterizing gun causing the testes, epididymis, and surrounding fat to be separated from the body and removed. The body wall and cutaneous wound was closed with tissue adhesive. To remove the ovaries, a dorsal midline incision was made. Like the adrenalectomies, this incision was used to expose the muscle wall lateral to the midline. Two incisions were made 4-5 cm below the last rib on both sides and the ovary and oviduct were removed through the flank incision. 4-0 gut sutures were used to close the incision in the muscle wall and metal clips were used to close the flank. The mice in the ADX/GDX group received both surgeries during the same session. In females the adrenals and the ovaries were removed through the same incisions. SHAM animals were anesthetized and had the appropriate incisions made, but no organs were removed. After surgery, all animals were allowed to recover on a heating pad, were administered a subcutaneous dose of 3 mg/kg

keterolac (Sigma-Aldrich, Saint Louis MO) for two 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 to insure that weight loss after surgery was minimal and that weight in all animals had recovered to SHAM levels.

HIC Scoring

HICs were scored according to a previously published scale (Crabbe et al., 1991). Briefly, a mild convulsion can be elicited by gently lifting a mouse by the tail and turning it 180 degrees if necessary. The HIC scoring ranges from 0 to 7; a score of 0 indicates no convulsions, a score including 1-3 indicates tonic or clonic convulsions obtained by a gentle turn, a score including 4-6 indicates convulsions elicited by only lifting the mouse by the tail, and a score of 7 indicates a spontaneous convulsion (See Table 1.1). Specifically, the animal is first briefly observed in the home cage for spontaneous convulsions, which would indicate a score of 7. If no spontaneous seizures are observed, the animal is lifted by the tail and briefly observed. If a seizure is elicited by lifting the animal alone, the HIC is scored as a 4-6, depending upon the severity of the convulsion and the animal is returned to its home cage. If no seizure is elicited from lifting the animal, the animal is gently turned 180 degrees by the tail. Seizures elicited by this turn are scored as 1-3, depending upon the severity. If no seizure is observed after one 180 degree spin, the HIC is scored as a zero and the animal is returned to the cage.

Blood Ethanol Concentrations (BEC)

A separate group of animals (B6 and D2 males and females) underwent surgeries, as described above, in another experiment. After 7-14 days of recovery, these animals were administered a 4 g/kg IP dose of EtOH. Immediately following EtOH administration, animals were tested for ethanol clearance rate using previously published methods (Gorin-Meyer et al., 2007; Shen et al., 1995). Briefly, retro-orbital blood samples were collected from each animal at 30, 60, 120, and 240 minutes post EtOH injection. Mice were restrained by hand while a 20-µl sample of blood was collected; eyes were alternated for each time point to minimize trauma. Between sampling, the mouse was returned back to its home cage. Samples were used to determine BECs at each time point. The blood samples were diluted into 500 µl of a matrix of 4mM n-propanol in deionized water. The 2.0 ml crimp top vial containing the blood sample in matrix was capped and vortexed thoroughly before analysis. Analysis was performed via ambient headspace sampling gas chromatography (Agilent 6890N GC, using a DB-ALC1 column, Wilmington, DE) on a 30 µl aliquot. Six pairs of EtOH standards (0.5 - 5.0 mg/ml), which included n-propanol (internal standard), were run before the samples (Finn et al., 2007). Recent work suggests that multiple retro-orbital blood sampling did not significantly alter BECs relative to animals that had received a single blood sampling (Kamens et al., 2006).

Data Analysis

Data are expressed as the mean ± the standard error. Analyses were conducted in animals with verified organ removal. Withdrawal severity was quantified by calculating the area under the curve (AUC) for each animal (hours 0-24), using the trapezoidal

method, as previously described (Crabbe et al., 1983; Metten and Crabbe, 2005). Peak withdrawal was found for each subject by taking the highest HIC score for each individual animal and averaging the peak score with the surrounding two scores. A multi-factorial ANOVA analyzing time as a within subjects factor and sex, genotype and surgery as between subjects factors was conducted. When appropriate (due to significant interactions), data were separated by sex and genotype 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 three way ANOVA (sex by genotype by surgery) and then followed by a one way ANOVA analyzing each sex and strain separately. Peak withdrawal was analyzed similarly. Baseline HIC scores were analyzed with a multi-factorial ANOVA and if necessary, a one way ANOVA due to interactions.

Linear regression analysis was performed on the retro-orbital BEC time course data for each repeatedly sampled animal. The linear portion of the regression line (60-240 min) was used to obtain an estimate of clearance rate (mg/ml/hr). An estimate of volume of distribution (ml) and volume of distribution accounting for body weight (ml/g) was determined by dividing the mgs of EtOH that each animal received by the estimated BEC at time = 0 (based on the regression slope and the y-intercept of the regression line). An estimate for total clearance time (min) was determined by the x-intercept of the regression line. A repeated measures ANOVA was used to assess the effects of strain, sex, surgery, and time on BECs, while a three-way ANOVA was used to assess the effects of strain, sex, and surgery on several of the clearance parameters. When

appropriate, clearance parameters where analyzed with a one way ANOVA on each sex and strain.

Tukey's post hoc test was used when appropriate. Significance was set at $p \leq 0.05$ for all analyses. All statistics were conducted with the SPSS statistical package (version 11, Chicago, IL).

Results

HIC scores

A multi factorial ANOVA revealed that there were significant main effects of genotype (D2 > B6) [$F(1,201)=353.22$, $p<0.001$] and surgery on hourly HIC score [$F(3,201)=10.15$, $p<0.001$], but no main effect of sex. However, this analysis also revealed that there was a trend for a significant four way interaction between time, sex, genotype and surgery [$F(14,25.35)=1.45$, $p=0.067$] and that all other interactions were significant [$Fs(14,25.357)>2.07$, $ps\leq0.001$]. Due to these highly significant interactions, further analysis on the hourly HIC scores was conducted separately on groups divided by sex and genotype.

Removal of the peripheral sources of steroids significantly increased the acute withdrawal profile from a 4 g/kg dose of EtOH in D2 male and female animals and in B6 male animals, measured by hourly HIC scores. In contrast, surgical status did not alter acute withdrawal severity in B6 female mice (See Figure 2.1 A-D). In D2 males, both

ADX and ADX/GDX mice had significantly elevated withdrawal profiles, when compared to SHAM and GDX mice. This conclusion is supported by the main effect of surgery [$F(3,43)=10.01$, $p<0.001$], main effect of time [$F(6,288)=142.61$, $p<0.001$], a significant interaction [$F(20,288)=1.94$, $p<0.001$] and post hoc tests. In D2 females, only ADX/GDX mice had an elevated withdrawal profile, when compared to the other surgical groups (main effect of surgery [$F(3,45)=3.40$, $p=0.024$], main effect of time [$F(7,307)=85.46$, $p<0.001$], a significant interaction [$F(20,307)=2.64$, $p<0.001$], and post-hoc tests). Similar to D2 males, B6 males had increased withdrawal in both ADX/GDX and ADX groups, when compared to SHAM and GDX mice. This conclusion is supported by the main effect of surgery [$F(3,56)=6.75$, $p=0.001$], main effect of time [$F(11,616)=23.99$, $p<0.001$] a significant interaction between surgical status and time [$F(33,616)=2.14$, $p<0.001$] and post-hoc tests. However, there was no effect of surgical status on the acute EtOH withdrawal profile in B6 females (main effect of time [$F(7,395)=39.07$ $p<0.001$], but no effect of surgery and no interaction).

Analysis was carried out on the baseline HIC scores in order to ascertain if baseline HIC scores were affected by surgical status. ANOVA revealed that there were significant main effects of genotype on the baseline HIC score (D2 > B6) [$F(1,198)= 354.35$, $p<0 .001$] and a main effect of surgery [$F(3,198)=2.86$, $p=0.038$]. However, there was also an interaction of genotype by surgery [$F(3,198)$, $p=0.047$]. In order to be comparable with the analysis on HICs during withdrawal, groups were separated by sex and genotype. In B6 males and females and D2 males, there were no effects of surgical status on baseline HIC score. There were effects of surgery on baseline HIC scores in D2 females [$F(3,42)=5.334$, $p=0.003$], with post-hoc tests revealing that baseline HIC score

was elevated in ADX/GDX and SHAM operated groups. Due to this finding, the previous analysis on HIC scores during withdrawal was repeated with baseline as a co-variate. This analysis produced results similar to that of the analysis run without baseline as a co-variate, indicating that the differences in baseline HIC did not significantly alter the acute EtOH withdrawal response measured by HIC.

To follow up on the analysis of the HIC time course with each sex and genotype, separate analyses were conducted at each time point to assess the effect of surgical status on HIC scores. In D2 male mice, there were significant effects of surgery on HIC score at hours six, eight, nine, ten and twelve [$Fs(3,47)>3.9$, $ps\leq0.05$]. Post-hoc tests revealed that at hour six, HIC scores in GDX animals were elevated above SHAM and ADX animals and that at hours eight, nine, ten and twelve, HIC scores in ADX and ADX/GDX animals were elevated over SHAM and GDX animals. In D2 female mice, there were effects of surgery at the baseline time point and at hours three, ten, eleven and twelve [$Fs(3,48)>3.61$, $ps\leq0.05$]. Post-hocs revealed that at the baseline, ADX/GDX and SHAM animals were elevated, when compared to ADX animals. At hour three, HIC scores in GDX animals were elevated over ADX and ADX/GDX animals; at hours ten and eleven, HIC scores in ADX/GDX animals were elevated over both ADX and GDX animals; and at hour twelve, HIC scores in ADX/GDX animals were elevated over all other groups. In B6 males there was a significant effect of surgery at hours five through ten [$Fs(3,59)>3.43$, $ps\leq0.05$]. Post-hocs revealed that at hour five, HIC scores in ADX/GDX animals were elevated over GDX and SHAM and that at hour six, HIC scores in ADX/GDX animals were elevated over all groups. At hour seven, HIC scores in ADX/GDX animals were elevated over GDX animals; and at hour eight, HIC scores in

ADX/GDX animals were elevated over GDX and HIC scores in both ADX/GDX and ADX animals were higher than SHAM. At hour nine, HIC scores in ADX and ADX/GDX animals were elevated over both GDX and SHAM animals; and finally, at hour ten, HIC scores in ADX/GDX animals were elevated over SHAM animals. There were no significant effects of surgery on HIC scores at any time point in B6 females.

AUC

In order to assess the effect of removal of peripheral sources of steroids on total withdrawal severity, the AUC was calculated for each group of animals (see Figure 2.2). A three way ANOVA revealed a main effect of genotype (D2 > B6) [$F(1,216)=220.85$, $p<0.001$], sex (male > female) [$F(1,216)=7.25$, $p<0.005$] and surgery [$F(3,216)=12.85$, $p<0.001$] on AUC. There was also a significant interaction of sex and surgery [$F(3,216)=4.25$, $p<0.005$] and a trend for an interaction between genotype and surgery [$F(1,216)=2.361$, $p<0.075$]. Due to these interactions, further analysis was conducted on groups, separated by sex and genotype. In D2 male mice, AUC was increased significantly in ADX/GDX and ADX mice, when compared with values in SHAM and GDX animals [$F(3,43)=10.01$, $p<0.001$ and post hoc tests]. The same pattern was observed in B6 male mice, with ADX/GDX and ADX mice having increased overall withdrawal when compared to SHAM and GDX animals [$F(3,56)=6.751$, $p<0.001$ and post hoc tests]. In D2 female mice, AUC was increased significantly only in the ADX/GDX mice when compared to SHAM animals [$F(3,45)=3.40$, $p<0.05$ and post hoc test]. There was no significant effect of surgery on AUC in B6 female mice [$F(3, 56)= 2.4$, $p=0.09$].

One way in which surgery could be increasing overall withdrawal severity is through increasing the peak HIC score that each animal reached. In order to investigate this possibility, average peak withdrawal was compared between surgical groups in each sex and genotype (see Figure 2.3). In each animal, the high dose of EtOH used in this experiment caused the animals to be completely sedated for several hours. As the EtOH was metabolized, withdrawal occurred with animals reaching peak withdrawal approximately 4-6 hours following EtOH administration. ANOVA revealed that in male D2 and B6 mice, surgical status significantly altered peak withdrawal severity [$F(3,56)= 8.46$, $p<0.05$ and $F(3,44)=6.95$, $p<0.05$, respectively]. Tukey's post hoc analysis revealed that both ADX and ADX/GDX surgical treatment significantly increased peak withdrawal when compared to the SHAM group. In contrast, there was no main effect of surgery in either female B6 or D2 mice.

BEC determination

A univariate ANOVA on body weight revealed a main effect of sex (male > female) [$F(1,73)=110.88$, $p<0.001$] and genotype (D2>B6) [$F(1,73)= 95.58$, $p<0.001$] with significant interactions between genotype and sex [$F(1,73)= 9.72$, $p<0.01$] and between surgery, genotype and sex [$F(3,73)= 3.27$, $p<0.05$] on body weight. When divided by sex and genotype, there were no effects of surgery in male and female D2 or female B6 mice. There was a significant effect of surgery on body weight in B6 males [$F(3,24)= 5.75$, $p<0.005$) with the ADX/GDX group weighing significantly less when compared to SHAM animals (see Table 2.1).

Repeated measures ANOVA of BECs measured 30, 60, 120 and 240 minutes after administration of a 4 g/kg dose of EtOH indicated that that BECs changed significantly across time [$F(3,216)=1003.31$, $p<0.001$]. There was a significant interaction between time and sex [$F(3,126)= 12.07$, $p<0.001$], but no interaction between time and surgery or time and genotype. When divided by sex and genotype, there was no effect of surgery on BEC in B6 or D2 male or female mice at any time point (see Figure 2.4 A-D).

Clearance rates were based on the linear portion of the curve (60-240 minutes post-EtOH injection). These estimated clearance rates were used to calculate several other clearance parameters (See Table 2.1). A three way ANOVA revealed that clearance (mg/ml/hr) was significantly affected by genotype (D2 > B6) [$F(1,88)=11.718$, $p<0.001$], sex (female>male) [$F(1,88)=34.36$, $p<0.001$], and surgery (ADX>SHAM) [$F(1,88)=3.30$, $p<0.05$ and post-hoc]. These changes in clearance rates represent a decrease of 9% in B6 compared to D2 mice, an 18% decrease in clearance in male compared to females and a 10% decrease in SHAM compared to ADX mice.

Other clearance parameters that were affected by both sex and genotype included the volume of distribution [$Fs(1,88)>7.5$, $ps<0.01$] and total clearance time [$Fs(1,88)>17.31$, $ps<.001$]. There were no effects of surgery on either of these parameters.

Discussion

The current experiments were undertaken in an effort to elucidate the effect of peripherally derived steroids on acute EtOH withdrawal severity. These experiments revealed that in male D2 and B6 mice, the removal of the adrenal glands significantly

increased the withdrawal profile following an acute exposure to EtOH, measured by hourly HIC scores, AUC and peak withdrawal. These measures were not significantly affected by GDX in male mice. In female D2 mice, the concurrent removal of the adrenals and the gonads significantly increased the withdrawal profile from an acute exposure to EtOH. Whereas hourly HIC score and AUC were increased in the ADX/GDX group, organ removal had no effect on peak withdrawal. The removal of the adrenal and/or gonads had no effect on acute withdrawal severity in B6 female mice. In general, these findings suggest that peripherally derived steroids are important during withdrawal from a high dose of EtOH. The fact that removal of the sources of these steroids significantly increased acute withdrawal severity in 3 out of 4 sexes/strains suggests that an endogenous anticonvulsant steroid (or steroids) contributes to the withdrawal profile exhibited in intact animals.

