ETHANOL ALTERS THE GABAERGIC NEUROACTIVE STEROID (3α,5α)-3-HYDROXYPREGNAN-20-ONE (3α,5α-THP or ALLOPREGNANOLONE) AT LOCAL BRAIN SITES: SIGNIFICANCE OF LOCAL 3α,5α-THP INCREASES IN THE VENTRAL TEGMENTAL AREA

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ABSTRACT

JASON B. COOK: Ethanol Alters the GABAergic Neuroactive Steroid (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or Allopregnanolone) at Local Brain Sites: Significance of Local 3α,5α-THP Increases in the Ventral Tegmental Area
(Under the direction of Dr. A. Leslie Morrow)

Neuroactive steroids are endogenous modulators of neuronal activity that modulate central nervous system inhibitory tone and influence motivation and emotional behaviors. The inhibitory neuroactive steroid (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) is a potent positive modulator of γ-aminobutyric acid type A ($GABA_A$) receptors. In rats, systemic ethanol administration increases 3α,5α-THP in the blood plasma, cerebral cortex, and hippocampus. Ethanol-induced increases of 3α,5α-THP contribute to the behavioral and neurophysiological effects of ethanol in rats and the subjective effects of alcohol in humans. Previous *in vivo* studies suggest that ethanol-induced increases of 3α,5α-THP are dependent on the adrenal glands, but *in vitro* studies suggest ethanol produces local synthesis of 3α,5α-THP. Furthermore, limitations in steroid assays have prevented the study of ethanol-induced changes of 3α,5α-THP in many of the brain regions implicated in alcoholism. The first aim of this project used 3α,5α-THP immunohistochemistry (IHC) to determine ethanol effects on cellular levels of 3α,5α-THP across the rat brain, and determine the role of the adrenal glands in these effects. We showed that ethanol produces divergent, brain region specific, effects on cellular 3α,5α-THP levels. We also showed that ethanol can increase or decrease local

brain levels of 3α,5α-THP independent of adrenal activation in subcortical brain regions, but increases of 3α,5α-THP in the medial prefrontal cortex (mPFC) are dependent on the adrenal glands. Previous studies have shown that systemic 3α,5α-THP can reduce ethanol reinforcement and consumption. However, systemic 3α,5α-THP is metabolized rapidly and can produce sedation, limiting therapeutic value. Therefore, in aim 2 we used gene delivery to drive local neuroactive steroid synthesis in the nucleus accumbens (NAc) or ventral tegmental area (VTA), which are both implicated in ethanol reinforcement and consumption. We showed that adeno-associated viral vector-mediated local increases of 3α,5α-THP in the VTA were associated with a long-term reduction in ethanol reinforcement and consumption. Further investigation showed that 3α,5α-THP is located in tyrosine hydroxylase (TH) positive and negative neurons, but not astrocytes in the VTA. These studies showed that ethanol can produce divergent effects on local brain levels of 3α,5α-THP and increasing local levels of 3α,5α-THP in the VTA is associated with reduced ethanol reinforcement and consumption.

Dedicated to my father Gary W. Cook and my grandmother Cleona Miller, both of which uniquely influenced my life.

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LIST OF ABBREVIATIONS

3α,5α-THDOC	(3α,5α)-3,21-dihydroxypregnan-20-one
3α,5α-THP	(3α,5α)-3-hydroxypregnan-20-one (allopregnanolone)
3α-HSD	3α-hydroxysteroid dehydrogenase
5α-DHP	5α-dihydroprogesterone
ADX	adrenalectomy
BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
DG	dentate gyrus
DMS	dorsomedial striatum
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HPA	hypothalamic-pituitary-adrenal axis
IHC	immunohistochemistry
LHb	lateral habenula
LTP	long-term potentiation
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
NeuN	Neuronal Nuclei
NMDA	N-Methyl-D-aspartic acid
P450scc	cytochrome P450 side chain cleavage
PVN	paraventricular nucleus of the hypothalamus
RIA	radioimmunoassay
rAAV2	recombinant adeno-associated virus serotype 2
StAR	steroidogenic acute regulatory protein
SEM	standard error of the mean
TH	tyrosine hydroxylase
VTA	ventral tegmental area

