# Mechanisms of Ethanol-Induced Steroidogenesis Following Acute and Chronic Ethanol Exposure

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Abstract
Kevin N Boyd: Mechanisms of Ethanol-Induced Steroidogenesis Following Acute and Chronic Ethanol Exposure
"Under the direction of Dr. A. Leslie Morrow, Ph.D."

The 3α,5α-reduced pregnan steroids, including (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC), are potent allosteric modulators of γ-aminobutyric acid type A ($GABA_A$) receptor activity. These neuroactive steroid levels are increased by acute ethanol administration at doses ≥ 1.5 g/kg in rats and mediate specific actions of ethanol in rodents and subjective effects of ethanol in humans. Acetaldehyde, a metabolite of ethanol, may also play a role in some of ethanol's actions. The first aim of this project examined if acetaldehyde plays a role in ethanol-induced increases in neuroactive steroids. We found that acetaldehyde is capable of increasing neuroactive steroid levels at high doses but does not seem to have effects when administered at doses observed after acute ethanol administration (2 g/kg). Manipulation of various ethanol and acetaldehyde metabolizing enzymes to alter acetaldehyde concentrations confirmed that the increases in neuroactive steroids observed after ethanol administration are not directly resulting from acetaldehyde. I also examined mechanisms by which acute ethanol administration elicits increases in neuroactive steroids. Focusing on key enzymes and signaling molecules involved in the steroid biosynthetic pathway, I found that pituitary adrenocorticotrophic hormone (ACTH) release and *de novo* adrenal steroidogenic acute regulatory (StAR) protein synthesis are each necessary, but not sufficient for ethanol-induced steroidogenesis. Furthermore, phosphorylation of StAR is markedly increased by acute ethanol administration and may be involved in StAR activity. Interestingly, tolerance to

ethanol-induced increases in neuroactive steroids occurs following chronic ethanol exposure. Thus, I investigated the biosynthetic enzymes and signaling molecules found to be important for ethanol-induced steroidogenesis to see if any were altered by chronic ethanol exposure. Indeed, chronic ethanol exposure elicited tolerance to ethanol-induced ACTH as well as plasma and brain steroids. StAR remained elevated following chronic ethanol exposure; however phosphorylation of StAR was no longer observed. ACTH replacement restored the neuroactive steroid response and enhanced phosphorylation of StAR protein following chronic ethanol exposure. Thus, the dysregulation of ethanol-induced ACTH release may lead to tolerance to ethanol-induced increases in neuroactive steroid levels and contribute to behavioral tolerance to ethanol. These mechanisms may provide a better understanding of ethanol sensitivity and factors that influence the progression towards alcoholism.

I would like to dedicate this dissertation to my family, for helping me to achieve everything I have to this point, and to all my friends that have made it an enjoyable experience along the way.

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Preface:

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3α,5α-THDOC…….. (3α,5α)-3,21-dihydroxypregnan-20-one

3α,5α-THP……. (3α,5α)-3-hydroxypregnan-20-one

ACTH…….. Adrenocorticotropic Hormone

ADH…… Alcohol dehydrogenase

ALDH……. Aldehyde dehydrogenase

AG…….. Aminoglutethimide

CHX……... Cycloheximide

CRF……..… Corticotropin Releasing Factor

EDTA……. Ethylenediaminetetraacetic Acid

HPA……..……. Hypothalamic-pituitary-adrenal axis

MLN64…….. Metastatic Lymph Node 64 Protein

P450scc…… Cytochrome P450 side chain cleavage

PBR……..…….. Peripheral Benzodiazepine Receptor

PBS……..…. Phosphate Buffered Saline

PKA……..…….. Protein Kinase A

PKC……..… Protein Kinase C

Preg……..…… Pregnenolone

Prog……..…… Progesterone

RIA……...…… Radioimmunoassay

RIPA……..…….. Radioimmunoprecipitation Buffer

SDS……. Sodium Dodecyl Sulfate

SDS-PAGE……..…….. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

StAR……..…….. Steroidogenic Acute Regulatory Protein

# Chapter I

# Introduction

## Significance of Alcoholism

Alcohol use has been prevalent throughout history and is a major health issue costing hundreds of billions of dollars each year. Alcohol is the most widely used psychoactive drug in the United States, yet only within the last 20-30 years have we made significant strides towards understanding the genetic and neurobiological changes associated with alcoholism. A study in 2002 by the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) found that roughly 18 million (8.5%) Americans either abuse alcohol or are alcohol dependent. Alcohol abuse is defined by one's drinking causing social and/or legal problems as well as interfering with their ability to fulfill obligations at work and home. Alcohol dependence, or alcoholism, defines a case where an individual cannot control their drinking, demonstrates a compulsion to drink, and elicits tolerance to alcohol as well as withdrawal symptoms. These conditions cannot be explained by any one particular factor and are likely the result of a combination of genetic and environmental influences. Interestingly, in the decade from 1992 to 2002, the rate of alcohol dependence declined while the rate of alcohol abuse increased to a greater extent (Grant et al., 2004).

Underage drinking is a major problem in the United States and is important to address because the rates of alcohol abuse are highest in 18-29 year olds (Grant et al., 2004). If an individual begins drinking at an earlier age they are much more likely to develop alcohol-related problems (Dawson et al., 2008). Furthermore, adolescents are less

sensitive than adults to the sedative and intoxicating effects of ethanol (Varlinskaya and Spear, 2006) increasing the likelihood of increased drinking. Not only does alcohol have differential effects in adolescents compared to adults, but differences exist between both men and women and between various populations and ethnicities. For example, men have higher rates of dependence than women (Grant et al., 2004), and Oriental populations have polymorphisms in ethanol metabolizing enzymes that protect against the development of alcoholism (Thomasson et al., 1991).

Until recently, alcoholism was viewed as a social problem rather than a disease. Recognition of alcoholism as a disease has helped in guiding efforts to develop effective treatments, but this remains a challenge without a clear understanding of the mechanisms of alcohol dependence. These mechanisms are difficult to determine because ethanol affects numerous cellular functions and neurotransmitter pathways. Currently, there are only three approved medications for alcohol dependence; disulfiram, naltrexone, and acamprosate. These medications, however, are not effective in most individuals, and many alcoholics never even seek treatment or utilize medication for alcohol use disorders. Thus, there is a clear need for more effective treatments for alcohol dependence, and research into the mechanisms of dependence are beginning to uncover potential targets.

**Ethanol Toxicity**

Ethanol is one of the most organ-toxic drugs as it can affect just about every part of the body. At the same time, it is also one of the least potent as it requires millimolar concentrations to exert its effects. One of the primary targets of ethanol toxicity is the liver where ethanol is directly toxic through its metabolism and production of reactive oxygen species and indirectly toxic through such mechanisms as cytochrome P450 2E1 (CYP2E1) induction (Lieber and DeCarli, 1970). Ethanol is first metabolized to acetaldehyde by alcohol dehydrogenase (ADH) isoforms. Acetaldehyde is extremely toxic, and alcoholics have blood levels of acetaldehyde that may contribute to ethanol toxicity (Korsten et al., 1975).

Acetaldehyde is then subsequently metabolized to acetate by aldehyde dehydrogenase (ALDH) isoforms and acetate is broken down to carbon dioxide and water for elimination. Ethanol may also be metabolized by CYP2E1, a common drug metabolizing enzyme whose activity is increased by chronic ethanol exposure. Not only does CYP2E1 metabolize many chemicals to reactive toxic intermediates, but chronic ethanol alterations of CYP2E1 can increase sensitivity to certain drugs as well as causing resistance to others in the absence of ethanol. Furthermore, ethanol metabolism by CYP2E1 significantly increases free radicals, consequently depleting glutathione levels and contributing to oxidative stress and tissue damage (Lieber, 2004).

In the brain, ethanol-induced neurotoxicity causes neurodegeneration and damages brain structures that regulate behavioral control leading to further ethanol consumption (Crews et al., 2004). Interestingly, data suggests that ethanol-induced damage occurs during intoxication, perhaps due to reactive oxygen species generated from ethanol metabolism, and/or from acetaldehyde toxicity. This suggests that glutamate excitotoxicity is not the mechanism of ethanol-induced brain damage (Crews and Nixon, 2009). An important factor in ethanol-induced neurotoxicity, however, is the stimulation of proinflammatory cytokines and oxidative stress (Collins et al., 1998). Indeed, inflammatory enzymes alter transcription factors such as CREB, and reduced CREB transcription contributes to ethanol neurotoxicity (Bison and Crews, 2003; Crews and Nixon, 2009). Furthermore, there are gene and age-related contributions to ethanol-induced neurodegeneration. For example, the alcohol-preferring P rat is genetically bred for alcohol preference and shows more ethanol-induced neurodegeneration than non-alcohol-preferring NP rats (Bowden et al., 2001). In humans, female alcoholics are more sensitive than their male counterparts to the damaging effects of ethanol, including ethanol-induced cirrhosis (Loft et al., 1987), cardiomyopathy (Fernandez-Sola et al., 1997), and neurotoxicity (Hommer et al., 2001). In addition, adolescent rats exhibited more damage than adult rats in

anterior portions of piriform and perirhinal cortices (Bowden et al., 2001). Thus, while adolescents may be less sensitive than adults to the intoxicating effects of ethanol, their developing brains are more susceptible to toxic insults and increased neurodegeneration. This may contribute to the progression to alcoholism.

**Ethanol Effects on Central Nervous System (CNS) Function**

Ethanol has a vast array of effects throughout the body including effects on the cardiovascular system, immune system, liver and CNS. The effects of ethanol on the brain are incredibly diverse and rely upon many factors and pre-existing co-morbid conditions. Ethanol can stimulate the release of several neurotransmitters including serotonin and dopamine that may contribute to the positive feelings and cravings to drink. Although ethanol affects multiple aspects of CNS function, a major site of acute ethanol action in the brain is at ion channels (see Crews et al., 1996, for review), specifically those of the glutamatergic and GABAergic systems. These ionotropic receptors are important for ethanol actions because they are able to elicit rapid changes in CNS activity. Glutamate is the major excitatory neurotransmitter and ethanol inhibits NMDA-glutamate receptor ion channels affecting functions such as memory. GABA is the major inhibitory neurotransmitter, and ethanol potentiates GABAergic transmission eliciting effects such as anxiolysis and sedation. Furthermore, the subunit combinations that make up these ion channel receptors play a role in determining sensitivity to ethanol.

**$GABA_A$ Receptors**

Various subtypes of $GABA_A$ receptors exist throughout the brain and compounds that potentiate $GABA_A$ function increase neuronal inhibition. There are close to twenty different GABA subunits and different receptor subunit compositions exist in different areas of the brain (Sieghart and Sperk, 2002). Various combinations of these subunits combine to make a heteropentameric receptor forming a chloride-gated ion channel. Activation of these receptors decreases cell excitability by opening the ion channel allowing negatively charged

chloride ions to flow into the cell. $GABA_A$ receptors are transmembrane receptors with the extracellular protein region responsible for GABA binding while the intracellular regions have phosphorylation sites allowing for kinases to regulate receptor function (Song and Messing, 2005). $GABA_A$ receptors are also the target of several clinically relevant drugs such as benzodiazepines, barbiturates, general anesthetics, and neuroactive steroids. These different classes of drugs have different binding sites on $GABA_A$ receptors, but share the general function of increasing chloride conductance. Thus, these compounds are effective as sedative/hypnotics and anxiolytics because they enhance $GABA_A$ mediated neuronal inhibition. Not surprisingly, alterations in $GABA_A$ receptor neurotransmission are implicated in many neuropsychiatric disorders.

**Ethanol Effects on $GABA_A$ Receptors**

Ethanol is known to interact with $GABA_A$ receptor function. $GABA_A$ receptor agonists enhance actions of ethanol's while antagonists diminish the ethanol response (Lister and Linnoila, 1991). Since $GABA_A$ receptors are present throughout the brain these effects would be presumed to be widespread. Recent evidence, however, has suggested that these effects are quite selective, as $GABA_A$ receptor responsiveness to ethanol is dependent upon receptor subunit composition. $GABA_A$ receptors composed of $\alpha 1$, $\beta$, and $\gamma$ subunits are benzodiazepine sensitive and are the most abundant receptors in the brain (Barnard et al., 1998). The $\alpha 1$ containing receptors are synaptic receptors mediating phasic inhibition and have low sensitivity to ethanol (Wallner et al., 2003; Wallner et al., 2006). In contrast, extrasynaptic or perisynaptic receptors mediate tonic inhibition, exhibit higher sensitivity to GABA than synaptic $GABA_A$ receptors, and respond to ethanol at pharmacologically relevant concentrations. Indeed, ethanol potentiates these receptors at concentrations of about 1-30 mM (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). These receptors have a more localized distribution in the brain and are composed of $\alpha 4$ or $\alpha 6$ subunits, $\beta$ subunits, and a

δ subunit, which replaces the γ2 subunit found on synaptic receptors (see Olsen et al., 2007, for review). Thus, extrasynaptic receptors appear to be important for mediating effects of low doses of ethanol.

$GABA_A$ receptors have been shown to mediate some of the effects of ethanol, but there is still not clear evidence as to whether or not this is a direct effect (Kumar et al., 2009). Studies with δ subunit containing extrasynaptic $GABA_A$ receptors have provided some evidence of direct ethanol binding in recombinant receptors, but this area of study is controversial (see Santhakumar et al., 2007, for review). Furthermore, there is not evidence of ethanol directly binding to synaptic $GABA_A$ receptors suggesting that some of ethanol's effects via $GABA_A$ receptors are mediated by other factors. Indeed, ethanol may alter kinases such as PKA and PKC (Kumar et al., 2006) to reduce $GABA_A$ receptor phosphorylation and enhance GABA-mediated Cl- flux (Kumar et al., 2005) or alter GABA binding to the receptor (Oh et al., 1999). Ethanol has also been shown to enhance presynaptic GABA release (Ariwodola and Weiner, 2004; Criswell and Breese, 2005; Roberto et al., 2003) that may contribute to behavioral effects of ethanol, and ethanol can increase the GABA agonist taurine (De Witte et al., 1994), which has been shown to enhance tonic inhibition (Jia et al., 2008). Furthermore, ethanol elevates neuroactive steroid levels (Barbaccia et al., 1999; Boyd et al., 2008; Gabriel et al., 2004; Khisti, 2005; Korneyev et al., 1993; Morrow et al., 1999; Morrow et al., 1998; O'Dell et al., 2004; Serra et al., 2003; VanDoren et al., 2000a) and these endogenous compounds are potent allosteric modulators of $GABA_A$ receptor activity capable of acting on multiple $GABA_A$ receptor subtypes (Herd et al., 2007). Moreover, these neuroactive steroids have similar pharmacological effects as ethanol.

**Neuroactive Steroids**

The discovery of neurosteroids is rather recent and denotes steroids that are synthesized *de novo* in the nervous system (Baulieu, 1981). Since steroids can also be produced peripherally, a more general term is neuroactive steroids, referring to those that have actions in the CNS regardless of their origin. Neuroactive steroids are synthesized to physiologically significant levels in rodents, monkeys, and humans (for review Morrow et al., 2006) and rapidly alter neuronal excitability through interactions with neurotransmitter membrane receptors rather than acting at nuclear receptors to affect gene expression. Specifically, these neuroactive steroids have been noted for their potency at $GABA_A$ receptors in the brain (for review Belelli and Lambert, 2005). The fact that these steroids can be synthesized both peripherally and centrally means that they can act via endocrine, paracrine, or even autocrine mechanisms.

Twenty years ago neuroactive steroids were shown to have multiple binding sites on $GABA_A$ receptors (Morrow et al., 1990). Recently, specific neuroactive steroid binding sites have been identified on the $\alpha$ subunit. One site on the $\alpha$ subunit mediates the potentiating effects of certain steroids, whereas another site at the interface of the $\alpha$ and $\beta$ subunits is affects direct gating of $GABA_A$ receptors by neuroactive steroids (Hosie et al., 2006). Indeed, high concentrations of neuroactive steroids can directly gate the $GABA_A$ channel in the absence of GABA (Ueno et al., 1997). Although the action of certain $GABA_A$ receptor agonists (e.g. benzodiazepines and some anesthetics) is highly dependent upon receptor subunit composition, the action of GABAergic neuroactive steroids is more promiscuous. Neuroactive steroids affect both $\delta$ subunit containing extrasynaptic receptors (Brown et al., 2002; Mihalek et al., 1999) and $\gamma 2$ containing synaptic receptors (Harrison et al., 1987; Majewska et al., 1986), possibly because their binding site involves $\alpha$ subunits and virtually all $GABA_A$ receptors contain this subunit type.

The most potent endogenous GABAergic neuroactive steroids are the 3α,5α–reduced metabolites of progesterone and deoxycorticosterone: (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC). The 3α reduction is vital for GABAergic activity whereas the C5 reduction can be either the α or β confirmation without much effect on activity. In regards to $GABA_A$ receptor potentiation, these 3α,5α-reduced endogenous neuroactive steroids are more efficacious than benzodiazepines (Wafford et al., 1993) and much more potent than barbiturates (Harrison and Simmonds, 1984).

**Synthesis of Neuroactive Steroids**

All steroids are derived from cholesterol that is metabolized to bile acids in the liver and steroid hormones in multiple organs. Steroids are classified into mineralocorticoids, glucocorticoids, and the androgenic and estrogenic sex hormones. All steroid synthesis, however, begins with cholesterol's conversion to pregnenolone by the cytochrome P450 side chain cleavage (P450scc) enzyme. The pathway of steroid synthesis is similar in all steroid producing tissues with differences in products dependent upon the enzymes that are expressed. For example, Leydig cells in the testis, and theca and granulosa cells in the ovary, primarily produce testosterone, estrogen, and progesterone respectively because they lack most of the enzymes necessary to synthesize mineralocorticoids and glucocorticoids. These latter two groups of steroids, along with neuroactive steroids, are primarily synthesized in the adrenal.

The adrenal gland is comprised of two distinct regions each responsible for different functions. The majority of the adrenal is the cortex and this region surrounds the adrenal medulla. The adrenal medulla receives direct innervations from the central nervous system and is involved with catecholamine synthesis. Conversely, the adrenal cortex relies on neuroendocrine signals from the pituitary and hypothalamus to regulate steroid biosynthesis.

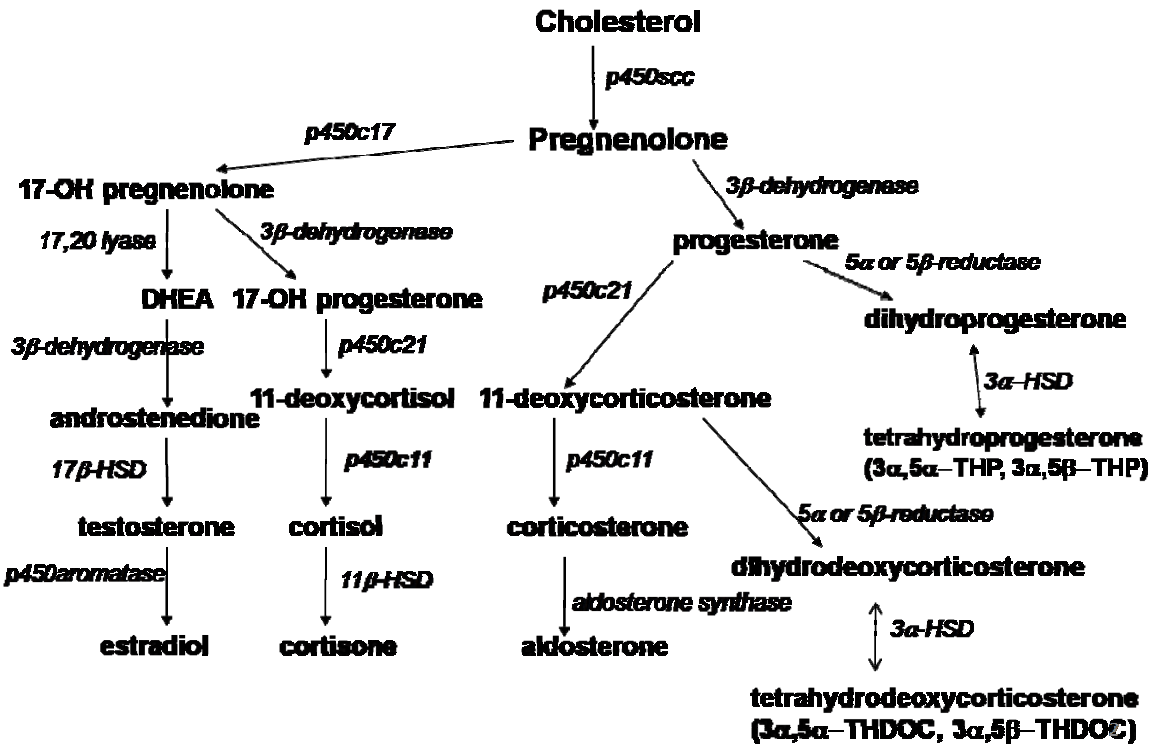


**Fig 1.1:** The steroid biosynthetic pathway and the associated enzymes. Cholesterol is the obligatory precursor.

The adrenal cortex is further divided into zones that are each responsible for production of a specific group of steroid hormones. The outermost zone is the zona glomerulosa, which is responsible for synthesizing mineralocorticoids like aldosterone to affect salt and water balance. The middle zone, the zona fasciculata, is primarily involved with glucocorticoid production that is implicated in glucose metabolism and immune suppression. The innermost zone, the zona reticularis, is the site of androgen synthesis and is important for developing and maintaining masculine features. The zona fasciculata and zona reticularis have similarities and both contain the necessary enzymes for neuroactive steroid synthesis (Compagnone and Mellon, 2000). Thus, the adrenal makes numerous steroids with important functions that must act all over the body. Moreover, since steroids are involved with a number of important processes in the brain, determining mechanisms of neuroactive steroid synthesis is pivotal to understanding and potentially treating numerous diseases and disorders.

Steroidogenesis in the adrenal is the result of a signaling cascade beginning in the hypothalamus. Activation of the hypothalamic-pituitary-adrenal (HPA) axis is critical for the body's response to stress (Chrousos and Gold, 1992; Spencer and Hutchison, 1999). Activation of this axis stimulates the release of corticotrophin releasing factor (CRF) from the hypothalamus. CRF activates cells in the pituitary to release adrenocorticotropic hormone (ACTH) that subsequently acts upon the adrenal leading to synthesis of steroids from the adrenal cortex. Thus, stress activates HPA axis function and synthesis of steroids is one mechanism of coping with stressful stimuli, either through direct anxiolytic actions of neuroactive steroids or through their ability to shut down the HPA axis. Both neuroactive steroids and glucocorticoids, primarily cortisol in humans and nonhuman primates, and corticosterone in rodents, provide negative feedback on the axis.

The ability of steroids to return HPA axis function to normal appears to be important for CNS function since this response is dysregulated in disorders such as depression, post-

traumatic stress disorder (PTSD) and premenstrual dysphoric disorder (PMDD) (Handwerger, 2009; Lombardi et al., 2004; Vermetten and Bremner, 2002; Young et al., 1993). Neuroactive steroids may also play a role in Parkinson's disease and other neurodegenerative disorders (Adibhatla and Hatcher, 2008). Indeed, alterations in neuroactive steroid levels may be due to changes in HPA axis activity. Socially isolated animals have an altered HPA axis response (Serra et al., 2005) accompanied by changes in the neuroactive steroid response to either stress or acute ethanol administration (Serra et al., 2003; Serra et al., 2000). Furthermore, the resulting stress from chronic ethanol administration alters HPA axis activity and decreases corticosterone levels (Spencer and McEwen, 1990). These alterations in HPA axis activity blunt the response of the axis to a subsequent ethanol challenge (Lee and Rivier, 1995) and are associated with a reduction in CRF and ACTH levels (Lee et al., 2001a). Similar changes in HPA axis function are also seen in human alcoholics who actively consume ethanol (Wand and Dobs, 1991).

While HPA axis activation is important for ACTH release and subsequent adrenal stimulation, there are many important biosynthetic proteins and enzymes responsible for the synthesis of steroids and conversion to their neuroactive metabolites. As mentioned above, steroids can be synthesized in both adrenal and brain, suggesting that central levels of neuroactive steroids are likely a combination of peripherally and centrally derived steroids. Indeed, steroids are lipophilic molecules capable of crossing the blood brain barrier and the enzymes necessary for neuroactive steroid synthesis exist in brain and adrenals. After adrenalectomy there is an attenuation of stimulus-induced increases in central steroid levels suggesting the importance of peripheral synthesis. Yet brain metabolism of peripherally derived precursors may still play a major role in contributing to central levels of neuroactive metabolites. In fact, administration of 5$\alpha$-dihydroprogesterone

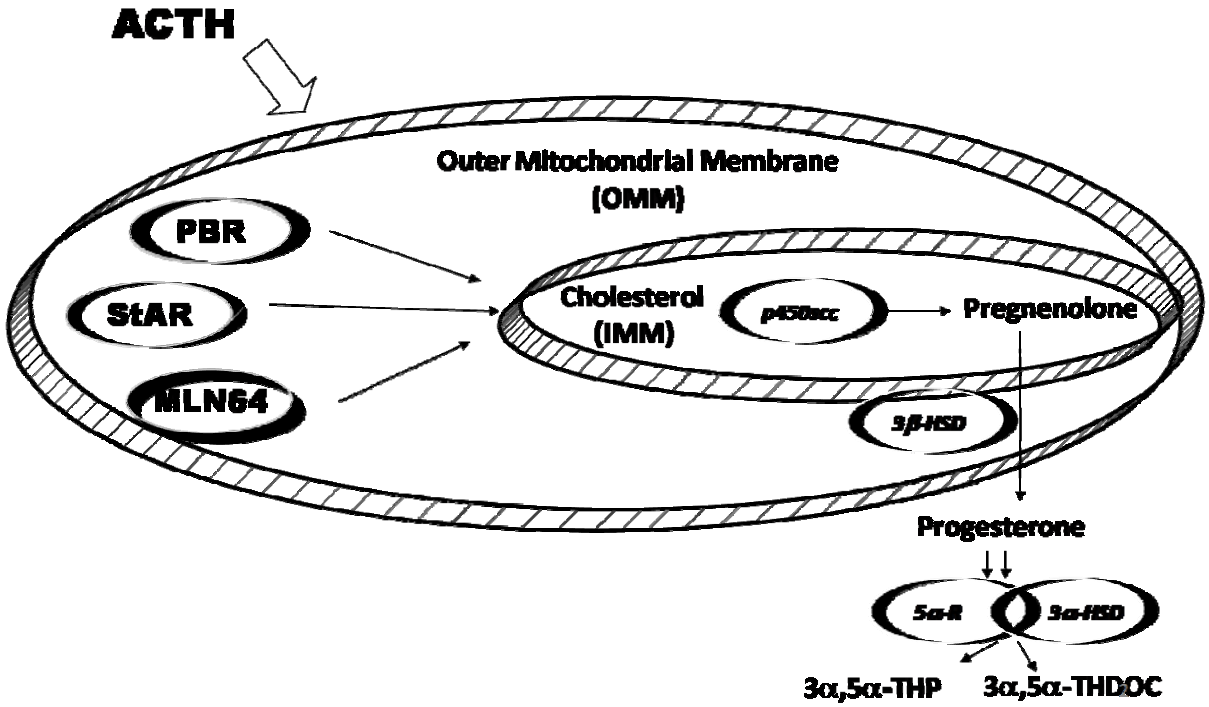


**Fig 1.2:** A schematic of the adrenal gland proteins and enzymes involved in neuroactive steroid synthesis. ACTH is released from the pituitary in response to HPA axis activation and stimulates the adrenal. StAR, PBR, and MLN64 are cholesterol transport proteins capable of delivering cholesterol to the P450scc enzyme on the inner mitochondrial membrane. Once P450scc converts cholesterol to pregnenolone, further synthesis can occur in the adrenal or these steroids can travel to the brain to be metabolized to GABAergic neuroactive steroids.

(5α-DHP), the immediate precursor of 3α,5α-THP, to adrenalectomized rats restores 3α,5α-THP levels in the brain (Khisti et al., 2003b).

As mentioned, all steroids are derived from cholesterol through conversion to pregnenolone by P450scc enzyme. The transfer of cholesterol from the outer mitochondrial membrane to the P450scc enzyme on the inner mitochondrial membrane is rate-limiting making it a potential site where peptide hormones and cAMP could regulate cholesterol transfer and steroid biosynthesis. The steroidogenic acute regulatory protein (StAR) is a cholesterol transport protein found in steroidogenic cells. Although numerous groups have studied StAR protein in Leydig cell steroidogenesis, our lab was the first to show that ethanol increases StAR protein expression in rat adrenal, and that these increases correlate with increased steroid levels (Khisti et al., 2003a). Furthermore, of the two enzymes required for conversion of steroid precursors to the GABAergic neuroactive metabolites, 5α-reductase enzyme activity was not affected by ethanol in adrenal or brain (unpublished data). Similarly, 3α-hydroxysteroid dehydrogenase enzyme activity was not affected by ethanol in adrenal (unpublished data) and would not be predicted to be affected in brain (Trauger et al., 2002). However, mRNA expression of these enzymes is observed in rodent brain in principal GABAergic output neurons, but not in cortical or hippocampal GABAergic interneurons (Agis-Balboa et al., 2006). Taken together, these data suggest the importance of cholesterol transport and subsequent steps prior to pregnenolone formation as important potential targets for ethanol's effects.

**Role of Neurosteroids in Ethanol Actions and Behavioral Sensitivity**

Acute ethanol administration increases GABAergic neuroactive steroids that contribute to specific actions of ethanol. Ethanol-induced increases in neuroactive steroids are time-dependent and correlate with some, but not all, effects of ethanol. For example, motor-incoordinating effects of ethanol appear prior to increases in neuroactive steroid

levels (Khisti et al., 2004). However, neuroactive steroids do modulate several effects of ethanol including anticonvulsant, anxiolytic, hypnotic, and antidepressant-like effects. Indeed, anxiolytic effects of ethanol measured by time spent in the open arm of an elevated plus maze were attenuated by inhibition of steroid synthesis with the 5α-reductase inhibitor finasteride (Hirani et al., 2005). In addition, ethanol increased the seizure threshold of the $GABA_A$ receptor antagonist bicuculline, but this effect was blocked by finasteride (VanDoren et al., 2000b). Neuroactive steroids also modulate sedative hypnotic effects of ethanol and adrenalectomy inhibits ethanol-induced increases in the duration of loss of righting reflex (Khisti et al., 2003b). Furthermore, the antidepressant like effect of ethanol, as measured by forced swim test, was blocked by inhibition of neuroactive steroid synthesis (Hirani et al., 2002). Moreover, in many of these examples the effects of neuroactive steroid inhibition are similar to that of $GABA_A$ receptor antagonism with bicuculline demonstrating that neuroactive steroids modulate specific ethanol actions via $GABA_A$ receptors. Taken together, these results suggest that neuroactive steroid synthesis contributes to the behavioral sensitivity of ethanol.

**Ethanol Effects on 3α,5α-THP and 3α,5α-THDOC Synthesis**

Much of the focus of ethanol effects on steroids has been directed towards the GABAergic neuroactive metabolites. However, ethanol effects on the biosynthesis of these neuroactive steroids is equally important as well as the pharmacological effects of precursor steroids. As early as the 1940s, it was found that DOC acetate and progesterone induced anesthetic effects in rats (Selye, 1941), and both DOC and progesterone had antiseizure effects (Selye, 1942), probably due to their 3α-reduced metabolites (Reddy and Rogawski, 2002; Rhodes and Frye, 2005). DOC (precursor of 3α,5α-THDOC) and progesterone (precursor of 3α,5α-THP) can readily cross the blood-brain barrier and distribute throughout the brain. Whereas small amounts of these steroids may be formed *de novo* in the brain,

ethanol-induced increases in neuroactive steroids are predominantly formed from adrenal precursors (Khisti et al., 2003a). Plasma and brain concentrations of pregnenolone and progesterone are increased more rapidly than 3α,5α-THP after acute ethanol administration (Korneyev and Costa, 1996; O'Dell et al., 2004). Furthermore, an intravenous injection of [1,2-3H]-DOC increased DOC levels across many brain regions (Kraulis et al., 1975). The temporal and regional associations found in these studies suggest that the steroids originate in the adrenals and are transported to the brain. Upon entering the brain, the steroids are metabolized by 5α-reductase and 3α-dehydrogenase enzymes whose regional and cell specific expression (Li et al., 1997) may regulate the distribution of steroid levels. Studies of ethanol's effects on neuroactive steroid precursors are important not only to determine the sources and synthesis of potent metabolites, but also to establish their role in physiological functions.