While removing the peripheral sources of steroids increased withdrawal from acute EtOH exposure in both D2 and B6 male mice and in D2 female mice, the method of increase was different between the sexes. In the male groups, the severity of peak withdrawal in addition to the duration of withdrawal were both significantly increased in the ADX and ADX/GDX groups. In contrast, the female D2 group that received ADX/GDX treatment exhibited a significant increase in the duration of withdrawal, with no change the peak withdrawal severity. Withdrawal in D2 female mice started sooner and lasted longer as compared to male mice. It is interesting to note that withdrawal started in D2 female animals several hours before the D2 and B6 males (See Figure 2.1). This is consistent with the fact that D2 females had a faster clearance time, when compared to the D2 and B6 males. This cannot, however, account for the fact that in D2

females, the AUC was greater in ADX/GDX animals. Nonetheless, these data suggest that removal of at least one anticonvulsant steroid significantly increased acute EtOH withdrawal severity and that male mice were more sensitive than the female mice to this manipulation (i.e., increases in peak and duration of withdrawal in D2 and B6 males vs. increases in duration in D2 females).

The differences between the male and female groups in the effect of surgical status may give a clue as to which peripherally derived (anticonvulsant) steroids are important in modulating withdrawal from an acute EtOH exposure. In males, only the removal of the adrenal gland was effective at increasing withdrawal severity, as there were no differences between the ADX and ADX/GDX groups or the GDX and SHAM groups. These findings suggest that testosterone and its neuroactive steroid metabolites, 5α-dihydrotestosterone and 3α-androstanediol, are not mediating the increase in withdrawal severity even though these neuroactive steroids are potent positive modulators of $GABA_A$ receptors and have seizure protective properties (Reddy, 2004a). Since both PROG and DOC are produced in the adrenals, it is possible that GABAergic metabolites of either or both of these steroids (i.e. 5α-THDOC or ALLO) are contributing to the present findings in male mice. Additionally, epinephrine is produced in the adrenals, and removal of epinephrine could be contributing to increased seizure susceptibility. This may be less likely, however, because as previously mentioned, epinephrine is still produced in the brain following ADX.

In contrast to the male group differences, withdrawal severity was only increased when both the adrenals and the ovaries were removed in the female D2 mice. The female

gonads produce a significant amount of PROG and estrogen in sexually mature mice such as those used in this study. While estrogen is pro-convulsant (Reddy, 2004b), PROG metabolites are anticonvulsant (Belelli et al., 1989) and represent some of the most potent endogenous positive $GABA_A$ receptor modulators (Gee, 1988; Morrow et al., 1987). Additionally, the adrenal glands also significantly contribute to the levels of PROG in females. An acute dose of EtOH can markedly increase PROG levels in ovariectomized (OVX) rats, suggesting that the adrenals represent an important contribution to hormone levels (Budec et al., 2002). Increases in PROG levels following an acute dose of EtOH also is seen in male rats and is attenuated by removal of the adrenals (O'Dell et al., 2004). Taken together, these data provide indirect evidence that PROG or a PROG metabolite, such as ALLO, may be important in mediating EtOH withdrawal severity in male and female mice and that these steroids are peripheral in origin.

Several studies have demonstrated that modest, acute doses of EtOH increased NAS levels in both the brain and plasma of rodents and that this increase was dose dependent (Barbaccia et al., 1999; Morrow et al., 1999; VanDoren et al., 2000). More recent studies have provided evidence that this rise could be attributed to peripheral origins (Budec et al., 2002; O'Dell et al., 2004), as removal of the adrenals and gonads attenuated the rise in NAS or their precursors following an acute dose of EtOH. Due to the time course of the rise and fall of NAS in response to a dose of EtOH, it has been hypothesized that NASs may be modulating some of EtOH's behavioral effects such as its anxiolytic (Hirani et al., 2005), anticonvulsant (VanDoren et al., 2000) and antidepressant (Hirani et al., 2002) properties. The anticonvulsant effect of EtOH can be prevented by administration of FIN, which blocks the metabolism of PROG, DOC and

testosterone, providing evidence that GABAergic neuroactive steroids can modulate some of EtOH's effects (VanDoren et al., 2000). FIN also reduced the antidepressant effects of EtOH in the forced swim test (Hirani et al., 2002). Additionally, it was recently shown that animals that had undergone ADX had reduced EtOH-induced loss of righting reflex (LORR) and that administering 5α-dihydroprogesterone (a precursor of ALLO) restored this behavior (Khisti et al., 2003). Finally, removal of the adrenal glands in male rats attenuated the rise in ALLO following an acute dose of EtOH (O'Dell et al., 2004). Thus, limited data suggest that NAS biosynthesis, especially that of ALLO, is important for some EtOH-related behaviors, and that this synthesis may occur in peripheral organs.

In addition to supporting the hypothesis that peripherally derived steroids can modulate withdrawal severity from an acute dose of EtOH, the present finding that acute EtOH withdrawal was significantly greater in D2 than in B6 mice is consistent with previous work (Gorin-Meyer et al., 2007; Roberts et al., 1992). The study by Roberts et al. (1992) suggested that CORT could be playing a role in the strain difference in acute withdrawal severity, as D2 mice had higher levels of CORT in response to an acute dose of EtOH and administration of high doses of CORT could mimic the acute withdrawal effect. One possibility is that the balance of steroid metabolism was shifted towards CORT as opposed to ALLO production in intact D2 mice and that D2 mice have higher withdrawal seizures because of lower production of ALLO in response to an acute dose of EtOH. However, the strain difference in withdrawal severity was maintained upon removal of the adrenals and gonads, suggesting the contribution of additional factors to the strain difference in acute withdrawal severity. Another possibility was that estrous cycle-related differences in seizure susceptibility (Finn and Gee, 1994) at the time of

testing contributed to the strain differences in withdrawal severity in the female D2 and B6 animals. While estrous cycle was not monitored in these animals, it is well documented that group-housed mice cycle together (Turner and Babnara, 1976). In our experience, upwards of 70% of group-housed mice have synchronized estrous cycles (unpublished observation). Importantly, the strain difference in acute withdrawal severity between the female genotypes was maintained after OVX, suggesting that estrous cycle-related fluctuations in seizure susceptibility did not contribute to the strain difference in acute EtOH withdrawal severity in female D2 and B6 mice.

Additional studies were conducted to ensure that the present findings on withdrawal severity were not due to an indirect effect of surgical status on ethanol metabolism. The current study represents the first report of the effects of ADX/GDX on EtOH metabolism in these strains of mice. While surgery caused significant changes in body weight only in B6 males, EtOH administration was based on body weight, and changes in body weight did not affect EtOH metabolism. These results agree with previous findings in our laboratory that an acute 4 g/kg dose of EtOH produced consistent BECs across male and female D2 and B6 mice (Gorin-Meyer et al., 2007). However, it was previously reported that GDX can increase the rate of EtOH elimination through altering the activity of liver alcohol dehydrogenase. If GDX was increasing the rate of EtOH elimination, one would expect a decrease in withdrawal severity or for animals to reach peak withdrawal sooner. However, neither of these effects was seen in the current study. In support of this conclusion, our study found no effect of surgery on total elimination time. Additional work indicated that metabolism of the 4 g/kg dose of EtOH was not affected in either OVX rats (Budec et al., 2002) or OVX B6 mice (Becker et al.,

1985), a conclusion supported by the current findings. With regard to ADX, there is contradictory data about the effects of ADX on EtOH metabolism. A study in which male rats were administered 0.8 g/kg of EtOH showed that ADX increased peak EtOH concentration without altering the rate of metabolism (Powis et al., 1977). In another study female C3H mice were administered 1.5 g/kg of EtOH after ADX and it was shown that these mice had a lowered peak BEC and unaltered rate of clearance (Wallis et al., 1984). The current study showed that while ADX alone did not affect clearance rates, the overall effect of ADX in combination with GDX significantly increased clearance rates by 10% when compared to SHAM animals (although there was no change in overall clearance time). Collectively, the current results in concert with previous studies indicate that the effects of surgery on EtOH metabolism cannot explain the changes in acute withdrawal severity.

The fact that removal of peripheral sources of neurosteroids did not seem to affect acute EtOH withdrawal in female B6 mice needs further exploration. It is possible to modulate the withdrawal profile of seizure resistant strains, as has been shown in the Withdrawal Seizure-Resistant selected line of mice with picrotoxin and pentylenetetrazol (Crabbe et al., 1991), suggesting that HICs can be increased under specific circumstances in resistant genotypes. However, in the present procedure, it is possible that B6 female mice are insensitive to NAS modulation. It has been shown that sensitivity to NAS differs between genotypes in male B6 and D2 mice (Finn et al., 2000; Finn et al., 1997), but this effect has not been investigated systematically in female animals. Although, it has been shown that there is an increase in whole brain ALLO levels in male B6 mice in response to a voluntary drinking paradigm, while the same paradigm elicited no change

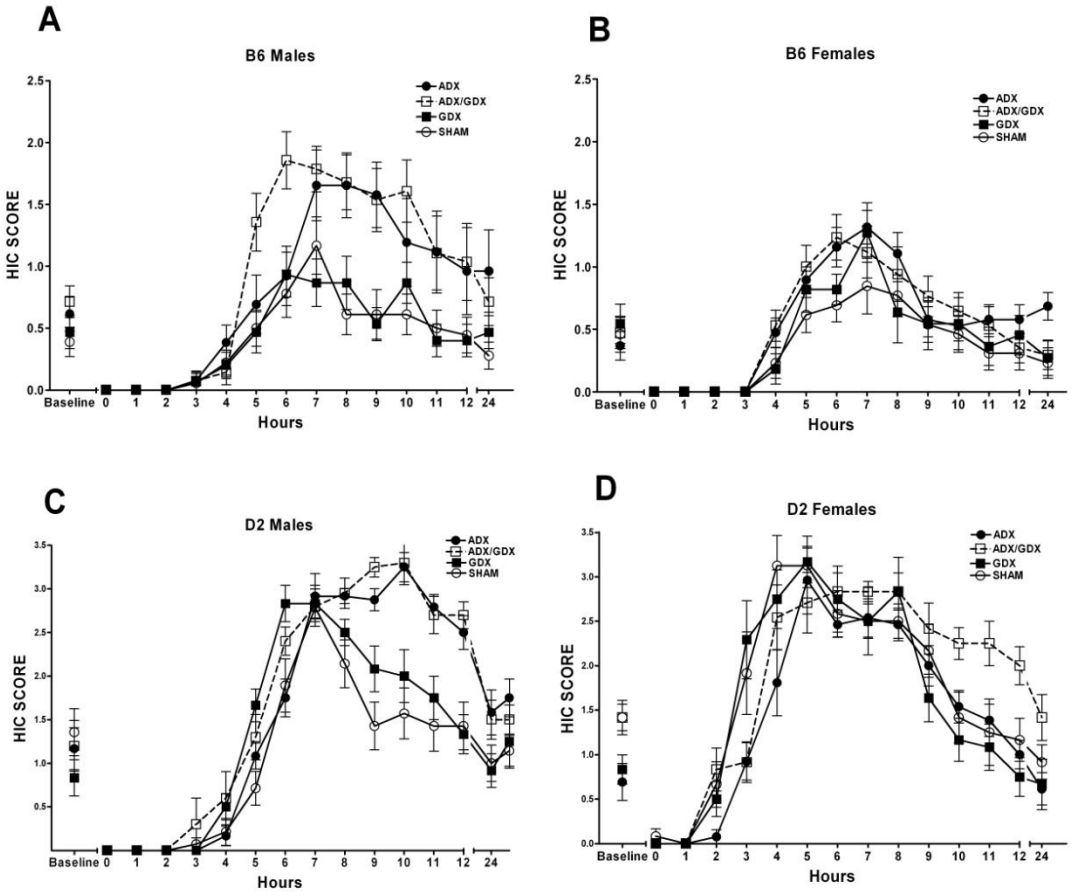
in brain ALLO levels in female B6 mice (Finn et al., 2004b) and that injections of ALLO increased EtOH consumption in male B6 mice (Ford et al., 2005; Sinnott et al., 2002), but had no effect on consumption in female B6 mice (Ford et al., 2008). Taken together, these data suggest that female B6 mice may be relatively insensitive to NAS modulation.

A limitation to the current findings is that hormone levels have not been analyzed in these animals. However, the removal of the adrenals and gonads was confirmed by visual inspection upon termination of the experiment, and animals with incomplete organ removal (< 5%) were removed from all analyses. Additionally, measuring ALLO levels in both the brain and the periphery of these animals would lend strength to the idea that the effect of ADX and ADX/GDX on acute EtOH withdrawal severity was being modulated by ALLO or another PROG metabolite. Although we were unable to measure ALLO levels in the present study due to decreased sensitivity of the antibody used in the ALLO assay after long term storage, we are exploring alternate methods of ALLO analysis for future studies.

In conclusion, the present findings provide important evidence that peripherally derived anticonvulsant steroids are modulating withdrawal severity from an acute dose of EtOH. These experiments represent important elucidation of factors involved in neuroadaptation and neuroexcitability from a high dose of EtOH. Notably, recent work indicated that acute (single dose) and chronic withdrawal have similar effects on $GABA_A$ receptor plasticity, with differences in the persistence of these changes (Liang et al., 2007). These findings lend support to the idea that examination of neuroadaptation following withdrawal from a single high dose of EtOH can provide insight regarding

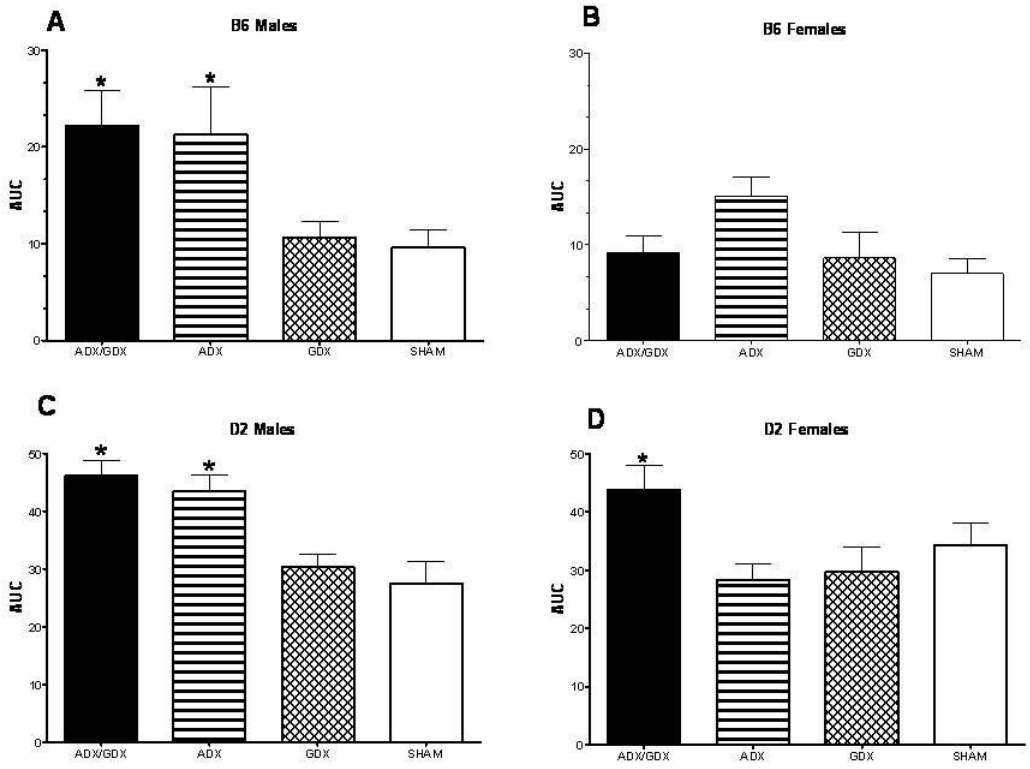
neuroadaptaion following chronic EtOH withdrawal. Therefore, neuroactive steroid synthesis may represent a potential important site for therapeutic intervention in the treatment of alcohol dependence. The interaction of EtOH and NAS warrants further investigation.