CHAPTER 1. **GENERAL INTRODUCTION**

ALCOHOLISM: SIGNIFICANCE OF THE PROBLEM

Alcohol drinking has been prevalent throughout human history and is often a part of social activities and celebrations. In the United States, most individuals will drink alcohol in their lifetime and moderate alcohol consumption is common. However, excessive alcohol use is currently one of the most costly public health issues in the United States, and indeed, throughout the world. The Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013) classifies alcohol use disorders as a problematic pattern of alcohol use leading to clinically significant impairment or distress that includes at least two of the following criteria within a 12 month period: alcohol tolerance, withdrawal symptoms, drinking more or longer than intended, craving or a strong desire for alcohol, inability to control use, a large amount of time spent seeking alcohol or recovering from use, missing important social/occupational/recreational activities due to alcohol use, physically hazardous use, continuing use despite knowledge of persistent or recurrent physical or psychological problems caused by alcohol, or alcohol use causes failure to fulfill major obligations at work, home, or school. The diagnostic results then specify if the alcohol use disorder is mild (2-3 symptoms), moderate (4-5 symptoms), or severe (6 or more symptoms). Based on the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC)

approximately 18 million people suffer from alcohol use disorders in the United States. Excessive alcohol consumption is the third leading preventable cause of death in the United States (Mokdad et al., 2004), and the economic impact of alcohol abuse has been estimated to be approximately $235 billion per year (Rehm et al., 2009).

The causes of alcohol use disorders are not known but likely are attributable to many genetic and environmental influences that increase an individual's susceptibility to develop these disorders. Genetic factors have been associated with an increased susceptibility for developing alcohol use disorders. For example, having a positive family history of alcoholism or low sensitivity to alcohol's effects are associated with an increased incidence of alcohol use disorders (Schuckit, 2000). However, in some Asian populations the presence of a polymorphism in the alcohol metabolizing enzymes, alcohol or aldehyde dehydrogenase, can be protective against developing alcohol use disorders (Thomasson et al., 1991). An early age of onset of drinking is also a predictor of future development of alcohol use disorders (Dawson et al., 2008). Finally, as with other drugs of abuse, the incidence of men with alcohol use disorders is higher than that of women (Grant et al., 2004).

Alcoholism is a chronic, lifelong disorder, which is inherently difficult to treat. Over the last several decades biomedical research has focused on determining the potential genetic and neurobiological mechanisms that contribute to excessive alcohol consumption. Although much has been learned about the neurobiology and genetics of alcoholism, the pharmacological treatments for alcohol use disorders are currently limited. The three available treatments in the United States are disulfuram, naltrexone, and acamprosate. These treatments are efficacious in some individuals, but many

alcoholics do not respond to any of these drugs. Therefore, it is crucial to develop many different treatments for alcohol use disorders, since treatments will most likely only be effective in some individuals. In the future, an individualized medicine approach appears promising since genetic differences can determine the effectiveness of a particular pharmacological treatment (Johnson et al., 2013; Thorsell, 2013).

EFFECTS OF ALCOHOLISM ON THE HUMAN BRAIN

Alcohol affects most cells of the body but the behavioral effects and addictive properties of alcohol are thought to be mediated by the central nervous system (CNS). Alcoholism produces brain dysfunction and structural abnormalities. It was originally observed in postmortem studies that alcoholism causes brain shrinkage. The reduction in brain volume in human alcoholics is thought to be due to reduced white matter predominantly, but there is also loss of grey matter in some regions (Harper, 1998). Advances in neuroimaging have led to increased knowledge about the effects of alcohol on brain structure and function in live alcoholics. Magnetic resonance imaging (MRI) has been used to show that chronic alcohol use decreases volume in the anterior hippocampus, caudate/putamen, nucleus accumbens (NAc), thalamus, corpus collosum, and cerebellum (Sullivan and Pfefferbaum, 2005). Furthermore, MRI and functional MRI studies suggest the deficits in brain volume and white matter underlie the cognitive and motor dysfunction observed in alcoholics (Sullivan and Pfefferbaum, 2005). The pathology of alcoholic neurodegeneration is not understood but is likely due to many different factors, such as neuroinflammation, excitotoxcicity, and changes in expression of myelin proteins.