**Neuroactive Steroids and Ethanol Consumption**

The use of drug discrimination procedures has shown that neuroactive steroids substitute for other $GABA_A$ receptor positive modulators including ethanol (see Shannon et al., 2005, for review). In addition, neuroactive steroids can also affect drinking behavior. These results are difficult to interpret, however, because 3$\alpha$,5$\alpha$-THP has rewarding properties at certain doses and may mediate some of the reinforcing effects of ethanol (Finn et al., 1997a). On the other hand, 3$\alpha$,5$\alpha$-THP reduces voluntary ethanol consumption in non-selected rats (Martin-Garcia et al., 2007) and dose-dependently decreases ethanol self-administration in ethanol-dependent P rats (see review, Morrow et al., 2001), and in mice (Ford et al., 2005). These results suggest that 3$\alpha$,5$\alpha$-THP may be protective against excessive drinking in dependent animals. This would have important therapeutic implications since steroid levels are suppressed in animals exposed to chronic ethanol.

**Potential Role of Neuroactive Steroids in Ethanol-Induced Neurotoxicity**

Although pro-inflammatory molecules derived through systemic and CNS signaling mechanisms contribute to ethanol neurotoxicity, antioxidants have been shown to be protective (Hamelink et al., 2005). Adrenal steroids also suppress the immune response and inhibit inflammation. Given that inflammation is critical for ethanol toxicity, neuroactive steroids may have neuroprotective effects. Arguing against this point is the fact that corticosterone administration decreased neurogenesis in the dentate gyrus while adrenalectomy promoted neurogenesis (Cameron and Gould, 1994). However, corticosterone did not inhibit neurogenesis when administered to adrenalectomized animals suggesting a complex interaction that has also been shown to involve NMDA receptor activation (Cameron et al., 1998). Furthermore, neuroactive steroids such as 3$\alpha$,5$\alpha$-THP have been shown to promote neurogenesis possibly through increases in intracellular calcium and activation of voltage-gated L-type calcium channels (Wang et al., 2005). In addition, neuroactive steroids have many neuroprotective functions including delaying neurodegeneration in a mouse model of Niemann-Pick's disease (Griffin et al., 2004), protecting against glutamate-mediated neurotoxicity (Kimonides et al., 1998), reducing damage and promoting neurological recovery from spinal chord injury (Guth et al., 1994; Thomas et al., 1999), inducing myelination (Schumacher et al., 2001) and promoting neuron survival in the face of excitotoxicity (Brinton, 1994). Furthermore, neuroactive steroids regulate growth factor expression (see Wang et al., 2008, for review) that could act to reverse neurodegenerative effects of a variety of diseases including alcoholism. Thus, it is plausible that the tolerance to ethanol-induced increases in neuroactive steroids following chronic ethanol administration (Janis et al., 1998; Khisti et al., 2005) decreases the protective effect of neuroactive steroids and contributes to ethanol-induced neurotoxicity.

Interestingly, using a variety of human brain cell lines, oligodendrocytes were the only cells capable of synthesizing steroids *de novo* (Brown et al., 2000). Loss of these cells

would be expected to decrease steroid production and may contribute to further neurodegeneration. Indeed, glial cells are more sensitive than neurons to the damaging effects of ethanol (Miguel-Hidalgo et al., 2002) and reductions in astrocytes and oligodendrocytes, but not neurons, were observed in human alcoholic hippocampus (Korbo, 1999). However, glial cells subsequently increase in response to alcohol damage and this may increase steroid synthesis that contributes to regeneration during abstinence. Indeed, oxidative stress can regulate dehydroepiandrosterone (DHEA) synthesis (Brown et al., 2000) and DHEA may act to prevent or reverse damage as it has been shown to be neuroprotective (Kimonides et al., 1998). Furthermore, PBR is involved in the regulation of cell proliferation and steroidogenesis and chronic ethanol increases the density of the PBR ligand [$_3$H]PK-11195 (Obernier et al., 2002; Syapin and Alkana, 1988). Thus, neuroactive steroids may play a role in preventing ethanol-induced neurotoxicity.

**Neuroactive Steroids and Chronic Ethanol Exposure**

Chronic ethanol consumption progressively leads to tolerance and dependence to ethanol. These phenomena are the result of numerous cellular adaptations in the brain, including changes in $GABA_A$ receptor subunit expression and activity. Ethanol dependence causes changes in GABAergic tone that lead to hyperexcitability and enhanced withdrawal symptoms, such as increased anxiety and seizure susceptibility. Interestingly, benzodiazepines are a commonly used therapeutic treatment for alcohol withdrawal symptoms, yet ethanol dependence exhibits cross tolerance to benzodiazepines and other $GABA_A$ receptor modulators.

Neuroactive steroids also play an important role in ethanol dependence and withdrawal as they have anxiolytic and anti-seizure properties. Chronic ethanol administration in rats leads to a suppression of the neuroactive steroid response. Following chronic ethanol administration, rats become tolerant to ethanol-induced increases in neuroactive steroids (Janis et al., 1998) and show a blunted steroid response to an ethanol

challenge (Khisti et al., 2005; Morrow et al., 2001). The loss of increased neuroactive steroid levels likely contributes to ethanol tolerance since these steroids are required for specific ethanol actions including anxiolytic and anticonvulsant actions. Furthermore, rats exposed to a chronic intermittent ethanol (CIE) paradigm in which they go through repeated withdrawals similar to a binge model, also fail to show ethanol-induced increases in neuroactive steroid levels (Cagetti et al., 2004). In addition, tolerance typically develops to the anticonvulsant effects of most GABA potentiating drugs but not 3$\alpha$,5$\alpha$-THP (Kokate et al., 1998). In fact, ethanol-dependent rats become sensitized to the anticonvulsant properties of neuroactive steroids (Cagetti et al., 2004; Devaud et al., 1996) making steroids potentially useful as a treatment for alcohol withdrawal.

**Neuroactive Steroids and Ethanol Sensitivity**

Individuals who are either insensitive to ethanol, or who quickly develop a tolerance, are at a higher risk for alcoholism than those who are sensitive to the effects of ethanol. Neuroactive steroids may play a role in ethanol sensitivity in rodents (see Morrow et al., 2006, for review) as well as humans (Pierucci-Lagha et al., 2005). Neuroactive steroids have similar pharmacological effects as ethanol, and the use of biosynthetic inhibitors has shown that neuroactive steroids are required for specific ethanol actions. Furthermore, the increases in plasma and brain levels of neuroactive steroids observed after acute ethanol administration are no longer present after chronic ethanol exposure. In turn, dependent rodents and humans are more susceptible to anxiety, seizures, and other withdrawal related phenotypes related to hyperexcitability. In addition, dependent rats challenged with ethanol can no longer mount the same steroidogenic response as a naïve animal administered ethanol. Taken together, these facts strongly support a pivotal role for neuroactive steroids in mediating the effects of ethanol, and suggest that the loss of the ethanol-induced steroid response following dependence is critical to the development of alcoholism and/or alcohol related disorders. Thus, variations in basal levels of neuroactive steroids may play a role in

ethanol sensitivity and risk for alcoholism. Manipulations of these steroid levels are a potential target for therapeutic intervention as neuroactive steroids may be beneficial for increasing ethanol sensitivity in dependent individuals, controlling drinking behavior and as a treatment for alcohol withdrawal.

**Rationale for Aims of Study**

Previous work in our lab and others had shown that acute ethanol administration increases neuroactive steroid levels to pharmacologically relevant concentrations in the brain. At some point during the development of ethanol tolerance and dependence this increase in neuroactive steroid levels is lost. The lack of neuroactive steroid response to ethanol following dependence is believed to contribute to ethanol tolerance since neurosteroids contribute to anxiolytic, sedative, and anticonvulsant effects of ethanol. Thus, the overall goal of these studies was to determine the mechanisms by which ethanol increases neuroactive steroid levels, and to identify the adaptations that occur in this response following chronic ethanol exposure. These studies are important because neuroactive steroid synthesis contributes to ethanol actions and is dysregulated following ethanol dependence. Thus, while the scope of this work is focused on ethanol action and dependence, the mechanisms identified can be applied to a variety of neuropsychiatric disorders where the use and/or regulation of neuroactive steroids is a potential therapeutic approach. Furthermore, the neuroprotective properties of neuroactive steroids may counteract some of the toxic effects of ethanol.

**Aim I Rationale**

Characterization of the acute neuroactive steroid response to ethanol is imperative in order to understand what adaptive changes occur during the transition to ethanol dependence. Ethanol is a non-specific drug affecting multiple systems and signaling molecules making studies of its effects extremely complex. Furthermore, ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) in liver and brain and

acetaldehyde is a biologically active molecule that can elicit similar behavioral responses as compared to ethanol (see Quertemont et al., 2005, for review). Thus, while the ability of ethanol to increase neuroactive steroids has been well documented, the first aim of these studies was to determine whether acetaldehyde played a role in the observed ethanol-induced increases in neuroactive steroids.

Alcohol dehydrogenase metabolizes alcohol to the very toxic metabolite acetaldehyde. In fact, the unpleasant effects of acetaldehyde are the reason behind the use of disulfiram as a preventative. Disulfiram inhibits ALDH leading to the buildup of acetaldehyde. Under normal circumstances acetaldehyde is rapidly metabolized to acetate and subsequently acetate is excreted as carbon dioxide and water. We take for granted that biochemical and behavioral effects observed after ethanol administration are actually due to ethanol itself and not a result of metabolites. Interestingly, some scientists believe that acetaldehyde, and perhaps acetate as well, is responsible for at least some of the effects of ethanol. While this has been debated in the literature, enzyme activity controlling acetaldehyde levels is predictive of drinking behaviors (Edenberg and Kranzler, 2005). Indeed, genetic variations that increase alcohol dehydrogenase activity or decrease aldehyde dehydrogenase activity are protective against the development of alcoholism in rodent models as well as human populations (Isse et al., 2005; Isse et al., 2002; Ocaranza et al., 2008; Quintanilla et al., 2005; Wall et al., 2003b). Furthermore, since neuroactive steroids are also postulated to be protective for alcoholism risk, and acetaldehyde administration may elicit behavioral effects similar to those of neuroactive steroids, there exists an unexplored link between acetaldehyde and neuroactive steroids.

**Aim II Rationale**

Ethanol-induced increases in neuroactive steroid levels could be achieved through three possible mechanisms. First, ethanol could initiate activation of steroid biosynthesis by affecting biosynthetic proteins. Second, increased steroid levels may be the result of

inhibition of steroid metabolism. Finally, ethanol may trigger the release of a sequestered steroid pool. A review of the literature does not lend much support to the latter two possibilities; however, numerous studies suggest a correlation between increased cholesterol transport, the rate-limiting step in steroidogenesis, and increased steroid levels (Clark et al., 1994; Crivello and Jefcoate, 1979; Khisti et al., 2003a; King et al., 1995; Krueger and Orme-Johnson, 1983; Papadopoulos et al., 2007). Thus, although I cannot rule out that ethanol-induced increases in neuroactive steroids are affected by a combination of these mechanisms, I focused the second aim studies on proteins involved in steroid biosynthesis and identifying how acute ethanol administration affects steroid biosynthesis.

**Aim III Rationale**

As mentioned, previous studies have shown that ethanol-dependent rats develop tolerance to the ethanol-induced increases in neuroactive steroids, but this work has not led to insight as to involved mechanisms. The loss of the steroid response is clearly important for behavioral phenotypes related to ethanol dependence and withdrawal as administration of these steroids attenuates withdrawal-like symptoms (Devaud et al., 1996; Martin-Garcia and Pallares, 2005). Thus, building upon the important mechanisms required for neuroactive steroid synthesis following acute ethanol, the third aim of these studies was to identify the adaptations that occur in the activity of these steroidogenic enzymes following ethanol dependence. Restoring neuroactive steroid responses to ethanol through either exogenous administration of steroids or modulation of proteins and biosynthetic enzymes appears likely to ameliorate some of the negative effects of ethanol dependence and withdrawal.

## Chapter II

## Materials and Methods

### Animals:

Male Sprague-Dawley rats weighing between 250-350 g were used for all experiments (Harlan, Indianapolis, IN). The animals were group housed (3 per cage) and maintained in standard light and dark (lights on, 7:00 A.M. to 7:00 P.M.) conditions with food and water available ad libitum. Rats were acclimated to the handling procedure for one week before the test day. Hypophysectomized and sham-operated animals were ordered from Harlan and housed for a week prior to experimentation. All experiments were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina Chapel Hill.

### Chronic Ethanol Liquid Diet Administration:

Rats were housed individually and administered a nutritionally complete liquid diet for the first 3 days (Dextrose diet, MP Biomedicals, Costa Mesa, CA). Rats then received ethanol (6 % v/v in liquid diet) for 7 days followed by ethanol (7.5 % v/v in liquid diet) for the duration of study. Control rats were fed the identical diet with dextrose substituted equicalorically for ethanol. Water was available *ad libitum* and dietary consumption was monitored daily. The mean body weights for the controls and ethanol diet rats were similar at the termination of the experiment. This procedure reliably results in physical dependence on ethanol (Morrow et al., 1992). Ethanol dependent rats had free access to ethanol diet up until the time of sacrifice.

Separate groups of rats that consumed ethanol by liquid diet for 14 days were injected with a challenge dose of ethanol (2 g/kg, 20 % v/v in saline) and tissue was collected after 60 minutes. These rats had their bottles removed and were put back onto standard chow for 24 hours prior to challenge. Rats receiving exogenous ACTH were administered two doses of ACTH (2 μg), one concurrently with the saline or ethanol challenge and one 30 minutes later. Tissue was collected 60 minutes after the saline or ethanol challenge. All rats were handled and habituated to saline injections and were sacrificed by decapitation.

**Drug Administration for Aim I Studies:**

For steroid measurements, animals were sacrificed by decapitation 45 minutes after i.p. administration of saline, ethanol (2 g/kg, 20% v/v), or acetaldehyde (20, 50, 75, 100 mg/kg). 4-methylpyrazole (200 mg/kg i.p.) was administered 60 minutes prior to ethanol to inhibit alcohol dehydrogenase. Animals receiving catalase inhibitor, similar to prior studies (Manrique et al., 2005), were administered sodium azide (10 mg/kg i.p.) 30 minutes prior to an acute ethanol injection (2 g/kg, 20% v/v). To inhibit aldehyde dehydrogenase, animals were administered cyanamide (50 mg/kg i.p.) 60 minutes prior to an acute ethanol injection (2 g/kg, 20% v/v) (Jamal et al., 2005). Saline pretreated animals were used for controls in both experiments. All experiments utilized a minimum of six animals per group.

**Drug Administration for Aim II Studies:**

Unless otherwise stated, animals were administered i.p. injections of saline or ethanol (2 g/kg, 20% v/v). The protein synthesis inhibitor cycloheximide (20 mg/ml i.p.) was dissolved in saline and administered as a 1 ml injection either concurrently with ethanol or vehicle, or 40 minutes post-administration of ethanol or vehicle. The PBR agonist, CB34 (15 mg/kg, i.p.), was dissolved in saline while the PBR antagonist PK11195 (1 mg/kg i.p.) was dissolved in Tween 80, diluted with saline, and given 30 minutes prior to ethanol or saline. The glucocorticoid receptor activator, dexamethasone 21-phosphate disodium salt (0.1 mg/kg

i.p.) was dissolved in saline and administered 90 minutes prior to ethanol or saline. The P450scc antagonist aminoglutethimide (10 mg/ml i.p.) was dissolved in propylene glycol, diluted with saline and administered as a 2 ml injection 1 hour prior to an ethanol or saline injection.

**Plasma Ethanol and Acetaldehyde Measurements by Gas Chromatography:**

A 6 μl aliquot of plasma was analyzed for ethanol levels using a SRI 8610c gas chromatograph (Torrance, CA). Acetaldehyde levels were determined using 100 μl of plasma and in both instances similar volumes were used to establish a standard curve. The ethanol standard curve ranged from 0 to 400 mg/dl and the acetaldehyde standard curve ranged from 0 to 250 μM. Samples were distributed to tubes containing 375 μl of water and 0.5 g NaCl. Samples were heated in a water bath at 60 °C for 10 minutes and a 1.5 ml sample of headspace gas was removed and injected directly into the GC. Samples were run at 140 °C through a Hayesep D column and detected with a flame ionization detector. Hydrogen gas, carrier gas, and internal air generator flow rates were 13.3, 25, and 250 ml/min respectively. Areas under the curve were analyzed with SRI PeakSimple software and converted to mg/dl for ethanol and μM for acetaldehyde based on the standard curves.

**Tissue and Protein Preparations:**

Mitochondrial membrane fractions from adrenal glands were prepared by homogenization, low speed centrifugation in 0.32 M sucrose and centrifugation of the supernatant at 17,000 x g for 30 minutes. The pellet (mitochondrial fraction) was resuspended in phosphate buffered saline (PBS). Individual cerebral cortices, as well as other brain regions, were homogenized directly in 2% sodium dodecyl sulfate (SDS). Protein measurement was conducted using a BCA protein assay (Thermo Fisher Scientific Inc, Rockford, IL).

**Western Blot Analysis:**

Adrenal mitochondrial fractions and cerebral cortical homogenate were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Novex Tris-Glycine gels (8-16%) and transferred to polyvinylidene diflouride membranes (Invitrogen, Carlsbad, CA). These membranes were probed with StAR, MLN64 (Abcam, Cambridge, MA) or PBR (Trevigen, Gaithersburg, MD) antibodies. Blots were subsequently exposed to a second primary antibody directed against β-actin to verify equivalent protein loading and transfer. Bands were detected by enhanced chemiluminescence (Amersham, UK), apposed to x-ray films under nonsaturating conditions, and analyzed by densitometric measurements using NIH Image 1.57. All comparisons were made within blots and statistical analysis was conducted using student's t-test or one-way ANOVA.

**Phospho-PKA Substrate Immunoprecipitation Analysis:**

Protein in the mitochondrial fraction was immunoprecipitated with phospho-PKA substrate antibody (Cell Signaling Technology Inc., Danvers, MA) similar to the method previously described (Kumar et al., 2002). Briefly, mitochondrial protein (200 μg) was solubilized and denatured in radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich, St. Louis, MO) with phosphatase inhibitor cocktail (Sigma-Aldrich), phenylmethylsulfonyl fluoride (1 mM), sodium fluoride (50 mM), sodium vanadate (200 μM), and EDTA (2 mM) to prevent protein degradation and dephosphorylation. Solubilized protein was centrifuged at 10,000*g* and supernatant (solubilized protein) was collected. Denaturation of protein in the supernatant was confirmed by SDS-PAGE analysis.

Immunoprecipitation of phosphoproteins for detection of phospho-StAR was performed using antibody conjugated to Dynal beads (Invitrogen, Carlsbad, CA) and western blot analysis of the immunoprecipitate. The optimal antibody and protein concentrations for immunoprecipitation were determined in pilot experiments to optimize the

conditions. The phospho-PKA substrate specific antibody or IgG (Rockland Inc., Gilbertsville, PA) was linked to Dynal beads by incubating 125 μl of Dynal beads with 100 μl of antibody (0.35 μg/μl) for 1 h at room temperature. The solubilized receptors were mixed with antibody-linked beads in a final volume of 500 μl and incubated in an orbital shaker overnight at 4°C. The receptor-antibody-bead complex was washed three times with PBS, resuspended in 50 μl of SDS, and boiled for 5 minutes. Phosphoprotein immunoprecipitates and adrenal mitochondrial fractions were analyzed by SDS-PAGE gel electrophoresis and western blotting from saline or ethanol-treated animals to examine the effects on immunoprecipitated phospho-StAR protein as well as total StAR protein.

**Radioimmunoassay (RIA) of Pregnenolone:**

Pregnenolone levels were measured by RIA as previously described (Porcu et al., 2006). Briefly, pregnenolone was extracted from 250 μl of plasma with 2 mls of diethyl ether three times. Extraction recovery was monitored by the addition of 1000 cpm of [$^3$H]pregnenolone. Samples were reconstituted and assayed in duplicate by the addition of [$^3$H]pregnenolone and anti-pregnenolone antibody (MP Biomedicals, Orangeburg, NY). Total binding was determined in the absence of unlabeled pregnenolone and nonspecific binding was determined in the absence of antibody. The antibody binding reaction was allowed to equilibrate for a minimum of 4 hours and cold dextran coated charcoal was used to separate bound from unbound steroid. Bound radioactivity was determined by liquid scintillation spectroscopy. Steroid levels in the samples were extrapolated from a concurrently run standard curve and corrected for their respective extraction efficiencies. The antiserum cross-reacts with 3α,5α-THP 16%, 3α,5β-THP 5.9%, progesterone 3.1%, 3α,5α-THDOC 1.1%. All of the following steroids had less than 1% cross-reactivity: 5α-dihydroprogesterone, 17α-hydroxyprogesterone, deoxycorticosterone, cortisol, 11-deoxycortisol, corticosterone, androsterone, 5α-dihydrotestosterone, cholesterol, 17β-

estradiol, estrone and estriol. The intra-assay and inter-assay coefficients of variation were 7.71% and 5.93% respectively.

**RIA of Progesterone and ACTH:**

Plasma progesterone and ACTH levels were measured using RIA kits according to the manufacturer's instructions (MP Biomedicals, Costa Mesa, CA). Total binding was determined in the absence of unlabeled progesterone or ACTH and nonspecific binding was determined in the absence of antibody. Steroid levels in the samples were extrapolated from a concurrently run standard curve. The intra-assay and inter-assay coefficients of variation for progesterone are 3.6% and 6.7% respectively and for ACTH they are 4.1% and 3.9%.

**RIA of Neuroactive Steroid 3α,5α-THP:**

RIAs were conducted as previously described (Janis et al., 1998). Briefly, brain samples were weighed and suspended in 2.5 ml of 0.3N NaOH, homogenized with a sonic dismembrator, and extracted three times with 3 ml aliquots of 10% ethyl acetate in heptane (vol/vol). Extraction recovery was monitored by the addition of 2000 cpm of [$^3$H]3α,5α-THP. The brain extracts were purified using solid phase silica columns (Burdick and Jackson, Muskegon, MI) and subsequently dried. Samples were reconstituted and assayed in duplicate by the addition of [$^3$H]3α,5α-THP and anti-3α,5α-THP polyclonal sheep antibody (Gift from Dr. Patrick Sluss). Total binding was determined in the absence of unlabeled 3α,5α-THP and nonspecific binding was determined in the absence of antibody. The antibody binding reaction was allowed to equilibrate for 2 hours and cold dextran-coated charcoal was used to separate bound from unbound steroid. Bound radioactivity was determined by liquid scintillation spectroscopy. Steroid levels in the samples were extrapolated from a concurrently run standard curve and corrected for their respective extraction efficiencies. The 3α,5α-THP antibody has minimal cross reactivity with other circulating steroids (Finn and Gee, 1994). 3α,5α-THP antiserum cross-reacts with

progesterone < 3%, 3α,5β-THP 6.6%, 3β,5α-THP 2.8%, 3β,5β-THP 0.5%, 5α-pregnan-3α,20α-diol 0.1% and 5α-pregnan-3,20-dione 3.5%. The antiserum cross-reacts with 3α-hydroxy-4-pregnen-20-one > 80% but there have been no significant levels of this steroid reported in circulation (Finn and Gee, 1994). The inter-assay coefficient of variation was 9.1% and the intra-assay coefficient of variation was not measured.

**Data analysis:**

Results are expressed as mean ± S.E.M. Steroid levels are expressed as ng/g for brain tissue. Plasma ethanol levels are expressed as mg/dl and plasma acetaldehyde concentrations are expressed in μM units. Western blot data is normalized to β-actin signals from the same blot and expressed as % control values taken from each blot. Significance was determined by ANOVA followed by post hoc Newman Keuls test or the Student's t test as appropriate. Analyses were performed using the software GraphPad Prism version 4.

## Chapter III

## The Role of Acetaldehyde in Ethanol-Induced Elevation of the Neuroactive Steroid 3α-hydroxy-5α-pregnan-20-one in Rats


### Summary

Systemic ethanol administration increases neuroactive steroid levels that increase ethanol sensitivity. Acetaldehyde is a biologically active compound that may contribute to behavioral and rewarding effects of ethanol. We investigated the role of acetaldehyde in ethanol–induced elevations of (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP) levels in cerebral cortex. Male Sprague-Dawley rats were administered ethanol and plasma acetaldehyde concentrations were measured by gas chromatography to determine relevant concentrations. Rats were then administered acetaldehyde directly, acetaldehyde plus cyanamide to block its degradation, or ethanol in the presence of inhibitors of ethanol metabolism, to determine effects on 3α,5α-THP levels in cerebral cortex. Ethanol administration (2 g/kg) to rats results in a peak acetaldehyde concentration of 6-7 μM at 10 minutes that remains stable for the duration of the time points tested. Direct administration of acetaldehyde eliciting this plasma concentration does not increase cerebral cortical 3α,5α-THP levels and inhibition of ethanol metabolizing enzymes to modify acetaldehyde formation does not alter ethanol–induced 3α,5α-THP levels. However, higher doses of acetaldehyde (75 and 100 mg/kg), in the presence of cyanamide to prevent its metabolism, are capable of increasing cortical 3α,5α-THP levels. Thus, physiological concentrations of acetaldehyde are not responsible for ethanol-induced increases in 3α,5α-THP, but a synergistic role for acetaldehyde with ethanol may contribute to increases in 3α,5α-THP levels and ethanol sensitivity.

## Introduction

Neuroactive steroids produce their effects on membrane receptors that regulate central nervous system activity rather than nuclear receptors that regulate gene expression. The 3α,5α-reduced pregnan steroids, including (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC), are endogenous modulators of GABA-A receptors and produce rapid changes in central nervous system activity (for review Belelli and Lambert, 2005). The GABA-A receptor system is the primary inhibitory receptor system in the brain and is responsible for many behavioral effects of ethanol. Systemic ethanol administration increases plasma and brain levels of neuroactive steroids (Morrow et al., 1999; VanDoren et al., 2000b) that can act with nanomolar potency on GABA-A receptors (Morrow et al., 1987; Puia et al., 1990).

Neuroactive steroids can be synthesized *de novo* in the brain or produced peripherally in the adrenals and gonads. While ethanol-induced increases in neuroactive steroids can originate from both adrenal glands and brain, the adrenal glands are a major source of neuroactive steroids and their precursors. Indeed, adrenalectomy reduces neuroactive steroid levels in plasma and brain and prevents ethanol-induced elevations. Conversely, administration of 5α-dihydroprogesterone, the immediate precursor of the potent GABAergic neurosteroid 3α,5α-THP, to adrenalectomized animals restores ethanol-induced elevations of cortical 3α,5α-THP (Khisti et al., 2003b) suggesting an important role for both adrenal and brain steroidogenesis. In addition, studies have demonstrated brain steroidogenesis in adrenalectomized animals given time for recovery (Follesa et al., 2006) as well as in cell culture (Hu et al., 1987). Indeed, all of the steroidogenic biosynthetic enzymes are present in brain and have been shown to colocalize in specific cell types (King et al., 2002).

Ethanol increases rat plasma and brain concentrations of the neuroactive steroids 3α,5α-THP and 3α,5α-THDOC, which are potent GABAergic modulators that can elicit many of the same effects as ethanol (Barbaccia et al., 1999; Morrow et al., 1999). The maximal effect of ethanol on neuroactive steroid levels is observed at 2.5 g/kg ethanol. Ethanol-induced increases in GABAergic neuroactive steroids are requisite for anxiolytic (Hirani et al., 2005) and anticonvulsant (VanDoren et al., 2000b) effects of ethanol and contribute to sedative-hypnotic actions (Khisti et al., 2003b) and spatial learning impairment (Matthews et al., 2002). In addition, electrophysiological effects of ethanol in medial septal and hippocampal neurons are dependent upon ethanol-induced increases in the GABAergic neuroactive steroids (Tokunaga et al., 2003; VanDoren et al., 2000b). These steroids substitute for ethanol in discrimination studies in rodents and monkeys (Grant et al., 1996; Hodge et al., 2001; Shannon et al., 2005) and exogenous administration can alter ethanol drinking patterns (Ford et al., 2007; Janak et al., 1998; Morrow et al., 2001). All these studies have suggested that neuroactive steroids mediate several behavioral effects of ethanol and contribute to ethanol sensitivity.

The primary metabolite of ethanol, acetaldehyde, can also produce behavioral effects that are similar to ethanol (for review Quertemont et al., 2005). For example, systemic acetaldehyde administration causes a depression in locomotor activity (Myers et al., 1987), impairment of spatial memory (Abe et al., 1999; Quertemont et al., 2004), ethanol-like discrimination (Redila et al., 2002) and hypnotic effects (Quertemont et al., 2004). These observations raise the possibility that acetaldehyde may contribute to GABAergic effects of ethanol mediated by neuroactive steroids. Although many behavioral effects have been studied, few studies have examined acetaldehyde's anxiolytic or anticonvulsant properties that are dependent upon elevations of neurosteroids. Moreover, many of the experiments involving acetaldehyde have relied upon direct administration of acetaldehyde into the brain or systemic administration of high concentrations unlikely to be

found physiologically. Thus, the present study was designed to determine if physiologically relevant concentrations of acetaldehyde contribute to the ethanol-induced increases in neuroactive steroid levels.

## Results

To determine the role of acetaldehyde in ethanol-induced increases in cerebral cortical 3α,5α-THP levels, we first sought to ascertain the concentrations of acetaldehyde in plasma following ethanol administration to rats. Rats were administered ethanol (2 g/kg) since this dose produces near maximal effects on GABAergic neuroactive steroids and produces prominent behavioral effects of ethanol. Plasma ethanol and acetaldehyde levels were measured at various time points following ethanol administration. Plasma ethanol levels peaked around 228 mg/dl (~50 mM) as quickly as 10 minutes post ethanol administration and steadily declined over time (Fig. 3.1A). Plasma acetaldehyde levels remained relatively constant at approximately 6-7 μM across the same time frame, while ethanol was continuously metabolized (Fig. 3.1B). Furthermore, plasma and cerebral cortical 3α,5α-THP levels were elevated at 45 minutes, corresponding to peak elevations in ethanol-induced acetaldehyde levels (Fig. 3.1C).