Figure 2.1. The effect of surgical status on acute EtOH withdrawal severity in male and female B6 and D2 mice, measured by hourly HICs.



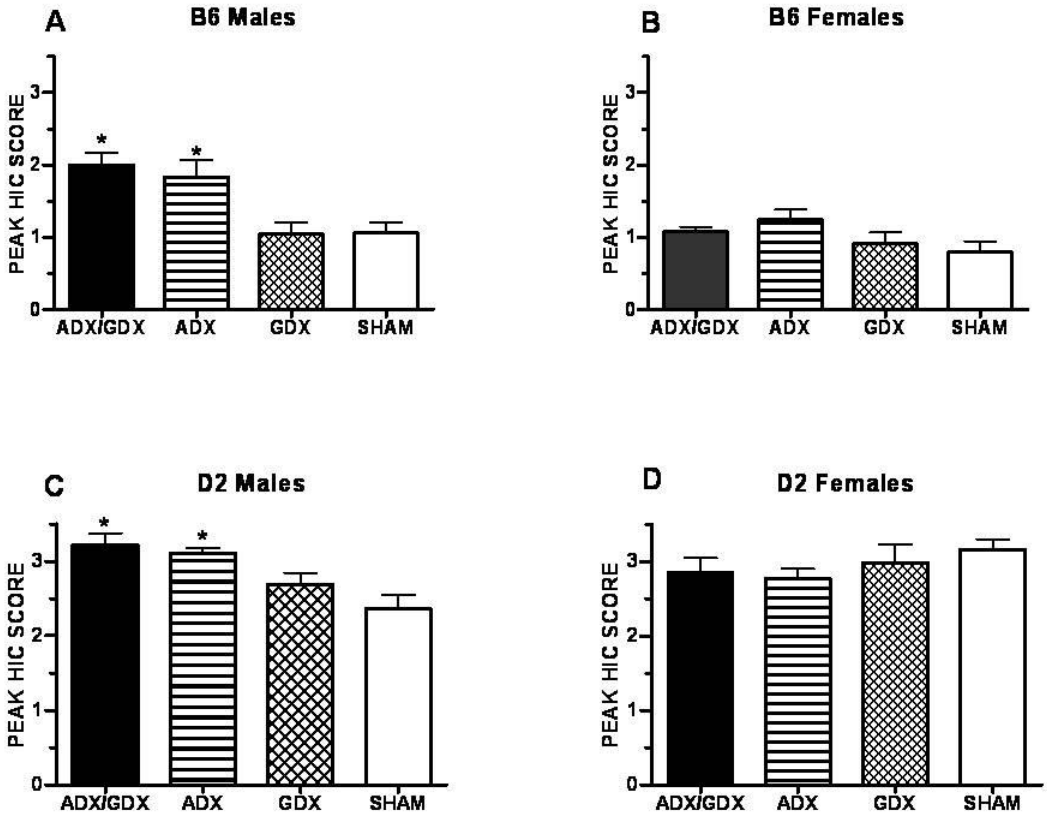
Overall, male B6 (panel A) and D2 (panel C) mice that had undergone ADX or ADX and GDX had a more severe withdrawal response from a high dose of EtOH. In D2 females (panel D) only ADX⁄GDX mice had a more severe withdrawal from an acute EtOH exposure; while there was no effect in B6 female mice (panel B). Values represent the mean ± SEM for 10 to 15⁄group. Note differences in y-axis between D2 and B6 mice.

Figure 2.2. Surgical status significantly alters acute EtOH withdrawal severity, measured by AUC.



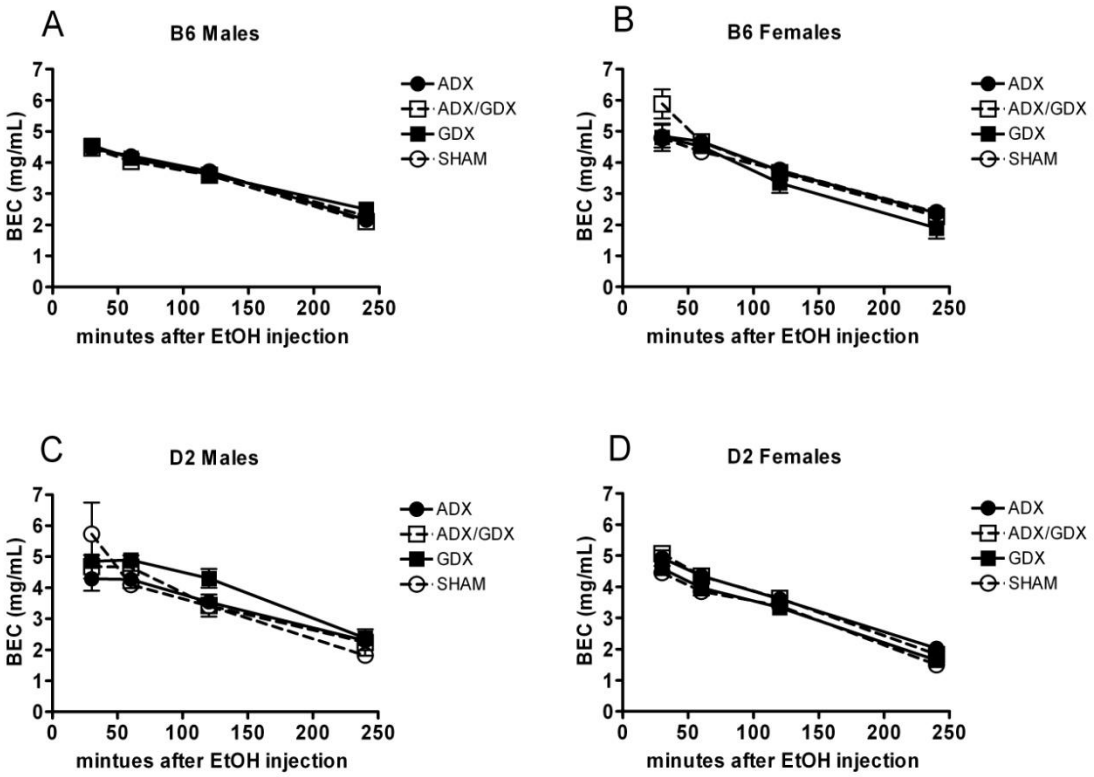
Tukey post hoc tests confirmed that AUC in the ADX and the ADX/GDX groups were significantly higher than in the GDX and no surgery (SHAM) groups in the B6 (panel A) and D2 (panel C) males. In the D2 females (panel D), AUC was significantly increased only in the ADX/GDX group, while in the B6 females (panel B), there were no group differences in AUC. Values represent the mean ± SEM for the animals depicted in Fig. 2.1. *Indicates significance of $p < 0.05$ versus SHAM. Note differences in y-axis between D2 and B6 mice.

Figure 2.3. Surgical status significantly affects peak withdrawal scores in male mice, but not in female mice.



Peak withdrawal is defined as the peak HIC score averaged with the 2 surrounding scores for each animal. In B6 males (panel A), peak withdrawal was significantly increased in groups that had undergone ADX and ADX/GDX surgeries. The same pattern was true for D2 males (panel C). There was no difference in peak withdrawal scores in D2 (panel D) or B6 (panel B) female mice. Values represent the mean ± SEM for the animals depicted in Fig. 2.1. *Indicates significance of $p \leq 0.05$ versus respective SHAM.

Figure 2.4. Surgical status has no significant effect on EtOH metabolism.



In B6 males (panel A), there was no significant effect of surgery on BEC at 30, 60, 120, or 240 minutes after EtOH administration. The same pattern was true for B6 females (panel B) and D2 male and female mice (Panels C and D, respectively). Values represent the mean ± SEM for 12 to 15/group.

Table 2.1. Clearance parameters

Clearance parameters	ADX	GDX	ADX/GDX	SHAM	ADX	GDX	ADX/GDX	SHAM
	C57BL/6J Males				C57BL/6J Females			
Weight (g)	25.18 ± 0.59	24.03 ± 0.59	23.15 ± 0.36	25.97 ± 0.49	20.00 ± 0.32	19.68 ± 1.31	20.82 ± 0.40	19.65 ± 0.24
BEC at time = 0 (mg/ml)	4.87 ± 0.19	4.78 ± 0.15	4.79 ± 0.12	4.86 ± 0.21	5.28 ± 0.23	5.24 ± 0.20	5.92 ± 0.28	5.08 ± 0.12
Clearance rate (mg/ml/h)	1.00 ± 0.03	0.86 ± 0.03	0.99 ± 0.02	0.95 ± 0.03	1.10 ± 0.10	1.28 ± 0.19	1.44 ± 0.15	1.01 ± 0.11
V_d (ml)	20.87 ± 1.16	20.29 ± 0.66	19.38 ± 0.42	21.76 ± 1.07	15.49 ± 0.92	15.80 ± 0.73	14.23 ± 0.57	15.47 ± 0.37
V_d/body weight (ml/g)	0.83 ± 0.04	0.85 ± 0.03	0.84 ± 0.02	0.84 ± 0.04	0.77 ± 0.04	0.82 ± 0.08	0.68 ± 0.03	0.79 ± 0.02
Total clearance time (minutes)	296.22 ± 20.81	335.19 ± 10.18	289.81 ± 4.85	306.66 ± 12.47	297.98 ± 20.81	250.50 ± 17.25	253.12 ± 13.70	304.62 ± 8.94
	DBA 2/J Males				DBA 2/J Females			
Weight (g)	25.55 ± 0.378	25.64 ± 0.33	25.53 ± 0.32	25.90 ± 0.20	22.67 ± 1.09	24.05 ± 0.41	21.97 ± 0.42	24.07 ± 0.95
BEC at time = 0 (mg/ml)	4.73 ± 0.29	5.51 ± 0.17	5.12 ± 0.34	5.01 ± 0.07	5.25 ± 0.17	4.92 ± 0.23	5.40 ± 0.12	4.83 ± 0.17
Clearance rate (mg/ml/h)	0.90 ± 0.08	1.23 ± 0.02	1.10 ± 0.06	1.20 ± 0.03	1.22 ± 0.08	1.13 ± 0.05	1.34 ± 0.05	1.23 ± 0.04
V_d (ml)	21.84 ± 0.89	18.70 ± 0.47	20.66 ± 1.77	20.71 ± 0.53	17.31 ± 0.65	19.72 ± 0.98	16.37 ± 0.49	20.00 ± 0.77
V_d/body weight (ml/g)	0.85 ± 0.03	0.82 ± 0.02	0.81 ± 0.07	0.80 ± 0.01	0.77 ± 0.02	0.73 ± 0.04	0.74 ± 0.01	0.83 ± 0.03
Total clearance time (minutes)	393.49 ± 17.84	293.96 ± 9.08	283.48 ± 24.53	250.56 ± 7.67	262.41 ± 11.70	241.58 ± 10.11	242.44 ± 8.02	236.22 ± 10.02

Values represent the mean ± SEM for 12 to 15/group. ADX, adrenalectomy; BEC, blood ethanol concentrations; GDX, gonadectomy.

CHAPTER 3: 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 (EtOH) 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 EtOH dose. Removal of the adrenals and gonads (ADX/GDX) increased withdrawal severity following 4 g/kg EtOH, 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 5α-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 EtOH 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, ganaxolone (a synthetic ALLO derivative), corticosterone (CORT), or vehicle. Animals were then administered a 4 g/kg EtOH 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 EtOH withdrawal severity.

Introduction

Each year in the United States, almost 5% of the population suffers from 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 it 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 (Devaud et al., 1997; Matsumoto et al., 2001; Mhatre and Ticku, 1994), 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 (Koob, 2003; Littleton, 1998). 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 interactions with neurotransmitter-gated ion channels (Paul and Purdy, 1992), and many NAS are potent allosteric agonists at the $GABA_A$ 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, 2002a; 2002b) . 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 [3α,5α-tetrahydroprogesterone (ALLO), 3α,5α-tetrahydrodeoxycorticosterone (THDOC) and 3α-androstanediol, respectively] through the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase (Compagnone and Mellon, 2000; Mellon, 1994). 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; Finn et al., 2004b; VanDoren et al., 2000). 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 (Gililland 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 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, ganaxolone (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 5α-reduced metabolites

would restore acute withdrawal severity back to the levels seen in intact animals and that co-administering FIN would abolish these effects.

Materials and Methods

Subjects

Drug naïve 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 ± 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). 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 below). Male and female D2 mice underwent SHAM surgery (see details below) and were allowed to recover for one week. Animals received either an injection of 20% cyclodextrin (VEH), or 50 mg/kg FIN in 20% cyclodextrin (both IP; 0.01 ml/gm). Animals then received a second injection of 50 mg/kg FIN 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 injections, 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 analyzed by radioimmunoassay (RIA).

Surgery

Surgeries were performed as previously described in Chapter 2. 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), PROG, FIN, ALLO, DOC, CORT, 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; purchased 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, San Diego, CA) 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. Please see Table 3.1 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 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. 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 (ALLO) extraction and Radioimmunoassay (RIA)

ALLO was extracted from whole brain according to the methods of Janis et al. (1998). Briefly, the whole brain was digested with 385 µl of 0.3N NaOH and then 100 µl [^{3}H] ALLO (2000 counts per minute (CPM)/100 µl in ethanol) 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 and combined. This solution was applied to solid silica phase columns (Honeywell, Burdick and Jackson, Muskegon, MI), which were then washed with heptane. 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 used [^{3}H] ALLO (10,000 cpm in 100 µl 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 *a priori* 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 a significant effects of sex [$F(1,107)=12.99$, $p<0.001$; F>M] and drug administration [$F(1,107)=49.27$, $p<0.001$;VEH>FIN], and no other interactions, indicating that FIN treatment had suppressed ALLO production during the test phase (see Figure 3.1).

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 3.2, while data in female mice are depicted in Figure 3.3.

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 test (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 3.2A and 3.2B 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 when compared to VEH treated animals, animals treated with PROG, DOC, GAN, CORT, and FIN had increased hourly HIC scores (see Figure 3.2C and 3.2D 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 3.3A and 3.3B 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 a lack of a main effect of drug treatment, no post-hoc tests were performed (See Figure 3.3C and 3.3D 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 [M>F; $F(1,530)=4.98$, $p=0.026$] 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, respectively.