EFFECTS OF ALCOHOL ON CNS FUNCTION

Alcohol (ethyl alcohol or ethanol) is a very promiscuous drug that produces a plethora of effects in the body. Animal models and *in vitro* cell and slice cultures have been used to investigate the actions of ethanol in the brain. Multiple lines of biomedical research have determined that ethanol alters many neurotransmitter systems including dopamine, serotonin, γ-aminobutyric acid (GABA), glycine, and glutamate. Determining sites where ethanol produces its pharmacological effects is difficult since it is such a small molecule and physiological effects require a large dose. Nonetheless, electrophysiological studies have shown that ethanol alters conductance at multiple cell membrane bound ion channels. At physiologically relevant doses, ethanol enhances GABA type A ($GABA_A$) receptor action (Suzdak et al., 1986) and inhibits excitatory glutamateric N-Methyl-D-aspartic acid (NMDA) receptors (Lovinger et al., 1989). Effects of ethanol at $GABA_A$ receptors are thought to mediate the anxiolytic and sedative effects of ethanol, while antagonism of NMDA receptors may contribute to ethanol's impairing effects on cognition and memory. Ethanol also produces actions at acetylcholine nicotinic (Yu et al., 1996), glycine (Ye et al., 2001), and serotonin receptors (Lovinger and White, 1991) as well as G-protein inwardly rectifying (GIRK) and big potassium (BK) potassium channels (Kobayashi et al., 1999; Chiou et al., 2002).

Ethanol alters neurotransmitter release, including glutamate, GABA, serotonin, and dopamine. A major focus has been on dopamine release in the mesolimbic pathway. The mesolimbic dopamine pathway consists of the dopamine cell bodies located in the ventral tegmental area (VTA) that project to the NAc. It is important to note that these dopamine neurons that classically have been implicated in motivation and drug

reinforcement, also have been shown to co-release glutamate in the NAc shell during optical stimulation (Stuber et al., 2010). Most drugs of abuse, including alcohol, increase dopamine release in the NAc when administered acutely. This increase in dopamine release is thought to contribute to drug reward and influence the addiction process through associative learning processes (Wise, 2004). Ethanol also regulates levels of neuroactive steroids (Morrow et al., 2001) that contribute to behavioral and physiological effects of ethanol. Considering the many sites of ethanol action and complex effects on neurophysiology it is challenging from a basic research perspective to unravel fundamental actions of ethanol and identify promising therapeutic targets for treating alcoholism.

$GABA_A$ RECEPTORS

GABA is the primary inhibitory neurotransmitter in the CNS, and $GABA_A$ receptors are the primary receptor family. The $GABA_A$ receptor is a heteropentameric protein complex that forms a chloride permeable ion channel. Activation of $GABA_A$ receptors produces neuronal inhibition by increasing chloride flux into the cell reducing the membrane potential. This results in a decreased likelihood of an action potential, and therefore, inhibition of neurotransmission. To date, 19 subunits have been identified (Sarto-Jackson and Sieghart, 2008), and the subunit composition of a particular $GABA_A$ receptor determines its pharmacological profile. The $GABA_A$ receptors are the site of action for several different compounds besides GABA including benzodiazepines, barbiturates, anesthetics, neuroactive steroids, and picrotoxin. Several clinically relevant drugs, including anxiolytics, sedatives/hypnotics, and general anesthetics as well as endogenous neuroactive steroids, produce their effects via allosteric modulation of the

$GABA_A$ receptor. $GABA_A$ receptors remain an attractive target for the development of new compounds for treating psychiatric disorders.

ETHANOL EFFECTS ON $GABA_A$ RECEPTORS

Some of the effects of ethanol appear to be mediated via actions at the $GABA_A$ receptor. $GABA_A$ receptor agonists enhance and antagonists reduce ethanol effects (Lister and Linnoila, 1991). Electrophysiological studies have shown ethanol can affect neurotransmission through actions at synaptic $GABA_A$ receptors that produce phasic inhibition and consist of two α, two β, and one γ, or at extrasynaptic $GABA_A$ receptors that produce tonic inhibition and consist of $\alpha4$ or $\alpha6$ and a δ instead of γ subunit. Extrasynaptic receptors respond to lower concentrations of ethanol, suggesting GABAergic effects at low doses of ethanol may be mediated via this population of receptors (Olsen et al., 2007). Ethanol's effect on $GABA_A$ receptors is mainly thought to be due to enhancement of chloride current through the receptor, but there are a few reports of ethanol inhibition of $GABA_A$ receptors (Marszalec et al., 1994; Aguayo et al., 2002). Evidence from transgenic animals suggests ethanol produces sedative/hypnotic effects largely via $\alpha1$ subunit containing receptors (Kralic et al., 2003; June et al., 2007), but specific ethanol effects mediated by other subunits remain unclear.