To determine if the concentration of acetaldehyde produced by ethanol (2 g/kg) is capable of producing an increase in 3α,5α-THP levels, we measured 3α,5α-THP in the cerebral cortices of rats administered various doses of acetaldehyde. Various doses of acetaldehyde were used because it was unknown what doses would produce relevant concentrations. Neuroactive steroid levels were measured at 45 minutes, as this represents

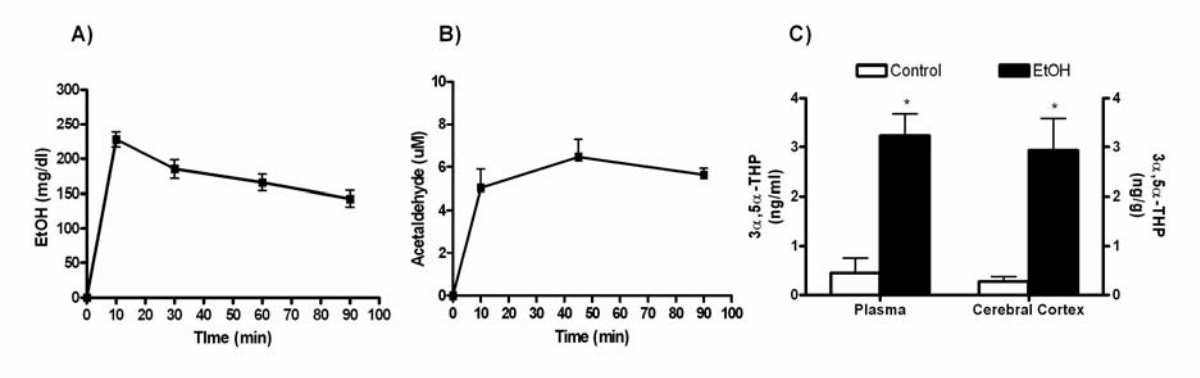


**Figure 3.1: Time-course of plasma ethanol and acetaldehyde concentrations following ethanol administration.** Animals were administered a 2 g/kg dose of ethanol and blood was collected at varying time points. (A) Plasma ethanol and (B) acetaldehyde levels were measured via gas chromatography. Ethanol and acetaldehyde are rapidly increased and then acetaldehyde levels stabilize as ethanol is metabolized. (C) 3$\alpha$,5$\alpha$-THP levels were increased in both the plasma and the cerebral cortex 45 minutes after ethanol administration. Plasma 3$\alpha$,5$\alpha$-THP levels are measured as ng/ml and brain as ng/g. *p<0.01 compared to controls, n=4-8 in duplicate.

the timeframe of the peak neurosteroid response to 2 g/kg ethanol. Figure 2 shows 3α,5α-THP and acetaldehyde levels 45 minutes post acetaldehyde administration from naïve animals (Fig. 3.2A,C) and animals pretreated with the aldehyde dehydrogenase (ALDH) inhibitor cyanamide (Fig. 3.2B,D). Cyanamide administration was necessary to delay acetaldehyde metabolism in order to achieve concentrations similar to those observed following ethanol administration. In this experiment, animals were pretreated with cyanamide 60 minutes prior to acetaldehyde administration and compared to control animals pretreated with the inhibitor prior to saline. Following cyanamide pretreatment, acetaldehyde administration increased 3α,5α-THP levels at both the 75 mg/kg and the 100 mg/kg doses of acetaldehyde. However, these treatments produced plasma acetaldehyde levels that were markedly higher than acetaldehyde concentrations found after ethanol administration alone (Fig. 3.2D). At 50 mg/kg, acetaldehyde administration produced blood acetaldehyde concentrations similar to ethanol, but there was no effect on cerebral cortical 3α,5α-THP levels.

Although direct acetaldehyde administration is important to assess acetaldehyde's effects, experiments examining its effects after ethanol administration are necessary to determine how acetaldehyde contributes to the ethanol response. Therefore, one strategy to study the role of acetaldehyde in ethanol-induced increases in neuroactive steroids is to alter its metabolism after ethanol administration. If acetaldehyde is involved, then systemic manipulation of the enzymes involved in its metabolism should alter affects on 3α,5α-THP levels following ethanol administration. Figure 3.3 shows cerebral cortical 3α,5α-THP levels and the corresponding plasma ethanol and acetaldehyde concentrations for animals receiving ethanol with and without prior inhibition of ALDH. 3α,5α-THP levels were increased following ethanol administration compared to their respective controls regardless of ALDH inhibition (Fig. 3.3A). Pretreatment with the ALDH inhibitor prior to ethanol

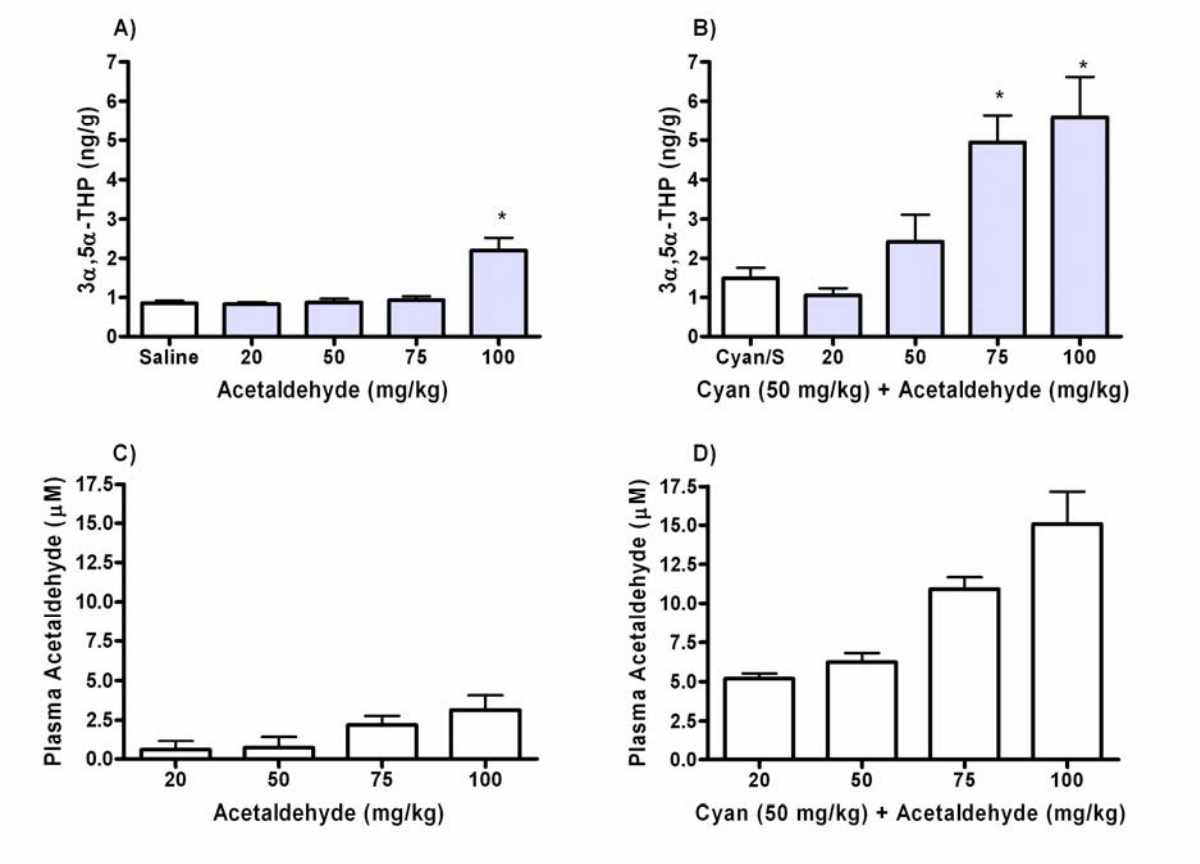


**Figure 3.2: Acetaldehyde administration to levels that mimic ethanol metabolism do not increase 3α,5α-THP levels in cortex.** (A) Acetaldehyde was administered in varying doses and brains were collected after 45 minutes to measure cortical levels of 3α,5α-THP. (B) The same concentrations of acetaldehyde were administered to rats pretreated with the ALDH inhibitor cyanamide (50 mg/kg) 60 minutes prior to acetaldehyde administration and cortical 3α,5α-THP was measured. Plasma acetaldehyde concentrations were measured in (C) animals receiving acetaldehyde alone and in (D) animals receiving the inhibitor prior to acetaldehyde administration. 3α,5α-THP levels were measured by RIA and acetaldehyde concentrations via gas chromatography. *$p<0.001$ compared to controls, n=6 for control and n=8 for acetaldehyde groups in duplicate.

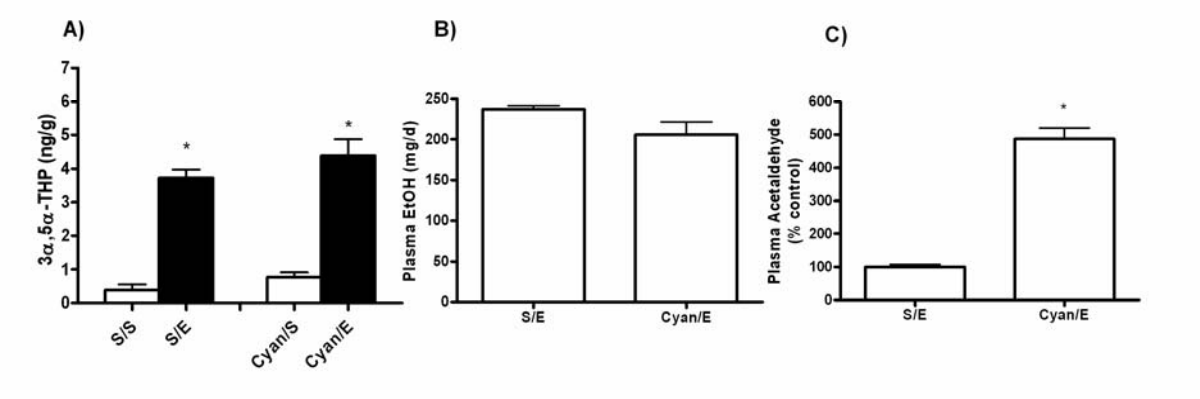


**Figure 3.3: Effect of pretreatment with the aldehyde dehydrogenase inhibitor cyanamide on ethanol-induced 3α,5α-THP elevation.** Animals were administered cyanamide (50 mg/kg, i.p.) 60 minutes prior to an acute injection of ethanol or saline and steroids were measured 45 minutes following the injections. (A) 3α,5α-THP levels were increased in the rat cerebral cortex after acute ethanol (2 g/kg, i.p.) administration and were not affected by treatment with an aldehyde dehydrogenase inhibitor. (B) Plasma alcohol levels were unaffected by aldehyde dehydrogenase inhibition while (C) plasma acetaldehyde levels were increased. *P < 0.001 compared to controls, n=6 in duplicate.

administration did not alter 3α,5α-THP levels compared to ethanol alone. In addition, the cyanamide pretreatment did not significantly alter steroid levels compared to saline pretreated controls. Acetaldehyde levels were measured to confirm that the inhibitor was effective. Acetaldehyde concentrations were increased 386.8 ± 35.1% following ALDH inhibition compared to ethanol alone (Fig. 3.3C). Plasma ethanol levels were not significantly altered by ALDH inhibition (Fig. 3.3B).

Next, we inhibited alcohol dehydrogenase and the catalase enzyme, which convert ethanol to acetaldehyde in the liver and brain, respectively. Inhibition of alcohol dehydrogenase with the competitive inhibitor 4-methylpyrazole (200 mg/kg) had no effect on 3α,5α-THP levels compared to ethanol alone (Figure 3.4A). However, there was no detectable change in plasma acetaldehyde concentrations, even though ethanol levels were increased from 195 mg/dl to 246 mg/dl and still elevated after 6 hours (data not shown). Furthermore, sodium azide (10mg/kg) had no effect on ethanol-induced increases in. 3α,5α-THP levels (Fig. 3.4A). In addition, sodium azide pretreatment did not significantly affect steroid levels compared to saline pretreated controls. Plasma ethanol concentrations were slightly increased and plasma acetaldehyde levels were decreased by 27.8 ± 8.8% following catalase inhibition when compared to ethanol alone (Fig. 3.4B and 3.4C).

## Discussion

The current study was performed to address a previously unexplored issue of whether acetaldehyde is involved in ethanol-induced increases in neuroactive steroids. Systemic administration of 2 g/kg ethanol elicits an increase in neuroactive steroids that is not altered by manipulation of ethanol metabolism. Although high concentrations of acetaldehyde can stimulate increases in neuroactive steroids, the administration of acetaldehyde at doses eliciting concentrations similar to those produced after ethanol

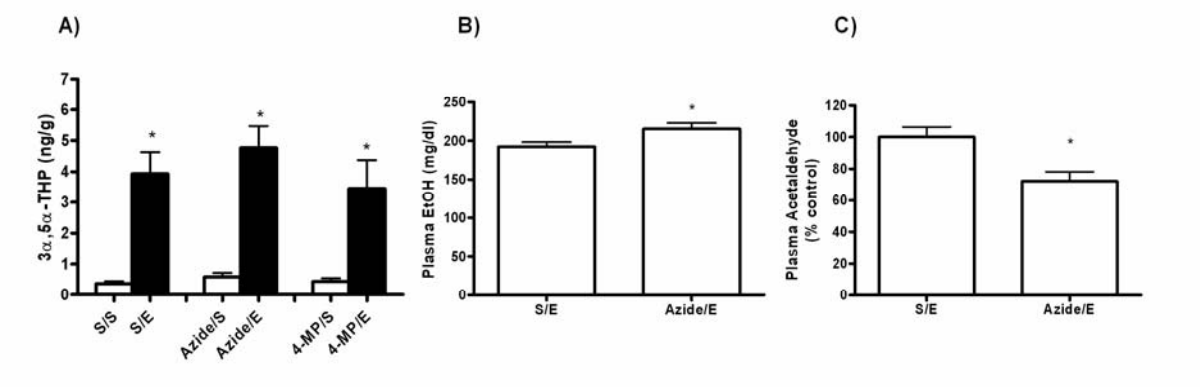


**Figure 3.4: Effects of pretreatment with 4-methylpyrazole and sodium azide on ethanol-induced 3α,5α-THP elevation**. Animals were administered 4-methylpyrazole (200mg/kg, i.p.) or sodium azide (10 mg/kg, i.p.) prior to an acute injection of ethanol or saline and steroids were measured 45 minutes following injections. (A) 3α,5α-THP levels were increased in the rat cerebral cortex after acute ethanol (2 g/kg, i.p.) administration and were not affected by treatment with either inhibitor. (B) Plasma alcohol levels were increased in animals receiving the catalase inhibitor and (C) plasma acetaldehyde concentrations were decreased when catalase was inhibited. *P < 0.05 compared to controls, n=6 in duplicate.

administration does not increase neuroactive steroid levels. Taken together, these results indicate that ethanol is primarily responsible for ethanol-induced increases in neuroactive steroids.

The most important factor in determining whether acetaldehyde contributes to ethanol-induced increases in neuroactive steroids is to establish what concentration of acetaldehyde exists in the blood following ethanol administration. Acetaldehyde levels remained relatively steady across time and were low due to the high level of ALDH activity. Since acetaldehyde is toxic, the body is very efficient at breaking it down and its clearance is much larger than that of ethanol (Fujimiya et al., 2002). Next, the effect of acetaldehyde administration at doses that produce similar concentrations was evaluated for their affect on neuroactive steroid levels. It is important to keep in mind that the acetaldehyde levels decline more rapidly following acetaldehyde vs ethanol administration since it is not continuously formed and metabolized. Pretreatment with cyanamide stabilized acetaldehyde levels across time to allow evaluation of acetaldehyde effects on 3$\alpha$,5$\alpha$-THP levels.

In the present study, plasma acetaldehyde concentrations were critical in determining the role of acetaldehyde in ethanol-induced increases in neuroactive steroids. We used a dose of ethanol (2g/kg) that produces peak increases in 3$\alpha$,5$\alpha$-THP levels. When plasma acetaldehyde concentrations following systemic acetaldehyde administration were lower than or comparable to levels observed following ethanol administration, no increase in 3$\alpha$,5$\alpha$-THP levels was observed. However, when plasma acetaldehyde concentrations were greater than levels following ethanol administration an increase in 3$\alpha$,5$\alpha$-THP levels was detected.

Previous studies with acetaldehyde administration involve various routes of administration as well as a variety of strains and species making it difficult to reliably compare results across studies. However, in studies with systemic acetaldehyde

administration, doses of at least 100 mg/kg were required to observe locomotor depression (Tambour et al., 2006) and sedative and hypnotic effects (Quertemont et al., 2004). This is the same dose required to elevate 3α,5α-THP levels in our study.

Indeed, we found that 100 mg/kg was the lowest acetaldehyde dose, without ALDH inhibition, that increased 3α,5α-THP levels, despite producing low acetaldehyde levels (approx 2.5 μM) compared to ethanol (7 μM). Since acetaldehyde is rapidly metabolized, its concentration would be expected to be low after 45 minutes without ALDH inhibition. Furthermore, when acetaldehyde levels were increased through ALDH inhibition, lower doses of acetaldehyde were capable of increasing 3α,5α-THP levels and produced plasma acetaldehyde concentrations greater than those seen after ethanol administration. This strongly suggests the importance of acetaldehyde concentration in producing or contributing to behavioral effects of ethanol.

When ethanol was administered to the rats, inhibition of ALDH increased acetaldehyde levels, but did not have any increased effect on 3α,5α-THP levels in the cortex when compared to animals treated with ethanol alone. However, the present results cannot rule out the possibility that acetaldehyde may contribute to the elevation of neuroactive steroids via synergistic actions with sub-maximal doses of ethanol. It is possible that very high concentrations of acetaldehyde alone are required in order to elicit increases in neuroactive steroids whereas lower doses, in conjunction with ethanol, may contribute to increased 3α,5α-THP levels. Indeed, acetaldehyde has been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis (Kinoshita et al., 2001), which is involved in neuroactive steroid synthesis. Furthermore, acetaldehyde is metabolized to acetate, which has also been shown to have CNS effects, although these appear to involve locomotor actions mediated by adenosine receptors (Carmichael et al., 1991).

The primary enzyme responsible for ethanol metabolism is alcohol dehydrogenase in the liver. In addition to alcohol dehydrogenase, CYP2E1 and catalase are also involved in ethanol metabolism. CYP2E1 is an inducible enzyme (Lieber and DeCarli, 1970) that plays a more important role in dependent individuals than after acute ethanol exposure. The catalase enzyme provides another pathway through which ethanol can be metabolized (Aragon et al., 1992) and plays a significant role in ethanol metabolism in the brain. Inhibition of catalase activity would be expected to have a marked effect on brain acetaldehyde concentrations, however, catalase inhibition did not affect cerebral cortical 3$\alpha$,5$\alpha$-THP levels in these experiments. Inhibition of alcohol dehydrogenase also failed to elicit any changes in 3$\alpha$,5$\alpha$-THP levels. However, inhibition of alcohol dehydrogenase activity is not as favorable for determining acetaldehyde's role in ethanol's effects because brain alcohol dehydrogenase activity is very low (Beisswenger et al., 1985).

The lack of effect of 4-methylpyrazole on acetaldehyde levels raises some concern. However, others have reported that inhibition of alcohol dehydrogenase fails to alter plasma acetaldehyde levels in the absence of an ALDH inhibitor (Quertemont and Didone, 2006). Since ethanol levels were increased up to 6 hrs following 4-methylpyrazole administration, we presume that alcohol dehydrogenase was inhibited. Changes in acetaldehyde levels may approach the limit of detection following 4-methylpyrazole administration. However, we are able to detect dose-dependent acetaldehyde levels following acetaldehyde administration as well as the expected increases when ALDH is inhibited. Hence, it is reasonable to conclude that ethanol-induced increases in 3$\alpha$,5$\alpha$-THP levels are independent of acetaldehyde. The lack of effect of 4-methylpyrazole on ethanol-induced increases in 3$\alpha$,5$\alpha$-THP levels also suggests that NADH/$NAD^+$ redox changes secondary to ethanol metabolism are not involved in this effect of ethanol. To date, there are not consistent results for alterations in alcohol dehydrogenase activity affecting ethanol consumption

patterns, suggesting that ALDH activity and acetaldehyde levels are important factors in regulating drinking behaviors.

Acetaldehyde has been implicated in some of the behavioral effects of ethanol although the precise role and mechanism of action remain unclear. Importantly, direct administration of acetaldehyde results in some of the same behavioral effects as ethanol providing a basis for the idea that acetaldehyde contributes to behavioral effects following ethanol administration. However, many experiments used higher concentrations of acetaldehyde then what would be expected from endogenous ethanol metabolism. Therefore, experiments conducted here focused on the effects of physiologically relevant acetaldehyde concentrations on 3$\alpha$,5$\alpha$-THP levels. Interestingly, when plasma acetaldehyde concentrations were comparable to levels found after ethanol administration there was no increase in cerebral cortical 3$\alpha$,5$\alpha$-THP levels suggesting that acetaldehyde is not responsible for ethanol-induced increases in neuroactive steroids.

Although these studies were performed in a rat model, the results may be applicable to humans. Adolescent males and females seen in the emergency room for alcohol intoxication had substantial increases in plasma levels of the neuroactive steroid 3$\alpha$,5$\alpha$-THP (Torres and Ortega, 2003; Torres and Ortega, 2004). Furthermore, various subjective effects of ethanol are diminished by prior administration of the neurosteroid biosynthesis inhibitor finasteride (Pierucci-Lagha et al., 2005). In contrast, laboratory administration of low or moderate ethanol doses had no effect on plasma 3$\alpha$,5$\alpha$-THP levels (Holdstock et al., 2006) or decreased 3$\alpha$,5$\alpha$-THP levels (Nyberg et al., 2005; Pierucci-Lagha et al., 2006). Though an explanation for these conflicting results is unresolved, the potential role of neurosteroids in human alcohol sensitivity has not been ruled out.

The present results support the theory that acetaldehyde modulates some of ethanol's effects rather than mediating them. However, the finding that high acetaldehyde

concentrations can increase neuroactive steroid levels leaves open the possibility that acetaldehyde could contribute to neurosteroid elevations after large quantities of ethanol consumption. Indeed, the extent of acetaldehyde's effects would vary between individuals depending upon the respective activities of their alcohol metabolizing enzymes and the amount of ethanol consumed. For example, human studies have noted that Native American populations, which have high rates of alcohol abuse and dependence, have polymorphisms in alcohol metabolizing enzymes that may account for drinking behaviors (Wall et al., 2003b). Other studies have suggested that high peripheral acetaldehyde concentrations are aversive and individuals with mutations in their ALDH2 gene metabolize acetaldehyde less rapidly and subsequently have a lower risk of developing alcoholism (for review Quertemont, 2004). In addition, animals with high ALDH activity have less acetaldehyde in their blood and tend to drink more ethanol (Quintanilla et al., 2005) while animals administered an adenoviral vector containing an ALDH2 antisense gene have reduced ALDH2 activity and reduced ethanol consumption (Ocaranza et al., 2008). Furthermore, ALDH2 knockout mice have increased blood acetaldehyde concentrations and drink less (Isse et al., 2005; Isse et al., 2002).

While speculative, it is intriguing to suggest a relationship between acetaldehyde and neuroactive steroids in risk for alcoholism. The production of neurosteroids is associated with an increased sensitivity to ethanol in rodents (for review Morrow et al., 2006) and possibly humans (Pierucci-Lagha et al., 2005). In the present study, high concentrations of acetaldehyde, which are associated with reduced drinking and risk for alcoholism, also increased neuroactive steroids. This effect may contribute to increased ethanol sensitivity and the decreased the risk for alcoholism. Therefore, while physiological concentrations of acetaldehyde do not appear to be responsible for ethanol-induced increases in neuroactive steroids in rats, the potential role of acetaldehyde cannot be excluded from contributing to

ethanol actions. Further studies will be necessary to clarify the importance of acetaldehyde in the effects of ethanol.

## Chapter IV

## Ethanol Induction of Steroidogenesis in Rat Adrenal and Brain is Dependent Upon Pituitary ACTH Release and *De Novo* Adrenal StAR Synthesis

### Summary

The mechanisms of ethanol actions that produce its behavioral sequelae involve the synthesis of potent GABAergic neuroactive steroids, specifically the GABAergic metabolites of progesterone, (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP), and deoxycorticosterone, (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC). I investigated the mechanisms that underlie the effect of ethanol on adrenal steroidogenesis. I found that ethanol effects on plasma pregnenolone, progesterone 3α,5α-THP and cortical 3α,5α-THP are highly correlated, exhibit a threshold of 1.5 g/kg, but show no dose dependence. Ethanol increases plasma ACTH, adrenal steroidogenic acute regulatory protein (StAR), and adrenal StAR phosphorylation, but does not alter levels of other adrenal cholesterol transporters including the peripheral benzodiazepine receptor (PBR), metastatic lymph node protein (MLN64) or the biosynthetic enzyme P450scc. Moreover, StAR and MLN64 levels are not altered by ethanol in brain, while PBR monomer levels are decreased. The inhibition of ACTH release, *de novo* adrenal StAR synthesis or P450scc activity prevents ethanol-induced increases in GABAergic steroids in plasma and brain. ACTH release and *de novo* StAR synthesis are independently regulated responses to ethanol administration and both are necessary, but not sufficient, for ethanol-induced elevation of plasma and brain steroids. These results suggest that ethanol enhances cholesterol transport via its effects on StAR protein and ACTH to stimulate increases in neuroactive steroids. Thus, both pituitary and

adrenal function are essential for ethanol-induced increases in circulating and brain neuroactive steroids. Since GABAergic steroids contribute to ethanol actions and ethanol sensitivity, the mechanisms of this effect of ethanol may be important factors that contribute to the behavioral actions of ethanol and risk for alcohol abuse disorders.

## Introduction

Neuroactive steroids are endogenous modulators of $GABA_A$ receptor function (see Belelli and Lambert, 2005, for review). They are allosteric modulators of $GABA_A$ activity and bind at specific sites on $\alpha$ subunits (Hosie et al., 2006). Neuroactive steroids act at both synaptic and extrasynaptic $GABA_A$ receptors and the most potent steroids are the 3$\alpha$-hydroxy ring-A reduced pregnane steroids (Paul and Purdy, 1992). Neuroactive steroid levels are rapidly altered following stress (Purdy et al., 1991) and may contribute to the behavioral effects of various psychoactive drugs including ethanol (see Morrow, 2007, for review).

Acute ethanol administration increases plasma and brain concentrations of GABAergic neuroactive steroids, including (3$\alpha$,5$\alpha$)-3-hydroxypregnan-20-one (3$\alpha$,5$\alpha$-THP) and (3$\alpha$,5$\alpha$)-3,21-dihydroxypregnan-20-one (3$\alpha$,5$\alpha$-THDOC) (Barbaccia et al., 1999; Morrow et al., 1999). These steroids are active at nanomolar concentrations and stimulate $GABA_A$ receptor mediated chloride conductance to potentiate the inhibitory actions of the receptor (Fodor et al., 2005; Majewska et al., 1986; Morrow et al., 1990; Morrow et al., 1987). In turn, these neuroactive steroids potentiate and/or mediate some of ethanol's actions. Indeed, inhibition of steroid biosynthetic enzymes, or the use of adrenalectomized rodents, has demonstrated that neuroactive steroids contribute to ethanol's inhibitory actions on medial septal and hippocampal neurons (Morrow et al., 2005; Tokunaga et al., 2003; VanDoren et al., 2000b), anxiolytic (Hirani et al., 2005), anticonvulsant (VanDoren et al., 2000b), hypnotic effects (Khisti et al., 2003b) and spatial learning deficits (Matthews et al.,

2002). Since ethanol-induced elevations in neuroactive steroids are important for ethanol actions, it is important to understand mechanisms that regulate the synthesis of these steroids.

Steroid levels fluctuate naturally and in response to various stressors and challenges. Steroidogenic organs include the adrenals, testis, ovaries, placenta, and brain. Biosynthesis of adrenal steroids is initiated upon stimulation by trophic hormones (Brownie et al., 1973). The cascade of signals that stems from trophic hormone stimulation increases cholesterol transport to the cytochrome P450 side chain cleavage (P450scc) enzyme that resides on the inner mitochondrial membrane. This is the rate-limiting step in steroidogenesis (Miller, 1988; Stocco, 2000) and is thought to be mediated by cholesterol transport proteins such as steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996) and the peripheral benzodiazepine receptor (PBR) (Lacapere and Papadopoulos, 2003). Indeed, mutations or deletions in the StAR gene disrupt steroid production causing congenital lipoid adrenal hyperplasia (Lin et al., 1995; Miller, 1997).

Since multiple glands can synthesize steroids, understanding mechanisms of steroidogenesis in adrenal and brain is critical for studying neuroactive steroids. Ethanol administration appears to mimic stress to activate the HPA axis and induce adrenal steroidogenesis. Indeed, previous studies have shown that ethanol-induced pituitary ACTH release appears to require both corticotrophin releasing factor (CRF) and vasopressin (Lee et al., 2004). Stress or ethanol-induction of neuroactive steroids in plasma and brain are completely prevented by adrenalectomy, although neuroactive steroids are still detectable in the brain of adrenalectomized rats (Khisti et al., 2003b; O'Dell et al., 2004; Porcu et al., 2004; Purdy et al., 1991). Further, administration of 5$\alpha$-dihydroprogesterone, the immediate precursor of 3$\alpha$,5$\alpha$-THP, to adrenalectomized animals restores the effect of ethanol on cortical 3$\alpha$,5$\alpha$-THP levels, demonstrating the possibility of brain steroidogenesis (Khisti et

al., 2003b). Moreover, ethanol-induced steroidogenesis has been directly demonstrated in hippocampal slices *in vitro* (Sanna et al., 2004) and recent studies have shown that ethanol increases StAR expression in rat brain (Serra et al., 2006). Therefore, whereas several studies have shown that ethanol increases neuroactive steroids, the adrenal mechanisms that are involved have not been elucidated and the role of the brain in ethanol-induced steroidogenesis *in vivo* remains unclear.

Using an *in vivo* rat model, this study examines the steroidogenic pathway including key enzymes, signaling molecules and cholesterol transport proteins to investigate which factors are critical for ethanol-induced increases of neuroactive steroids in adrenals and brain. Moreover, by concurrently measuring both plasma and brain steroid concentrations, I examined the importance of adrenal steroid synthesis for regulating brain neuroactive steroid levels.

## Results

First, I investigated the threshold and dose dependence of ethanol-induced increases in plasma pregnenolone and progesterone and as well as cerebral cortical 3$\alpha$,5$\alpha$-THP. There were significant differences between groups for plasma pregnenolone [$F(6,21) = 30.72$, $P < 0.0001$] and progesterone [$F(4,13) = 20.24$, $P < 0.0001$]. Ethanol (0.5 g/kg to 3.0 g/kg) induction of the 3$\alpha$,5$\alpha$-THP precursors pregnenolone and progesterone in plasma was only observed at doses of 1.5 g/kg ethanol and above (Fig. 4.1A,B). Interestingly, there does not appear to be a dose dependent effect of ethanol on plasma pregnenolone and progesterone as the 1.5 g/kg dose elicited a maximal response that was not increased by greater doses of ethanol. In cerebral cortex, 1.5 g/kg ethanol was also the lowest dose that increased 3$\alpha$,5$\alpha$-THP levels [$F(5,15) = 7.23$, $P = 0.0013$] demonstrating the same threshold response as plasma steroid levels (Fig. 4.1C). In addition, the plasma levels of pregnenolone and progesterone were highly correlated with one another ($r^2=0.94$, $p=0.0003$)

and plasma progesterone was correlated with cortical levels of the neuroactive steroid 3α,5α-THP ($r^2$=0.82, $p<0.05$) (Fig. 4.1D). Therefore, in subsequent experiments, either pregnenolone or progesterone was measured in plasma while 3α,5α-THP was measured in cerebral cortex.

ACTH is released from the pituitary to stimulate steroid production from the adrenals. To determine the role of ACTH in ethanol-mediated steroidogenesis, I investigated the effects of hypophysectomy and dexamethasone in adult rats. Ethanol (2 g/kg) increased ACTH levels of intact animals 18-fold (Fig. 4.2A). There was a main effect of both ethanol [$F(1,14) = 50.18$, $P < 0.0001$] and hypophysectomy [$F(1,14) = 58.06$, $P < 0.0001$] as well as an interaction between ethanol and hypophysectomy [$F(1,14) = 5058$, $P < 0.0001$]. Ethanol also increased plasma progesterone levels of intact animals 36-fold (Fig. 4.2B). There was a main effect of both ethanol [$F(1,14) = 228.8$, $P < 0.0001$] and hypophysectomy [$F(1,14) = 249.3$, $P < 0.0001$] as well as an interaction between ethanol and hypophysectomy [$F(1,14) = 233.7$, $P < 0.0001$]. Furthermore, ethanol increased cerebral cortical 3α,5α-THP levels of intact animals 9-fold (Fig. 4.2C). There was a main effect of both ethanol [$F(1,12) = 34.56$, $P < 0.0001$] and hypophysectomy [$F(1,12) = 23.68$, $P = 0.0004$] as well as an interaction between ethanol and hypophysectomy [$F(1,12) = 34.96$, $P < 0.0001$]. Hypophysectomized animals did not exhibit any changes in plasma ACTH, plasma progesterone, or cerebral cortical 3α,5α-THP levels (Fig. 4.2A,B,C).