In order to analyze the effects of surgical status, sex and drug treatment on total withdrawal severity, the AUC was calculated. A three way ANOVA on AUC revealed

main effects of sex [M>F; $F(1,530)=3.856$, $p=0.05$], 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 3.4). 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 3.4A). Similarly to the results in ADX/GDX females (Figure 3.4C), administration of PROG and DOC significantly decreased AUC. Additionally, the administration of FIN significantly increased AUC in these animals (Figure 3.4A). 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 3.4B). A one-way ANOVA comparing the SHAM VEH treated males to ADX/GDX PROG and DOC treated males revealed a 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 3.4C), 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 3.4D; $F(8,138)=0.86$, $p=0.56$]. 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 (Gililland and Finn, 2007). The present 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, and 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 has 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 5α-reduction of PROG and DOC, indicating that the GABAergic NAS metabolites of PROG and DOC are 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 5α-reduced NAS.

While these results indicate that 5α-reduced anticonvulsant NAS modulate the rebound neuronal hyperexcitability seen during acute EtOH withdrawal, they also indicate 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 (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) 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 5α-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 HICs 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; Finn et al., 2004b; Morrow et al., 1999; VanDoren et al., 2000). 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 3β-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 it was likely that using this drug would have produced the same interpretational difficulties as with ALLO administration. A final consideration is that administration of PROG and DOC may allow cells in the brain to determine the optimal production of NAS for anticonvulsant activity.

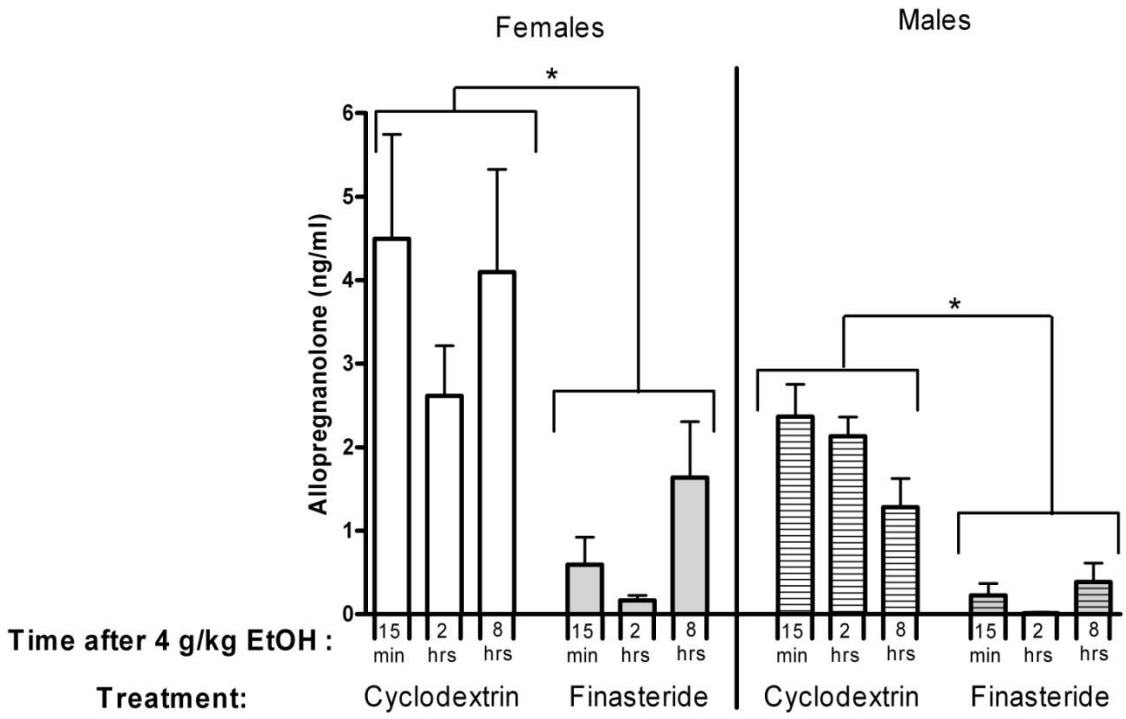
While the current experiment was designed to try and imitate the rise in NAS seen in intact animals in response to an acute dose of EtOH, it is possible that being deprived

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 5α-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 (Belelli et al., 1990; Morrow et al., 1987). 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.

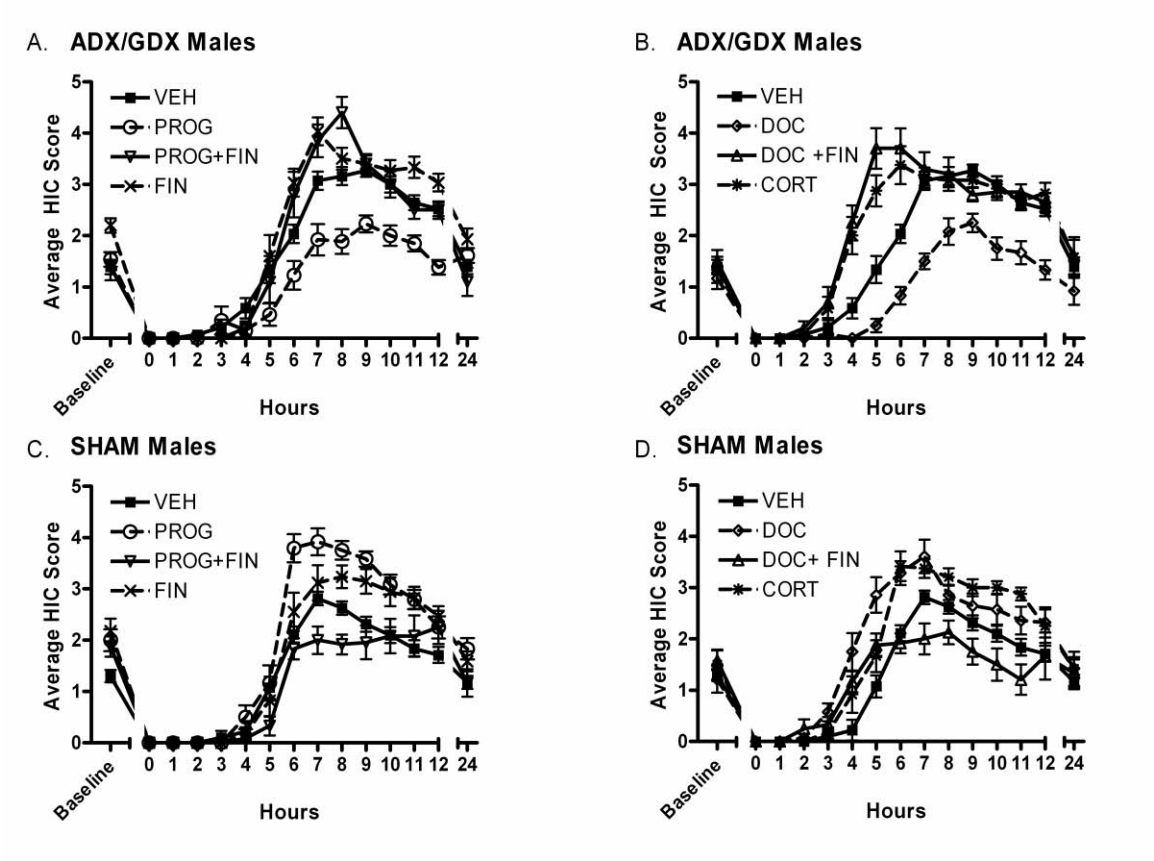
Overall, removing the peripheral sources of NAS during EtOH withdrawal increases the severity of the withdrawal. Restoring either the PROG or DOC arm of the biosynthetic NAS pathway can restore the withdrawal profile to that seen in SHAM operated animals. The current results indicate that the increase 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 5α-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 underlying 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.

Figure 3.1. Two doses of 50 mg/kg FIN reliably decreased ALLO production over the 8 hour period of measurement.



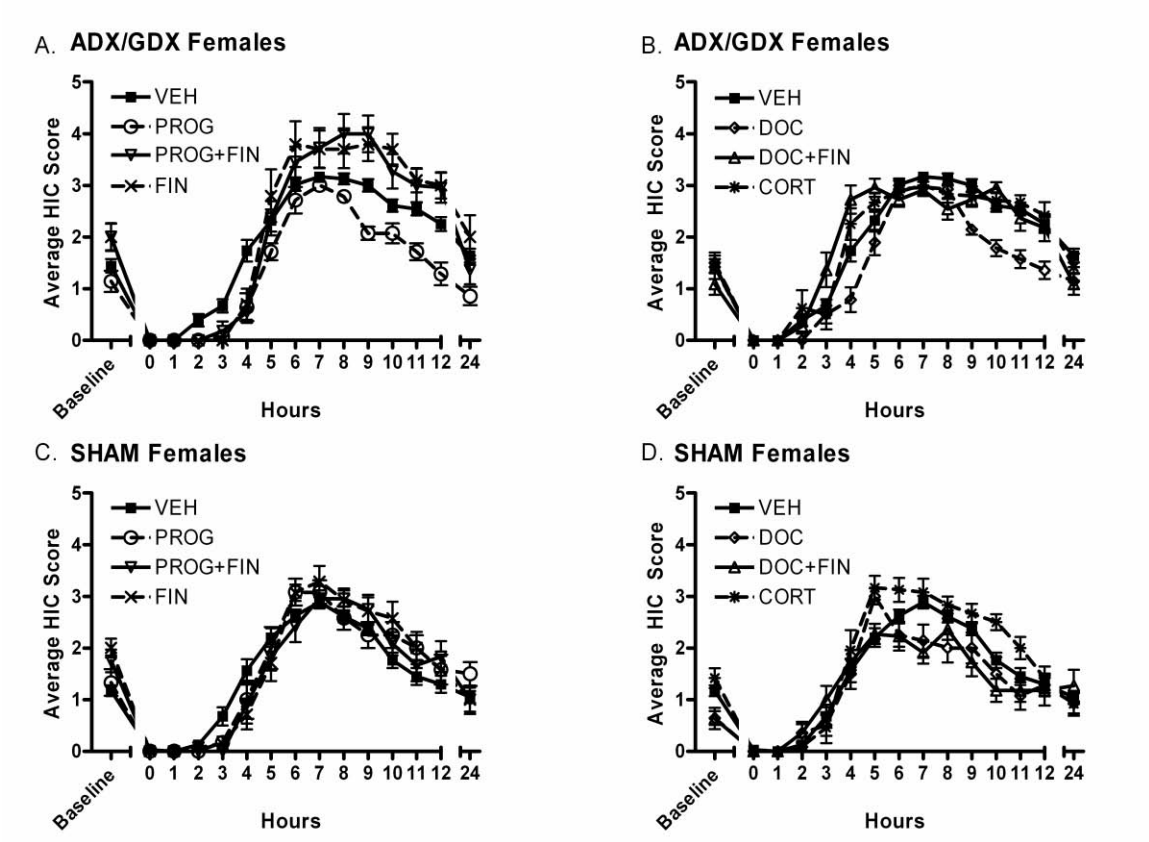
Male and female animals were dosed with either 50 mg/kg FIN or 20% cyclodextrin 24 hours before and with a 4 g/kg dose of EtOH. Different groups of animals were euthanized at 15 minutes, 2 hours and 8 hours following the second injection. FIN administration significantly decreased ALLO production during the entire 8 hour period when compared to VEH treated animals. Bars represent the mean + the SEM for 8-10 animals/group. * $p<0.05$.

Figure 3.2. The effects of steroid replacement on acute EtOH withdrawal severity in male D2 mice, measured by hourly HICs.



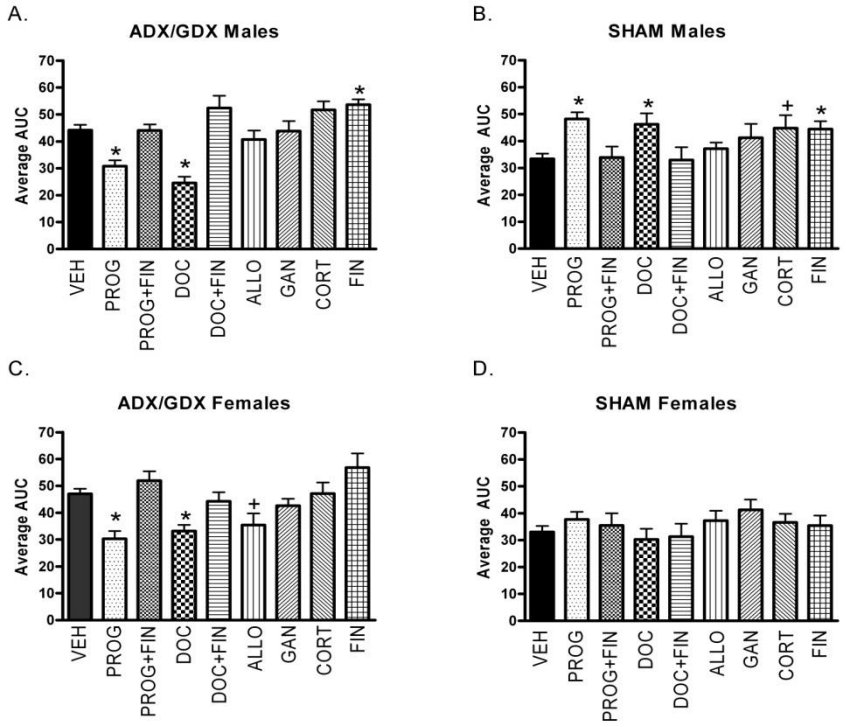
Overall, in ADX/GDX males (panels A and B), replacement with PROG or DOC decreased withdrawal severity. Co-administration of FIN blocked this effect. In SHAM males (panels C and D), administration of PROG or DOC increased withdrawal severity, and co-administration of FIN blocked this effect. Points represent the mean +/- the SEM for 9-16 for treatment groups and 36-40 for VEH treated groups that were collapsed across experimental passes.

Figure 3.3. 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 +/- the SEM for 12-16 for treatment groups and 28-40 for VEH treated groups that were collapsed across experimental passes.

Figure 3.4. 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 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 +/- the SEM for the animals depicted in Figures 3.2 and 3.3. + $p < 0.10$, * $p < 0.05$ vs. respective VEH treated group.

Table 3.1. Treatments administered and the references supporting the doses chosen

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 x 2, 24 hours before and with EtOH	Hirani et al., 2005; Gorin et al., 2005 and current experiment**s**
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 x 2, 24 hours before and with EtOH	Reddy and Rogawski, 2002; Gorin et al., 2005 and current experiments
Finasteride (FIN)	50 mg/kg x 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

CHAPTER 4: EFFECTS OF STEROID ADMINISTRATION PRIOR TO ACUTE ETHANOL WITHDRAWAL ON EXPRESSION OF $GABA_A$ RECEPTOR SUBUNITS AND STAR PROTEIN IN INTACT AND ADRENALECTOMIZED/GONADECTOMIZED DBA/2J MALE AND FEMALE MICE

Katherine R. Kaufman

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Abstract

Recent work documented that neural rebound hyperexcitability seen during withdrawal from an acute dose of ethanol (EtOH) was modulated by neuroactive steroids, which are potent positive modulators of the $GABA_A$ receptor. This conclusion is based on the finding that adrenalectomy (ADX) and gonadectomy (GDX) significantly increased acute EtOH withdrawal severity and that pretreatment with the neuroactive steroid precursors progesterone (PROG) and deoxycorticosterone (DOC) restored the acute withdrawal profile in ADX/GDX mice to that seen in intact animals. In order to examine the mechanism for this effect, we measured the expression of eight $GABA_A$ receptor subunits and a protein (steroidogenic acute regulatory protein, StAR protein) that is involved in the rate limiting step in neuroactive steroid biosynthesis.