It is not clear whether the effects of ethanol on $GABA_A$ receptors are produced via direct or indirect activation of the receptor. Although there is some evidence for direct activation of δ subunit containing recombinant extrasynaptic receptors, these results are controversial (Santhakumar et al., 2007). There are many possibilities of indirect effects of ethanol on $GABA_A$ receptor function. One method of ethanol induced changes is by altering protein kinase A (PKA) or C (PKC), which can enhance $GABA_A$ receptor

function (Kumar et al., 2005; Choi et al., 2008). Ethanol can increase presynaptic GABA release (Roberto et al., 2003; Ariwodola and Weiner, 2004) in many but not all brain regions (Kelm et al., 2011). The GABA agonist taurine also can be increased by ethanol (De Witte et al., 1994), which at low levels can increase tonic inhibition (Jia et al., 2008). Ethanol also increases levels of potent GABAergic neuroactive steroids (Barbaccia et al., 1999; Morrow et al., 1999; Khisti et al., 2003; O'Dell et al., 2004; Sanna et al., 2004; Boyd et al., 2010; Tokuda et al., 2011), which have similar pharmacologic effects as ethanol and are thought to contribute to some neurophysiological and behavioral effects of ethanol.

NEUROACTIVE STEROIDS

Neuroactive steroids are endogenous neuromodulators that alter neuronal activity. This effect on neurotransmission is due to their actions at membrane bound receptors where they alter currents through ion channels, unlike the classic genomic steroid effects via regulation of gene expression. The term neurosteroid was coined over 30 years ago (Baulieu, 1981) to describe steroids which are synthesized *de novo* in the nervous system. These same steroids are also synthesized in the periphery where they are more generally referred to as neuroactive steroids. Levels of neuroactive steroids fluctuate depending on environmental factors like stress (Purdy et al., 1991b), biological factors including estrous/menstrual cycle (Purdy et al., 1990b) and pregnancy (Paul and Purdy, 1992), following drug administration (Marx et al., 2000; Porcu et al., 2004; Grobin et al., 2005; Pinna et al., 2006; Tokuda et al., 2010), and may be altered in some psychiatric disorders. Therefore, neuroactive steroids have garnered a lot of attention as potential therapeutic agents for multiple psychiatric disorders including depression (Uzunova et al., 1998),

anxiety disorders (Strohle et al., 2002), post-traumatic stress disorder (Rasmusson et al., 2006), and schizophrenia (Marx et al., 2006).

Depending on the particular neuroactive steroid, the effects on neuronal activity can be excitatory or inhibitory. The sulfated derivatives of pregnenolone and dehydroepiandrosterone (DHEA) as well as the 3α,5α- and 3α,5β-reduced metabolites of cortisol are considered excitatory neuroactive steroids due to their ability to enhance NMDA receptor activity (Farb and Gibbs, 1996), or inhibit $GABA_A$ receptor activity (Penland and Morrow, 2004). There are several inhibitory neuroactive steroids, often referred to as GABAergic neuroactive steroids. These include the 3α,5α- and 3α,5β-reduced metabolites are of progesterone, deoxycorticosterone, DHEA and testosterone. The most widely studied GABAergic neuroactive steroids are (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC), which are metabolites of progesterone and deoxycorticosterone, respectively. 3α,5α-THP and 3α,5α-THDOC are potent positive allosteric modulators of $GABA_A$ receptors, where they bind to two known neuroactive steroid transmembrane binding sites on α subunits (Hosie et al., 2006). One site on the α subunit potentiates the receptor, while the other site located at the interface of α and β subunits activates the receptor directly at high concentrations. Therefore, they positively modulate synaptic and extrasynaptic receptors since all $GABA_A$ receptors likely contain α subunits. Furthermore, at high concentrations 3α,5α-THP and 3α,5α-THDOC can directly activate $GABA_A$ receptors in the absence of GABA (Ueno et al., 1997). Exogenous administration of 3α,5α-THP and 3α,5α-THDOC produces pharmacological

effects that are similar to ethanol including anxiolytic, sedative, anticonvulsant, and cognitive impairing effects (for review see, Morrow et al., 2001).