Pretreatment with the synthetic steroid dexamethasone also prevented ethanol-induced increases in plasma ACTH, plasma progesterone, and cerebral cortical and hippocampal 3α,5α-THP. There were significant differences between groups for plasma

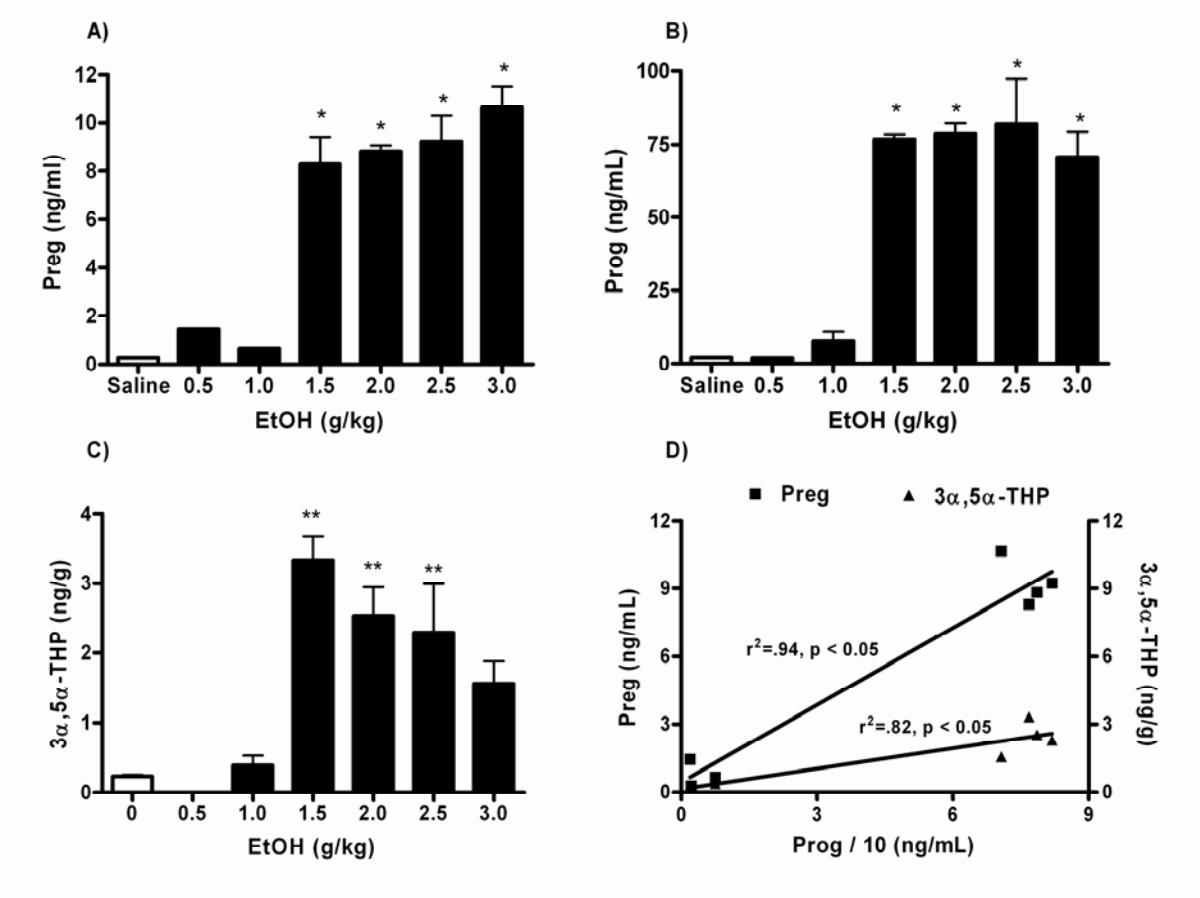


**Figure 4.1: Threshold for ethanol-induced increases in plasma steroids.** Ethanol was administered at varying doses and plasma and cerebral cortex were collected after 60 minutes to measure steroid levels. (A) plasma pregnenolone, (B) plasma progesterone and (C) cerebral cortical 3α,5α-THP. (D) Correlation between plasma precursors and cortical 3α,5α-THP. * $p < 0.001$ and ** $p < 0.05$ compared to saline control (one-way ANOVA followed by Newman-Keuls test), n = 4-5 for each group in duplicate.

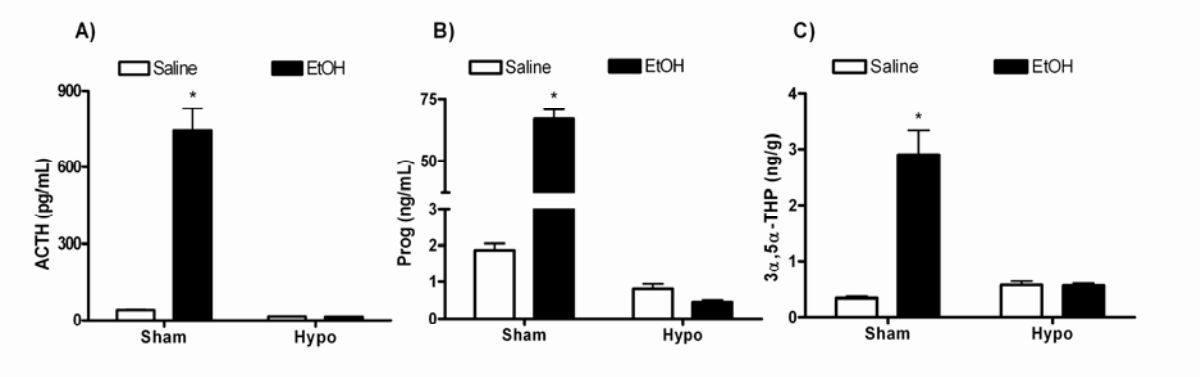


**Figure 4.2: Hypophysectomy abolishes ethanol-induced increases in ACTH release as well as plasma and brain steroid levels.** (A) plasma ACTH (B) plasma progesterone and (C) cerebral cortical 3$\alpha$,5$\alpha$-THP levels following hypophysectomy compared to sham-operated controls. * $p < 0.001$ compared to all groups, (Two-way ANOVA followed by Bonferroni test) n = 8-9 in duplicate.

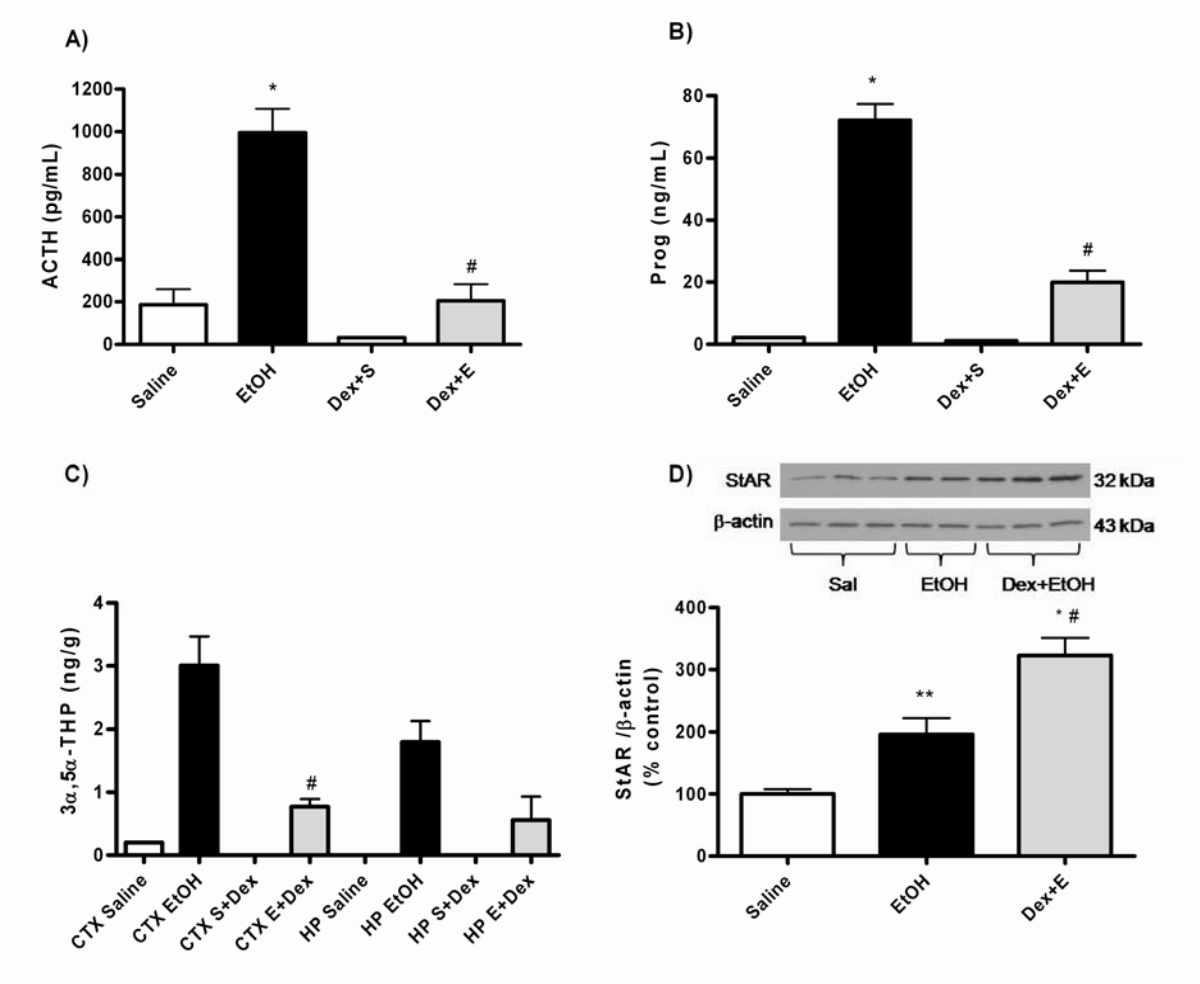


**Figure 4.3: Dexamethasone inhibits ACTH release as well as ethanol-induced increases in steroid levels.** Effect of dexamethasone (0.1 mg/kg, i.p.) for 90 minutes on the ethanol-induced increase in (A) plasma ACTH (B) plasma progesterone (C ) 3α,5α-THP in the cerebral cortex and hippocampus and (D) adrenal StAR protein expression. A representative blot is shown for adrenal StAR protein. StAR protein was normalized to β-actin and presented as % control. * $p < 0.001$ compared to control, ** $p < 0.01$ compared to control, # $p < 0.001$ compared to EtOH, (ANOVA, followed by Newman-Keuls test) n = 6 in duplicate.

ACTH [$F(3,19) = 28.52$, $P < 0.0001$; Fig. 4.3A], plasma progesterone [$F(3,19) = 110.5$, $P < 0.0001$; Fig. 4.3B], and cerebral cortical and hippocampal 3$\alpha$,5$\alpha$-THP levels [$F(3,19) = 9.07$, $P = 0.001$; Fig. 4.3C]. In contrast, dexamethasone enhanced ethanol-induced increases in adrenal StAR protein expression by 65% [$F(2,43) = 26.61$, $P < 0.0001$; Fig 3D], showing that ethanol-induced increases in StAR protein are not sufficient to promote steroidogenesis. Together, these results suggest the importance of ACTH for ethanol-mediated steroidogenesis.

In order to evaluate the importance of StAR protein in ethanol-induced steroidogenesis I investigated the time course and effects of ethanol dose on adrenal StAR expression. Similar to the plasma steroid levels seen in Figure 4.1, there was a threshold of 1.5 g/kg ethanol required to elicit increases in adrenal StAR expression (Fig. 4.4A). To investigate the temporal effects of ethanol, rats were administered ethanol (2 g/kg) at various time points and plasma progesterone levels were measured along with adrenal StAR protein levels. Adrenal StAR protein (32kDa) levels were elevated as early as twenty minutes and remained elevated across the two hour testing period (Fig. 4.4B) corresponding with increased steroid levels [$F(6,20) = 28.13$, $P < 0.0001$; Fig. 4.4D]. The maximal steroidogenic response occurred at 60 minutes corresponding to previous data examining the temporal response of progesterone in plasma and 3$\alpha$,5$\alpha$-THP in cerebral cortex (VanDoren et al., 2000b). No effect of ethanol on StAR (32 kDa) expression was observed in cerebral cortex or any brain region tested (Table 4.1). The 37 kDa form of StAR was not detected by the antibody under any condition in adrenal mitochondria or brain homogenates.

I also examined the effect of ethanol on other cholesterol transporters that may be involved in steroidogenesis. Rats were administered 2 g/kg dose of ethanol and PBR and metastatic lymph node 64 (MLN64) protein levels were measured 60 minutes later in adrenal and cerebral cortical fractions. Ethanol administration decreased expression of the

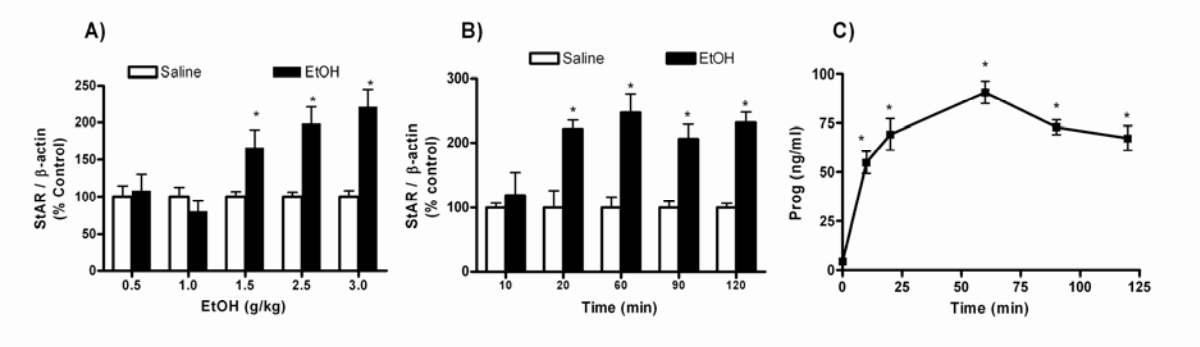


**Figure 4.4: Ethanol exhibits a threshold for adrenal StAR induction and StAR expression and plasma progesterone levels are rapidly increased.** Rats were administered ethanol and blood was collected at varying time points. (A) Adrenal StAR protein expression was measured by western blot analysis. Each group was compared to saline controls on separate blots, normalized to β-actin and converted to % control values (B) Time course of StAR protein induction (C) Time course of plasma progesterone induction. * $p < 0.01$ compared to control, (Student's *t* test or ANOVA followed by Newman-Keuls test where appropriate) n = 4-6 for each group in duplicate.

**Table 4.1: Ethanol decreases PBR expression in cerebral cortex but does not alter StAR or MLN64 expression.**

| | StAR (32kDa) | PBR (18kDa) | MLN64 (53kDa) |
|---|---|---|---|
| Saline | 100 ± 4.95 | 100 ± 5.88 | 100 ± 18.34 |
| EtOH | 101.5 ± 7.27 | 75.37 ± 4.79 * | 108.5 ± 26.63 |

Data represent mean values ± S.E.M. and are normalized to β-actin and presented as % control values from each western blot. * $p < 0.01$ compared to saline control (Student's *t* test), n = 8-16

PBR monomer (18 kDa) in the cerebral cortex (Table 4.1), but did not alter PBR or MLN64 levels in the adrenal (Fig. 4.5A,B) or MLN64 in brain (Table 4.1).

To determine if ethanol altered the activity of PBR, I investigated the effect of the antagonist, PK11195, on ethanol-mediated steroidogenesis by measuring effects on plasma progesterone. PK11195 (1 mg/kg) had no effect on ethanol-induced increases in steroid levels, but a selective high affinity ligand of PBR, CB34 (15 mg/kg), increased plasma steroid levels similar to ethanol [$F(4,12) = 168.3$, $P < 0.0001$; Fig. 4.5C]. Higher doses of PK11195 (5 and 10 mg/kg) also had no effect on ethanol-induced increases in steroid levels (data not shown).

A previous study suggested that only newly formed StAR is active and supports steroidogenesis (Artemenko et al., 2001). In order to determine if ethanol-induced elevation of neurosteroids is dependent upon *de novo* StAR synthesis, I tested the effect of the general protein synthesis inhibitor cycloheximide on ethanol-induced steroidogenesis. Simultaneous administration of cycloheximide with a 2 g/kg dose of ethanol 60 minutes before sacrifice prevented the ethanol-induced increases in StAR protein and plasma progesterone levels. There was a significant difference between groups for StAR protein [$F(3,24) = 15.29$, $P < 0.0001$; Fig. 4.6A] and plasma progesterone levels [$F(3,17) = 230$, $P < 0.0001$; Fig. 4.6B]. Ethanol-induced increases in cortical 3$\alpha$,5$\alpha$-THP levels were no longer detectable after simultaneous administration of cycloheximide (data not shown). In another experiment, cycloheximide was administered 40 minutes following ethanol but 20 minutes prior to sacrifice. This strategy more clearly prevented *de novo* StAR synthesis, and cycloheximide decreased ethanol-induced StAR protein expression and reduced steroid levels by 60%. There was a significant difference between groups for adrenal StAR protein expression [$F(2,20) = 6.36$, $P = 0.0073$; Fig. 4.7A], plasma progesterone levels

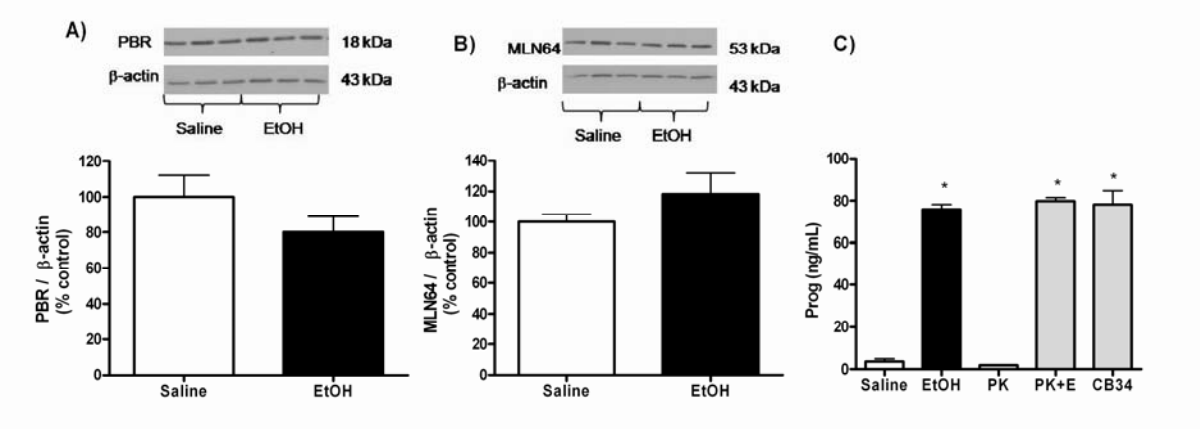


**Figure 4.5: Ethanol did not alter adrenal PBR or MLN64 expression, and inhibition of PBR did not affect ethanol-induced plasma progesterone levels.** Ethanol (2 g/kg) was administered and adrenals were collected after 60 minutes. (A) Adrenal PBR (18 kDa) and (B) MLN64 (53 kDa) levels were measured by western blot analysis. (C) Rats were pretreated with the PBR antagonist PK11195 (1 mg/kg, i.p.) 30 minutes prior to ethanol administration and steroid levels were measured 30 minutes post ethanol. Protein levels were normalized to β-actin and presented as % control * $p < 0.001$ compared to control (ANOVA followed by Newman Keuls test), n = 6-8 for each group in duplicate.

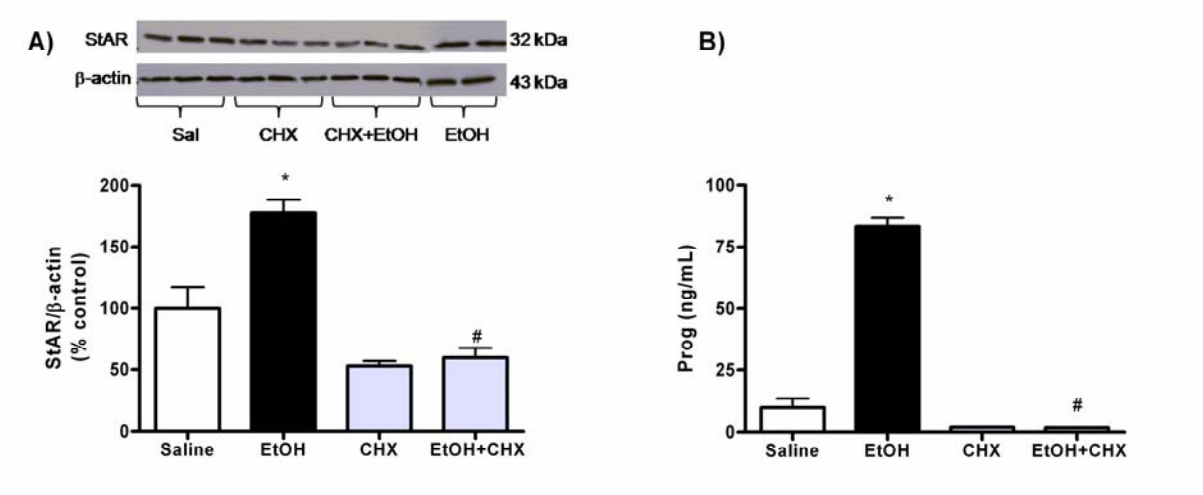


**Figure 4.6: Simultaneous administration of cycloheximide (CHX) with ethanol prevents ethanol-induced increases in StAR protein as well as plasma and brain steroid levels.** Cycloheximide (20 mg, i.p.) was administered with ethanol (2 g/kg) and tissue was collected 60 minutes later. (A) Adrenal StAR protein expression was measured by western blot analysis as shown in a representative blot. StAR was normalized to β-actin and presented as % control values (B) plasma progesterone levels * $p < 0.001$ compared to control and # $p < 0.001$ compared to ethanol (ANOVA followed by Newman Keuls test), n = 6 in duplicate.

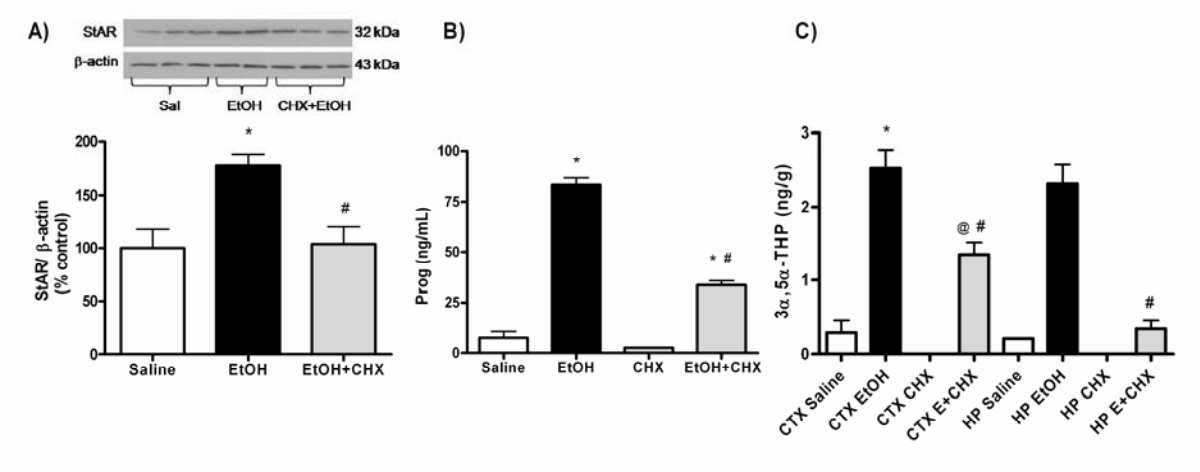


**Figure 4.7: CHX administration following ethanol arrests ethanol-induced increases in StAR as well as plasma and brain steroid levels.** Cycloheximide (20 mg, i.p.) was administered for the final 20 minutes of a 60 minute ethanol exposure (A) adrenal StAR expression was normalized to β-actin and presented as % control (B) plasma progesterone levels, and (C) 3α,5α-THP levels in cortex and hippocampus. * $p < 0.001$ compared to control, @ $p < 0.05$ compared to control and # $p < 0.01$ compared to ethanol (ANOVA followed by Newman Keuls tests), n = 6 in duplicate.

[F(3,20) = 205.8, P < 0.0001; Fig. 4.7B] and cerebral cortical and hippocampal 3α,5α-THP levels [F(4,20) = 19.23, P < 0.0001; Fig. 4.7C]. In addition to blocking the elevation in plasma steroids, cycloheximide inhibition also attenuated 3α,5α-THP increases in the cerebral cortex and the hippocampus.

StAR protein phosphorylation by PKA has been shown to be important for steroidogenesis (Arakane et al., 1997; Jo et al., 2005). In order to determine if ethanol alters phosphorylation of StAR, I examined phosphorylated StAR levels by immunoprecipitation with a phospho-PKA substrate antibody. First, the specificity of the phospho-PKA substrate antibody for phosphorylated proteins was confirmed by SDS-PAGE following incubation of adrenal fractions with Lambda phosphatase to dephosphorylate proteins (Fig. 4.8A). In addition, after solubilization and denaturation in RIPA buffer, SDS-PAGE analysis confirmed that StAR protein was not bound to any non-specific proteins that may be immunoprecipitated by the phospho-PKA substrate antibody (Fig. 4.8B). Furthermore, no band for StAR peptide was visible via Western blot from adrenal fractions immunoprecipitated with rabbit IgG signifying that immunoprecipitation with phospho-PKA substrate antibody is specific (Fig. 4.8C). Following confirmation of the antibody specificity, adrenal mitochondrial fractions were immunoprecipitated with the phospho-PKA substrate specific antibody, separated by SDS-PAGE, and probed for StAR. Ethanol administration to rats (2 g/kg) increased phosphorylation of adrenal StAR 5.7-fold compared to saline treated rats (Fig. 4.9). Furthermore, in the same adrenal fraction, ethanol-induced increases in total StAR increased 2.1-fold indicating that ethanol-mediated increases of StAR phosphorylation exceed its effect on StAR protein synthesis (p = 0.0095, Student's *t* test).

StAR protein is important for steroidogenesis, but the only enzyme known to convert cholesterol to pregnenolone is the P450scc enzyme (Miller, 2007b). Therefore, inhibition of this enzyme should prevent the formation of pregnenolone and neuroactive

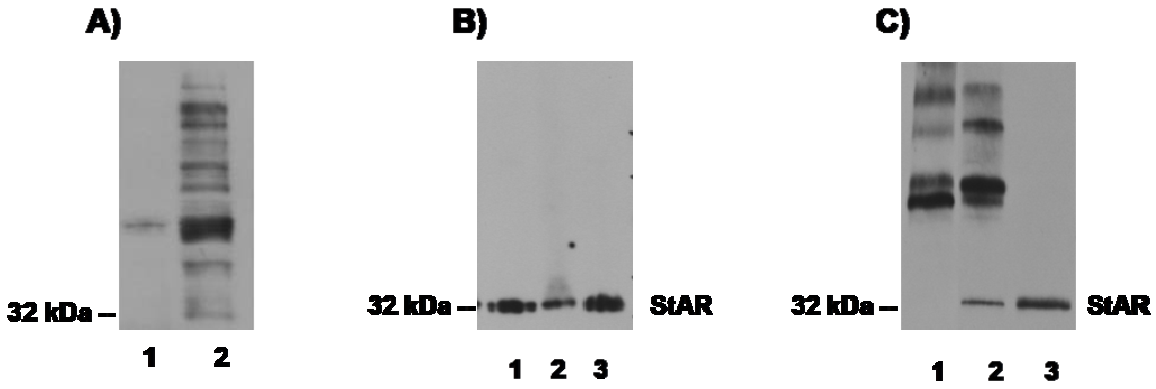


**Figure 4.8: Immunoprecipitation of phosphorylated StAR protein.** Adrenal mitochondrial fractions were prepared and (A) the specificity of phospho-PKA substrate antibody was tested by incubation of adrenal fraction (30 μg) with λ-phosphatase (50,000 units) to dephosphorylate all proteins (Lane 1), or phosphatase inhibitor (1:100, Lane 2). (B) Adrenal mitochondrial fractions were solubilized in RIPA buffer and run on SDS-PAGE to show that StAR protein is not bound to other proteins. Lane 1 shows heat denatured protein following RIPA solubilization, lane 2 shows RIPA solubilization without heat and Lane 3 shows the adrenal fraction denatured in SDS with no RIPA solubilization. (C) Phospho-PKA substrate antibody immunoprecipitated phosphorylated StAR. Lane 1 shows the negative control demonstrating that IgG antibody from the same species does not immunoprecipitate StAR. Lane 2 shows phospho-PKA substrate antibody immunoprecipitate of phosphorylated StAR and Lane 3 shows a positive control for adrenal StAR protein.

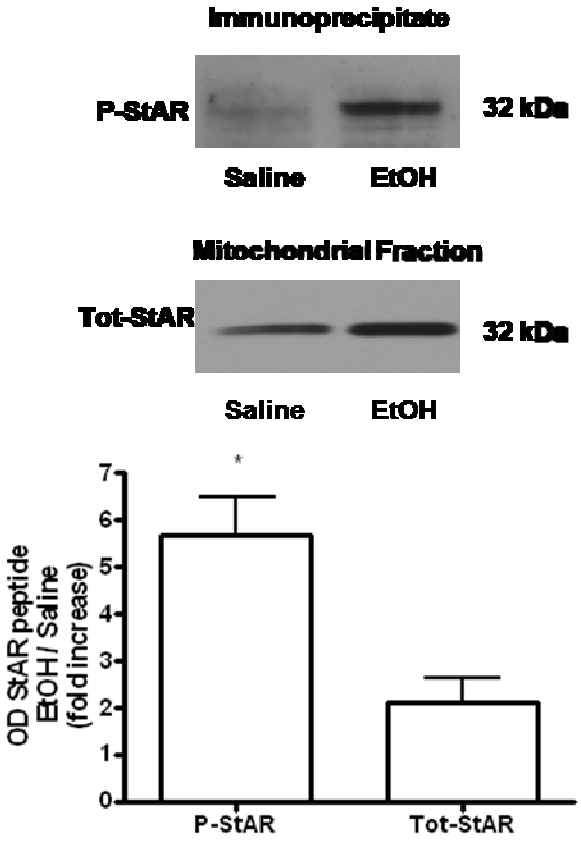


**Figure 4.9: Acute ethanol administration increases phosphorylation of adrenal StAR protein.** Rats were administered ethanol (2 g/kg) and tissues were collected 60 minutes later. Adrenal fractions were immunoprecipitated with phospho-PKA substrate antibody, run on SDS-PAGE and probed with StAR. Results are reported as fold increase of phospho-StAR (EtOH vs. saline) compared to fold increase of total StAR (EtOH vs saline) * $p < 0.01$ (Student's *t* test), n=4.

steroids irrespective of StAR protein levels. Aminoglutethimide was used to inhibit the P450scc enzyme and blocked ethanol-induced increases in plasma steroid levels by 58% [$F(2,29) = 39.83$, $P < 0.0001$; Fig. 4.10A]; although there was a slight increase in steroid levels from the inhibitor alone. Aminoglutethimide had no effect on ethanol-induced increases in adrenal StAR protein levels (Fig. 4.10B).