Male and female DBA/2J mice underwent ADX/GDX or SHAM surgery. One to two weeks after surgery, separate groups received pretreatment of vehicle or of various steroids (tested in Chapter 3) prior to an injection of 4 g/kg EtOH. Mice were scored for EtOH withdrawal hourly for 12 hours and then at 24 hour using handling-induced convulsions. After the 24 hour measurement, mice were euthanized, and the hippocampus was rapidly dissected and frozen for subsequent examination of gene expression using quantitative Real-Time reverse transcriptase polymerase chain reaction.

An unsupervised hierarchical cluster analysis that analyzed all genes, sexes and treatments together found no highly associated changes. However, when each gene was analyzed separately, it was found that expression of the $GABA_A$ receptor α1 subunit mRNA in male and female ADX/GDX mice was decreased in all groups that had

received pretreatments that did not restore the withdrawal profile to that in intact animals. A similar finding was revealed in ADX/GDX female mice when expression of StAR protein mRNA was analyzed. These results suggest that $GABA_A$ receptor α1 subunit expression and StAR protein expression may be an important reflection of some of the effects seen during acute EtOH withdrawal.

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Introduction

Every year, over 10 million Americans meet criteria for alcohol (ethanol; EtOH) abuse or dependence as defined by the DSM-IV (Grant et al., 2004). Two percent or over 200,000 of these individuals end up in the hospital being treated for EtOH withdrawal related symptoms such as tremors, hallucinations or seizures (Kozak et al., 2002). While treatment for EtOH withdrawal is fairly straight forward with supportive care and benzodiazepine administration, this disease is heavily taxing on already over-worked hospital staff and has an enormous public cost (Holbrook et al., 1999; Mark et al., 2000). Understanding the underlying etiology of EtOH withdrawal is vital in order to prevent its occurrence and reduce this burden of EtOH abuse. However, understanding this disease is complicated as EtOH has effects on many receptor systems, and chronic EtOH exposure can lead to up- and down-regulation in many of these systems (Koob, 2003; Wallner et al., 2006).

One system that has been implicated in the etiology of EtOH addiction and withdrawal is the γ-aminobutyric acid$_A$ ($GABA_A$) system. It has been shown that EtOH can have both direct and indirect effects at $GABA_A$ receptors, with the indirect effects being modulated through a class of steroids known as neuroactive steroids (FIN; Sanna et al., 2004). NAS are the most potent positive allosteric agonist modulators of the $GABA_A$ receptor, allowing chloride to flux into the cell and causing general neural depression (Belelli et al., 1990; Kumar et al., 2009; Majewska et al., 1986). Given that both EtOH and NAS are positive modulators of $GABA_A$ receptors, many lines of evidence point to the fact that NAS may alter sensitivity to, or the duration of, some behavioral effects of EtOH (Follesa et al., 2006).

An interesting more recent development is based on evidence suggesting that exposure to and withdrawal from both EtOH and NAS can alter $GABA_A$ receptor subunit expression and in turn the physiology of GABAergic inhibition in the brain (Follesa et al., 2004). The $GABA_A$ receptor is comprised of five subunits, and there are 19 known different subunit proteins, although these 19 subunits can only assemble into a few dozen receptor conformations (Whiting et al., 1999). Each of these conformations of $GABA_A$ receptors has a specific anatomical distribution (Pirker et al., 2000) and specific pharmacological and physiological properties (Hevers and Luddens, 1998).

Much research has focused on the fact that chronic exposure to EtOH can change $GABA_A$ subunit expression. Expression of α1 and α2 subunit mRNA levels were significantly decreased, while α4 expression was significantly increased, in the cerebral cortex of dependent animals (Devaud et al., 1997; Montpied et al., 1991; Morrow et al., 1990). In the hippocampus, a different time course for the change in the expression was observed with the α1 subunit still decreased, the α4 subunit also increased, but no change in the α2 subunit (Matthews et al., 1998). It has been theorized that these changes are responsible for the increased seizure severity that is seen during withdrawal as the increase in the α4 and decrease in the α1 subunits persisted during withdrawal (along with an increase in the β2, β3 and γ1 subunits) (Devaud et al., 1997).

Administration of and withdrawal from steroids and NAS has also been shown to modulate $GABA_A$ receptor subunit expression. Withdrawal from progesterone (PROG) increased expression of the α4 (Smith et al., 1998a; Smith et al., 1998b) and δ subunits (Sundstrom-Poromaa et al., 2002). Withdrawal of steroids through adrenalectomy (ADX) increased hippocampal expression of the α1, α2 and γ2 subunits, but decreased β2

subunit expression (Orchinik et al., 1994). While much has been done elucidating the effects of chronic ethanol and NAS separately on $GABA_A$ receptor subunit expression, little has been done to elucidate the effects of these substances in concert. Additionally, little research has focused on the effects of acute EtOH withdrawal on $GABA_A$ receptor subunit expression.

Recent work in our laboratory showed that endogenous NAS were important modulators of acute EtOH withdrawal in mice (Gililland and Finn, 2007). Male and female mice that had undergone removal of the peripheral sources of NAS (the adrenals and the gonads) had increased withdrawal severity from a 4 g/kg dose of EtOH, as measured by handling induced convulsions (HICs). Replacement with PROG and deoxycorticosterone (DOC), which are metabolized to the NAS allopregnanolone (ALLO) and tetrahydrodeoxycorticosterone (THDOC) restored the withdrawal profile in ADX/GDX animals to that seen in intact animals. Inhibiting the metabolism of PROG and DOC by co-administering the 5α-reductase inhibitor finasteride (FIN) blocked this effect. However, there was a paradoxical effect in which the administration of PROG and DOC increased acute EtOH withdrawal severity in intact male, but not female mice.

The purpose of the current experiments was twofold. First, we wanted to determine whether there were effects of sex or surgical status on the expression of specific $GABA_A$ receptor subunits (α1, α2, α4, α5, β1, β2, γ2, δ) or in steroidogenic acute regulatory (StAR) protein. These subunits were chosen based on their involvement in sensitivity to either NAS or EtOH and their involvement in synaptic vs. extrasynaptic $GABA_A$ receptor mediated transmission. StAR protein was chosen based on its involvement as a rate limiting step in NAS production and due to its ability to be

modulated by some hormone administrations. Second we wanted to determine whether changes in the expression of specific $GABA_A$ receptor subunits or in StAR protein corresponded to the changes in acute EtOH withdrawal that we observed in our earlier experiments. Specifically, we predicted that acute EtOH withdrawal would increase the expression of the α4 and δ subunits and that steroid replacement in ADX/GDX animals that restored their behavior to intact levels would display subunit expression patterns similar to those seen in intact animals. In order to accomplish these goals, hippocampal gene expression from animals that had previously participated in our behavioral acute withdrawal paradigm was examined using quantitative Real-Time reverse transcriptase polymerase chain reaction (qRT-PCR). The hippocampus was chosen as it is an important site for both EtOH and NAS action and an important component of seizure circuitry (Ryabinin et al., 1997).

Materials and Methods

Animals

Drug naïve male and female DBA/2J (D2) mice were purchased from Jackson West Laboratories (Davis, CA). All mice were 8-12 weeks old at the time of experiment. Animals were group housed four to a cage (separated by sex), allowed free access to rodent chow (Labdiet 5001 rodent diet; PMI International, Richmond, IN) and tap 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 ± 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.

Experimental Procedure

Animals were assigned to one of two groups: ADX/GDX surgeries (in which both the adrenals and the gonads were removed; surgery detailed in Chapters 2 and 3) or SHAM surgery (in which no organs were removed). After the surgery was performed, animals were allowed to recover for 7-14 days. Once recovered, animals were assigned to one of nine treatment groups: vehicle (VEH), PROG, FIN, ALLO, DOC, corticosterone (CORT), ganaxolone (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 by intraperitoneal (IP) injection. VEH, PROG (5 mg/kg; Sigma; St. Louis, MO), ALLO (10 mg/kg; Purchase from Dr. Robert Purdy), DOC (50 mg/kg; Sigma), CORT (20 mg/kg; Sigma) and GAN (10 mg/kg; Purchased from Dr. Robert Purdy, San Diego, CA) 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 for a total of four injections. See Table 3.1 for references for supporting the doses chosen.

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 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. Although in the original experiment there was ~12/group, a subset of 4 randomly selected animals from each treatment group was used for this experiment.

Tissue Collection and qRT-PCR

The hippocampus was dissected on ice and immediately frozen in liquid nitrogen. Samples were stored at -80°C until use. Total RNA was isolated from dissected hippocampal tissues using the RNA Stat-60 kit (Tel-Test, Inc, Friendswood, TX.). Contaminating DNA was removed by Zymo-spin column purification following manufacturer's recommendations (Zymo Research, Orange, CA). RNA was quantified by spectrophotometric methods; lack of degradation was confirmed by agarose gel electrophoresis followed by staining with SYBR Gold. Twenty ng RNA was reverse transcribed and amplified in a 25 μl reaction mix using a one-step QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Valencia, CA) and 0.5 μM of each primer. Primers were purchased from Qiagen (Valencia, CA). $GABA_A$ receptor subunit transcript expression (α1, α2, α4, α5, β1, β2, γ2 and δ) and StAR protein expression was performed using qRT-PCR with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Relative expression of the RT-PCR product was

determined using the comparative $\Delta\Delta C_t$ method (Winer et al., 1999), after normalizing expression to total RNA concentration employing the RNA-specific binding dye RiboGreen® [Molecular Probes, Carlsbad, CA; (Hashimoto et al., 2004)]. Real-time qRT-PCR efficiency was determined for each primer set using a five-fold dilution series of total RNA. Following PCR, specificity of the PCR reaction was confirmed with melt curve analysis.

Data Analysis

Once relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method, fold increase over control was calculated. Data are expressed as the mean fold change. Individual gene expression differences were analyzed by either two-tailed *t*-test between SHAM animals and ADX/GDX animals or between males and females, or a one-way ANOVA comparing treatment effects within each sex and surgical status. One-way ANOVAs were followed by Dunnet's post-hoc tests comparing each treatment group to the VEH treated animals. Significance was set at $p<0.05$. An exploratory analysis was performed using an unsupervised hierarchical cluster analysis (average linkage, Euclidian distance) with 100 resampling iterations to determine Bootstrap values for each node using the TIGR Multiple Experiment Viewer software (The Institute for Genomic Research).

Results

Each gene was analyzed separately. When looking at the differences between VEH treated males and females and surgical status, several significant results were found (see Table 4.1). In summary, regardless of surgical status, females had significantly

increased expression of StAR protein mRNA when compared to males. In males, ADX/GDX significantly decreased StAR protein expression versus SHAM animals. In animals that had undergone ADX/GDX surgery, there was a significant decrease in expression of both the α2 and α5 subunits in females when compared to males. There were no differences in expression of mRNA for the α1, α4, β1, β2, γ2, or δ $GABA_A$ receptor subunits between males and females or SHAM surgery compared to ADX/GDX surgery. When all genes, sexes and treatments where analyzed together using an unsupervised hierarchical cluster analysis, no highly associated changes were found.

An analysis of expression of the $GABA_A$ receptor subunit α1 transcript by one way ANOVAs showed that there were significant effects of treatment [$Fs(8,33) \geq 5.64$, $ps \leq 0.0004$; see Figure 4.1 and Tables 4.2-4.5]. Post-hoc analysis comparing each pretreatment to the appropriate VEH treated animals revealed that in male SHAM animals, all steroid treatments (PROG, PROG+FIN, DOC, DOC+FIN, ALLO, GAN and CORT), except for FIN treatment alone, decreased α1 expression (Table 4.5 and Figure 4.1C). When the same analysis was done on the levels of α1 mRNA in SHAM surgery females, significant decreases in α1 expression occurred in groups that received all pretreatments except for ALLO (Table 4.3, Figure 4.1A). In ADX/GDX males, pretreatment with PROG+FIN, DOC+FIN, GAN and CORT significantly decreased expression of the α1 subunit transcript (Table 4.4, Figure 4.1D). Results in ADX/GDX female mice were similar, with PROG+FIN, DOC+FIN, GAN, CORT pretreatments all decreasing α1 subunit expression as well as the FIN alone group (Figure 4.1B, Table 4.2).

Examination of the $GABA_A$ receptor α2 subunit transcript revealed that pretreatment significantly altered α2 expression in ADX/GDX males and females and

SHAM males and females [Fs(8,33)≥2.99, ps≤0.016; see Tables 4.2-4.5]. In ADX/GDX males, α2 subunit expression was significantly decreased in groups pretreated with GAN, CORT and FIN, while there was a significant increase in expression in the group pretreated with DOC+FIN (Table 4.4). In ADX/GDX females, there was a highly significant 34- and 30-fold increase in expression of the α2 transcript in animals pretreated with DOC+FIN and ALLO, respectively (Table 4.2). In SHAM males, α2 subunit expression was significantly decreased in animals treated with DOC, GAN, CORT and FIN (Table 4.5). In SHAM females, there was highly significant 35-fold increase in α2 subunit expression in animals that had been treated with ALLO (Table 4.3).

Expression of the $GABA_A$ receptor α4 subunit was significantly altered by steroid treatments in ADX/GDX males, ADX/GDX females and SHAM males (Fs(8,33)≥8.35, ps≤0.0001] (see Tables 4.2-4.5). In ADX/GDX males, post-hoc tests revealed a significant increase in the α4 subunit transcript of 35-fold in animals that had been administered GAN and an increase of 13-fold in animals administered FIN (Table 4.4). In ADX/GDX females there was a significant increase in α4 subunit mRNA expression only in animals that were administered GAN (Table 4.2). Finally, in SHAM males, there were significant increases in α4 subunit expression in animals that were administered DOC or GAN (Table 4.5).

Examination of the $GABA_A$ receptor α5 transcript revealed that there were significant effects of treatment on α5 expression in ADX/GDX and SHAM males and females [Fs(8,33)≥7.95, ps≤0.0001, see Tables 4.2-4.5]. In ADX/GDX males, there were significant decreases in α5 subunit mRNA expression in all treatment groups when

compared to VEH treated animals, except for GAN pretreatment, which significantly increased α5 expression (Table 4.4). Conversely, in ADX/GDX females, there was only a significant 22-fold increase in α5 subunit mRNA expression in GAN treated animals (Table 4.2). This was similar to the results in SHAM males in which the only significant effect was an increase in α5 subunit mRNA expression in GAN treated animals, although it was only a 2.5-fold increase (Table 4.5). This result also generalized to SHAM females, where there was a significant increase in α5 subunit mRNA expression in GAN treated animals, with an additional increase in α5 subunit expression in DOC+FIN treated animals (Table 4.3).

When comparing β1 mRNA expression between VEH treated and steroid treated animals, there were significant effects in all four groups [$Fs(8,33) \geq 5.36$, $ps \leq 0.0008$, see Tables 4.2-4.5]. In ADX/GDX males, there were large significant increases in β1 mRNA expression in animals that had been pretreated with DOC+FIN or CORT (i.e. 96- and 148-fold, respectively; Table 4.4). Results were similar in ADX/GDX females; with increased β1 mRNA expression in DOC+FIN and CORT pretreated animals when compared to VEH treated ADX/GDX females (Table 4.2). In SHAM males and females, there were significant increases in β1 mRNA expression in animals that had been pretreated with CORT (Tables 4.3 and 4.5). In SHAM females, there were additional increases in β1 subunit expression in animals that had been pretreated with DOC or FIN, although not in the combination (Table 4.3).