NEUROACTIVE STEROID SYNTHESIS

Steroids are derived from cholesterol and are synthesized in the nervous system, adrenal glands, and gonads. Steroid synthesis begins in the mitochondria where the P450 side chain cleavage (P450scc) enzyme converts cholesterol to pregnenolone (Fig. 1.1). Most steroidogenic cholesterol is derived from circulating lipoproteins, and there are multiple cholesterol transporters that move cholesterol from the outer mitochondrial membrane to the inner membrane where P450scc is located. Pregnenolone is then available to be converted to other steroid products. Steroid hormones are classified into five major classes: mineralocorticoids (aldosterone), glucocorticoids (corticosterone, cortisol), progestens (progesterone), androgens (testosterone, dihydrotestosterone) and estrogens (estradiol). Steroid synthesis is similar in most tissues and the steroid products depend on the biosynthetic enzymes present in that particular cell, which ultimately influences cell function. For instance, Leydig cells in the testis and ganulosa cells in the ovary predominantly produce testosterone and estrogens (Rommerts and Brinkman, 1981; Garzo and Dorrington, 1984), respectively. This is due to the fact that Leydig and granulosa cells express enzymes responsible for the synthesis of testosterone and estrogens. The adrenal glands are important steroidogenic sites that regulate many aspects of physiology. The structure of the adrenal gland consists of a large outer cortex region, which encompasses the inner adrenal medulla region. The medulla produces the catecholamines epinephrine and norepinephrine, and receives information directly from the sympathetic nervous system via preganglionic fibers of the thoracic spinal cord.

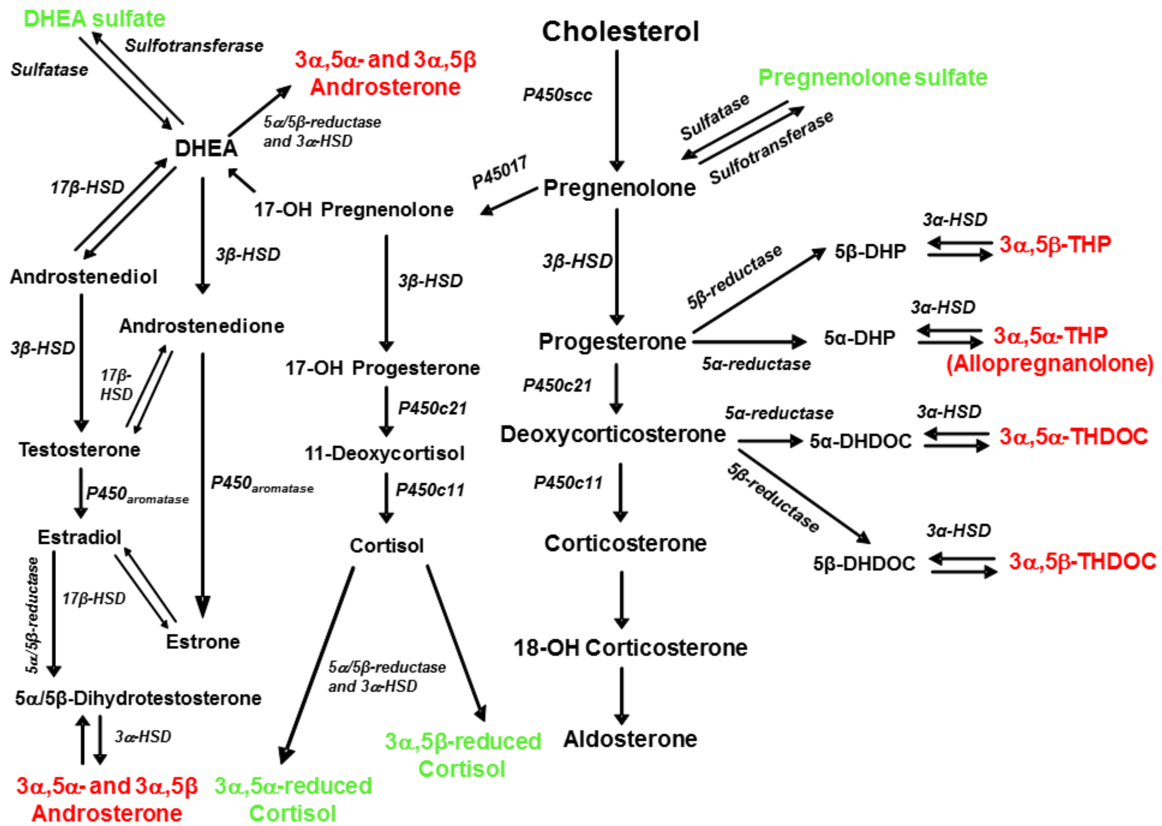


Figure 1.1: Steroid biosynthetic pathway including synthetic enzymes. Inhibitory neuroactive steroids are shown in red and excitatory neuroactive steroids are shown in green.