## Discussion

Although numerous studies have established that acute ethanol administration increases neuroactive steroids in plasma and brain, it has not been clear where ethanol acts to increase steroidogenesis. Because previous studies showed that adrenal integrity was required for ethanol-induced steroidogenesis in plasma and brain, the current study examined key steps in the adrenal steroidogenic pathway to attempt to determine which factors are required for ethanol-induced increases in neuroactive steroids. I hypothesized that adrenal and brain steroidogenesis would be differentially regulated, yet I found no ethanol-induced increases in cholesterol transport proteins in brain.

The results indicate that both pituitary-derived ACTH and *de novo* StAR synthesis in the adrenals are required for ethanol-induced increases in circulating and cerebral cortical levels of 3$\alpha$,5$\alpha$-THP, as well as circulating levels of pregnenolone and progesterone. Further, I show that ethanol increases adrenal StAR phosphorylation, which has previously been shown to enhance steroidogenesis in COS-1 and Leydig cells (Arakane et al., 1997; Jo et al., 2005). Although cholesterol transport to the inner mitochondrial membrane is the rate-limiting step in steroidogenesis, the ability of ethanol to increase StAR protein levels, independent of ACTH, appears to be necessary, but not sufficient for ethanol-induced steroidogenesis. Hypophysectomy and dexamethasone both prevented ethanol-induced increases in neuroactive steroids, even while StAR protein was enhanced by

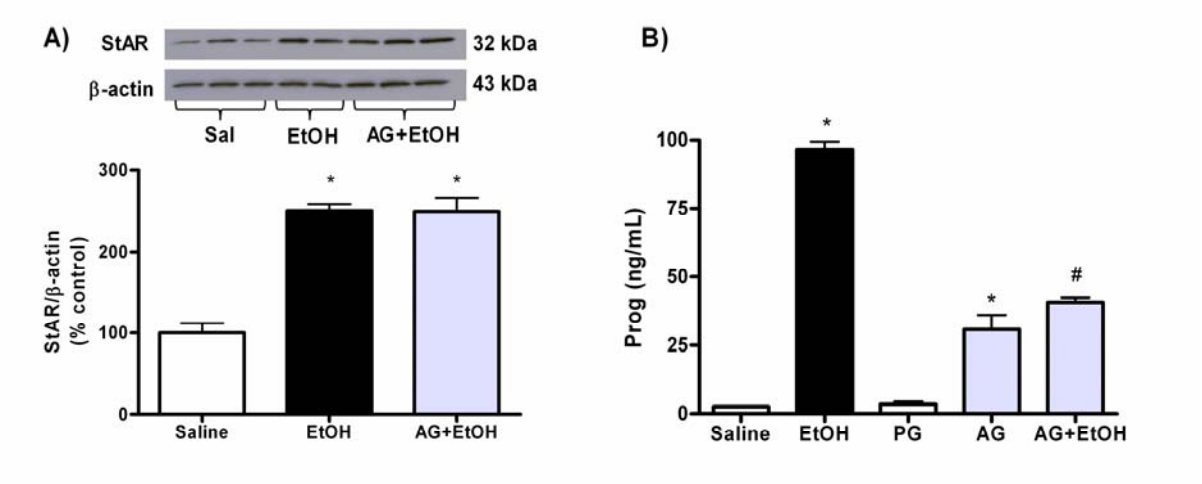


**Figure 4.10: Inhibition of P450scc by aminoglutethimide (AG) attenuates ethanol-induced increases in plasma steroids.** Rats were administered aminoglutethimide (20 mg, i.p.) or propylene glycol (PG) vehicle 60 minutes prior to ethanol or saline vehicle and tissue was collected 60 minutes post ethanol administration. (A) Adrenal StAR protein levels were measured by western blot analysis. StAR levels are normalized to β-actin and presented as % control (B) Plasma progesterone levels. * $p < 0.001$ compared to control and # $p < 0.001$ compared to ethanol (ANOVA followed by Newman Keuls test), n = 6 in duplicate.

ethanol. Ethanol-induced increases in brain 3α,5α-THP were also prevented by hypophysectomy and dexamethasone inhibition of ACTH as well as cycloheximide inhibition of adrenal StAR synthesis. Therefore, it appears that ethanol-induced increases in GABAergic neuroactive steroids in both plasma and brain are dependent upon pituitary activation to release ACTH and independent *de novo* StAR formation to promote adrenal steroidogenesis.

The findings of the present study suggest that ethanol-induced increases in neuroactive steroids result from stimulation of the HPA axis as well as effects on the adrenal independent of HPA axis activity. Indeed, ethanol activates the HPA axis (Ogilvie and Rivier, 1997) and increases ACTH release (Rivier et al., 1996). Since the adrenal is part of the HPA axis it can be difficult to separate these effects. Alterations in HPA axis signaling through inhibition of ACTH markedly altered steroidogenesis but did not block ethanol's effect on the adrenal steroidogenic protein StAR. This suggests that ethanol is capable of increasing adrenal StAR protein by a mechanism independent of ACTH. Another study, however, found that ACTH can regulate StAR synthesis in adrenal fasciculata cells (Nishikawa et al., 1996). ACTH may also act to free up cholesterol for use in steroid biosynthesis (Jefcoate, 2002) or stimulate StAR phosphorylation. Previous studies have shown that CRF and vasopressin are necessary for ethanol-induced pituitary ACTH release (Lee et al., 2004). CRF replacement, however, does not reverse the effect of dexamethasone on corticosterone synthesis although ACTH administration does (Cole et al., 2000). Therefore, while CRF is clearly critical in coordinating HPA axis responses (Sarnyai et al., 2001), and ethanol may activate signaling upstream of ACTH, CRF does not appear to stimulate adrenal steroidogenesis without the presence of ACTH.

The use of hypophysectomized animals further demonstrated the significance of ACTH signaling on ethanol-induced increases in adrenal steroidogenesis. One caveat of the hypophysectomized animals is that the adrenals are atrophied due to lack of stimulation.

Nevertheless, the results in hypophysectomized rats are consistent with the effects of dexamethasone, suggesting that ACTH signaling is needed for ethanol-induced steroidogenesis. Furthermore, hypophysectomy and dexamethasone administration not only inhibited ethanol-induced increases in plasma neuroactive steroids and but also similarly blocked the elevation of cerebral cortical and hippocampal neuroactive steroid levels. Many studies have examined the effects of ethanol on ACTH but the present study demonstrates that ACTH is necessary, but not sufficient, for ethanol-induced steroidogenesis.

Although ACTH stimulation of adrenal is important for steroidogenesis, cholesterol transport to P450scc is the rate-limiting step (Miller, 1988; Stocco, 2000). I investigated the effects of ethanol on the cholesterol transport proteins StAR, PBR, and MLN64 in adrenal and brain. Although StAR is initially synthesized as a 37 kDa pre-protein I was only able to measure the processed 32 kDa mitochondrial form. Although the 37 kDa form can mediate cholesterol uptake in nonsteroidogenic COS-1 cells, it is barely detectable in steroidogenic cells (Artemenko et al., 2001), and its processed form may be the major factor in cholesterol transport to P450scc (for review Jefcoate, 2002). I found that ethanol-induced increases in adrenal StAR protein expression corresponded with increases in plasma steroid levels. In addition to the temporal association, I looked for a dose-dependent response of StAR and steroid levels. Rather than a dose response, I found a threshold effect where ethanol induction of adrenal StAR protein and plasma steroid levels required a dose greater than 1.0 g/kg ethanol. Interestingly, this finding agrees with studies in humans where blood alcohol levels less than 0.1 mg% (comparable to a 1.0 g/kg injection), did not increase plasma steroid levels (Holdstock et al., 2006; Jenkins and Connolly, 1968; Pierucci-Lagha et al., 2006; Waltman et al., 1993). Rodent studies, however, have elicited mixed results, with studies finding steroids increased at ethanol doses ≥ 1.3 g/kg (VanDoren et al., 2000b) and others observing effects at 1.0 g/kg (Barbaccia et al., 1999; Serra et al., 2003). Genetic differences in rat strains may underlie these results. Still, it is worth noting that when a lower

dose of ethanol (1.0 g/kg) increases steroids, there is a corresponding increase in StAR protein (Serra et al., 2006). Both the temporal and dose relationship of StAR with steroid levels following ethanol administration suggest the importance of StAR protein in mediating ethanol-induced steroidogenesis.

MLN64 was examined in this study because it has been shown to have StAR-like activity in cells (Bose et al., 2000; Watari et al., 1997), but its expression in adrenals and brain was not changed by ethanol. It has been suggested that MLN64 plays a role in the placenta, where StAR is absent but steroidogenesis still occurs. Perhaps a more intriguing example is PBR, recently named the mitochondrial translocator protein. It has been suggested that StAR and PBR may work together to promote steroidogenesis (Miller, 2007a; Papadopoulos et al., 2007). In the present study, I did not detect any change in adrenal PBR expression following ethanol administration; possibly because PBR is already highly expressed in steroidogenic cells. PBR agonists have been shown to increase steroid levels (Serra et al., 1999) and the present study also found an increase comparable to that seen following ethanol administration. Further, I found no effect of the PBR antagonist PK11195 (1, 5, 10 mg/kg) on ethanol-induced increases in neuroactive steroids, suggesting that PBR does not mediate ethanol-induced steroidogenesis. Interestingly, 1 mg/kg of PK11195 has been shown to attenuate ethanol-induced anxiolysis (Hirani et al., 2005); a behavior commonly associated with neuroactive steroids. The Hirani et al. study, however, only measured behavioral effects, and not neuroactive steroid levels, so the lack of anxiolysis may be linked to a pharmacological effect of PBR antagonism unrelated to neuroactive steroid synthesis. Therefore, while PBR is clearly capable of steroidogenesis, it does not appear to be necessary for ethanol-induced steroidogenesis. Taken together, these studies also suggest, albeit indirectly, that StAR is the cholesterol transporter involved in ethanol-induced steroidogenesis.

A convincing role for StAR's importance in steroidogenesis is also evident in other animal and human studies. Generation of a StAR knockout mouse showed the absence of StAR to be lethal (Caron et al., 1997) and humans with mutations in the StAR gene have congenital lipoid adrenal hyperplasia (Miller, 1997). Thus, I investigated the importance of StAR for ethanol-induced steroidogenesis in more detail. If ethanol is increasing steroid levels by increasing cholesterol transfer to the P450scc, then inhibition of *de novo* StAR synthesis, a cholesterol transport protein, should diminish this ethanol effect. In fact, administration of cycloheximide concurrently with ethanol completely inhibited ethanol-induced increases in StAR protein expression and both plasma and brain steroid levels. It is important to note that cycloheximide is a general protein synthesis inhibitor so many proteins could be inhibited and potentially affect steroid synthesis.

To minimize inhibition of other proteins, I took advantage of the fact that StAR is rapidly synthesized and administered cycloheximide 40 minutes post ethanol administration and 20 minutes prior to sacrifice. This experiment showed that inhibition of *de novo* StAR synthesis arrested steroid production, although steroid levels were not completely blocked. When cycloheximide was administered with ethanol, there was complete blockade of steroid production. Together, these experiments demonstrate that inhibition of *de novo* StAR synthesis dramatically affects steroid levels and strongly supports its role as a major component of increases in ethanol-induced neuroactive steroid levels. Furthermore, synthetic hydroxycholesterols, which can cross the membrane freely, stimulate steroid synthesis in cells where StAR has been inhibited (Kim et al., 1997) providing further support for the essential role of cholesterol transport in steroidogenesis.

Although ethanol's ability to increase adrenal StAR protein is important for ethanol mediated steroidogenesis, StAR phosphorylation is critical for full steroidogenic activity of StAR (Arakane et al., 1997). In the present study, ethanol not only increased adrenal StAR protein expression but also increased StAR phosphorylation. Although the present study

cannot definitively conclude that this is PKA phosphorylation, a point mutation of the PKA phosphorylation site diminishes StAR activity (Arakane et al., 1997). Furthermore, experiments in Leydig cells demonstrated that protein kinase C (PKC) activation can elicit increases in StAR protein expression but PKC does not increase steroid levels or phosphorylate StAR. On the other hand, addition of a cAMP analogue increases StAR phosphorylation and steroid levels (Jo et al., 2005). Studies to determine if StAR phosphorylation is required for ethanol steroidogenesis *in vivo* are underway.

Although cholesterol transport is the rate-limiting step in steroidogenesis, steroid synthesis is still dependent upon the conversion of cholesterol to pregnenolone and subsequent metabolism to neuroactive metabolites. To date, the only known enzyme capable of converting cholesterol to pregnenolone is the P450scc enzyme (Miller, 2007c). Therefore, inhibition of this enzyme should prevent ethanol-induced increases in steroid levels. In the present study, inhibition of P450scc abolished ethanol-induced increases in steroid levels without having any effect on increases in StAR expression. This suggests that cholesterol was still being transported to the inner mitochondrial membrane and may account for the low level of steroid formation as cholesterol accumulated. Cholesterol accumulates at the inner mitochondrial membrane following P450scc inhibition and at the outer mitochondrial membrane following cycloheximide administration (Privalle et al., 1983). I therefore conclude that ethanol enhancement of cholesterol transport from the outer to inner mitochondrial membrane is necessary for its effect on steroidogenesis.

There is no doubt that the brain is a steroidogenic organ, but the results of the present study suggest that the increased neuroactive steroid levels measured by RIA after acute ethanol administration are dependent upon adrenal steroidogenesis. Indeed, neuroactive steroids are lipophilic and capable of crossing the blood brain barrier. Although the neuroactive metabolites can be synthesized peripherally and travel to the brain, it is likely that adrenal precursors also contribute to the central levels of GABAergic steroids

(Khisti et al., 2005; Kraulis et al., 1975; Wang et al., 1997). These precursors can be synthesized in the adrenals and metabolized to neuroactive metabolites in the brain with their regional distribution dependent upon steroidogenic enzyme expression (Li et al., 1997). Furthermore, no increases in cholesterol transport proteins were detected in the brain following ethanol administration. In fact, PBR levels of the 18 kDa monomer were decreased in the cerebral cortex. PBR monomers, however, can polymerize and I detected increased expression of bands corresponding to the molecular weight of a PBR dimer and trimer (data not shown). Thus, the decreased expression of PBR monomers may actually be the result of polymerization. Interestingly, PBR monomers are better suited for cholesterol binding, but reactive oxygen species, possibly as a result of ethanol metabolism, lead to polymerization (Delavoie et al., 2003).

Basal StAR protein expression (32 kDa) was observed in multiple brain regions, but I found no evidence for ethanol-induced increases in cortex or hippocampus, possibly because ethanol-induced increases in neuroactive steroids are dependent upon adrenal biosynthesis. Inhibition of *de novo* StAR synthesis, with cycloheximide, and ACTH production, with dexamethasone, both show that inhibition of adrenal steroid biosynthesis prevents ethanol-induced increases in brain 3$\alpha$,5$\alpha$-THP levels. Prior adrenalectomy experiments further emphasize the importance of peripheral steroidogenesis for controlling brain steroid levels. Adrenalectomized animals subjected to either stress, GHB, ethanol, nicotine, morphine, olanzapine or clozapine administration fail to show increases in cortical levels of the potent GABAergic neuroactive steroids (Khisti et al., 2003b; O'Dell et al., 2004; Porcu et al., 2004; Purdy et al., 1991, Concas, 2006 #5534, Marx, 2003 #4412). Thus, an interesting dynamic exists where the brain is dependent upon the periphery for increases in neuroactive steroids, yet, may still ultimately control its levels of GABAergic neuroactive steroids through HPA axis activation.

Since neuroactive steroids have been postulated to affect ethanol sensitivity and the risk for alcoholism, it is important to understand how ethanol affects neuroactive steroid synthesis. Chronic ethanol administration elicits ethanol tolerance in both rodents and humans. Although studies in humans are limited, ethanol dependent rodents do not exhibit increased neuroactive steroid levels (Janis et al., 1998) and have a blunted response to ethanol challenge (Khisti et al., 2005; Morrow et al., 2001). Furthermore, adrenalectomy, which reduces peripheral and central neuroactive steroids, increases ethanol withdrawal severity in mice (Gililland and Finn, 2007). Thus, the present study demonstrates the importance of adrenal StAR protein and plasma ACTH in regulating ethanol-induced increases in neuroactive steroids. Moreover, the results of this study will also be utilized as a comparison for the effects of chronic ethanol exposure to examine ethanol-induced alterations in steroidogenesis.

In conclusion, pituitary and adrenal function are essential for ethanol-induced increases in circulating and brain neuroactive steroids. Future studies manipulating proteins to control steroid biosynthesis may be beneficial for treatment of alcoholism and alcohol-related diseases, as well as various other neuropsychiatric disorders involving altered steroidogenesis.

## Chapter V

## Effects of Chronic Ethanol Exposure on Ethanol-Induced Increases in ACTH, *De Novo* StAR Synthesis and StAR Phosphorylation

### Summary

Acute ethanol administration increases potent GABAergic neuroactive steroids, specifically the GABAergic metabolites of progesterone, (3$\alpha$,5$\alpha$)-3-hydroxypregnan-20-one (3$\alpha$,5$\alpha$-THP), and deoxycorticosterone, (3$\alpha$,5$\alpha$)-3,21-dihydroxypregnan-20-one (3$\alpha$,5$\alpha$-THDOC). In addition, neuroactive steroids mediate and/or contribute to some of ethanol's actions. Chronic ethanol exposure results in tolerance to many effects of ethanol including ethanol-induced increases in neuroactive steroid levels. I investigated critical steroid biosynthetic enzymes and signaling molecules that may be altered by chronic ethanol exposure. Male Sprague-Dawley rats were administered ethanol via liquid diet for two weeks and protein expression and steroid levels were measured. I show that chronic ethanol exposure elicits tolerance to ethanol effects on plasma ACTH and the steroids pregnenolone and progesterone. Adrenal steroidogenic acute regulatory (StAR) protein expression is important for steroidogenesis, but chronic ethanol exposure does not result in tolerance to ethanol-induced increases in adrenal StAR protein. However, StAR phosphorylation is decreased when compared to the effect of acute ethanol administration. Rats exposed to chronic ethanol diet and subsequently challenged with ethanol (2 g/kg) had no changes in plasma ACTH but exhibited a blunted elevation of progesterone and cerebral cortical 3$\alpha$,5$\alpha$-THP. Administration of ACTH with the ethanol challenge restores the phosphorylation of adrenal StAR protein as well as plasma ACTH, progesterone, and

cerebral cortical 3α,5α-THP to levels observed in naïve rats administered ethanol. Thus, chronic ethanol exposure disrupts ACTH release that leads to tolerance to ethanol-induced increases in neuroactive steroid levels. Loss of the ethanol-induced increases in neuroactive steroids may contribute to behavioral tolerance to ethanol and influence the progression towards alcoholism.

## Introduction

Neuroactive steroids produce their effects on membrane receptors that regulate central nervous system activity rather than nuclear receptors that regulate gene expression. Thus, these steroid hormones are capable of eliciting rapid changes in neuronal excitability primarily through their enhancement of $GABA_A$ receptor activity (for review Belelli and Lambert, 2005). Neuroactive steroids can be synthesized *de novo* in the brain or produced peripherally in the adrenals and gonads. Potent GABAergic neuroactive steroids (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC) positively modulate $GABA_A$ receptor activity (Majewska et al., 1986; Morrow et al., 1990; Morrow et al., 1987) and can elicit many of the same effects as ethanol.

Systemic ethanol administration increases plasma and brain levels of neuroactive steroids that contribute to several of the behavioral effects of ethanol. In fact, experiments with administration of steroid biosynthetic enzyme inhibitors, or adrenalectomized animals, have demonstrated that neuroactive steroids are required for specific ethanol actions. Indeed, GABAergic neuroactive steroids potentiate and/or mediate ethanol's inhibitory actions on medial septal and hippocampal neurons (Morrow et al., 2005; Tokunaga et al., 2003; VanDoren et al., 2000b), as well as antidepressant-like actions in the forced swim test (Hirani et al., 2002), anxiolytic effects in the elevated plus maze (Hirani et al., 2005), anticonvulsant effects on bicuculine-induced seizures (VanDoren et al., 2000b), hypnotic effects measured by the duration of the loss of the righting reflex (Khisti et al., 2003b) and

spatial learning deficits in the water maze test where spatial cues map the location of a submerged platform (Matthews et al., 2002). Furthermore, the GABA agonist-like steroids substitute for ethanol in discrimination studies in rodents and monkeys (Grant et al., 1996; Hodge et al., 2001; Shannon et al., 2005) and exogenous administration can alter ethanol drinking patterns (Ford et al., 2007; Janak et al., 1998; Morrow et al., 2001; O'Dell et al., 2005). Thus, determining how GABAergic neuroactive steroids are synthesized and regulated following ethanol administration is important for understanding various ethanol actions.

We have recently reported that mechanisms of ethanol-induced increases in neuroactive steroids following acute ethanol administration are dependent upon pituitary ACTH release, *de novo* adrenal steroidogenic acute regulatory (StAR) protein synthesis, and cytochrome P450 side chain cleavage (P450scc) enzyme activity (Boyd et al., 2009). Indeed, ACTH release is important for adrenal stimulation (Rivier et al., 1984) and acute ethanol administration activates the HPA axis to synthesize the stress hormone corticosterone in rodents (Ellis, 1966; Ogilvie and Rivier, 1997; Rivier, 1996). Furthermore, HPA axis stimulation of adrenal steroidogenesis is also important for neuroactive steroid synthesis. Indeed, disruptions or alterations in HPA axis signaling markedly affect neuroactive steroid levels, and studies involving adrenalectomized animals have demonstrated the necessity of adrenals for ethanol-induced increases in plasma and brain neuroactive steroids (Khisti et al., 2003b; O'Dell et al., 2004; Porcu et al., 2004).

Although activation of the HPA axis is important for steroidogenesis, the rate-limiting step is the transfer of cholesterol from the outer mitochondrial membrane to P450scc enzyme residing on the inner mitochondrial membrane (Miller, 1988; Stocco, 2000). Cholesterol cannot pass freely across the mitochondrial membranes and must be assisted by protein transport. Acute ethanol administration has been shown to increase adrenal StAR protein concomitantly with plasma steroid levels (Khisti et al., 2003a), and we recently

demonstrated that *de novo* adrenal StAR synthesis is essential for ethanol-induced increases in plasma and brain neuroactive steroids (Boyd et al., 2009). In addition, a knockout of the StAR gene is lethal in mice (Caron et al., 1997) and mutations in humans disrupt steroid production causing congenital lipoid adrenal hyperplasia (Lin et al., 1995; Miller, 1997) further demonstrating its importance.

Chronic ethanol exposure results in a number of adaptive changes in central nervous system activity leading to tolerance and hyperexcitability. Although chronic ethanol exposure does not affect basal levels of neuroactive steroids, tolerance develops to ethanol-induced increases in steroid levels. Indeed, whereas acute ethanol administration increases neuroactive steroid levels, chronic ethanol exposure eliminates this effect (Janis et al., 1998) and blunts the steroid response to a subsequent ethanol challenge (Khisti, 2005; Morrow et al., 2001). In addition, adrenalectomized rodents have reduced levels of neuroactive steroids, exhibit tolerance to the sedative-hypnotic effects of ethanol (Khisti et al., 2003b) and display an increased ethanol withdrawal severity (Gililland and Finn, 2007). Further, during withdrawal from chronic ethanol exposure, withdrawal seizure-prone mice display tolerance to the anticonvulsant effects of 3$\alpha$,5$\alpha$-THP (Gililland-Kaufman et al., 2008). However, $GABA_A$ receptor sensitivity to 3$\alpha$,5$\alpha$-THP and 3$\alpha$,5$\alpha$-THDOC is enhanced in ethanol dependent rats (Devaud et al., 1996).

Since behavioral effects of ethanol are partially dependent on ethanol-induced steroidogenesis, the loss of this effect following chronic ethanol exposure may contribute to ethanol tolerance. Focusing on the mechanisms of steroidogenesis recently reported by our lab to be critical for increases in neuroactive steroids following acute ethanol administration, the present study, using an *in vivo* rat model, investigated alterations resulting from chronic ethanol exposure. Tolerance to ethanol-induced increases in neuroactive steroids may alter ethanol sensitivity and contribute to alcoholism and alcohol use disorders.

## Results

To determine if chronic ethanol administration elicits changes in steroid biosynthesis I first measured levels of the neuroactive steroids pregnenolone and progesterone. Rats were chronically exposed to ethanol through a nutritionally complete liquid diet and plasma steroid levels were measured. Following chronic ethanol administration, there were no changes in plasma ACTH, pregnenolone, or progesterone (Fig. 5.1A,B,C) compared to pair-fed control rats. Our lab has previously shown that there are also no increases in plasma or cerebral cortical 3$\alpha$,5$\alpha$-THP in dependent rats (Janis et al., 1998).

Next, I investigated the steroid response to an ethanol challenge (2 g/kg) in rats chronically exposed to ethanol. In rats administered control diet, ethanol challenge resulted in increased plasma ACTH levels compared to rats administered a saline challenge [$F(3,31) = 6.92$, $P = 0.0011$; Fig. 5.2A]. However, rats exposed to chronic dietary ethanol exhibited reduced plasma ACTH levels compared to the control diet group challenged with ethanol, and no change in plasma ACTH levels compared to rats fed control diet and challenged with saline. Furthermore, ethanol challenge in rats exposed to chronic ethanol resulted in no changes in plasma ACTH compared to chronic ethanol exposed rats administered a saline challenge. Similar results were also observed for plasma progesterone levels [$F(3,15) = 27.28$, $P < 0.0001$; Fig. 5.2B] as well as cerebral cortical 3$\alpha$,5$\alpha$-THP levels [$F(3,14) = 14.2$, $P = 0.0002$; Fig. 5.2C]. Indeed, ethanol administration to rats administered control diet increased steroid levels in plasma and brain that were lost following chronic ethanol exposure. Further, chronic ethanol exposure produced a blunted steroid response to ethanol challenge in both plasma and brain, but these levels were not significantly increased over saline challenge. Importantly, there does not appear to be any effect of the control diet on

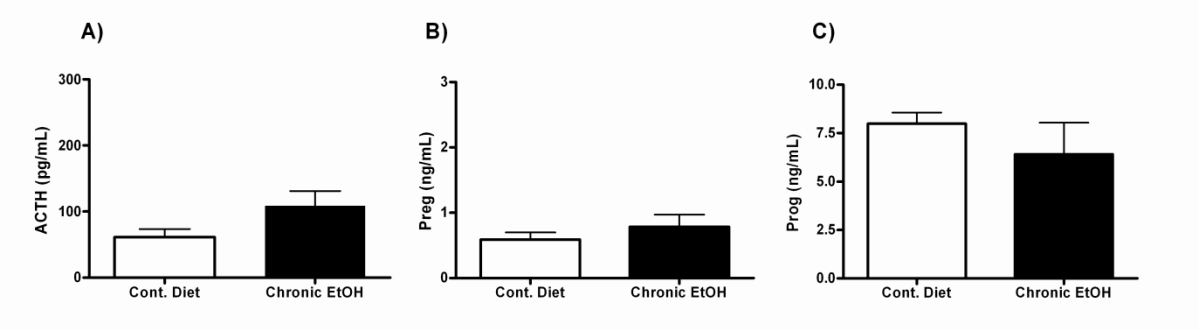


**Figure 5.1: Chronic ethanol exposure does not elicit changes in ACTH, pregnenolone or progesterone.** Chronic ethanol administration was via liquid diet and (A) plasma ACTH as well as plasma (B) pregnenolone and (C) progesterone were measured. n = 8-10 in duplicate.

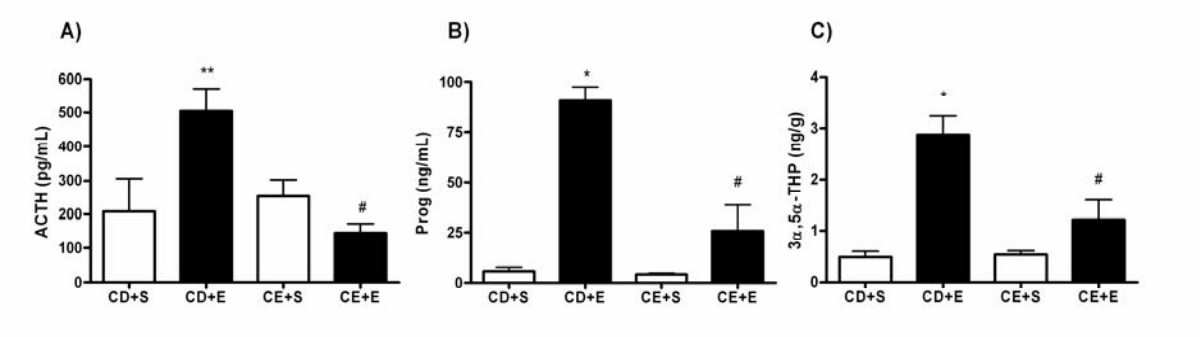


**Figure 5.2: Chronic ethanol exposure elicits no change in ACTH and a blunted steroid response to ethanol challenge.** Groups of rats administered control or ethanol diet for two weeks received a challenge injection of saline or ethanol (2 g/kg, i.p.), tissue was collected 60 minutes post-ethanol administration and plasma (A) ACTH and (B) progesterone, as well as (C) cerebral cortical 3$\alpha$,5$\alpha$-THP were measured. * $p < 0.001$ and **$p < 0.05$ compared to control diet + saline (CD+S), and #$p < 0.01$ compared to control diet + ethanol (CD+E) (ANOVA followed by Newman-Keuls test), n = 5-13 in duplicate.

ACTH or steroid levels and rats in this group respond to the ethanol challenge to the same extent as a naïve animal.

Since chronic ethanol exposure appeared to produce tolerance to ethanol-induced increases in pregnenolone and progesterone, I hypothesized that adaptations in the steroid biosynthetic pathway had occurred in the adrenals. Thus, I investigated the effects of chronic ethanol exposure on the expression of the cholesterol transport protein StAR. Chronic ethanol administration increases adrenal StAR protein 3.26-fold compared to control diet [$t(22) = 4.95$, $p < 0.0001$; Fig. 5.3A]. This increase in adrenal StAR protein expression is similar to that observed following acute ethanol administration (Fig. 5.4). However, a 2 g/kg ethanol challenge does not have any further effect on adrenal StAR protein compared to a chronic ethanol exposed rat challenged with saline (Fig. 5.3B). Furthermore, while acute ethanol administration increases StAR phosphorylation [$t(6) = 3.75$, $p = 0.0096$], chronic ethanol administration does not alter StAR phosphorylation compared to rats administered control diet, suggesting tolerance to ethanol enhanced StAR phosphorylation (Fig. 5.4).