There were significant effects of treatments in ADX/GDX males and females and in SHAM males and females in expression of the $GABA_A$ receptor β2 subunit [$Fs(8,33) \geq 2.55$, $ps \leq 0.0338$, see Tables 4.2-4.5]. In ADX/GDX males there was a

significant 117-fold decrease in β2 subunit mRNA in animals that had been pretreated with GAN (Table 4.4). There was a similar large decrease (117-fold) in β2 $GABA_A$ subunit mRNA expression in ADX/GDX female animals that had been pretreated with GAN, while ALLO pretreated animals in this group had a 2-fold increase in β2 expression (Table 4.2). In SHAM males, $GABA_A$ receptor β2 subunit mRNA was decreased 142- and 150-fold in DOC and GAN pretreated animals, respectively, while there was an increase in expression in animals were that were pretreated PROG+FIN (Table 4.5). In SHAM females, there was an increases in $GABA_A$ receptor β2 subunit mRNA only in animals that had been pretreated with ALLO (Table 4.3).

Expression of the $GABA_A$ receptor γ2 subunit was significantly affected by treatment only in ADX/GDX males and females [$Fs(8,32) \geq 8.13$, $ps < 0.0001$, see Tables 4.2-4.5]. In both ADX/GDX males and females, there was a significant increase in γ2 subunit mRNA expression only in animals that had been administered GAN (Tables 4.2 and 4.4).

Significant treatment affects were seen in the expression of the $GABA_A$ receptor δ subunit only in ADX/GDX males and females [$Fs(8,32) \geq 2.67$, $ps \leq 0.028$] (see Tables 4.2-4.5). In ADX/GDX males, δ subunit mRNA expression was increased in CORT pretreated animals and decreased in mice pretreated with PROG+FIN (Table 4.4). Conversely, in ADX/GDX females, there were significant decreases in δ subunit mRNA expression in PROG+FIN, GAN and FIN treated animals (Table 4.2).

StAR protein expression was significantly affect by treatment in ADX/GDX males and females and in SHAM males [$Fs(8,33) \geq 7.44$, $ps \leq 0.0001$] (see Figure 4.2 and

Tables 4.2-4.5). In ADX/GDX males, StAR protein expression was significantly increased in animals that had been pretreated with FIN alone (Figure 4.2D and Table 4.4). Similar results were observed in SHAM males where StAR protein expression was significantly increased only in FIN pretreated mice (Figure 4.2C and Table 4.5). In ADX/GDX females there were significant decreases in mRNA expression for StAR protein in PROG+FIN, DOC+FIN, ALLO, GAN, CORT and FIN pretreated mice (Figure 4.2B and Table 4.2).

Discussion

Previous work in our lab has shown that NAS are an important modulator of acute EtOH withdrawal (Gililland and Finn, 2007). NAS steroids interact with the $GABA_A$ receptor and specifically on residues that are conserved on the α subunit (Hosie et al., 2009; Hosie et al., 2006; Hosie et al., 2007). EtOH withdrawal is thought to be mitigated, at least in part, by reduced function of $GABA_A$ receptors in the brain (Kumar et al., 2009). Several mechanism could underlie this reduced $GABA_A$ receptor function, including change in the density or the affinity of the $GABA_A$ receptors, post-translational modifications, trafficking of the $GABA_A$ receptor, synaptic localization, or phosphorylation status of the receptor (Kumar et al., 2004). Another postulated mechanism is that altered expression of $GABA_A$ subunits is mitigating this effect (Devaud et al., 1997). In order to investigate if the changes in withdrawal severity that we have previously seen in our lab would be reflected by changes in $GABA_A$ receptor subunit expression 24 hours after acute EtOH withdrawal, we measured gene expression of 8 different $GABA_A$ receptor subunits and StAR protein, based on evidence that these

transcripts might be important in modulating $GABA_A$ receptor sensitivity to NAS and EtOH.

We choose to measure hippocampal gene expression for a variety of reasons. First, research examining the interaction between NAS and EtOH has produced strong evidence for the involvement of GABAergic steroids as endogenous modulators of some of the behavioral effects of EtOH (Hirani et al., 2002; Hirani et al., 2005; Khisti et al., 2003; VanDoren et al., 2000) and for the importance of the CA1 region of the hippocampus for some of these interactions (Martin-Garcia and Pallares, 2005a; 2005b). More specifically to EtOH withdrawal, data from our lab has indicated that manipulation of the NAS environment of the CA1 region of the hippocampus produced bidirectional effect on chronic EtOH withdrawal severity (Gililland-Kaufman et al., 2008). Second, immediate early genes in the hippocampus have been shown to be bi-directionally modulated during EtOH intoxication and withdrawal. Both acute and chronic administration EtOH administration suppress immediate-early gene expression in the hippocampus of rats and mice (Ryabinin et al., 1997; Ryabinin et al., 2003; Sharpe et al., 2005). However, during the peak of behavioral signs of withdrawal from both acute and chronic EtOH administration, expression of immediately early genes increases, which cannot be totally attributed to withdrawal seizures as genotypes that do not have withdrawal seizures (as measured by HICs) still show increases in immediate early genes (Borlikova et al., 2006; Knapp et al., 1998; Kozell et al., 2005; Olive et al., 2001; Putzke et al., 1996). Finally, many lines of research (including *in-vivo* and *in-vitro*) have shown that EtOH administration and withdrawal, as well as NAS withdrawal, can alter $GABA_A$ receptor subunit expression. For example, a chronic intermittent withdrawal paradigm, in

which animals were subjected to numerous withdrawal periods, was shown to increase mRNA for the α4 subunit in the hippocampus (Mahmoudi et al., 1997). Additionally, chronic EtOH administration was shown to increase mRNA for the α4 subunit, while decreasing mRNA for the α1 subunit in the hippocampus (Matthews et al., 1998). NAS withdrawal elicits similar changes in $GABA_A$ receptor subunit mRNA, especially with increases in the α4 subunit (Follesa et al., 2000; Smith et al., 1998a; Smith et al., 1998b).

Although mice with a null mutation in the $GABA_A$ receptor α1 subunit (i.e., α1 knockout, KO) did not show differences in HICs during withdrawal from a chronic EtOH diet (Blednov et al., 2003), receptors containing the α1 subunit are highly sensitive to the actions of ALLO (Belelli et al., 1996) and chronic EtOH administration itself decreases α1 mRNA expression and α1 peptide in the hippocampus, cerebral cortex and the cerebellum (Devaud et al., 1997; Matthews et al., 1998; Montpied et al., 1991). Therefore, the current experiments measured α1 subunit mRNA expression. The results are interesting: groups of ADX/GDX males and females that were administered steroid treatments that did *not* restore their withdrawal profile to that of SHAM animals (i.e PROG+FIN, DOC+FIN, etc.) generally had decreased α1 mRNA expression versus vehicle treatment, while α1 subunit mRNA expression was unchanged in the groups where steroid administration *did* restore their withdrawal profile to that of SHAM animals. Decreases in the expression of the α1subunit mRNA may indicate decreases in expression of the α1 subunit protein, which could shift receptor kinetics towards tonic $GABA_A$ receptor activation, as the α1 subunit clusters in the synaptic receptors (Mody, 2001; Olsen and Sieghart, 2009; Somogyi et al., 1996). As phasic $GABA_A$ receptor activation is required for rapid changes in GABAergic tone (Farrant and Nusser, 2005),

this may give insight to a mechanism behind the results that PROG and DOC administration restored the withdrawal profile in ADX/GDX mice.

Previous data indicated that withdrawal from chronic EtOH exposure in cerebellar granule cells increased α2 subunit mRNA expression (Follesa et al., 2003), which was similar to that seen when these cells were exposed to NAS (Follesa et al., 2001). Additionally, it has been shown that the $GABA_A$ receptor α2 subunit may confer sensitivity to some of EtOH's subjective effects in humans (Pierucci-Lagha et al., 2005). We investigated if these results generalized to the model used in the current experiments. Our results indicate that α2 mRNA expression was not affected by one to two weeks of steroid deprivation, as SHAM and ADX/GDX animals did not differ in their expression of this $GABA_A$ receptor subunit. While the α2 subunit did not seem to be conferring susceptibility to withdrawal seizures, several treatments did induce robust changes in mRNA expression (see Tables 4.2 and 4.3).

$GABA_A$ receptors that contain the α4 subunit are insensitive to modulation by benzodiazepines (Barnard et al., 1998). Withdrawal from PROG increases the expression of the α4 subunit protein in the hippocampus (Smith et al., 1998a; Smith et al., 1998b), and it has been hypothesized that this increase in expression corresponds to many of the effects induced by neurosteroid withdrawal (Follesa et al., 2004; Smith et al., 1998a). Exposure to chronic EtOH increased expression of α4 subunit mRNA in the cerebral cortex and the hippocampus (Devaud et al., 1995; Mahmoudi et al., 1997), while withdrawal from chronic EtOH exposure also increased the expression of α4 subunit mRNA in the cerebral cortex (Devaud et al., 1997; Follesa et al., 2003). With these results in mind, it is surprising that the current investigations found minimal changes in

α4 subunit mRNA expression. There were no changes in SHAM and ADX/GDX operated animals, which can be considered a model of NAS withdrawal, especially in female mice. It is possible that since all animals in these experiments underwent EtOH withdrawal, they all had an elevated expression of α4 mRNA, washing out any effects of steroid deprivation. However, α4 mRNA expression was not at ceiling levels, as it was still possible for GAN administration in ADX/GDX males and females and SHAM males to further increase mRNA expression.

Receptors containing the α5 subunit exhibit the highest affinity for GABA (Sigel et al., 1990), and the expression of α5 subunits are increased in the hippocampus during chronic EtOH exposure (Charlton et al., 1997). The α5 subunits are expressed almost exclusively in the hippocampus and are thought to mediate some of learning deficits seen during intoxication (Collinson et al., 2002; Sur et al., 1999). In the current experiments, there was a sex difference in the ADX/GDX VEH-treated animals, with females having a 14-fold decrease in α5 subunit mRNA expression when compared to males. Since EtOH withdrawal is lower in female versus male mice, the decrease in α5 subunit mRNA expression in females is consistent with the idea that increased α5 subunit expression corresponds to increased withdrawal. However, when ADX/GDX males were treated with steroids, enzyme inhibitors, or both, they all experienced a suppression of α5 subunit mRNA expression that did not correspond to a change in withdrawal. Thus, it seems unlikely that α5 expression alone is conferring susceptibility to EtOH withdrawal seizures.

In contrast to most of the $GABA_A$ receptor α subunits, it has been shown that the β subunits increase in response to both chronic EtOH treatment and withdrawal (Devaud

et al., 1997; Marutha Ravindran et al., 2007; Mhatre and Ticku, 1994; Mhatre and Ticku, 1992). The current experiments examined mRNA expression for both the β1 and β2 subunits. There were no changes in mRNA expression 24 hours after acute EtOH administration in either subunit due to sex or surgery in the VEH-treated animals and minimal changes in steroid-treated animals. Overall, these results indicate that β subunit mRNA expression is not expressing changes that may be reflecting withdrawal severity in the current model utilized.

The $GABA_A$ receptor δ subunit is important for conferring sensitivity of $GABA_A$ receptors to NAS (Belelli et al., 2002), and NAS withdrawal increases the expression of the δ subunit (Sundstrom-Poromaa et al., 2002). Ethanol exposure decreases δ subunit expression on the cell surface (Liang et al., 2007), which may make cells less sensitive to NAS modulation. The $GABA_A$ receptor δ subunit is almost exclusively located in extrasynaptic $GABA_A$ receptors that are involved in tonic inhibition (Farrant and Nusser, 2005; Nusser et al., 1998; Serwanski et al., 2006). There were minimal changes in these experiments with regard to δ subunit mRNA expression, suggesting that the animals in the present study would be sensitive to manipulations in steroid levels.

The γ2 subunit is required for sensitivity to benzodiazepines (Pritchett et al., 1989), and long term exposure to EtOH causes an increase in γ2 subunit mRNA expression (Follesa et al., 2003). Additionally, the γ2 subunit is thought to be required for clustering of the $GABA_A$ receptor at the synapse, and therefore, required for phasic GABA signaling (Schweizer et al., 2003). There were minimal effects of the current pretreatments on γ2 subunit mRNA expression. One exception was in male and female ADX/GDX mice, where GAN administration significantly increased γ2 subunit mRNA

expression. These results are interesting, as withdrawal of GAN in cultured granule cells resulted in a decrease in γ2 subunit (Mascia et al., 2002), indicating that GAN may be able to bi-directionally modulate expression of γ2 subunit mRNA.

StAR protein may play an important role in the interaction of NAS and $GABA_A$ receptors, as it is thought to be the rate limiting step in steroid biosynthesis (Stocco, 2000), although 5α-reductase plays an important role as a unidirectional step toward NAS production (Melcangi et al., 1998). Results suggest that brain expression of StAR protein was maintained during ADX/GDX surgery, and that acute EtOH administration increased this expression in several brain areas, including the hippocampus (Joon Kim et al., 2003). Although the experiments in Chapter 2 indicated that peripheral sources of NAS were important during acute EtOH, we hypothesized that the pretreatments in our model that were decreasing withdrawal seizure severity could be functioning through increasing StAR protein mRNA expression, with a subsequent increase in StAR protein, local NAS production and GABAergic inhibition. It has been shown that PROG administration stimulated its own synthesis (Rothchild, 1981) and increased expression of 3α-hydroxysteroid dehydrogenase (Dimattina et al., 1986; Tanaka et al., 1993).While there has been no evidence that PROG or DOC administration can regulate the expression of StAR protein, estrogen administration increased expression of StAR protein in granulose cells (Townson et al., 1996) and StAR protein mRNA is decreased during steroid depletion in granulose cells (Chaffin et al., 2000). These results indicate that steroid administration could regulate StAR protein expression in brain; therefore, it is possible that PROG or DOC administration could be increasing StAR protein and local NAS concentrations.

The results from these experiments indicated that female animals, regardless of surgical status, had increased levels of StAR protein mRNA versus males, and that there was a very slight, albeit significant, decrease in StAR protein mRNA expression in ADX/GDX males when compared to SHAM males. In both ADX/GDX and SHAM males, there was a significant increase in StAR protein mRNA in animals that were treated with FIN. In female ADX/GDX animals, there were significant decreases in StAR protein mRNA in all treatments that did not rescue withdrawal severity to that of SHAM surgical animals, while there were no changes in SHAM females. In female animals, this fits with our hypothesis very well, indicating that replacement with NAS precursors may be serving to increase StAR protein mRNA, and thereby local NAS production and may reflect modulation during withdrawal severity.

Some tentative conclusions can be made from the results of the current experiments. First, the results suggest that expression of both the α1 subunit and StAR protein mRNA at 24 hours following EtOH administration may reflect the modulation of withdrawal following an acute 4 g/kg dose of EtOH under some circumstances. In male and female animals that had undergone steroid withdrawal (through ADX/GDX surgery), steroid replacement that restored withdrawal severity to that seen in SHAM animals (notably PROG and DOC replacement) did not significantly alter α1 mRNA levels when compared with VEH treated animals. Treatments that did not restore the withdrawal profile to SHAM levels significantly decreased α1 mRNA levels. Maintaining α1 levels during acute EtOH withdrawal may be important, as $GABA_A$ receptors that contain the α1 subunit are highly sensitive to modulation by NAS (Belelli et al., 1996). A similar pattern was seen in ADX/GDX females with regard to StAR protein mRNA expression.