The adrenal cortex receives neuroendocrine hormones from the pituitary gland, which regulates function and steroid synthesis. Structurally, the adrenal cortex is divided into three layers (i.e. zones). The zones of the cortex are functionally distinct based on the steroids they produce, which is due to the biosynthetic enzymes present in each zone. The zona glomerulosa is the outermost layer and is responsible for production of the mineralocorticoid aldosterone, which regulates sodium concentrations and blood volume. The zona fasciculata is the middle zone and the site of glucocorticoid production, which are involved in glucose metabolism, stress responses, and regulation of other hormones. The innermost layer of the adrenal cortex is the zona reticularis where androgens are produced, which produce many sex-specific effects. The enzymes for neuroactive steroid synthesis are located in the zona fasciculate and zona reticularis (Compagnone et al., 1995).

In the adrenal glands, neuroactive steroid synthesis is observed following hypothalamic-pituitary-adrenal (HPA) axis activation. Acute stress (Tsigos and Chrousos, 2002) and some drug administrations, including alcohol (Boyd et al., 2010), activate the HPA axis. HPA activation increases release of corticotropin releasing factor (CRF) from the hypothalamus, which signals to neuroendocrine cells in the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH signals to the adrenal cortex to release GABAergic neuroactive steroids, neuroactive steroid precursors, and glucocorticoids. GABAergic neuroactive steroids can then provide negative feedback on the HPA by reducing CRF and ACTH release in rats (Owens et al., 1992; Patchev et al., 1994; Patchev et al., 1996). Recent evidence suggests that the GABAergic neuroactive steroid

3α,5α-THDOC can also initiate the HPA stress response in C57BL/6J mice (Sarkar et al., 2011).

The synthesis of neuroactive steroids in the adrenals can be due to hormonal peptide-induced increases of cyclic adenosine monophosphate (cAMP), increased availability of cholesterol, increased expression of the cholesterol transporter steroidogenic acute regulatory protein (StAR), and increased expression of P450 hydroxylase and other steroidogenic enzymes. The transcription factor steroidogenic factor 1 (SF-1) regulates gene expression of many of the enzymes that increase steroidogenesis (Omura and Morohashi, 1995). Brain synthesis of neuroactive steroids is not well understood, but the prominent idea is that it is under the control of expression of biosynthetic enzymes (Baulieu, 1998; Compagnone and Mellon, 2000). Many of the neurosteroidogenic enzymes are expressed in the brain including StAR, P450scc, 5α-reductase type I and II, 3α-hydroxysteroid dehydrogenase (3α-HSD), and 3β-hydroxysteroid dehydrogenase (3β-HSD). Furthermore, the steroidogenic enzymes display brain regional specificity in their expression. In the case of the GABAergic neuroactive steroids 3α,5α-THP and 3α,5α-THDOC and their biosynthetic enzymes 5α-reductase and 3α-HSD are located in the principal projecting neurons (Agis-Balboa et al., 2006; Saalmann et al., 2007). Is has also been shown that environmental stress, which increases 3α,5α-THP in the cerebral cortex, also increases protein levels of 5α-reductase in the mPFC (Sanchez et al., 2009). Therefore, changes in brain expression of biosynthetic enzyme may underlie changes in these potent GABAergic neuroactive steroids.