Since rats chronically exposed to ethanol appear to be tolerant to increases in ACTH and neuroactive steroid levels following an ethanol challenge, I examined if exogenous ACTH replacement could restore ethanol-induced steroidogenesis. Separate groups of rats were administered liquid diet as described above. However, in this experiment, all groups received chronic dietary ethanol and I compared the effect of ACTH (2 μg) replacement combined with ethanol challenge to the effect of ethanol challenge alone. Ethanol challenge in combination with exogenous ACTH administration increased plasma ACTH [$F(3,29) = 10.24$, $P < 0.0001$; Fig. 5.5A] and progesterone [$F(3,30) = 25.35$, $P < 0.0001$; Fig. 5.5B], as well as cerebral cortical 3α,5α-THP levels [$F(3,30) = 7.39$, $P = 0.0008$; Fig. 5.5C]. There was no effect of ethanol challenge alone, in agreement with the experiments shown in Figure

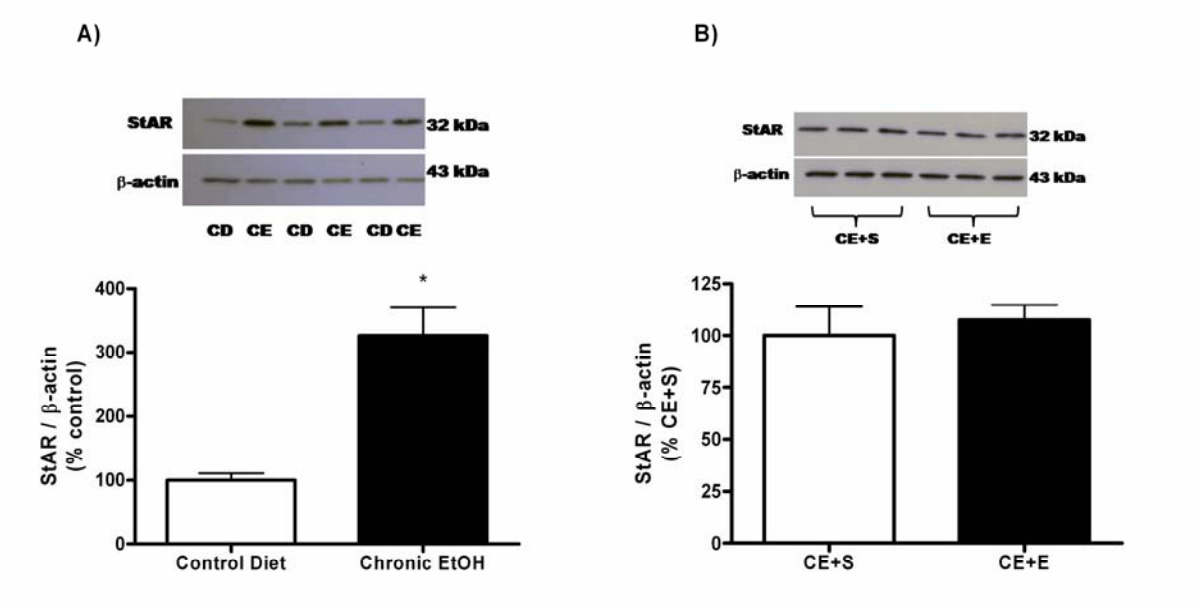


**Figure 5.3: Adrenal StAR protein is elevated following chronic ethanol exposure but is not further increased by an ethanol challenge.** (A) Adrenal StAR protein expression was measured via western blot analysis in rats exposed to liquid ethanol diet, normalized to β-actin and presented as % control diet. (B) Adrenal StAR protein expression was also measured in separate groups of rats were exposed to liquid ethanol diet and subsequently challenged with saline or ethanol (2 g/kg). Results are normalized to β-actin and presented as % chronic ethanol + saline. Representative blots are shown above their respective bar graphs. * $p < 0.0001$ compared to control diet (Student's *t* test), n = 8-12 in duplicate.

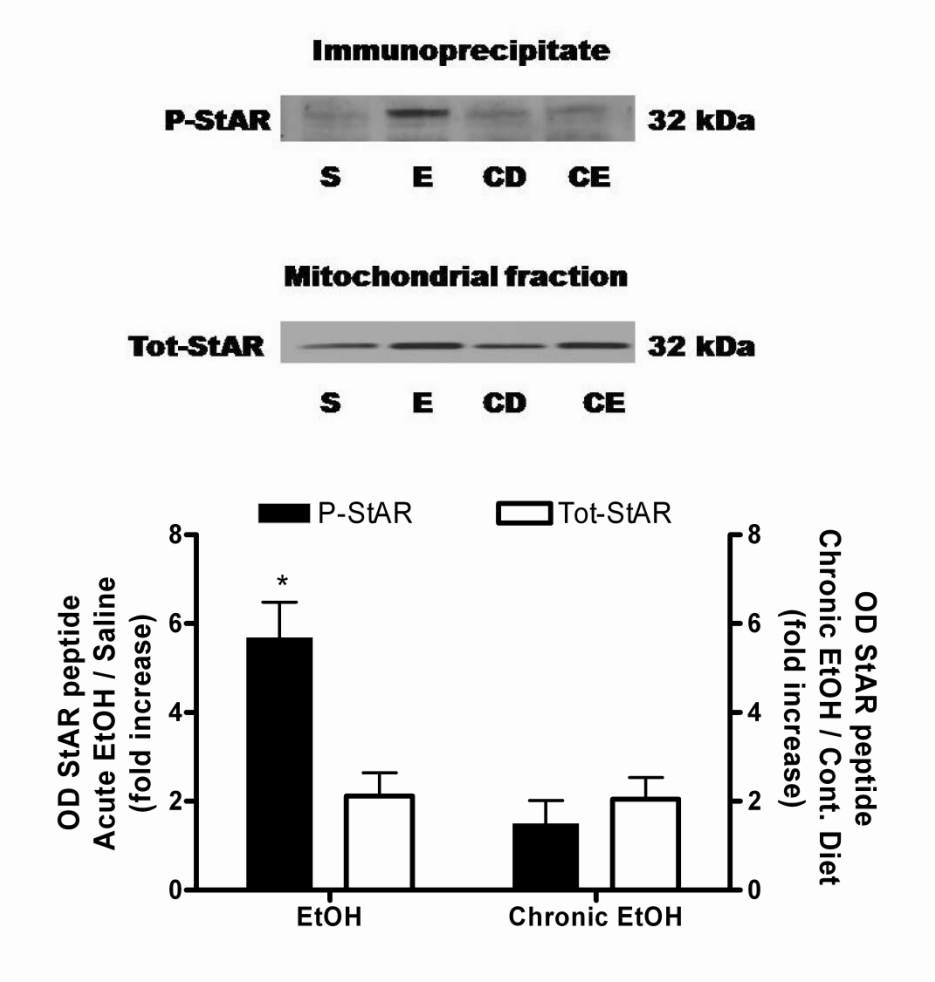


**Figure 5.4: Chronic ethanol exposure results in tolerance to increased phosphorylation of adrenal StAR protein.** Adrenal fractions were immunoprecipitated with phospho-PKA substrate antibody, separated by SDS-PAGE and probed with StAR. A representative blot is shown above the bar graph. For acute ethanol, results are reported as fold increase of phospho-StAR compared to saline. For chronic ethanol exposure, results are reported as fold increase of phospho-StAR compared to control diet. The fold increase of total StAR for both acute and chronic ethanol was also measured and compared to their respective controls. *$p < 0.01$ compared to total star for acute ethanol (Students *t* test), n = 4.

5.2. Remarkably, ACTH restored the effect of ethanol on steroid levels to similar concentrations as those found in naïve animals administered ethanol (Fig. 5.2).

Chronic ethanol exposed rats administered saline + ACTH have elevated ACTH levels compared to ethanol exposed rats administered saline without ACTH. Moreover, chronic ethanol exposed rats challenged with ethanol + ACTH show increased plasma ACTH compared to chronic ethanol exposed rats challenged with ethanol alone, as well as rats challenged with saline + ACTH. These rats also showed similar results for plasma progesterone levels as well as cerebral cortical 3$\alpha$,5$\alpha$-THP levels. Indeed, plasma progesterone and cerebral cortical 3$\alpha$,5$\alpha$-THP were increased in chronic ethanol exposed rats challenged with saline + ACTH compared to those challenged with saline alone. Furthermore, plasma progesterone and cerebral cortical 3$\alpha$,5$\alpha$-THP were increased in chronic ethanol exposed rats challenged with ethanol + ACTH compared to those challenged with ethanol alone, as well as those challenged with saline + ACTH.

Since ACTH administration following chronic ethanol exposure in rats restored the ethanol-induced increases in plasma and brain steroid levels, I also examined whether it reinstated the phosphorylation of StAR. Adrenals are from the same rats used for steroid measurements in figure 5.5 and results are presented as fold increase of phosphorylated or total StAR for each group compared to chronic ethanol exposed rats administered a saline challenge. Although not significantly different from each other, chronic ethanol exposed rats receiving either ethanol challenge, saline + ACTH challenge, or ethanol + ACTH challenge, all exhibited increases in phosphorylated StAR protein (Fig. 5.6). By comparison, total StAR was not elevated in any groups suggesting that the observed increases in phosphorylated StAR cannot be attributed to an increase in total StAR.

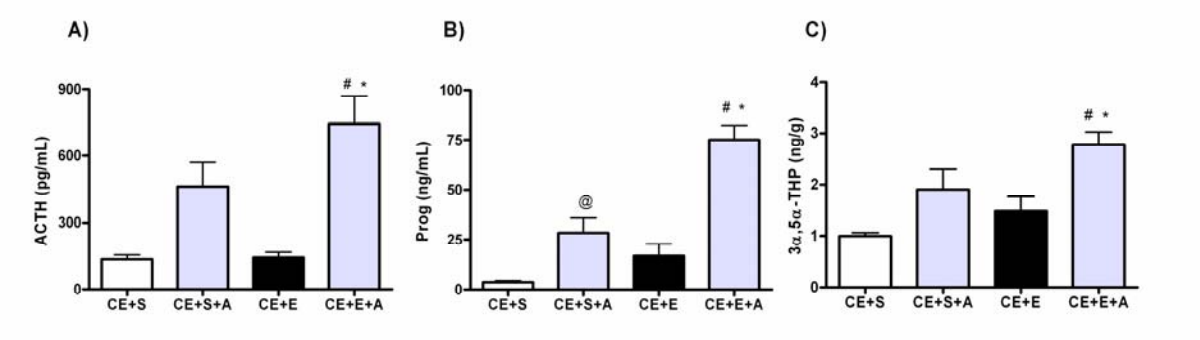


**Figure 5.5: Exogenous ACTH replacement restores plasma and brain neuroactive steroid levels following chronic ethanol exposure.** Rats exposed to chronic dietary ethanol were challenged with saline or ethanol (CE+S and CE+E respectively). ACTH (2 μg, i.p.) was also administered along with the saline or ethanol challenge (CE+S+A and CE+E+A respectively). Tissues were collected 60 minutes after challenge and plasma (A) ACTH and (B) progesterone, as well as (C) cerebral cortical 3α,5α-THP were measured. *p < 0.001 compared to CE+S, #p < 0.05 compared to CE+S+A and CE+E, and @p < 0.05 compared to CE+S (ANOVA followed by Newman-Keuls test), n = 8-9 in duplicate.

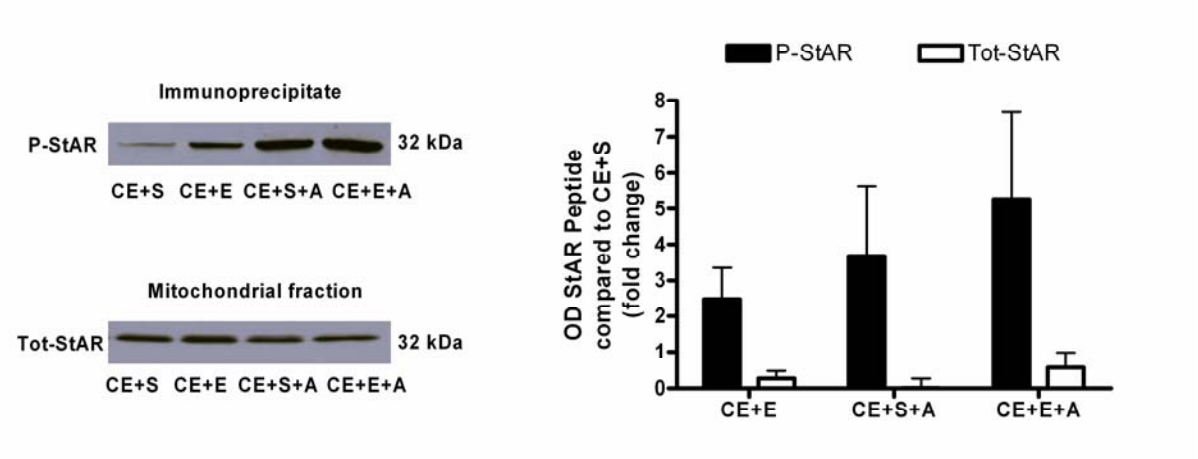


**Figure 5.6: Phosphorylation of adrenal StAR protein is increased by ethanol and ACTH following chronic dietary ethanol exposure.** Adrenal fractions were immunoprecipitated with phospho-PKA substrate antibody, separated by SDS-PAGE and probed with StAR antibody. A representative blot is shown above the bar graph. Results are reported as the fold increase of phospho-StAR for each group compared to the chronic ethanol + saline (CE+S) group. Total StAR was also measured and reported as fold increase for each group compared to fold increase of total StAR for CE+S group. Chronic ethanol + ethanol (CE+E), chronic ethanol + saline + ACTH (CE+S+A) and chronic ethanol + ethanol + ACTH (CE+E+A), n = 6.

## Discussion

Changes in $GABA_A$ receptor activity contribute to tolerance and dependence following prolonged exposure to ethanol (for review Grobin et al., 1998). The ethanol-induced increases in neuroactive steroids, which positively modulate $GABA_A$ receptor activity, following acute ethanol administration are lost after chronic ethanol exposure. The lack of neuroactive steroid production may influence $GABA_A$ receptor function and contribute to tolerance of ethanol's effects. The present study examined mechanisms important for ethanol-induced steroidogenesis that may be altered by chronic ethanol exposure to explain the attenuated steroid response. The results indicate that chronic ethanol exposure produces tolerance to ethanol-induced increases in ACTH that dramatically alter plasma and brain neuroactive steroid levels. Further, these alterations affect the ability of chronic ethanol exposed rats to respond normally to a subsequent ethanol challenge. The availability of StAR protein to transport cholesterol is not diminished following chronic ethanol exposure, but phosphorylation of StAR is no longer increased. In addition, exogenous ACTH replacement restores the neuroactive steroid response in plasma and brain as well as increasing StAR phosphorylation. Together, these results suggest that the inability of chronic ethanol exposed rats to synthesize sufficient amounts of neuroactive steroids is a result of a blunted ACTH response leading to diminished neuroactive steroid synthesis in adrenals and brain.

HPA axis activity is important for steroidogenesis and CNS functions as dysregulation of the HPA axis is associated with altered steroid responses as well as depression and various other mood disorders (McQuade and Young, 2000). When the HPA axis is functioning properly, steroids, including GABAergic neuroactive steroids, are increased by HPA axis activation but also provide negative feedback to inhibit CRF production and release, ACTH release, and corticosterone levels in rodents (Owens et al., 1992; Patchev et al., 1996; Patchev et al., 1994). In the present study, the blunted steroid

response observed after chronic ethanol exposure appears to be related to dysregulation of the HPA axis as I also observed blunted ACTH levels following chronic ethanol exposure. Indeed, ACTH stimulation of adrenal function is essential for steroidogenesis as well as adrenal integrity. In fact, the tolerance to increased ACTH levels I observed following chronic ethanol exposure was concomitant with tolerance to increased neuroactive steroid levels. These results are congruent with previous data where hypophysectomized rats displayed atrophied adrenals characterized by reduced weight and less steroidogenic output (Boyd et al., 2009; Colby et al., 1974) demonstrating the importance of ACTH signaling. Furthermore, the fact that tolerance to ethanol-induced increases in neuroactive steroids occurred in both plasma and brain further exemplifies the significance of HPA axis signaling and the adrenal for regulating circulating and central levels of neuroactive steroids.

The levels of pregnenolone, progesterone, and 3$\alpha$,5$\alpha$-THP are all blunted after chronic ethanol exposure suggesting that alterations occur early in the steroidogenic pathway. StAR protein, which mediates cholesterol transport required for steroidogenesis, was increased by acute ethanol administration and correlated with increased steroid levels (Khisti et al., 2003a). Since steroid levels are blunted following chronic ethanol exposure I hypothesized that I would observe changes in StAR protein expression as well. Conversely, adrenal StAR protein remained elevated following chronic ethanol exposure. However, tolerance to ethanol-induced phosphorylation of StAR was observed after chronic ethanol exposure and loss of StAR phosphorylation may contribute to reduced StAR activity (Arakane et al., 1997) irrespective of increased expression (Jo et al., 2005). Decreased StAR activity could reduce cholesterol transport to the P450scc for steroidogenesis and ultimately account for the reduction of neuroactive steroid levels.

Alterations in ACTH following chronic ethanol exposure may explain the blunted neuroactive steroid responses directly, due to adrenal stimulation, and indirectly, through phosphorylation of StAR. In the present study, exogenous ACTH administration to

dependent rats restored the ethanol-induced steroid response suggesting that ACTH is critical. ACTH is rapidly broken down, yet it is still present 60 minutes post ethanol administration in a naïve animal (Fig. 4.3) suggesting that it is continuously formed. Furthermore, while StAR was still increased following chronic ethanol exposure, steroid levels remained blunted without exogenous ACTH administration. This result suggests that ACTH might be involved in freeing up cholesterol for steroidogenesis or in activating the adrenal and cholesterol transport by StAR. However, chronic ethanol exposed rats challenged with ethanol still show increases in phosphorylated StAR despite the blunted ACTH release. This suggests that ACTH is not the only factor controlling phosphorylation of StAR and that ethanol may stimulate StAR phosphorylation via another mechanism. Nevertheless, when exogenous ACTH is administered with an ethanol challenge, the combination of increased StAR phosphorylation and restoration of ACTH levels elicits a full steroidogenic response. Thus, following chronic ethanol exposure, increases in adrenal StAR protein expression are not sufficient for steroidogenesis and require ACTH release and StAR phosphorylation.

Although the present study did not explore effects of chronic ethanol exposure on HPA axis function upstream of ACTH, these effects likely regulate ethanol-induced ACTH release. Indeed, knocking out the type 1 CRF receptor in mice blunts the ACTH response to ethanol, and repeated alcohol exposures blunts CRF and type 1 CRF receptor activity as well as ACTH (Lee et al., 2001a; Lee et al., 2001b). However, regardless of where proper functioning is altered, blunted ACTH release is an important consequence of chronic ethanol exposure. Following repeated ethanol exposures to rats, the ACTH response to an acute ethanol challenge has been shown to remain blunted for at least 21 days, which would correspond to over 1 year in humans (Rivier and Lee, 2001). Interestingly, in a subsequent study, the blunting of the ACTH response to acute ethanol challenge was not permanent (Lee and Rivier, 2003). However, if a short duration of repeated ethanol exposures can alter

ACTH function for an extended period of time then ethanol dependence might cause even more pronounced, or even permanent, effects. Furthermore, after repeated exposures to a stimulus such as ethanol, the HPA axis does not respond to the same stimulus, but remains capable of mounting a full response to different stimuli (Rivier and Lee, 2001). This result suggests that the HPA axis is extremely complex as ethanol is specific in blunting the axis responses to future ethanol exposures, while at the same time maintaining full HPA axis activation to new stressors. Perhaps ethanol is targeting extrahypothalamic mechanisms to initiate HPA axis activity.

Different laboratories employ various paradigms for chronic ethanol administration with differences in lengths and routes of exposure. Our method of two weeks of ethanol in a liquid diet reliably results in physical dependence on ethanol (Morrow et al., 1992). In addition, extending the ethanol diet to six weeks produced the same effect in terms of blunted ACTH and steroid levels (unpublished result). In the present study, ACTH and neuroactive steroid levels were blunted whether I challenged the animal on the 15$^{th}$ day of liquid diet or if I withdrew the ethanol diet and waited 24 hours before administering the ethanol challenge. Similar alterations in neuroactive steroid levels have been seen in other chronic models where the loss of neuroactive steroid production was linked to behavioral effects such as increased anxiety and seizure susceptibility (Cagetti et al., 2004; Devaud et al., 1995).

Since neuroactive steroids are required for many of the effects of ethanol, tolerance to ethanol-induced increases in neuroactive steroids may lead to increased consumption of alcohol. Indeed, chronic ethanol exposed rats that no longer have elevated 3$\alpha$,5$\alpha$-THP levels readily self-administer ethanol, but this can be reduced by 3$\alpha$,5$\alpha$-THP administration (Morrow et al., 2001). Conversely, 3$\alpha$,5$\alpha$-THP appears to be rewarding in non-dependent rats (Janak et al., 1998) suggesting that tolerance to increased steroid levels drives the rat

to drink more in order to try and achieve the same pharmacological effects. Genetics plays a factor in alcohol dependence as well as neuroactive steroid sensitivity (Finn et al., 1997b) and may explain the variability in steroid levels of chronic ethanol exposed rats challenged with ethanol.

Since neuroactive steroids are also postulated to contribute to ethanol sensitivity (Morrow et al., 2006) and HPA axis activation contributes to steroidogenesis, it is conceivable that proper HPA axis functioning plays a role in ethanol sensitivity. In fact, clinical studies have shown a link between alcoholism and alterations in HPA axis functioning (Adinoff et al., 2005; Lovallo et al., 2000). In rats, it has been suggested that increased drinking results from the loss of elevated ACTH and steroid responses once drinking begins rather than being attributed to low basal hormone levels (Richardson et al., 2008). In other words, alterations in HPA axis functioning and steroid levels appear to be an effect of chronic ethanol exposure rather than a predictor of increased alcohol consumption. In fact, the opioid antagonist naltrexone, which reduces relapse risk in human alcoholics, activates the HPA axis and increases ACTH and steroid levels (O'Malley et al., 2002). Naltrexone has also been shown to reverse the suppression of testosterone seen after chronic ethanol exposure (Emanuele et al., 1999). In addition, plasma levels of the HPA antagonist leptin are elevated in human alcoholics, correlating with ethanol consumption (Nicolas et al., 2001), and leptin injections in mice increase alcohol intake (Kiefer et al., 2001). Furthermore, leptin decreases the expression of StAR in adrenocortical cells and inhibits steroid synthesis (Cherradi et al., 2001). Together, these results suggest a link between HPA axis activity, StAR protein, neuroactive steroids and ethanol consumption.

Thus, proper HPA axis functioning, including pituitary ACTH release, and adrenal StAR protein activity, are critical for ethanol-induced increases in neuroactive steroids. Chronic ethanol exposure alters the HPA axis and blunts steroid responses to subsequent ethanol challenges. These phenomena contribute to ethanol tolerance and dependence and

restoring ACTH levels in dependent individuals may be therapeutic for alcoholism and associated withdrawal symptoms.

## Chapter VI

## Conclusions and Future Directions

The work carried out for this dissertation built upon previous findings in the field of neuropharmacology and evolved into an exploration into the mechanisms of neuroactive steroid synthesis following acute and chronic ethanol administration. Prior findings had demonstrated that neuroactive steroids are positive modulators of $GABA_A$ receptor activity that increase following acute ethanol administration and contribute to specific behavioral effects of ethanol. Furthermore, tolerance to some of the behavioral effects of ethanol, as well as tolerance to increased neuroactive steroid levels, develops following prolonged ethanol exposure suggesting that alterations in neuroactive steroid levels may play a role in ethanol tolerance. Thus, the present body of work attempts to identify the mechanisms by which acute ethanol administration increases neuroactive steroids, and to then use that knowledge to determine which of these processes are altered by chronic ethanol exposure.

I investigated ethanol-induced mechanisms of steroidogenesis using male Sprague-Dawley rats as our model. While animal models have their limitations for mechanistic studies, they can also provide valuable data, and were particularly useful in the present studies in order to examine the relative contributions to steroidogenesis from both adrenal and brain. Furthermore, the *in vivo* model maintains signaling from the CNS to the periphery so that important pathways involved in steroidogenesis, such as HPA axis signaling, remain intact. Hence, while the results of the present *in vivo* studies are important in their own right, they may also be further utilized as a guide for subsequent *in vitro* experiments delving further into critical mechanisms of steroidogenesis. The overall

conclusions of this project are that ethanol, possibly with some contributions from its primary metabolite acetaldehyde, increases neuroactive steroid levels by activating the HPA axis to initiate ACTH release. The effect of ethanol on ACTH is necessary, but not sufficient for ethanol-induced steroidogenesis. At the same time, ethanol increases the cholesterol transport protein StAR, as well as its phosphorylation, to facilitate increased cholesterol substrate for P450scc conversion to pregnenolone. Furthermore, while both the adrenals and brain are capable of synthesizing neuroactive steroids, the ethanol-induced increases in central and circulating neuroactive steroid levels are primarily driven by adrenal steroidogenesis. Consequently, the blunted steroid response observed after chronic ethanol exposure appears to be the result of alterations in HPA axis signaling leading to a blunted ACTH response and diminished StAR phosphorylation. Correcting for the loss of ethanol-induced increases in ACTH by administration of exogenous ACTH restores neuroactive steroid levels to those seen in a naïve animal and increases StAR phosphorylation. Understanding the mechanism of tolerance to ethanol-induced steroidogenesis may lead to strategies to reverse the effects of chronic ethanol exposure to alleviate alcohol withdrawal symptoms as well as increase ethanol sensitivity.

Acetaldehyde administration is capable of eliciting some of the same effects as ethanol but its ability to increase neuroactive steroid levels had never been investigated. I found that acetaldehyde can stimulate increases in neuroactive steroid levels at high concentrations but has no effect at concentrations commonly observed after ethanol administration. Furthermore, I also found that high concentrations of acetaldehyde that increase neuroactive steroid levels also increased adrenal StAR protein further supporting the importance of this protein for steroidogenesis. Although I cannot rule out the possibility that acetaldehyde contributes to ethanol-induced steroidogenesis, the main conclusion drawn from these experiments is that ethanol is responsible for the observed increases in

steroid levels. Nevertheless, these experiments suggest that acetaldehyde may increase ethanol-induced steroidogenesis that would be expected to increase ethanol sensitivity.

Neuroactive steroids have been associated with increased sensitivity to ethanol (Morrow et al., 2006) and changes in blood acetaldehyde levels are linked to ethanol consumption in rodents and humans. For example, studies in Native American populations, where there is a high prevalence of alcohol dependence, have found that certain polymorphisms are protective (Ehlers et al., 2004; Wall et al., 2003a). Interestingly, these polymorphisms lead to faster metabolism of ethanol and therefore a more rapid production of acetaldehyde. Furthermore, mutations affecting ALDH2 activity, such as those seen in Oriental populations, lead to increased acetaldehyde concentrations and are protective against the development of alcoholism (Thomasson et al., 1991). Hence, it is conceivable that the increased acetaldehyde levels observed in these situations are increasing neuroactive steroid levels thereby contributing to ethanol sensitivity and diminishing the risk for alcoholism. On the other hand, one may argue that the reduced drinking and protection from alcoholism in people with these polymorphisms are the result of the toxic profile of acetaldehyde and its negative associations to drinking. However, disulfiram is a drug used for the treatment of alcoholism and works by inhibiting acetaldehyde metabolism. Interestingly, the ALDH2 isozyme is resistant to disulfiram (Yoshida et al., 1984) suggesting that the protection inferred upon individuals with mutations in ALDH2 activity may not be due to the same factors as disulfiram; namely negative feelings from acetaldehyde accumulation. Therefore, future studies could investigate the relationship of not only neuroactive steroids, but also acetaldehyde, in regards to ethanol sensitivity.

Ethanol sensitivity is critical for predicting risk for alcoholism. This concept is intuitive as individuals who are sensitive to the effects of ethanol will tend to drink less than those who are insensitive, and lower consumption levels are less likely to lead to the development of alcoholism. Since neuroactive steroids play a role in ethanol sensitivity and chronic

ethanol exposure elicits tolerance to increased steroid levels, a big focus of this work was allocated to investigating the mechanisms regulating ethanol-induced steroidogenesis. Through either surgical removal of the pituitary gland or the administration of various inhibitors, I found that neuroactive steroid synthesis following acute ethanol administration is dependent upon ACTH release from the pituitary as well as *de novo* synthesis of adrenal StAR protein and P450scc activity. In addition, phosphorylation of adrenal StAR protein is increased following acute ethanol administration.

The mechanisms identified as being critical for ethanol-induced steroidogenesis following acute ethanol administration are exciting in their own right, but also serve as a guide for interpreting results obtained following chronic ethanol exposure. In other words, an adequate understanding of the acute effects of ethanol on steroidogenesis allows for us to observe what is altered by chronic ethanol for the ultimate purpose of identifying a therapeutic target. I found that chronic ethanol exposure leads to the development of tolerance to increases in ACTH as well as both plasma and brain steroid levels. Moreover, rats that underwent chronic ethanol exposure demonstrate a blunted ACTH and steroid response to a 2 g/kg ethanol challenge whether it is administered on the final day of ethanol diet or following 24 hour withdrawal. Interestingly, StAR protein remains elevated following chronic ethanol exposure suggesting that cholesterol transport could still occur. However, the increase in StAR phosphorylation observed after acute ethanol administration is lost following chronic ethanol exposure. Conceivably, the most striking result is that administration of ACTH to rats that underwent chronic ethanol exposure increases StAR phosphorylation and restores the neuroactive steroid response in both plasma and brain. Thus, this project has identified a critical mechanism for ethanol-induced steroidogenesis that is altered by chronic ethanol exposure, yet, when corrected, restores the steroid response to a similar level as that seen in a naïve animal.

While the results of these experiments are exciting, there is always more work to be done. By focusing our chronic ethanol experiments on steps in the steroidogenic pathway that were affected by acute ethanol administration, and choosing to ignore those that were not altered by acute ethanol administration, I may have missed certain effects of chronic ethanol exposure. For example, the cholesterol transport protein PBR did not appear to be altered in connection with ethanol-induced steroidogenesis and I detected no changes in its adrenal protein expression. However, PBR ligand binding is increased in response to neurotoxicity (Gavish et al., 1999) and thus future studies should examine PBR expression and function following chronic ethanol exposure.

Another example involves P450scc activity. I demonstrated that P450scc activity is important for ethanol-induced steroidogenesis but found no changes in protein expression following acute ethanol exposure. Considering that P450scc is the only known enzyme that converts cholesterol to pregnenolone (Miller, 2007b), it was somewhat surprising to find that very few studies exist on the effects of ethanol on P450scc. Interestingly, many studies in rodents and cells measure one particular steroid (i.e. corticosterone) and draw conclusions about P450scc activity based on whether they observed increases or decreases in steroid levels. Hence, if steroid levels are decreased then it is concluded that P450scc activity was decreased. However, these results can be somewhat misleading as the experiments are not designed to measure enzyme activity, and there is no control or knowledge of available substrate levels (i.e. cholesterol). Therefore, following chronic ethanol exposure when steroid levels are comparable to basal levels, it would be interesting to know if ethanol directly alters P450scc enzyme activity or whether it is indirectly affected because ethanol alters cholesterol availability or delivery to the enzyme. In other words, since P450scc expression remains unchanged, is the blunted steroid response due to ethanol-induced alterations in P450scc activity or would the enzyme function properly if cholesterol substrate was supplied.

At first glance, one might presume that chronic ethanol decreases P450scc activity compared to acute ethanol because pregnenolone levels are decreased. Furthermore, StAR levels remain elevated following chronic ethanol exposure suggesting that cholesterol can still be transported to P450scc on the inner mitochondrial membrane. However, based on the present study, as well as data in the literature, it is likely that chronic ethanol exposure causes alterations in HPA axis functioning that lead to reduced steroid levels. Moreover, tolerance to HPA axis activation would diminish ACTH release and ACTH is postulated to contribute to cholesterol availability (Jefcoate, 2002). Furthermore, following repeated ethanol exposures, HPA axis activation is tolerant to an ethanol challenge but can mount a full response to a footshock (Rivier and Lee, 2001). Thus, P450scc activity seems to remain intact and the lack of an ethanol-induced steroid response appears to be due to HPA axis tolerance. Although the focus of this project was on *in vivo* mechanisms of adrenal steroidogenesis, future studies could take the important steps identified, such as P450scc activity, and use an *in vitro* system to do more in depth mechanistic studies. Indeed, some studies are underway in the lab to examine the effects of increasing P450scc enzyme expression, and presumably its activity, on neuroactive steroid levels.