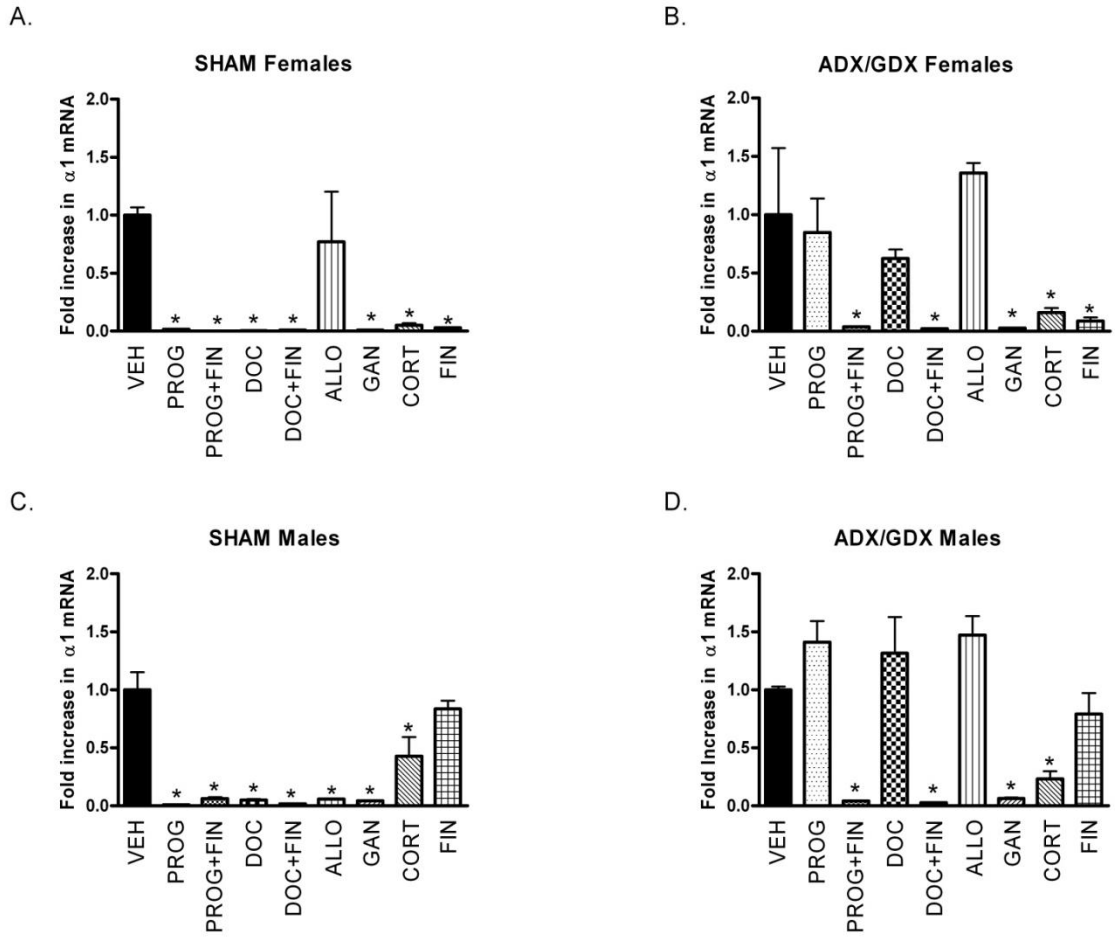
While the current experiments did not measure protein levels of the α1 subunit or StAR, these results are consistent with the idea that alterations in expression of the α1 subunit and StAR protein may be an important reflection of modulation during the rebound neural hyperexcitability seen during acute EtOH withdrawal and warrant further studies.

The data from the current experiments far from explain the behavioral results we previous observed with ADX/GDX increasing the severity of acute EtOH withdrawal and administration of PROG or DOC restoring the acute withdrawal profile. These results could be strengthened by inclusion of no EtOH control groups as well as samples at one hour after EtOH administration and at peak withdrawal. Additionally, the present results provided no insight into the interesting effect that PROG and DOC administration was pro-convulsant in SHAM males during EtOH withdrawal. These results could be investigated further through the examination of $GABA_A$ subunit expression in a different area of the brain, such as the frontal cortex, cerebellum, or other areas important to EtOH related behaviors. It is also possible that these animals may have a different phosphorylation status of their $GABA_A$ receptors, which would cause them to be insensitive to steroid modulation (Harney et al., 2003). Another possibility is that this model may be activating pre-synaptic $GABA_A$ receptors, which in some cases can cause efflux of chloride into the cell (Haage et al., 2002).

In conclusion, these present results further explore the idea that rebound neural hyperexcitability seen during the withdrawal from an acute dose of EtOH is modulated by an interaction between NAS and the $GABA_A$ receptor. Fully elucidating this interaction would be an important step in understanding how EtOH works in the normally functioning brain, and perhaps more importantly, could provide insight into how EtOH

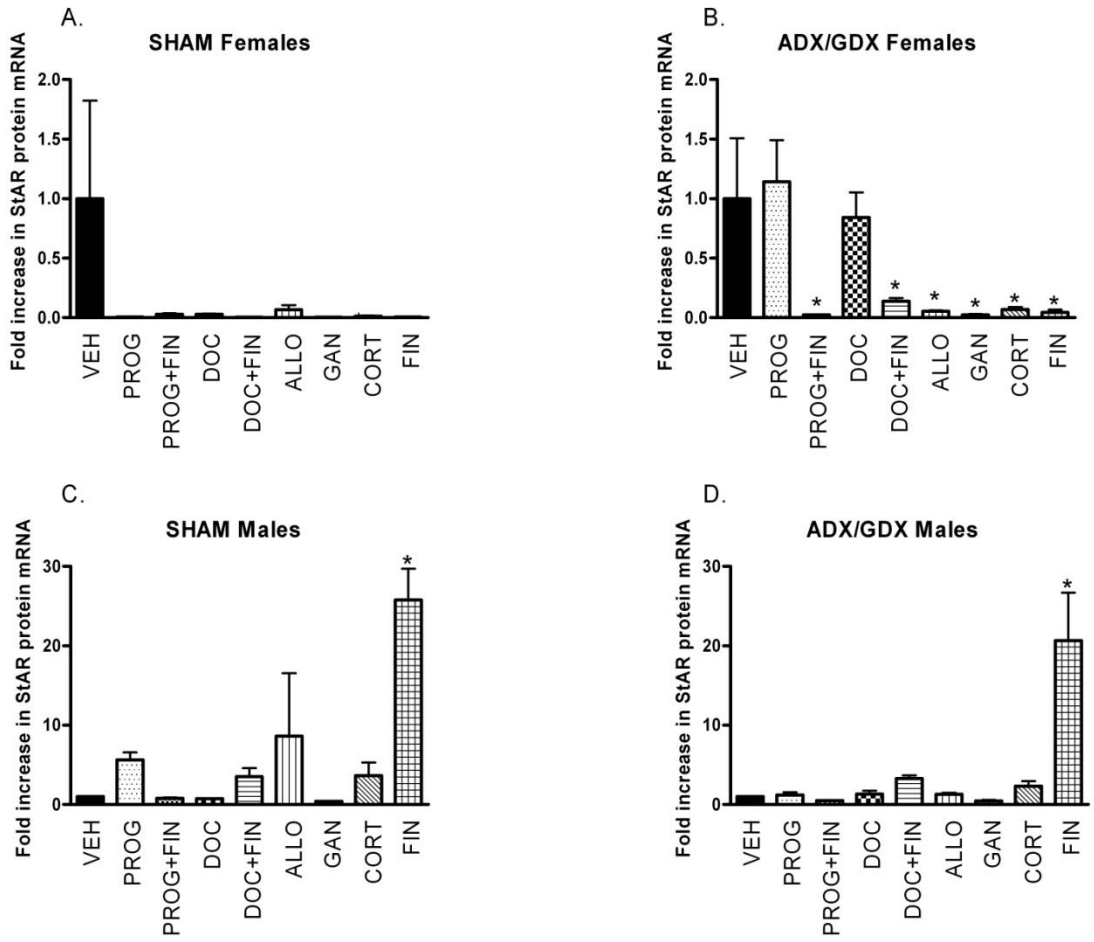
functions in the alcoholic brain. These insights could lead to future treatments for EtOH disorders.

Figure 4.1. The effects of steroid administration during acute EtOH withdrawal on expression of the $GABA_A$ receptor α1 subunit mRNA.



Male and female animals that underwent ADX/GDX surgery had decreased α1 mRNA expression in groups that were administered pretreatments that would have blocked the 5α-reduction of the pretreatment (panels B and D). Male and female animals that had undergone SHAM surgery had decreased expression in almost all groups (panels A and C). Bars represent the mean ±SEM for 2-4 animals/group. *$p<0.05$ vs. respective VEH treated group.

Figure 4.2. The effects of steroid administration during acute EtOH withdrawal on expression of StAR protein mRNA.



Female animals that underwent ADX/GDX surgery had decreased StAR protein mRNA expression in groups that were administered pretreatments that would have blocked the 5α-reduction of the pretreatment (panel B). There were no significant effects of pretreatments on StAR protein mRNA expression in SHAM females (panel A), perhaps due to high variability in the VEH group. FIN pretreatment significantly increased StAR protein mRNA in male ADX/GDX and SHAM males (panels C and D). Bars represent the mean ±SEM for 2-4 animals/group. *$p<0.05$ vs. respective VEH treated group.

Table 4.1. Gene expression changes in VEH treated mice: Effects of surgery and sex

	SHAM vs. ADX/GDX		Male vs. Female	
GENE	Male	Female	SHAM	ADX/GDX
α1	=	=	=	=
α2	=	=	=	↓20 fold
α4	=	=	=	=
α5	=	=	=	↓14.5 fold
β1	=	=	=	=
β2	=	=	=	=
γ2	=	=	=	=
δ	=	=	=	=
StAR protein	↓ 1 fold	=	↑9 fold	↑22 fold

Cells represent fold change from comparison group. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method, fold increase over control was calculated. = indicates no change, ↑ indicates a significant fold increase, and ↓ indicates a significant fold decrease.

Table 4.2. Gene expression changes in female ADX/GDX mice: Effects of steroid pretreatments versus VEH

GENE	PROG	PROG+FIN	DOC	DOC+FIN	ALLO	GAN	CORT	FIN
α1	=	↓ 24 fold	=	↓48 fold	=	↓35 fold	↓6 fold	↓11 fold
α2	=	=	=	↑34 fold	↑30 fold	=	=	=
α4	=	=	=	=	=	↑3 fold	=	=
α5	=	=	=	=	=	↑22 fold	=	=
β1	=	=	=	↑43 fold	=	=	↑37 fold	=
β2	=	=	=	=	↑2 fold	↓117 fold	=	=
γ2	=	=	=	=	=	↑2 fold	=	=
δ	=	↓4 fold	=	=	=	↓6 fold	=	↓3 fold
StAR	=	↓40 fold	=	↓9 fold	↓18 fold	↓40 fold	↓14 fold	↓21 fold

Cells represent fold change from comparison group. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method, fold increase over control was calculated. = indicates no change, ↑ indicates a significant fold increase, and ↓ indicates a significant fold decrease.

Table 4.3. Gene expression changes in female SHAM mice: Effects of steroid pretreatments versus VEH.

	PROG	PROG+FIN	DOC	DOC+FIN	ALLO	GAN	CORT	FIN
α1	↓14 fold	↓98 fold	↓40 fold	↓20 fold	=	↓20 fold	↓4 fold	↓7 fold
α2	=	=	=	=	↑35 fold	=	=	=
α4	=	=	=	=	=	=	=	=
α5	=	=	=	↑5 fold	=	↑6 fold	=	=
β1	=	=	↑7 fold	=	=	=	↑10 fold	↑6 fold
β2	=	=	=	=	↑10 fold	=	=	=
γ2	=	=	=	=	=	=	=	=
δ	=	=	=	=	=	=	=	=
StAR	=	=	=	=	=	=	=	=

Cells represent fold change from comparison group. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method, fold increase over control was calculated. = indicates no change, ↑ indicates a significant fold increase, and ↓ indicates a significant fold decrease.

Table 4.4. Gene expression changes in male ADX/GDX Mice: Effects of steroid pretreatment versus VEH

	Male ADX/GDX VEH vs. Male ADX/GDX treated							
	PROG	PROG+FIN	DOC	DOC+FIN	ALLO	GAN	CORT	FIN
α1	=	↓ 24 fold	=	↓36 fold	=	↓15 fold	↓4 fold	=
α2	=	=	=	↑2 fold	=	↓66 fold	↓63 fold	↓15 fold
α4	=	=	=	=	=	↑35 fold	=	↑13 fold
α5	↓ 3 fold	↓ 17 fold	↓ 2 fold	↓ 39 fold	↓ 2 fold	↑2 fold	↓23 fold	↓32 fold
β1	=	=	=	↑96 fold	=	=	↑148 fold	=
β2	=	=	=	=	=	↓117 fold	=	=
γ2	=	=	=	=	=	↑7 fold	=	=
δ	=	↓4 fold	=	=	=	=	↑2 fold	=
StAR	=	=	=	=	=	=	=	↑20 fold

Cells represent fold change from comparison group. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method, fold increase over control was calculated. = indicates no change, ↑ indicates a significant fold change, and ↓ indicates a significant fold decrease.

Table 4.5. Gene expression changes in male SHAM mice: Effects of steroid pretreatment versus VEH.

	PROG	PROG+FIN	DOC	DOC+FIN	ALLO	GAN	CORT	FIN
α1	↓106 fold	↓16 fold	↓19 fold	↓56 fold	↓17 fold	↓22 fold	↓2 fold	=
α2	=	=	↓57 fold	=	=	↓80 fold	↓26 fold	↓14 fold
α4	=	=	↑33 fold	=	=	↑41 fold	=	=
α5	=	=	=	=	=	↑2.5 fold	=	=
β1	=	=	=	=	=	=	↑312 fold	=
β2	=	↑2 fold	↓142 fold	=	=	↓150 fold	=	=
γ2	=	=	=	=	=	=	=	=
δ	=	=	=	=	=	=	=	=
StAR	=	=	=	=	=	=	=	↑25 fold

Cells represent fold change from comparison group. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method, fold increase over control was calculated. = indicates no change, ↑ indicates a significant fold change, and ↓ indicates a significant fold decrease.

CHAPTER 5: GENERAL DISCUSSION

The overall goal of this dissertation was to explore the contribution of endogenous GABAergic NAS to the expression of the acute EtOH withdrawal profile in male and female mice. In brief, the first set of experiments showed that an anticonvulsant NAS was important during acute EtOH withdrawal, as removal of the peripheral sources of NAS (through ADX/GDX surgery) resulted in an increase in withdrawal severity as measured by HICs. These results supported the hypothesis that the anticonvulsant 5α-reduced neurosteroids are protective during neuronal rebound hyperexcitability. The second set of experiments further explored this effect and documented the importance of the 5α-reduced metabolites of DOC and PROG, as replacement with DOC and PROG restored withdrawal severity in ADX/GDX animals to that seen in SHAM animals, an effect that was ameliorated when FIN was co-administered. These findings provided further support for the hypothesis that the 5α-reduced NAS are important during acute EtOH withdrawal. The final set of experiments revealed that expression levels of the α1 subunit of the $GABA_A$ receptor and expression of StAR protein may be an important reflection of the modulation in the behavioral effects seen in the previous experiments.