EFFECTS OF ETHANOL ON GABAERGIC NEUROACTIVE STEROID SYNTHESIS

Ethanol activates the HPA axis (Boyd et al., 2010) causing the adrenal glands to increase steroids and steroid precursors into the peripheral bloodstream. In rats, ethanol doses of ~1.3g/kg and higher produce physiologically relevant increases of the GABAergic neuroactive steroids 3α,5α-THP and 3α,5α-THDOC in the blood plasma (Porcu et al., 2010). Ethanol also increases levels of 3α,5α-THP and 3α,5α-THDOC in the whole cerebral cortex (VanDoren et al., 2000), frontal cortex (O'Dell et al., 2004) and hippocampus (Barbaccia et al., 1999). In humans, 0.8g/kg ethanol administered orally fails to elevate 3α,5α-THP or 3α,5α-THDOC levels in the blood plasma of healthy men (Porcu et al., 2010). Due to ethical concerns, higher doses of ethanol cannot be administered to humans in the laboratory. However, previous studies have shown that adolescents requiring emergency services for acute alcohol intoxication displayed elevated levels of plasma 3α,5α-THP (Torres and Ortega, 2003, 2004). Therefore, similar to what is seen in rats, doses of ethanol higher than 0.8g/kg may increase levels of 3α,5α-THP in humans.

Both 3α,5α-THP and 3α,5α-THDOC readily cross the blood brain barrier, and adrenalectomy prevents ethanol-induced elevations of 3α,5α-THP in the frontal cortex (O'Dell et al., 2004) and whole cerebral cortex (Khisti et al., 2003). Importantly, the administration of the immediate precursor of 3α,5α-THP, 5α-dihydroprogesterone (5α-DHP), restores ethanol-induced increases of 3α,5α-THP in the cerebral cortex as well as corresponding effects of ethanol on loss of righting reflex following adrenalectomy (Khisti et al., 2003). This suggests that ethanol-induced increases of 3α,5α-THP in the

cerebral cortex are dependent on adrenal gland activation to provide 3α,5α-THP precursor. Ethanol-induced increases of 3α,5α-THP in the cerebral cortex are also dependent on *de novo* adrenal synthesis of the cholesterol transporter StAR (Boyd et al., 2010). StAR transports cholesterol to the inner mitochondrial membrane where it is converted to pregnenolone by P450scc. Taken together, StAR synthesis in the adrenals may be necessary to provide precursor to the cerebral cortex where 3α,5α-THP is increased following ethanol. *In vitro* studies have shown that ethanol can increase levels of 3α,5α-THP in hippocampal slices (Sanna et al., 2004; Tokuda et al., 2011) in the absence of adrenal glands. It is not known *in vivo*, however, if ethanol can alter brain synthesis of 3α,5α-THP independent of the adrenal glands.

Studies investigating ethanol-induced changes of 3α,5α-THP in the brain have been limited to large brain regions like whole cerebral cortex or the hippocampus, which is highly enriched in 3α,5α-THP. Recently, our lab has been using immunohistochemistry (IHC) to measure levels of 3α,5α-THP in multiple brain regions that are implicated in alcohol use disorders. We have shown that ethanol (2g/kg) produces both increases and decreases of cellular 3α,5α-THP in the rat brain (Cook et al., 2013). Specifically, we have shown that ethanol increases cellular levels of 3α,5α-THP in the medial prefrontal cortex (mPFC), pyramidal cell layer of the CA1 hippocampus, polymorphic cell layer of the dentate gyrus (DG), bed nucleus of the stria terminalis, and paraventricular nucleus of the hypothalamus (PVN). Surprisingly, we also observed a decrease of cellular 3α,5α-THP in the NAc “shore” (core-shell border) and the central nucleus of the amygdala (CeA). Furthermore, there was no change in cellular levels of 3α,5α-THP in the granule cell layer of the DG, dorsomedial striatum, basolateral or lateral amygdala, or VTA.

Therefore, it appears that ethanol dynamically regulates cellular levels of 3α,5α-THP in the rat brain.

GABAERGIC NEUROACTIVE STEROIDS CONTRIBUTE TO THE BEHAVIORAL AND NEUROPHYSIOLOGICAL EFFECTS OF ETHANOL

As mentioned above, ethanol administration in rats increases plasma, cerebral cortical, and hippocampal levels of the GABAergic neuroactive steroids 3α,5α-THP and 3α,5α-THDOC. The ethanol-induced increases in GABAergic steroids are dose as well as time dependent and levels of steroids reach concentrations that are pharmacologically significant (VanDoren et al., 2000). A large body of evidence suggests the ethanol-induced elevations of GABAergic neuroactive steroids contribute to many of the behavioral effects of ethanol in rats. For example, preventing GABAergic neuroactive steroid synthesis with the 5α-reductase inhibitor finasteride or performing adrenalectomy reduces the hypnotic (Khisti et al., 2003), anxiolytic-like, (Hirani et al., 2005), antidepressant-like (Hirani et al., 2002), and anticonvulsant (VanDoren et al., 2000) effects of ethanol. Importantly, in humans finasteride prevents some of the subjective effects of alcohol (Pierucci-Lagha et al., 2005).