Phosphorylation of StAR had previously been shown to be important for full steroidogenic activity in COS-1 cells (Arakane et al., 1997) and was linked to steroidogenesis in our studies as well. If we examine the role of ACTH for a moment, it is possible that phosphorylation of StAR is directly related to increased ACTH secretion as ACTH stimulates cAMP production necessary for activation of PKA (Jefcoate, 2002). Without a commercially available antibody for phospho-StAR protein I used a phospho-PKA substrate antibody for immunoprecipitation experiments and then probed for StAR. While there is no doubt that acute ethanol administration increases StAR phosphorylation, and that this effect is lost following chronic ethanol exposure, I cannot say with absolute certainty that this is due to PKA. However, this appears to be likely as a point mutation of a PKA

consensus site in COS-1 cells inhibits steroidogenic output (Arakane et al., 1997). Nevertheless, I also attempted, to no avail, to demonstrate the importance of PKA phosphorylation for steroidogenesis by inhibiting PKA with H-89. However, while being marketed as a potent and selective inhibitor of PKA, H-89 affects at least eight other kinases (Lochner and Moolman, 2006) and kinase inhibition is extremely difficult to assess in an *in vivo* system. Interestingly, rolipram administration, which is a phosphodiesterase inhibitor that can prevent cAMP metabolism, was able to elicit increases in neuroactive steroids, albeit to a much lesser extent than ethanol. These results suggest that PKA activity plays a role in steroid biosynthesis, and this relationship should be further explored in an *in vitro* system where kinase activity can be easily manipulated. Furthermore, PKA RII $\beta$ subunit knockout mice consume more ethanol than wild-type controls and are less sensitive to the sedative effects of ethanol (Ferraro et al., 2006; Thiele et al., 2000). Moreover, the specific PKA inhibitor KT 5720 attenuated the anticonvulsant effects of ethanol (Lai et al., 2007). Thus, it would be interesting to determine if these effects are due to decreased steroid levels as a result of decreased phosphorylation of StAR.

Although there is at least enough evidence to cause speculation that ACTH directly leads to phosphorylation of StAR and contributes to steroidogenesis, data from this project suggests that ethanol-induced increases in adrenal StAR protein are distinct from increases in ACTH. However, both ACTH and adrenal StAR protein are required for full steroidogenic activity. Thus, a complex relationship exists where ethanol independently regulates two molecules that must both be present in order to achieve ethanol's full steroidogenic potential. The dexamethasone experiment demonstrated that inhibiting ACTH reduced steroid levels but did not diminish StAR expression. Furthermore, cycloheximide experiments demonstrated that StAR was critical for steroidogenesis, although ACTH was still elevated in those experiments. Taken together, these data suggest that ethanol acts on the HPA axis to stimulate ACTH release while also acting directly on the adrenal to increase

adrenal StAR expression; and both processes are necessary for ethanol induction of the steroidogenic response. This project identified StAR as a critical component of ethanol-induced steroidogenesis and future studies could explore exactly how ethanol increases StAR. Ethanol may act by increasing transcription through steroidogenic factor 1 (SF-1) but it may also affect StAR breakdown as well. Furthermore, StAR activity appears to be dependent upon its conformation (Miller, 2007a; Roostaee et al., 2009) and ethanol may be able to affect conformational changes to increase cholesterol transfer.

Perhaps one of the more intriguing findings of these studies is that the adrenals appear to control neuroactive steroid levels in periphery and brain. Given the fact that the brain is a steroidogenic organ, I initially hypothesized to find differential effects on adrenal and brain steroidogenesis. However, no matter how I manipulated adrenal steroidogenic proteins I always saw similar alterations in cerebral cortical 3$\alpha$,5$\alpha$-THP levels. In support of these findings, previous studies in adrenalectomized animals observed no increases in central neuroactive steroid levels following a variety of drugs that increase steroid levels in intact animals (Concas et al., 2006; Khisti et al., 2003b; Marx et al., 2003; O'Dell et al., 2004; Porcu et al., 2004). Yet, systemic administration of 5$\alpha$-DHP, the immediate precursor of 3$\alpha$,5$\alpha$-THP, can be converted to the neuroactive metabolite in brain of adrenalectomized animals (Khisti et al., 2003b). Furthermore, *de novo* steroid synthesis has been observed in brain slices at very high ethanol concentrations (Sanna et al., 2004) and in oligodendrocyte cell cultures given cholesterol substrate (Hu et al., 1987).

Interestingly, studies in human cell lines suggest that oligodendrocytes are the only CNS cell type capable of *de novo* steroidogenesis (Brown et al., 2000). They also found that, although not capable of converting cholesterol to pregnenolone, neurons could synthesize neuroactive metabolites provided precursors were supplied. Thus, it's likely that the brains steroidogenic capability allows it to respond and control specific processes under

normal conditions, but the adrenals are required to respond to challenges such as ethanol. In addition, one of the limitations of RIA for neuroactive steroid measurements in brain is that we measure steroid levels using large amounts of tissue. Therefore we may not be able to detect smaller or more discrete, localized changes in neuroactive steroid levels. Moreover, since adrenals are important for steroidogenesis, neuroactive steroids may also act at sites other than brain when delivered via systemic circulation. Nevertheless, this project identified key mechanisms of adrenal steroidogenesis that mediate ethanol-induced increases in neuroactive steroids in plasma and brain.

When interpreting the results of this project and comparing to studies in the literature, I would suggest that the two most interesting questions that immediately arise for future experiments are: 1) where does the cholesterol come from for steroidogenesis and 2) how is the HPA axis tolerant to one stimulus but able to fully respond to another. Cholesterol is abundant in cells but cholesterol available for steroid synthesis is postulated to be distinct from that used for structural integrity. In addition, StAR is only active on the outer mitochondrial membrane (Bose, 2002) and it is uncertain as to how cholesterol is transported to StAR. START proteins are proteins with a similar sequence as StAR and may be involved in transporting cholesterol to StAR (Alpy and Tomasetto, 2005). Uncovering this mechanism would be very interesting and may help clarify what is happening after chronic ethanol exposure. Maybe StAR is still increased because ethanol directly affects StAR expression but there is no cholesterol available to transport. Alternatively, the results of the present studies suggest that the loss of StAR phosphorylation after chronic ethanol exposure alters its function. Moreover, the ACTH replacement experiment suggests that ACTH regulates StAR phosphorylation and it may also affect cholesterol availability. Interestingly, cholesterol availability could also be linked to ethanol metabolism. Indeed, ethanol is metabolized to acetaldehyde and then acetate and acetate is a precursor for cholesterol.

As mentioned, another interesting topic for future studies is the idea that the HPA axis can become tolerant to a specific stimulus while still being able to respond to a new stressor (Dallman, 2007). Our studies demonstrated that there is tolerance to the ACTH response after chronic ethanol exposure. Interestingly, another study demonstrated that repeated ethanol exposure blunts the ACTH response to ethanol challenge but not footshock (Rivier and Lee, 2001). This suggests that there are important signaling pathways, possibly upstream of the HPA axis, where ethanol acts to blunt HPA axis activation. Indeed, in experiments with repeated restraint stress, tolerance develops in hypothalamic paraventricular nuclei (PVN) (Girotti et al., 2006). However, lesions of the paraventricular thalamus block this habituation (Bhatnagar et al., 2002). Furthermore, the limbic system can play a role in the stress response without directly innervating the PVN (Herman et al., 2004) demonstrating that upstream signaling pathways are involved in HPA axis activation. The present studies did not investigate mechanisms upstream of the pituitary and it would be of great interest to determine exactly how ethanol activates the HPA axis to initiate ACTH release and steroidogenesis.

The results uncovered in our rat model may also be applicable in humans. To date, the role of neuroactive steroids in alcohol actions in humans has elicited mixed results. Indeed, adolescent males and females seen in the emergency room for alcohol intoxication had elevated 3$\alpha$,5$\alpha$-THP levels (Torres and Ortega, 2003; Torres and Ortega, 2004). However, administration of alcohol to humans in a laboratory setting did not increase 3$\alpha$,5$\alpha$-THP and even decreased levels in some cases (Holdstock et al., 2006; Nyberg et al., 2005; Pierucci-Lagha et al., 2006). These differences may be explained by genetic factors or the amount of ethanol consumed. Indeed, $GABA_A$ receptor subunit polymorphisms have been identified and shown to have associations with ethanol dependence (for review Kumar et al., 2009). For example, a $GABA_A$ receptor $\alpha$2 subunit polymorphism associated with alcoholism

reduces sensitivity to alcohol and finasteride (Pierucci-Lagha et al., 2005). The blood alcohol concentrations of indivudals in these studies may also explain the observed effects on neuroactive steroid levels. Although not measured until hours later, admittance to the emergency room for alcohol intoxication would likely require very high blood alcohol levels. In contrast, humans administered alcohol in the laboratory setting had peak blood alcohol levels of about 0.08 mg% but levels below 0.1 mg% do not activate the HPA axis or steroidogenesis (Jenkins and Connolly, 1968; Waltman et al., 1993). Nevertheless, the steroid biosynthetic inhibitor finasteride can block various subjective effects of ethanol including sedative and anesthetic effects (Pierucci-Lagha et al., 2005). Thus, while not conclusive, these results suggest a role for neuroactive steroids in human ethanol sensitivity. Moreover, humans synthesize different neuroactive steroids than the rat and many of these steroids have never been measured in humans.

Taken as a whole, this project identified key mechanisms of ethanol-induced adrenal steroidogenesis and demonstrated that ACTH administration can restore the steroid response after chronic ethanol exposure. While this research is only a small microcosm of the alcoholism field, it is nonetheless important as neuroactive steroids may be beneficial for treatment of alcoholism and alcohol withdrawal. In fact, following chronic ethanol exposure, there is cross-tolerance between ethanol and benzodiazepines but increased sensitivity to neuroactive steroids (Devaud et al., 1996). There is a continuing need to develop effective treatments for alcoholism, but it is an extremely difficult challenge. Ethanol is a progressive disease making it difficult to correct damage and abnormalities that have been accumulating over time. Perhaps the lack of a good target has delayed the development of treatments. For example, a treatment that makes an individual more sensitive to the effects of ethanol may cause them to drink less, but would be tough to effectively market since it could also make the individual feel more intoxicated. On the other hand, a drug that blocks the effects of ethanol may cause an individual to drink more and that would have negative

consequences. One advantage of neuroactive steroids is that they are endogenous molecules likely to have very low toxicity. Thus, utilizing steroids for their anxiolytic and anticonvulsant properties as an effective treatment for alcohol withdrawal disorders is likely to be well tolerated. Moreover, neuroactive steroids would be predicted to reduce ethanol consumption, prevent or reverse inflammation and neurotoxicity, and restore normal stress responsiveness. The combination of these properties may have therapeutic utility for alcoholism.

Although the findings of these studies are focused on neuroactive steroids in relation to alcohol and the potential development of alcoholism or alcohol use disorders, they have much broader applications. Indeed, neuroactive steroids are also involved in anxiety, mood disorders, Parkinson's disease, and many other neuropsychiatric disorders. Since I studied the mechanisms of steroidogenesis, similar alterations to those discovered following ethanol may also exist for a variety of other disorders where steroid levels are altered. Therefore, not only can these results be used to understand and develop potential treatments for alcoholism, but StAR, ACTH, and other processes shown to be critical to steroidogenesis in this project could now be examined in some of these other neuropsychiatric disorders.

## References


Abe K, Yamaguchi S, Sugiura M and Saito H (1999) The ethanol metabolite acetaldehyde inhibits the induction of long-term potentiation in the rat dentate gyrus *in vivo*. *British Journal of Pharmacology* **127**:1805-1810.

Adibhatla RM and Hatcher JF (2008) Altered Lipid Metabolism in Brain Injury and Disorders. *Subcell Biochem* **49**:241-268.

Adinoff B, Junghanns K, Kiefer F and Krishnan-Sarin S (2005) Suppression of the HPA axis stress-response: implications for relapse. *Alcohol Clin Exp Res* **29**:1351-5.

Agis-Balboa RC, Graziano P, Zhubi A, Maloku E, Veldic M, Costa E and Guidotti A (2006) Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **103**:14602-14607.

Alpy F and Tomasetto C (2005) Give lipids a START: the StAR-related lipid transfer (START) domain in mammals. *J Cell Sci* **118**:2791-801.

Aragon CM, Rogan F and Amit Z (1992) Ethanol metabolism in rat brain homogenates by a catalase-$H_2O_2$ system. *Biochem.Pharmacol.* **44**:93-98.

Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM and Strauss III JF (1997) Phosphorylation of Steroidogenic Acute Regulatory Protein (StAR) Modulates its Steroidogenic Activity. *The Journal of Biological Chemistry* **272**:32656-32662.

Ariwodola OJ and Weiner JL (2004) Ethanol potentiation of GABAergic synaptic transmission may be self-limiting: role of presynaptic $GABA_B$ receptors. *J Neurosci* **24**:10679-86.

Artemenko I, Zhao D, Hales DB, Hales KH and Jefcoate CR (2001) Mitochondrial Processing of Newly Synthesized Steroidogenic Acute Regulatory Protein (StAR), but Not Total StAR, Mediates Cholesterol Transfer to Cytochrome P450 Side Chain Cleavage Enzyme in Adrenal Cells. *The Journal of Biological Chemistry* **276**:46583-46596.

Barbaccia ML, Affricano D, Trabucchi M, Purdy RH, Colombo G, Agabio R and Gessa GL (1999) Ethanol markedly increases "GABAergic" neurosteroids in alcohol-preferring rats. *Eur J Pharmacol* **384**:R1-R2.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN and Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* **50**:291-313.

Baulieu EE (1981) Steroid hormone regulation of the brain, in *Proceedings of International Symposium at Wenner-Gren Center, Stockholm* (Fuxe K, Gustafsson JA and Wetterber L eds) pp 3-14, Pergamon Press, Oxford.

Beisswenger T, Holmquist B and Vallee B (1985) chi-ADH is the sole alcohol dehydrogenase isozyme in mammalian brains: implications and inferences. *Proc Natl Acad Sci U S A* **24**:8369-8373.

Belelli D and Lambert JJ (2005) Neurosteroids: endogenous regulators of the $GABA_A$ receptor. *Nat Rev Neurosci* **6**:565-75.

Bhatnagar S, Huber R, Nowak N and Trotter P (2002) Lesions of the posterior paraventricular thalamus block habituation of hypothalamic-pituitary-adrenal responses to repeated restraint. *J Neuroendocrinol* **14**:403-10.

Bison S and Crews F (2003) Alcohol withdrawal increases neuropeptide Y immunoreactivity in rat brain. *Alcohol Clin Exp Res* **27**:1173-83.

Bose H, Lingappa, V.R., Miller, W.L. (2002) The Steroidogenic Acute Regulatory Protein, StAR, Works Only at the Outer Mitochondrial Membrane. *Endocrine Research* **28**:295-308.

Bose HS, Whittal RM, Huang M, Baldwin MA and Miller WL (2000) N-218 MLN64, a Protein with StAR-like Steroidogenic Activity, Is Folded and Cleaved Similarly to StAR. *Biochemistry* **39**:11722-11731.

Bowden SC, Crews FT, Bates ME, Fals-Stewart W and Ambrose ML (2001) Neurotoxicity and neurocognitive impairments with alcohol and drug-use disorders: potential roles in addiction and recovery. *Alcohol Clin Exp Res* **25**:317-21.

Boyd KN, Kumar S, O'Buckley TK and Morrow AL (2009) Ethanol Induction of Steroidogenesis is Dependent upon Pituitary ACTH Release and *De Novo* Adrenal StAR Synthesis. *Neuropsychopharmacology*:Submitted.

Boyd KN, O'Buckley TK and Morrow AL (2008) Role of acetaldehyde in ethanol-induced elevation of the neuroactive steroid 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one in rats. *Alcohol Clin Exp Res* **32**:1774-81.

Brinton RD (1994) The neurosteroid 3α-hydroxy-5α-pregnan-20-one induces cytoarchitectural regression in cultured fetal hippocampal neurons. *Journal of Neuroscience* **14**:2763-2774.

Brown N, Kerby J, Bonnert TP, Whiting PJ and Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human α4β3δ $GABA_A$ receptors. *Br J Pharmacol* **136**:965-74.

Brown RC, Cascio C and Papadopoulos V (2000) Pathways of neurosteroid biosynthesis in cell lines from human brain: regulation of dehydroepiandrosterone formation by oxidative stress and ß-amyloid peptide. *Journal of Neurochemistry* **74**:847-859.

Brownie AC, Alfano J, Jefcoate CR, Orme-Johnson W, Beinert H and Simpson ER (1973) Effect of ACTH on adrenal mitochondrial cytochrome P-450 in rat. *Ann N Y Acad Sci* **212**:344-360.

Cagetti E, Pinna G, Guidotti A, Baicy K and Olsen RW (2004) Chronic intermittent ethanol (CIE) administration in rats decreases levels of neurosteroids in hippocampus, accompanied by altered behavioral responses to neurosteroids and memory function. *Neuropharmacology* **46**:570-9.

Cameron HA and Gould E (1994) Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* **61**:203-9.

Cameron HA, Tanapat P and Gould E (1998) Adrenal steroids and N-methyl-D-aspartate receptor activation regulate neurogenesis in the dentate gyrus of adult rats through a common pathway. *Neuroscience* **82**:349-54.

Carmichael F, Israel Y, Crawford M, Minhas K, Saldivia V, Sandrin S, Campisi P and Orrgeo H (1991) Central nervous system effects of acetate: contribution to the central effects of ethanol. *Journal of Pharmacology & Experimental Therapeutics* **259**:403-408.

Caron KM, Soo SC, Wetsel WC, Stocco DM, Clark BJ and Parker KL (1997) Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. *Proc Natl Acad Sci U S A* **94**:11540-5.

Cherradi N, Capponi AM, Gaillard RC and Pralong FP (2001) Decreased expression of steroidogenic acute regulatory protein: a novel mechanism participating in the leptin-induced inhibition of glucocorticoid biosynthesis. *Endocrinology* **142**:3302-8.

Chrousos GP and Gold PW (1992) The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *Jama* **267**:1244-52.

Clark BJ, Wells J, King SR and Stocco DM (1994) The Purification, Cloning, and Expression of a Novel Luteinizing Hormone-induced Mitochondrial Protein in MA-10 Mouse Leydig Tumor Cells. *The Journal of Biological Chemistry* **269**:28314-28322.

Colby HD, Malendowicz LK, Caffrey JL and Kitay JI (1974) Effects of hypophysectomy and ACTH on adrenocortical function in the rat. *Endocrinology* **94**:1346-50.

Cole MA, Kim PJ, Kalman BA and Spencer RL (2000) Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies. *Psychoneuroendocrinology* **25**:151-67.

Collins MA, Zou JY and Neafsey EJ (1998) Brain damage due to episodic alcohol exposure in vivo and in vitro: furosemide neuroprotection implicates edema-based mechanism. *Faseb J* **12**:221-30.

Compagnone NA and Mellon SH (2000) Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol* **21**:1-56.

Concas A, Sogliano C, Porcu P, Marra C, Brundu A and Biggio G (2006) Neurosteroids in nicotine and morphine dependence. *Psychopharmacology (Berl)* **186**:281-92.

Crews F, Morrow AL, Criswell H and Breese G (1996) Effects of Ethanol on Ion Channels, in *International Review of Neurobiology, Vol 39* (Bradley RJ and Harris RA eds) pp 283-367, Academic Press, New York.

Crews FT, Collins MA, Dlugos C, Littleton J, Wilkins L, Neafsey EJ, Pentney R, Snell LD, Tabakoff B, Zou J and Noronha A (2004) Alcohol-induced neurodegeneration: when, where and why? *Alcohol Clin Exp Res* **28**:350-64.

Crews FT and Nixon K (2009) Mechanisms of neurodegeneration and regeneration in alcoholism. *Alcohol Alcohol* **44**:115-27.

Criswell HE and Breese GR (2005) A conceptualization of integrated actions of ethanol contributing to its GABAmimetic profile: A commentary. *Neuropsychopharmacology* **30**:1407-1425.

Crivello JF and Jefcoate CR (1979) The effects of cytochalasin B and vinblastine on movement of cholesterol in rat adrenal glands. *Biochem Biophys Res Commun* **89**:1127-34.

Dallman MF (2007) Modulation of stress responses: how we cope with excess glucocorticoids. *Exp Neurol* **206**:179-82.

Dawson DA, Goldstein RB, Chou SP, Ruan WJ and Grant BF (2008) Age at first drink and the first incidence of adult-onset DSM-IV alcohol use disorders. *Alcohol Clin Exp Res* **32**:2149-60.

De Witte P, Dahchour A and Quertemont E (1994) Acute and chronic alcohol injections increase taurine in the nucleus accumbens. *Alcohol Alcohol Suppl* **2**:229-33.

Delavoie F, Li H, Hardwick M, Robert JC, Giatzakis C, Peranzi G, Yao ZX, Maccario J, Lacapere JJ and Papadopoulos V (2003) In Vivo and in Vitro Peripheral-Type Benzodiazepine Receptor Polymerization: Functional Significance in Drug Ligand and Cholesterol Binding. *Biochemistry* **42**:4506-4519.

Devaud LL, Purdy RH, Finn DA and Morrow AL (1996) Sensitization of $\gamma$-aminobutyric $acid_A$ receptors to neuroactive steroids in rats during ethanol withdrawal. *J. Pharmacol. Exp. Ther.* **278**:510-517.

Devaud LL, Purdy RH and Morrow AL (1995) The neurosteroid, 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one, protects against bicuculline-induced seizures during ethanol withdrawal in rats. *Alcoholism, Clinical and Experimental Research* **19**:350-355.

Edenberg HJ and Kranzler HR (2005) The contribution of genetics to addiction therapy approaches. *Pharmacol Ther* **108**:86-93.

Ehlers CL, Spence JP, Wall TL, Gilder DA and Carr LG (2004) Association of ALDH1 promoter polymorphisms with alcohol-related phenotypes in southwest California Indians. *Alcohol Clin Exp Res* **28**:1481-6.

Ellis FW (1966) Effect of ethanol on plasma corticosterone levels. *Journal of Pharmacology and Experimental Therapeutics* **153**:121-127.

Emanuele NV, LaPaglia N, Steiner J, Kirsteins L and Emanuele MA (1999) Reversal of chronic ethanol-induced testosterone suppression in peripubertal male rats by opiate blockade. *Alcohol Clin Exp Res* **23**:60-6.

Fernandez-Sola J, Estruch R, Nicolas JM, Pare JC, Sacanella E, Antunez E and Urbano-Marquez A (1997) Comparison of alcoholic cardiomyopathy in women versus men. *Am J Cardiol* **80**:481-5.

Ferraro FM, 3rd, Sparta DR, Knapp DJ, Breese GR and Thiele TE (2006) Increased consumption but not operant self-administration of ethanol in mice lacking the RIIbeta subunit of protein kinase A. *Alcohol Clin Exp Res* **30**:825-35.

Finn DA and Gee KW (1994) The estrus cycle, sensitivity to convulsants and the anticonvulsant effect of a neuroactive steroid. *Journal of Pharmacology and Experimental Therapeutics* **271**:164-170.

Finn DA, Phillips TJ, Okorn DM, Chester JA and Cunningham CL (1997a) Rewarding effect of the neuroactive steroid 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one in mice. *Pharmacol. Biochem. Behav.* **56**:261-264.

Finn DA, Roberts AJ, Lotrich F and Gallaher EJ (1997b) Genetic differences in behavioral sensitivity to a neuroactive steroid. *J. Pharmacol. Exp. Ther.* **280**:820-828.

Fodor L, Biro T and Maksay G (2005) Nanomolar allopregnanolone potentiates rat cerebellar $GABA_A$ receptors. *Neuroscience Letters* **383**:127-130.

Follesa P, Biggio F, Talani G, Murru L, Serra M, Sanna E and Biggio G (2006) Neurosteroids, $GABA_A$ receptors, and ethanol dependence. *Psychopharmacology* **186**:267-280.

Ford MM, Mark GP, Nickel JD, Phillips TJ and Finn DA (2007) Allopregnanolone influences the consummatory processes that govern ethanol drinking in C57BL/6J mice. *Behav Brain Res* **179**:265-272.

Ford MM, Nickel JD, Phillips TJ and Finn DA (2005) Neurosteroid modulators of $GABA_A$ receptors differentially modulate ethanol intake patterns in male C57BL/6J mice. *Alcohol Clin Exp Res* **29**:1630-1640.

Fujimiya T, Yamaoka K, Ohbora Y, Aki T and Shinagawa H (2002) Michaelis-Menten elimination kinetics of acetaldehyde during ethanol oxidation. *Alcoholism: Clinical and Experimental Research* **26**:49S-54S.

Gabriel KI, Cunningham CL and Finn DA (2004) Allopregnanolone does not influence ethanol-induced conditioned place preference in DBA/2J mice. *Psychopharmacology (Berl)* **176**:50-6.

Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G and Weizman A (1999) Enigma of the peripheral benzodiazepine receptor. *Pharmacol.Rev.* **51**:629-650.

Gililland-Kaufman KR, Tanchuck MA, Ford MM, Crabbe JC, Beadles-Bohling AS, Snelling C, Mark GP and Finn DA (2008) The neurosteroid environment in the hippocampus exerts bi-directional effects on seizure susceptibility in mice. *Brain Res* **1243**:113-23.

Gililland KR and Finn DA (2007) 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. *Alcoholism: Clinical and Experimental Research* **31**:1846-1857.

Girotti M, Pace TW, Gaylord RI, Rubin BA, Herman JP and Spencer RL (2006) Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. *Neuroscience* **138**:1067-81.

Grant BF, Dawson DA, Stinson FS, Chou SP, Dufour MC and Pickering RP (2004) The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991-1992 and 2001-2002. *Drug Alcohol Depend* **74**:223-34.

Grant KA, Azarov A, Bowen CA, Mirkis S and Purdy RH (1996) Ethanol-like discriminative stimulus effects of the neurosteroid 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one in female *Macaca fascicularis* monkeys. *Psychopharmacology* **124**:340-346.

Griffin LD, Gong W, Verot L and Mellon SH (2004) Niemann-Pick type C disease involves disrupted neurosteroidogenesis and responds to allopregnanolone. *Nat Med* **10**:704-11.

Grobin AC, Matthews DB, Devaud LL and Morrow AL (1998) The role of $GABA_A$ receptors in the acute and chronic effects of ethanol. *Psychopharmacology* **139**:2-19.

Guth L, Zhang Z and Roberts E (1994) Key role for pregnenolone in combination therapy that promotes recovery after spinal cord injury. *Proc Natl Acad Sci U S A* **91**:12308-12.

Hamelink C, Hampson A, Wink DA, Eiden LE and Eskay RL (2005) Comparison of cannabidiol, antioxidants, and diuretics in reversing binge ethanol-induced neurotoxicity. *J Pharmacol Exp Ther* **314**:780-8.

Handwerger K (2009) Differential patterns of HPA activity and reactivity in adult posttraumatic stress disorder and major depressive disorder. *Harv Rev Psychiatry* **17**:184-205.

Harrison NL, Majewska MD, Harrington JW and Barker JL (1987) Structure-activity relationships for steroid interaction with the gamma-aminobutyric acid-A receptor complex. *J Pharmacol Exp Ther* **241**:346-353.

Harrison NL and Simmonds MA (1984) Modulation of the GABA receptor complex by an steroid anaesthetic. *Brain Research* **323**:287-292.

Herd MB, Belelli D and Lambert JJ (2007) Neurosteroid modulation of synaptic and extrasynaptic $GABA_A$ receptors. *Pharmacol Ther* **116**:20-34.

Herman JP, Mueller NK and Figueiredo H (2004) Role of GABA and glutamate circuitry in hypothalamo-pituitary-adrenocortical stress integration. *Ann N Y Acad Sci* **1018**:35-45.

Hirani K, Khisti RT and Chopde CT (2002) Behavioral action of ethanol in Porsolt's forced swim test: modulation by 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one. *Neuropharmacology* **43**:1339-1350.

Hirani K, Sharma AN, Jain NS, Ugale RR and Chopde CT (2005) Evaluation of GABAergic neuroactive steroid 3$\alpha$-hydroxy-5$\alpha$-pregnane-20-one as a neurobiological substrate for the anti-anxiety effect of ethanol in rats. *Psychopharmacology* **180**:267-278.

Hodge CW, Nannini MA, Olive MF, Kelley SP and Mehmert KK (2001) Allopregnanolone and pentobarbital infused into the nucleus accumbens substitute for the discriminative stimulus effects of ethanol. *Alcohol Clin Exp Res* **25**:1441-7.

Holdstock L, Penland SN, Morrow AL and De Wit H (2006) Moderate doses of ethanol fail to increase plasma levels of neurosteroid 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one-like immunoreactivity in healthy men and women. *Psychopharmacology* **186**:442-450.

Hommer D, Momenan R, Kaiser E and Rawlings R (2001) Evidence for a gender-related effect of alcoholism on brain volumes. *Am J Psychiatry* **158**:198-204.

Hosie AM, Wilkins ME, da Silva HM and Smart TG (2006) Endogenous neurosteroids regulate $GABA_A$ receptors through two discrete transmembrane sites. *Nature* **444**:486-489.

Hu ZY, Bourreau E, Jung-Testas I, Robel P and Baulieu E-E (1987) Neurosteroids: Oligodendrocyte mitochondria convert cholesterol to pregnenolone. *Proceedings of the National Academy of Sciences of the United States of America* **84**:8215-8219.

Isse T, Matsuno K, Oyama T, Kitagawa K and Kawamoto T (2005) Aldehyde Dehydrogenase 2 Gene Targeting Mouse Lacking Enzyme Activity Shows High Acetaldehyde Level in Blood, Brain, and Liver after Ethanol Gavages. *Alcoholism: Clinical and Experimental Research* **29**:1959-1964.

Isse T, Oyama T, Kitagawa K, Matsuno K, Matsumoto A, Yoshida A, Nakayama K, Nakayama K and Kawamoto T (2002) Diminished alcohol preference in transgenic mice lacking aldehyde dehydrogenase activity. *Pharmacogenetics* **12**:621-626.

Jamal M, Ameno K, Wang W, Kumihashi M, Ameno S, Ikuo U, Shinji A and Ijiri I (2005) Inhibition of acetaldehyde metabolism decreases acetylcholine release in medial frontal cortex of freely moving rats. *Brain Research* **1039**:90-96.

Janak PH, Redfern JEM and Samson HH (1998) The reinforcing effects of ethanol are altered by the endogenous neurosteroid, allopregnanolone. *Alcohol. Clin. Exp. Res.* **22**:1106-1112.

Janis GC, Devaud LL, Mitsuyama H and Morrow AL (1998) Effects of chronic ethanol consumption and withdrawal on the neuroactive steroid 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one in male and female rats. *Alcohol. Clin. Exp. Res.* **22**:2055-2061.

Jefcoate CR (2002) High-flux mitochondrial cholesterol trafficking, a specialized function of adrenal cortex. *The Journal of Clinical Investigation* **110**:881-890.

Jenkins JS and Connolly J (1968) Adrenocortical Response to Ethanol in Man. *British Medical Journal* **2**:804-805.

Jia F, Yue M, Chandra D, Keramidas A, Goldstein PA, Homanics GE and Harrison NL (2008) Taurine is a potent activator of extrasynaptic GABA(A) receptors in the thalamus. *J Neurosci* **28**:106-15.

Jo Y, King SR, Khan SA and Stocco DM (2005) Involvement of Protein Kinase C and Cyclic Adenosine 3',5'-Monophosphate-Dependent Kinase in Steroidogenic Acute Regulatory Protein Expression and Steroid Biosynthesis in Leydig Cells. *Biology of Reproduction* **73**:244-255.

Khisti RT, Boyd KN, Kumar S and Morrow AL (2005) Systemic ethanol administration elevates deoxycorticosterone levels and chronic ethanol exposure attenuates this response. *Brain Res* **1049**:104-11.