A Role for Neuroactive Steroids during EtOH Withdrawal

The first set of experiments (presented in Chapter 2) established that peripherally produced NAS were important in modulating the neural rebound hyperexcitability seen during acute EtOH withdrawal. The experiments were specifically designed so that the importance of either pro or anticonvulsant steroids could be detected and so that a clue toward which steroid was important could be garnered. The results indicated that an

anticonvulsant steroid was important, since removing the peripheral sources of NAS increased acute EtOH withdrawal severity in D2 males and females and B6 males. In order to increase acute withdrawal severity in D2 females, both the adrenals and the ovaries had to be removed, while in males only removing the adrenals was effective at increasing withdrawal; there was no apparent effect of removing the testis. These results are consistent with the idea that a steroid that is synthesized in both the adrenal and ovary contributes to the acute EtOH withdrawal profile in intact animals. In addition to agreeing with previous results indicating that peripherally produced NAS were important in modulating several EtOH intoxication-related behaviors (Hirani et al., 2002; Hirani et al., 2005; Khisti et al., 2003; VanDoren et al., 2000), the present findings extended those results by indicating that the NAS also were important in modulating EtOH withdrawal-related convulsive behaviors.

The results from the first experiment suggested that trying to further elucidate the step or steps along the NAS biosynthetic pathway that were modulating acute EtOH withdrawal severity was a feasible undertaking. Specifically, the first experiment provided insight into which NAS pathways seemed wise to investigate. As the experiment revealed that an anticonvulsant NAS with a primary origin of the ovaries and the adrenals (but not the testis) was modulating acute withdrawal severity, we were left with 2 obvious choices: the 5α-reduced NAS derivatives of PROG and DOC. It also was possible that the 5α-reduced metabolite of dihydroepiandrosterone (DHEA), androsterone could be involved, as DHEA is produced both in the adrenal gland and *de novo*. However, the testicles are also a significant source of androsterone as androstanediol (a testosterone derivative) that can be metabolized into androsterone via 17β-HSD (see

Figure 1.1). While we had not ruled out androsterone, especially because of its apparent anticonvulsant activity (Rafal et al., 2005), the lack of an effect of GDX in male mice suggested that the subsequent investigation should focus on the PROG and DOC metabolites.

The purpose of the second set experiments (presented in Chapter 3) was to identify specific steps along the NAS biosynthetic pathway that were necessary or sufficient to modulate the neuronal rebound hyperexcitability following a high dose of EtOH. These experiments confirmed my hypothesis that replacing ADX/GDX operated mice with DOC or PROG during EtOH withdrawal would restore withdrawal severity back down to levels seen in SHAM mice, and that preventing the 5α-reduction of these steroids would abrogate this effect. The results in these experiments did not rule out the possibility that other NAS could also modulate these behaviors, but only that these substances were sufficient. While co-administering FIN to the ADX/GDX animals abolished the effects of DOC and PROG, administration of ALLO or GAN did not replicate the effects of DOC and PROG in the ADX/GDX animals. While speculative, I hypothesize that this was due to timing of the administration and in the case of ALLO, rapid metabolism.

It was interesting that in male SHAM operated mice, PROG and DOC administration actually increased withdrawal severity, an unexpected finding. PROG and DOC have previously been shown to be anticonvulsant in intact male animals, although in different models (Barbaccia et al., 1999; Belelli et al., 1989; Frye et al., 2002; Lonsdale and Burnham, 2003; Perez-Cruz et al., 2007; Reddy and Rogawski, 2002). It has also been shown that there can be a withdrawal profile when very high (supra-

physiological; 75 mg/kg) doses of ALLO are administered in male Withdrawal Seizure-Prone mice (Reilly et al., 2000). However, it is unlikely that doses of PROG and DOC that were administered to SHAM male mice in the current experiment were high enough to achieve withdrawal from the steroid treatments themselves.

Although highly speculative, it is possible that administration of PROG and DOC in male intact animals (which would already have circulating levels of these hormones) could have saturated the 5α-reductase enzyme, which would favor biosynthesis toward the production of CORT (see Figure 1.1) rather than the reduction of PROG or DOC. While there have been no studies to date showing sex differences in the brain levels of 5α-reductase, male rats and mice have significantly lower levels of circulating ALLO when compared to female rodents (Finn et al., 2004b; Janis et al., 1998; Paul and Purdy, 1992). Additional research has shown that increases in 5α-reductase mRNA corresponded to increases in 5α-reductase activity at times during the estrus cycle when ALLO levels were high in female rats (Holzbauer, 1975; Ichikawa et al., 1974; Lephart et al., 1990; Lephart et al., 1992; Paul and Purdy, 1992). These data could suggest that lower ALLO levels would correspond to lower levels of 5α-reductase. If this were the case, males may have lower levels of 5α-reductase, and the doses of PROG and DOC that were administered to intact animals may have saturated the enzyme. Thus, the saturation of the 5α-reductase enzyme could cause the PROG and DOC to be metabolized into CORT, which as discussed in Chapter 1, is proconvulsant (Karst et al., 1999; Roberts et al., 1994; Roberts and Keith, 1995).

The Role of $GABA_A$ Receptor Subunits in Acute EtOH Withdrawal

The purpose of the experiments presented in Chapter 4 was to see if the expression of specific $GABA_A$ receptor subunit mRNA and of StAR protein mRNA changed in a manner that corresponded to the behavioral results in seen in Chapters 2 and 3. As previously discussed in Chapter 1 and 4, the subunit composition of the $GABA_A$ receptor can affect the receptor's sensitivity to EtOH, NAS and whether it is located in the synapse or extra-synaptically. I hypothesized that $GABA_A$ subunit conformation in animals that had undergone ADX/GDX surgery and had steroid pretreatments that restored their behavior to intact levels would be similar to the $GABA_A$ subunit conformation in SHAM operated animals.

The results described in Chapter 4 show that this hypothesis may be partially true. Expression of the $GABA_A$ receptor α1 subunit mRNA in ADX/GDX males and females was decreased in groups that had received pretreatments that did *not* restore their withdrawal profile to that of intact (SHAM) animals. ADX/GDX animals that had received pretreatments that did restore their withdrawal profile had $GABA_A$ receptor α1 subunit mRNA levels that were similar to VEH treated ADX/GDX animals. Decreases in the expression of the $GABA_A$ receptor α1 subunit could represent a shift toward tonic GABergic actions, as the α1 subunit is important in phasic inhibition (Farrant and Nusser, 2005; Mody, 2001). While the results in $GABA_A$ receptor α1 subunit mRNA expression nicely follow the pattern of behavior seen in Chapters 2 and 3, it is puzzling that the ADX/GDX animals administered PROG and DOC were not different from the ADX/GDX VEH treated groups. The results in $GABA_A$ receptor α1 subunit mRNA were similar to the pattern of results seen in ADX/GDX female mice with respect to expression of StAR protein mRNA. Again, these results could have physiological relevance, as a

decrease in StAR protein mRNA should correspond to a decrease in the *de novo* production of NAS. Lowered NAS production could decrease endogenous GABAergic inhibitory tone, with a resultant increase in seizure susceptibility. Overall, it seemed that these results provided insight into why certain treatments may not have been effective, but did not provide much insight into why certain treatments did work.

It is important to note that these experiments were carried out in only hippocampus tissue from each animal. While this area was chosen for a variety of reasons detailed in Chapter one, it is important to point out that the hippocampus may be a unique brain area during EtOH exposure and these results may not generalize to other areas. Every other area in the brain tested to date shows c-fos activation in response to EtOH, while the hippocampus shows overall reduction (Vilpoux et al., 2009). An additional caveat to these experiments would be to keep in mind that the methods used in these experiments resulted in many different cells types being included in the analysis. More specific results may have been found if individual cell types were analyzed through cell culture or flow cytometry. For example, astrocytes are a major source of NAS synthesis in the brain (Akwa et al., 1993) and it may be that regulation of StAR protein specifically in this cell type is important during acute withdrawal.

Future Directions

The future directions that could be explored from this project are numerous. In reference to the data presented in Chapters 2 and 3, there are several directions in which

this project could be taken in order to fully elucidate the contributions of NAS during acute EtOH withdrawal.

In the steroid replacement studies described in Chapter 3, a single administration of NAS or NAS precursors was chosen in an attempt to model the discrete increase in NAS levels seen after administration of EtOH (or to discretely block this increase). However, it could be that basal levels of NAS, in addition to the increase seen after EtOH, might also be important during acute EtOH withdrawal. It would be interesting to alter the dosing regimen used in this model. Osmotic minipumps could be used to provide continual release of the steroids prior to acute withdrawal and then an acute dose of steroids could be administered to mimic the increase seen in response to an acute dose of EtOH. These experiments could provide insight to the contributions of NAS basal tone to the acute EtOH withdrawal profile.

Second, it would be worthwhile to investigate the contributions of the 5α-reduced metabolite of DHEA. This NAS has been less studied than other NAS, although it has been shown to have anticonvulsant properties (Rafal et al., 2005). Additionally, it would be interesting to elucidate the effects of combined NAS treatments in ADX/GDX mice. Would administering both PROG and DOC be additive or synergistic on acute withdrawal severity? These results could provide additional insights into the contributions of these hormones during withdrawal in intact animals, as it is unlikely that the effects seen are due to just one NAS, but more likely a concert of effects working together. Additionally, it would be worthwhile to work out the timing issues for ALLO administration in this paradigm in order to show conclusively that this 5α-reduced NAS can modulate acute EtOH withdrawal severity in ADX/GDX animals. It may be that

through a different administration paradigm, such as timed release via osmotic minipumps, that ALLO administration could effectively restore the withdrawal profile in ADX/GDX mice back down to that seen in SHAM animals.

The data presented in Chapter 4 indicate that mRNA expression of $GABA_A$ subunits and StAR protein does not fully explain the differences in behavior seen in Chapters 2 and 3. However, I think that redesigning this experiment could yield more valuable information on the contribution of specific $GABA_A$ receptor subunits during acute EtOH withdrawal. The experiments presented in Chapter 4 represent data from animals at 24 hours after withdrawal, a time point when differences in behavior have dissipated. I think that in order to more fully elucidate the contributions of $GABA_A$ receptor subunit composition, measurements of $GABA_A$ receptor subunits should be quantified before EtOH and steroid pretreatment and again at peak withdrawal when behavior is the most different between the groups. This information would yield results that could more conclusively show the contributions of $GABA_A$ receptor subunit expression in the hippocampus during acute EtOH withdrawal and allow for comparisons with EtOH naïve animals. Additionally, these experiments could be replicated in additional brain regions, as the hippocampus is only one brain region that has been shown to be important for withdrawal from EtOH or NAS. The cortex could be an important region to investigate, since acute EtOH administration increases levels of ALLO in the frontal cortex (Barbaccia et al., 1999; O'Dell et al., 2004), chronic EtOH exposure decreases expression of $GABA_A$ receptor subunit α1 while increasing α4 in the cortex (Grobin et al., 2000), and human alcoholics have been found to have decreased expression of the $GABA_A$ receptor α1 subunit in frontal cortex (Lewohl et al., 1997). It

also would be important to determine whether any increases or decreases in mRNA levels corresponded to changes in protein.

I think the most worthwhile future experiments would be those further elucidating the underlying mechanisms involved in NAS modulation of acute EtOH withdrawal. There are many ways in which $GABA_A$ receptors can be modulated besides the expression of subunits. $GABA_A$ receptors have phosphorylation sites that can be phosphorylated through the actions of protein kinase C (PKC) and protein kinase A (PKA) (Brandon et al., 2000; McDonald and Moss, 1997), and the phosphorylation status can modify GABA binding at the receptor (Oh et al., 1999). Acute EtOH administration can alter the phosphorylation status of $GABA_A$ receptors, which can alter the receptor function without altering receptor expression (Kumar et al., 2006). It would be interesting to measure the phosphorylation status of $GABA_A$ receptors in our model. Western blots for the phosphorylated and unphosphorylated state of the receptors could show that while $GABA_A$ receptor subunit composition is not necessarily changing, the receptor is being modified nonetheless. The phosphorylation status of $GABA_A$ receptors during acute EtOH withdrawal has not been investigated, and this data would yield important information about $GABA_A$ receptor function during withdrawal.

Another interesting aspect that could be investigated is that acute EtOH withdrawal could be affecting presynaptic GABA release. Exposure to EtOH enhances presynaptic GABA release from cells in the hippocampus (Ariwodola and Weiner, 2004), among other areas (Criswell and Breese, 2005; Roberto et al., 2003; Roberto et al., 2004). Through electrophysiological techniques, the contribution of presynaptic release could be elucidated during acute EtOH withdrawal. Research in this area has focused on EtOH

administration and very little attention had been paid to levels of GABA release during EtOH withdrawal, a mechanism by which endogenous GABAergic inhibitory tone could be altered.

A final direction that cannot be ignored is that some of the results seen in this dissertation could be a function of EtOH or NAS modulating EtOH withdrawal severity through another neurotransmitter system, such as NMDA. As discussed in Chapter one, NAS can both positively and negatively modulate the NMDA receptor (Irwin et al., 1994; Park-Chung et al., 1994; Wu et al., 1991), and EtOH administration decreases glutamate release [and subsequent action at NMDA receptors (Diamond and Gordon, 1997)]. Additionally, glutamate release is dramatically increased during withdrawal from chronic EtOH exposure, a circumstance that would increase seizure susceptibility (Rossetti and Carboni, 1995). As results in Chapter 2 indicated that ADX/GDX removed an anticonvulsant substance that was important during withdrawal, it would be interesting to see what effect replacing ADX/GDX animals with the negative NMDA modulatory NAS pregnanolone sulfate and epipregnanalone sulfate would have.

Overall Implications

In this dissertation, I provide evidence for the critical involvement of NAS during the neural rebound hyperexcitability seen during acute EtOH withdrawal. These NAS were protective as their removal increased withdrawal-induced convulsive behavior and their replacement restored withdrawal severity to levels in intact animals. These results are important in light of data that has been found during withdrawal from chronic EtOH exposure. During withdrawal from chronic EtOH, NAS production is decreased and

$GABA_A$ receptor sensitivity to modulation by NAS is also decreased in seizure-prone genotypes (Beckley et al., 2008; Finn et al., 2004b; Morrow et al., 2001). Taken together, this may indicate that decreased NAS production and sensitivity to NAS action during withdrawal may put individuals at risk for EtOH withdrawal seizures. Since one of the major factors in alcoholics continuing to drink is withdrawal avoidance, this could be an important finding. Restoring both levels and sensitivity to NAS could be a viable treatment option. However, it is important to note that the experiments in this dissertation were carried out in a model of acute EtOH withdrawal. While some research has shown that changes seen during acute EtOH withdrawal are mirrored during chronic EtOH withdrawal (Liang et al., 2007), it may be that the effects seen in these experiments do not relate to withdrawal from chronic EtOH. However, it is important to understand the etiology of withdrawal in an acute model, so that the dysregulation seen during chronic withdrawal can be understood.

In addition to the potential contribution of lower levels of, and reduced sensitivity to, GABAergic NAS with regard to increasing withdrawal-related convulsive behavior, it is possible that a reduction in GABAergic NAS also could reduce sensitivity to EtOH's sedative, anxiolytic and antidepressant effects (as was shown with the use of FIN to decrease ALLO levels; Hirani et al., 2002; 2005; Khisti et al., 2003; VanDoren et al., 2000). In human studies individuals with reduced sensitivity to EtOH's ataxic effect resulted in increased administration and increased risk for the development of alcoholism (Schuckit, 1994; Schuckit and Smith, 1996). Fully elucidating the contributions of NAS levels and sensitivity to NAS in EtOH addiction will further the search for viable treatments options for this disease.

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