Khisti RT, Boyd, K.N., Kumar, S., Morrow, A. L. (2005) Differential effects of acute and chronic ethanol exposures on plasma and brain deoxycorticosterone levels, pp Online, Society for Neuroscience, 2005 Abstract Viewer/Itinerary.

Khisti RT, Kumar S and Morrow AL (2003a) Ethanol rapidly induces steroidogenic acute regulatory protein expression and translocation in rat adrenal gland. *Eur J Pharmacol* **473**:225-7.

Khisti RT, VanDoren MJ, Matthews DB and Morrow AL (2004) Ethanol-induced elevation of 3alpha-hydroxy-5alpha-pregnan-20-one does not modulate motor incoordination in rats. *Alcohol Clin Exp Res* **28**:1249-56.

Khisti RT, VanDoren MJ, O'Buckley TK and Morrow AL (2003b) Neuroactive steroid 3$\alpha$-hydroxy-5$\alpha$-pregnan-20-one modulates ethanol-induced loss of righting reflex in rats. *Brain Res* **980**:255-265.

Kiefer F, Jahn H, Wolf K, Kampf P, Knaudt K and Wiedemann K (2001) Free-choice alcohol consumption in mice after application of the appetite regulating peptide leptin. *Alcohol Clin Exp Res* **25**:787-9.

Kim Y, Ariyoshi N, Artemenko I, Elliot ME, Bhattacharyya KK and Jefcoate CR (1997) Control of cholesterol access to cytochrome P450scc in rat adrenal cells mediated by regulation of the steroidogenic acute regulatory protein. *Steroids* **62**:10-20.

Kimonides VG, Khatibi NH, Svendsen CN, Sofroniew MV and Herbert J (1998) Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) protect hippocampal neurons against excitatory amino acid-induced neurotoxicity. *Proc Natl Acad Sci U S A* **95**:1852-7.

King SR, Manna PR, Ishii T, Syapin PJ, Ginsberg SD, Wilson K, Walsh LP, Parker KL, Stocco DM, Smith RG and Lamb DJ (2002) An essential component in steroid synthesis, the steroidogenic acute regulatory protein, is expressed in discrete regions of the brain. *J Neurosci* **22**:10613-20.

King SR, Ronen-Fuhrmann T, Timberg R, Clark BJ, Orly J and Stocco DM (1995) Steroid Production after *in Vitro* Transcription, Translation, and Mitochondrial Processing of Protein Products of Complementary Deoxyribonucleic Acid for Steroidogenic Acute Regulatory Protein. *Endocrinology* **136**:5165-5176.

Kinoshita H, Jessop DS, Finn DP, Coventry TL, Roberts DJ, Ameno K, Jiri I and Harbuz MS (2001) Acetaldehyde, a metabolite of ethanol, activates the hypothalamic-pituitary-adrenal axis in the rat. *alcohol and alcoholism* **36**:59-64.

Kokate TG, Yamaguchi S, Pannell LK, Rajamani U, Carroll DM, Grossman AB and Rogawski MA (1998) Lack of anticonvulsant tolerance to the neuroactive steroid pregnanolone in mice. *J Pharmacol Exp Ther* **287**:553-8.

Korbo L (1999) Glial cell loss in the hippocampus of alcoholics. *Alcohol Clin Exp Res* **23**:164-8.

Korneyev A and Costa E (1996) Allopregnanolone (THP) mediates anesthetic effects of progesterone in rat brain. *Horm Behav* **30**:37-43.

Korneyev AY, Costa E and Guidotti A (1993) During anesthetic-induced activation of hypothalamic pituitary adrenal axis, blood borne steroids fail to contribute to the anesthetic effect. *Neuroendocrinology* **57**:559-565.

Korsten MA, Matsuzaki S, Feinman L and Lieber CS (1975) High blood acetaldehyde levels after ethanol administration. Difference between alcoholic and nonalcoholic subjects. *N Engl J Med* **292**:386-9.

Kraulis I, Foldes G, Traikov H, Dubrovsky B and Birmingham MK (1975) Distribution, metabolism and biological activity of deoxycorticosterone in the central nervous system. *Brain Research* **88**:1-14.

Krueger RJ and Orme-Johnson R (1983) Acute Adrenocorticotropic Hormone Stimulation of Adrenal Corticosteroidogenesis. *The Journal of Biological Chemistry* **258**:10159-10167.

Kumar S, Khisti RT and Morrow AL (2005) Regulation of native $GABA_A$ receptors by PKC and protein phosphatase activity. *Psychopharmacology* **183**:241-7.

Kumar S, Lane BM and Morrow AL (2006) Differential effects of systemic ethanol administration on PKC$\varepsilon$, $\gamma$ and $\beta$ isoform expression, translocation to membranes and target phosphorylation: Reversal by chronic ethanol exposure. *J Pharmacol Exp Ther* **319**:1366-1375.

Kumar S, Porcu P, Werner DF, Matthews DB, Diaz-Granados JL, Helfand RS and Morrow AL (2009) The role of GABA(A) receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology (Berl)*.

Kumar S, Sieghart W and Morrow AL (2002) Association of protein kinase C with $GABA_A$ receptors containing $\alpha$1 and $\alpha$4 subunits in the cerebral cortex: selective effects of chronic ethanol consumption. *J Neurochem* **82**:110-7.

Lacapere JJ and Papadopoulos V (2003) Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. *Steroids* **68**:569-585.

Lai CC, Kuo TI and Lin HH (2007) The role of protein kinase A in acute ethanol-induced neurobehavioral actions in rats. *Anesth Analg* **105**:89-96.

Lee S and Rivier C (1995) Altered ACTH and corticosterone responses to interleukin-1$\beta$ in male rats exposed to an alcohol diet: possible role of vasopressin and testosterone. *Alcohol Clin Exp Res* **19**:200-208.

Lee S and Rivier C (2003) Long-term influence of an initial exposure to alcohol on the rat hypothalamic-pituitary axis. *Alcohol Clin Exp Res* **27**:1463-70.

Lee S, Schmidt ED, Tilders FJ and Rivier C (2001a) Effect of repeated exposure to alcohol on the response of the hypothalamic-pituitary-adrenal axis of the rat: I. Role of changes in hypothalamic neuronal activity. *Alcohol Clin Exp Res* **25**:98-105.

Lee S, Selvage D, Hansen K and Rivier C (2004) Site of action of acute alcohol administration in stimulating the rat hypothalamic-pituitary-adrenal axis: comparison between the effect of systemic and intracerebroventricular injection of this drug on pituitary and hypothalamic responses. *Endocrinology* **145**:4470-9.

Lee S, Smith GW, Vale W, Lee KF and Rivier C (2001b) Mice that lack corticotropin-releasing factor (CRF) receptors type 1 show a blunted ACTH response to acute alcohol despite up-regulated constitutive hypothalamic CRF gene expression. *Alcohol Clin Exp Res* **25**:427-33.

Li X, Bertics PJ and Karavolas HJ (1997) Regional distribution of cytosolic and particulate 5alpha-dihydroprogesterone 3alpha-hydroxysteroid oxidoreductases in female rat brain. *J Steroid Biochem Mol Biol* **60**:311-318.

Lieber CS (2004) The discovery of the microsomal ethanol oxidizing system and its physiologic and pathologic role. *Drug Metab Rev* **36**:511-29.

Lieber CS and DeCarli LM (1970) Hepatic Microsomal Ethanol-oxidizing System. *Journal of Biological Chemistry* **245**:2505-2512.

Lin D, Sugawara T, Strauss III JF, Clark BJ, Stocco DM, Saenger P, Rogol A and Miller WL (1995) Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267**:1828-1831.

Lister RG and Linnoila M (1991) Alcohol, the chloride ionophore and endogenous ligands for benzodiazepine receptors. *Neuropharmacology* **30**:1435-1440.

Lochner A and Moolman JA (2006) The many faces of H89: a review. *Cardiovasc Drug Rev* **24**:261-74.

Loft S, Olesen KL and Dossing M (1987) Increased susceptibility to liver disease in relation to alcohol consumption in women. *Scand J Gastroenterol* **22**:1251-6.

Lombardi I, Luisi S, Quirici B, Monteleone P, Bernardi F, Liut M, Casarosa E, Palumbo M, Petraglia F and Genazzani AR (2004) Adrenal response to adrenocorticotropic hormone stimulation in patients with premenstrual syndrome. *Gynecol Endocrinol* **18**:79-87.

Lovallo WR, Dickensheets SL, Myers DA, Thomas TL and Nixon SJ (2000) Blunted stress cortisol response in abstinent alcoholic and polysubstance-abusing men. *Alcohol Clin Exp Res* **24**:651-8.

Majewska MD, Harrison NL, Schwartz RD, Barker JL and Paul SM (1986) Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* **232**:1004-1007.

Manrique H, Miquel M and Aragon CM (2005) Brain catalase mediates potentiation of social recognition memory produced by ethanol in mice. *Drug and Alcohol Dependence* **79**:343-350.

Martin-Garcia E, Darbra S and Pallares M (2007) Intrahippocampal allopregnanolone decreases voluntary chronic alcohol consumption in non-selected rats. *Prog Neuropsychopharmacol Biol Psychiatry* **31**:823-31.

Martin-Garcia E and Pallares M (2005) Effects of intrahippocampal nicotine and neurosteroid administration on withdrawal in voluntary and chronic alcohol-drinking rats. *Alcohol Clin Exp Res* **29**:1654-63.

Marx CE, VanDoren MJ, Duncan GE, Lieberman JA and Morrow AL (2003) Olanzapine and clozapine increase the GABAergic neuroactive steroid allopregnanolone in rodents. *Neuropsychopharmacology* **28**:1-13.

Matthews DB, Morrow AL, Tokunaga S and McDaniel JR (2002) Acute ethanol administration and acute allopregnanolone administration impair spatial memory in the Morris water task. *Alcohol. Clin. Exp. Res.* **26**:1747-51.

McQuade R and Young AH (2000) Future therapeutic targets in mood disorders: the glucocorticoid receptor. *Br J Psychiatry* **177**:390-5.

Miguel-Hidalgo JJ, Wei J, Andrew M, Overholser JC, Jurjus G, Stockmeier CA and Rajkowska G (2002) Glia pathology in the prefrontal cortex in alcohol dependence with and without depressive symptoms. *Biol Psychiatry* **52**:1121-33.

Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW and Homanics GE (1999) Attenuated sensitivity to neuroactive steroids in $\gamma$-aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad Sci U S A* **96**:12905-10.

Miller WL (1988) Molecular biology of steroid hormone synthesis. *Endoc Rev* **9**:295-318.

Miller WL (1997) Congenital lipoid adrenal hyperplasia: the human gene knockout for the steroidogenic acute regulatory protein. *Journal of Molecular Endocrinology* **19**:227-240.

Miller WL (2007a) Mechanism of StAR's regulation of mitochondrial cholesterol import. *Molecular and Cellular Endocrinology* **265-266**:46-50.

Miller WL (2007b) StAR Search -- What We Know About How the Steroidogenic Acute Regulatory Protein Mediates Mitochondrial Cholesterol Transport. *Molecular Endocrinology* **21**:589-601.

Miller WL (2007c) Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. *Biochimica et Biophysica Acta* **1771**:663-676.

Morrow AL (2007) Recent developments in the significance and therapeutic relevance of neuroactive steroids - Introduction to the special issue. *Pharmacol Ther* **116**:1-6.

Morrow AL, Herbert JS and Montpied P (1992) Differential effects of chronic ethanol administration on $GABA_A$ receptor $\alpha$1 and $\alpha$6 subunit mRNA levels in rat cerebellum. *Mol Cell Neurosci* **3**:251-258.

Morrow AL, Janis GC, VanDoren MJ, Matthews DB, Samson HH, Janak PH and Grant KA (1999) Neurosteroids mediate pharmacological effects of ethanol: A new mechanism of ethanol action? *Alcohol Clin Exp Res* **23**:1933-1940.

Morrow AL, Pace JR, Purdy RH and Paul SM (1990) Characterization of steroid interactions with γ-aminobutyric acid receptor-gated chloride ion channels: evidence for multiple steroid recognition sites. *Mol Pharmacol* **37**:263-270.

Morrow AL, Penland S, Khisti RT, Porcu P and Matthews DB (2005) GABAergic neuroactive steroids contribute to ethanol action in the CNS. *Alcoholism Clinical and Experimental Research* **29**:173A.

Morrow AL, Porcu P, Boyd KN and Grant KA (2006) Hypothalamic-pituitary-adrenal axis modulation of GABAergic neuroactive steroids influences ethanol sensitivity and drinking behavior. *Dialogues Clin Neurosci* **8**:463-477.

Morrow AL, Suzdak PD and Paul SM (1987) Steroid hormone metabolites potentiate GABA receptor-mediated chloride ion flux with nanomolar potency. *Eur J Pharmacol* **142**:483-485.

Morrow AL, VanDoren MJ and Devaud LL (1998) Effects of progesterone or neuroactive steroid? *Nature* **395**:652-653.

Morrow AL, VanDoren MJ, Penland SN and Matthews DB (2001) The role of GABAergic neuroactive steroids in ethanol action, tolerance and dependence. *Brain Res Brain Res Rev* **37**:98-109.

Myers WD, Gibson S, Ng KT and Singer G (1987) Sex differences in acetaldehyde on body temperature and open-field performance in the rat. *Drug Alcohol Depend* **19**:1-6.

Nicolas JM, Fernandez-Sola J, Fatjo F, Casamitjana R, Bataller R, Sacanella E, Tobias E, Badia E and Estruch R (2001) Increased circulating leptin levels in chronic alcoholism. *Alcohol Clin Exp Res* **25**:83-8.

Nishikawa T, Sasano H, Omura M and Suematsu S (1996) Regulation of Expression of the Steroidogenic Acute Regulatory (StAR) Protein by ACTH in Bovine Adrenal Fasciculata Cells. *Biochemical and Biophysical Research Communications* **223**:12-18.

Nyberg S, Andersson A, Zingmark E, Wahlstrom G, Backstrom T and Sundstrom-Poromaa I (2005) The effect of a low dose of alcohol on allopregnanolone serum concentrations

across the menstrual cycle in women with severe premenstrual syndrome and controls. *Psychoneuroendocrinology* **30**:892-901.

O'Dell LE, Alomary AA, Vallee M, Koob GF, Fitzgerald RL and Purdy RH (2004) Ethanol-induced increases in neuroactive steroids in the rat brain and plasma are absent in adrenalectomized and gonadectomized rats. *Eur J Pharmacol* **484**:241-7.

O'Dell LE, Purdy RH, Covey DF, Richardson HN, Roberto M and Koob GF (2005) Epipregnanolone and a novel synthetic neuroactive steroid reduce alcohol self-administration in rats. *Pharmacol. Biochem. Behav.* **81**:543-50.

O'Malley SS, Krishnan-Sarin S, Farren C, Sinha R and Kreek MJ (2002) Naltrexone decreases craving and alcohol self-administration in alcohol-dependent subjects and activates the hypothalamo-pituitary-adrenocortical axis. *Psychopharmacology (Berl)* **160**:19-29.

Obernier JA, White AM, Swartzwelder HS and Crews FT (2002) Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. *Pharmacol Biochem Behav* **72**:521-32.

Ocaranza P, Quintanilla M, Tampier L, Karahanian E, Sapag A and Israel Y (2008) Gene Therapy Reduces Ethanol Intake in an Animal Model of Alcohol Dependence. *Alcoholism: Clinical and Experimental Research* **32**:52-57.

Ogilvie KM and Rivier C (1997) Gender differences in hypothalamic-pituitary-adrenal axis response to alcohol in the rat: activational role of gonadal steroids *Brain Research* **766**:19-28.

Oh S, Jang CG, Ma T and Ho IK (1999) Activation of protein kinase C by phorbol dibutyrate modulates $GABA_A$ receptor binding in rat brain slices. *Brain Res* **850**:158-165.

Olsen RW, Hanchar HJ, Meera P and Wallner M (2007) $GABA_A$ receptor subtypes: the "one glass of wine" receptors. *Alcohol* **41**:201-9.

Owens MJ, Ritchie JC and Nemeroff CB (1992) 5$\alpha$-Pregnane-3$\alpha$,21-diol-20-one (THDOC) attenuates mild stress-induced increases in plasma corticosterone via a non-glucocorticoid mechanism: comparison with alprazolam. *Brain Res.* **573**:353-355.

Papadopoulos V, Liu J and Culty M (2007) Is there a mitochondrial signaling complex facilitating cholesterol import? *Molecular and Cellular Endocrinology* **265-266**:59-64.

Patchev VK, Hassan AHS, Holsboer F and Almeida OFX (1996) The neurosteroid tetrahydroprogesterone attenuates the endocrine response to stress and exerts glucocorticoid-like effects on vasopressin gene transcription in the rat hypothalamus. *Neuropsychopharmacology* **15**:533-540.

Patchev VK, Shoaib M, Holsboer F and Almeida OFX (1994) The neurosteroid tetrahydroprogesterone counteracts corticotropin-releasing hormone-induced anxiety and alters the release and gene expression of corticotropin-releasing hormone in the rat hypothalamus. *Neuroscience* **62**:265-271.

Paul SM and Purdy RH (1992) Neuroactive steroids. *FASEB Journal* **6**:2311-2322.

Pierucci-Lagha A, Covault J, Feinn R, Khisti RT, Morrow AL, Marx CE, Shampine LJ and Kranzler HR (2006) Subjective effects and changes in steroid hormone concentrations in humans following acute consumption of alcohol. *Psychopharmacology* **186**:451-461.

Pierucci-Lagha A, Covault J, Feinn R, Nellissery M, Hernandez-Avila C, Oncken C, Morrow AL and Kranzler HR (2005) GABRA2 alleles moderate the subjective effects of alcohol, which are attenuated by finasteride. *Neuropsychopharmacology* **30**:1193-203.

Porcu P, Rogers LSM, Morrow AL and Grant KA (2006) Plasma pregnenolone levels in cynomolgus monkeys following pharmacological challenges of the hypothalamic-pituitary-adrenal axis. *Pharmacol Biochem Behav* **84**:618-627.

Porcu P, Sogliano C, Ibba C, Piredda M, Tocco S, Marra C, Purdy RH, Biggio G and Concas A (2004) Failure of γ-hydroxybutyric acid both to increase neuroactive steroid concentrations in adrenalectomized-orchiectomized rats and to induce tolerance to its steroidogenic effect in intact animals. *Brain Res* **1012**:160-8.

Privalle C, Crivello J and Jefcoate CR (1983) Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. *Pro Nat Acad Sci U A* **80**:702-706.

Puia G, Santi M, Vicini S, Pritchett DB, Purdy RH, Paul SM, Seeburg PH and Costa E (1990) Neurosteroids act on recombinant human $GABA_A$ receptors. *Neuron* **4**:759-765.

Purdy RH, Morrow AL, Moore PH, Jr. and Paul SM (1991) Stress-induced elevations of gamma-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc Natl Acad Sci U. S. A.* **88**:4553-4557.

Quertemont E (2004) Genetic polymorphism in ethanol metabolism: acetaldehyde contribution to alcohol abuse and alcoholism. *Molecular Psychiatry* **9**:570-581.

Quertemont E and Didone V (2006) Role of acetaldehyde in mediating the pharmacological and behavioral effects of alcohol. *Alcohol Res Health* **29**:258-265.

Quertemont E, Tambour S, Bernaerts P, Zimatkin SM and Tirelli E (2004) Behavioral characterization of acetaldehyde in C57BL/6J mice: locomotor, hypnotic, anxiolytic and amnesic effects. *Psychopharmacology* **177**:84-92.

Quertemont E, Tambour S and Tirelli E (2005) The role of acetaldehyde in the neurobehavioral effects of ethanol: A comprehensive review of animal studies. *Progress in Neurobiology* **75**:247-274.

Quintanilla M, Tampier L, Sapag A and Israel Y (2005) Polymorphisms in the mitochondrial aldehyde dehydrogenase gene (Aldh2) determine peak blood acetaldehyde levels and voluntary ethanol consumption in rats. *Pharmacogenetics and Genomics* **15**:427-431.

Reddy DS and Rogawski MA (2002) Stress-induced deoxycorticosterone-derived neurosteroids modulate $GABA_A$ receptor function and seizure susceptibility. *J Neurosci* **22**:3795-805.

Redila VA, Aliatas E, Smith BR and Amit Z (2002) Effects of ethanol on an acetaldehyde drug discrimination with a conditioned taste aversion procedure. *Alcohol* **28**:103-109.

Rhodes ME and Frye CA (2005) Attenuating 5alpha-pregnane-3alpha-ol-20-one formation in the hippocampus of female rats increases pentylenetetrazole-induced seizures. *Epilepsy Behav.* **6**:140-146.

Richardson HN, Lee SY, O'Dell LE, Koob GF and Rivier CL (2008) Alcohol self-administration acutely stimulates the hypothalamic-pituitary-adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state. *Eur J Neurosci* **28**:1641-53.

Rivier C (1996) Alcohol stimulates ACTH secretion in the rat: mechanisms of action and interactions with other stimuli. *Alcohol Clin Exp Res* **20**:240-254.

Rivier C, Bruhn T and Vale W (1984) Effect of ethanol on the hypothalamic-pituitary-adrenal axis in the rat: role of corticotropin-releasing factor (CRF). *Journal of Pharmacology and Experimental Therapeutics* **229**:127-131.

Rivier C and Lee S (2001) Effect of repeated exposure to alcohol on the response of the hypothalamic-pituitary-adrenal axis of the rat: II. Role of the length and regimen of alcohol treatment. *Alcohol Clin Exp Res* **25**:106-11.

Rivier C, Rivier J and Lee S (1996) Importance of pituitary and brain receptors for corticotrophin-releasing factor in modulating alcohol-induced ACTH secretion in rat. *Brain Research* **721**:83-90.

Roberto M, Madamba SG, Moore SD, Tallent MK and Siggins GR (2003) Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. *Proc Natl Acad Sci U S A* **100**:2053-8.

Roostaee A, Barbar E, Lavigne P and LeHoux JG (2009) The mechanism of specific binding of free cholesterol by the steroidogenic acute regulatory protein: evidence for a role of the C-terminal alpha-helix in the gating of the binding site. *Biosci Rep* **29**:89-101.

Sanna E, Talani G, Busonero F, Pisu MG, Purdy RH, Serra M and Biggio G (2004) Brain steroidogenesis mediates ethanol modulation of $GABA_A$ receptor activity in rat hippocampus. *J Neurosci* **24**:6521-30.

Santhakumar V, Wallner M and Otis TS (2007) Ethanol acts directly on extrasynaptic subtypes of GABAA receptors to increase tonic inhibition. *Alcohol* **41**:211-21.

Sarnyai Z, Shaham Y and Heinrichs SC (2001) The role of corticotropin-releasing factor in drug addiction. *Pharmacol Rev* **53**:209-43.

Schumacher M, Guennoun R, Mercier G, Desarnaud F, Lacor P, Benavides J, Ferzaz B, Robert F and Baulieu EE (2001) Progesterone synthesis and myelin formation in peripheral nerves. *Brain Res Brain Res Rev* **37**:343-59.

Selye H (1941) The anesthetic effect of steroid hormones. *Proc Soc Exp Biol Med* **46**:116-121.

Selye H (1942) The antagonism between anesthetic steroid hormones and pentamethylenetetrazol. *J Lab Clin Med* **27**:1051-1053.

Serra M, Madau P, Chessa MF, Caddeo M, Sanna E, Trapani G, Franco M, Liso G, Purdy RH, Barbaccia ML and Biggio G (1999) 2-Phenyl-imidazo[1,2-a]pyridine derivatives as ligands for peripheral benzodiazepine receptors: stimulation of neurosteroid synthesis and anticonflict action in rats. *British Journal of Pharmacology* **127**:177-187.

Serra M, Mostallino MC, Talani G, Pisu MG, Carta M, Mura ML, Floris I, Maciocco E, Sanna E and Biggio G (2006) Social isolation-induced increase in $\alpha 4$ and $\delta$ subunit gene expression is associated with a greater efficacy of ethanol on steroidogenesis and $GABA_A$ receptor function. *J. Neurochem.* **98**:122-133.

Serra M, Pisu MG, Floris I and Biggio G (2005) Social isolation-induced changes in the hypothalamic-pituitary-adrenal axis in the rat. *Stress* **8**:259-264.

Serra M, Pisu MG, Floris I, Cara V, Purdy RH and Biggio G (2003) Social isolation-induced increase in the sensitivity of rats to the steroidogenic effect of ethanol. *J Neurochem* **85**:257-63.

Serra M, Pisu MG, Littera M, Papi G, Sanna E, Tuveri F, Usala L, Purdy RH and Biggio G (2000) Social isolation-induced decreases in both the abundance of neuroactive steroids and $GABA_A$ receptor function in rat brain. *J. Neurochem.* **75**:732-740.

Shannon EE, Porcu P, Purdy RH and Grant KA (2005) Characterization of the discriminative stimulus effects of neuroactive steroid pregnanolone in DBA/2J and C57BL/6J inbred mice. *J. Pharmacol. Exp. Ther.* **314**:675-85.

Sieghart W and Sperk G (2002) Subunit composition, distribution and function of GABA(A) receptor subtypes. *Curr Top Med Chem* **2**:795-816.

Song M and Messing RO (2005) Protein kinase C regulation of $GABA_A$ receptors. *Cellular and Molecular Life Sciences* **62**:119-127.

Spencer RL and Hutchison KE (1999) Alcohol, aging, and the stress response. *Alcohol Res Health* **23**:272-83.

Spencer RL and McEwen BS (1990) Adaptation of the hypothalamic-pituitary-adrenal axis to chronic ethanol stress. *Neuroendocrinology* **52**:481-489.

Stocco DM (2000) The role of StAR protein in steroidogenesis: challenges for the future. *Journal of Endocrinology* **164**:247-253.

Stocco DM and Clark BJ (1996) Regulation of the acute production of steroids in steroidogenic cells. *Endocr Rev* **17**:221-244.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K and Smith S (2002) Hormonally regulated $\alpha_4\beta_2\delta$ $GABA_A$ receptors are a target for alcohol. *Nat. Neurosci.* **5**:721-722.

Syapin PJ and Alkana RL (1988) Chronic ethanol exposure increases peripheral-type benzodiazepine receptors in brain. *Eur J Pharmacol* **147**:101-9.

Tambour S, Didone V, Tirelli E and Quertemont E (2006) Locomotor effects of ethanol and acetaldehyde after peripheral and intraventricular injections in Swiss and C57BL/6J mice. *Behavioral Brain Research* **172**:145-154.

Thiele TE, Willis B, Stadler J, Reynolds JG, Bernstein IL and McKnight GS (2000) High ethanol consupmtion and low sensitivity to ethanol-induced sedation in protein kinase A-mutant mice. *J Neurosci* **20**:RC75.

Thomas AJ, Nockels RP, Pan HQ, Shaffrey CI and Chopp M (1999) Progesterone is neuroprotective after acute experimental spinal cord trauma in rats. *Spine* **24**:2134-8.

Thomasson HR, Edenberg HJ, Crabb D, Mai X, Jerome R, Li T-K, Wang S, Lin Y, Lu R and Yin S (1991) Alcohol and aldehyde dehydrogenase genotypes and alcoholism in chinese men. *American Journal of Human Genetics* **48**:677-681.

Tokunaga S, McDaniel JR, Morrow AL and Matthews DB (2003) Effect of acute ethanol administration and acute allopregnanolone administration on spontaneous hippocampal pyramidal cell neural activity. *Brain Res* **967**:273-280.

Torres JM and Ortega E (2003) Alcohol intoxication increases allopregnanolone levels in female adolescent humans. *Neuropsychopharmacology* **28**:1207-9.

Torres JM and Ortega E (2004) Alcohol intoxication increases allopregnanolone levels in male adolescent humans. *Psychopharmacology* **172**:352-5.

Trauger JW, Jiang A, Stearns BA and LoGrasso PV (2002) Kinetics of allopregnanolone formation catalyzed by human 3 alpha-hydroxysteroid dehydrogenase type III (AKR1C2). *Biochemistry* **41**:13451-9.

Ueno S, Bracamontes J, Zorumski C, Weiss DS and Steinbach JH (1997) Bicuculline and gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. *J.Neurosci.* **17**:625-634.

VanDoren MJ, Johnson CJ and Morrow AL (2000a) Alcohol preference in ethanol preferring P rats is associated with altered HPA axis and neurosteroid responses to alcohol. *Alcoholism, Clinical and Experimental Research* **24**:49A.

VanDoren MJ, Matthews DB, Janis GC, Grobin AC, Devaud LL and Morrow AL (2000b) Neuroactive steroid 3α-hydroxy-5α-pregnan-20-one modulates electrophysiological and behavioral actions of ethanol. *J Neurosci* **20**:1982-1989.

Varlinskaya EI and Spear LP (2006) Ontogeny of acute tolerance to ethanol-induced social inhibition in Sprague-Dawley rats. *Alcohol Clin Exp Res* **30**:1833-44.

Vermetten E and Bremner JD (2002) Circuits and systems in stress. II. Applications to neurobiology and treatment in posttraumatic stress disorder. *Depress Anxiety* **16**:14-38.

Wafford KA, Whiting PJ and Kemp JA (1993) Differences in affinity and efficacy of benzodiazepine receptor ligands at recombinant gamma-aminobutyric $acid_A$ receptor subtypes. *Molecular Pharmacology* **43**:240-244.

Wall TL, Carr LG and Ehlers CL (2003a) Protective association of genetic variation in alcohol dehydrogenase with alcohol dependence in Native American Mission Indians. *Am J Psychiatry* **160**:41-6.

Wall TL, Carr LG and Ehlers CL (2003b) Protective associaton of genetic variation in alcohol dehydrogenase with alcohol dependence in Native American Mission Indians. *American Journal of Psychiatry* **160**:41-46.

Wallner M, Hanchar HJ and Olsen RW (2003) Ethanol enhances α4β3δ and α6β3δ γ-aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci U S A* **100**:15218-23.

Wallner M, Hanchar HJ and Olsen RW (2006) Low dose acute alcohol effects on $GABA_A$ receptor subtypes. *Pharmacol Ther*.

Waltman C, Blevins LSJ, Boyd G and Wand GS (1993) The effects of mild ethanol intoxication on the hypothalamic-pituitary-adrenal axis in non-alcoholic men. *J Clin Endocrinol Metab* **77**:518-522.

Wand GS and Dobs AS (1991) Alterations in the hypothalamic-pituitary-adrenal axis in actively drinking alcoholics. *J. Clin. Endocrinol. Metab.* **72**:1290-5.

Wang JM, Johnston PB, Ball BG and Brinton RD (2005) The neurosteroid allopregnanolone promotes proliferation of rodent and human neural progenitor cells and regulates cell-cycle gene and protein expression. *J Neurosci* **25**:4706-18.

Wang JM, Liu L, Irwin RW, Chen S and Brinton RD (2008) Regenerative potential of allopregnanolone. *Brain Res Rev* **57**:398-409.

Wang MD, Wahlstrom G and Backstrom T (1997) The regional brain distribution of the neurosteroids pregnenolone and pregnenolone sulfate following intravenous infusion. *J Steroid Biochem Mol Biol* **62**:299-306.

Watari H, Arakane F, Moog-Lutz C, Kallen CB, Tomasetto C, Gerton GL, Rio M, Baker ME and Strauss III JF (1997) MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **94**:8462-8467.

Yoshida A, Huang IY and Ikawa M (1984) Molecular abnormality of an inactive aldehyde dehydrogenase variant commonly found in Orientals. *Proc Natl Acad Sci U S A* **81**:258-61.

Young EA, Kotun J, Haskett RF, Grunhaus L, Greden JF, Watson SJ and Akil H (1993) Dissociation between pituitary and adrenal suppression to dexamethasone in depression. *Arch Gen Psychiatry* **50**:395-403.