GABAergic neuroactive steroids also contribute to the neurophysiological effects of ethanol. The neurophysiological effects of ethanol-induced increases of GABAergic neuroactive steroids have been best characterized in hippocampal pyramidal cells. Ethanol-induced increase of 3α,5α-THP in the hippocampus may contribute to ethanol-induced impairment of memory and cognitive processes. In hippocampal pyramidal cells ethanol inhibits long-term potentiation (LTP), which is a type of synaptic plasticity thought to be a model of the physiological processes underlying learning and memory.

The same dose of ethanol that reduces LTP in CA1 pyramidal cells also increases 3α,5α-THP immunoreactivity in these cells (Tokuda et al., 2011). Furthermore, inhibiting GABAergic steroid synthesis with finasteride prevents ethanol inhibition of LTP and increased 3α,5α-THP immunoreactivity. This suggests that ethanol inhibition of LTP may be mediated by 3α,5α-THP. Ethanol also increases 3α,5α-THP in isolated hippocampal tissue with concomitant increases in amplitude of $GABA_A$ receptor mediated spontaneous and evoked inhibitory postsynaptic currents in CA1 pyramidal cells, the latter of which was prevented by finasteride (Sanna et al., 2004). Effects of ethanol-induced increases of 3α,5α-THP on pyramidal cell physiology are also observed *in vivo*. For example, systemic ethanol administration (1.5g/kg) reduces spontaneous firing of hippocampal pyramidal neurons in anesthetized rats, which is prevented by inhibiting GABAergic steroid synthesis with finasteride (Tokunaga et al., 2003b). Ethanol-induced increases of 3α,5α-THP also modulate neurophysiology outside the hippocampus as well. Ethanol (1.5g/kg) reduces spontaneous firing of medial septal/diagonal band of Broca neurons, which is prevented by finasteride (VanDoren et al., 2000). Ethanol-induced changes in 3α,5α-THP that contribute to behavioral effects of ethanol are likely due to effects of 3α,5α-THP at $GABA_A$ receptors across many brain regions. Therefore, it will be important to examine the neurophysiological effects of ethanol-induced changes of 3α,5α-THP in other brain regions. Since we recently showed that ethanol produces divergent effects on cellular levels of 3α,5α-THP in the rat brain (Cook et al., 2013), the neurophysiological effects of ethanol-induced changes in 3α,5α-THP levels are presumably functionally distinct depending on brain region.

NEUROACTIVE STEROIDS AND ETHANOL SELF-ADMINISTRATION

Systemic administration of endogenous and synthetic neuroactive steroids has been shown to reduce ethanol self-administration in rodents. The first steroid produced from cholesterol, pregnenolone, reduces operant ethanol self-administration in alcohol-preferring (P) rats (Besheer et al., 2010a). In this same study, the authors showed that one of the effective doses of pregnenolone (50mg/kg) increased 3α,5α-THP levels in the cerebral cortex of P rats that had been trained to self-administer ethanol, but not in ethanol naive P rats. These data suggest that increased brain levels of 3α,5α-THP contribute to pregnenolone's ability to reduce ethanol self-administration, and that a history of ethanol self-administration may produce adaptations in neuroactive steroid biosynthesis.

Systemic and intracerebroventricular (ICV) administration of 3α,5α-THP have been shown to produce biphasic effects on ethanol consumption using both homecage (Morrow et al., 2001; Ford et al., 2005; Ford et al., 2007) and operant self-administration of ethanol (Janak et al., 1998; Janak and Michael Gill, 2003). However, the effects of low dose systemic administration of 3α,5α-THP on ethanol consumption may depend on whether the animal is ethanol dependent. For example, a 5 mg/kg dose of 3α,5α-THP was shown to increase ethanol consumption in non-dependent P rats, but decreased consumption in dependent P rats (Morrow et al., 2001). Producing a reduction in ethanol consumption only in ethanol dependent animals is a desired outcome for a potential pharmacological treatment, since the treatment may be acting on neuroadaptations involved in ethanol dependence.

The therapeutic value of exogenous 3α,5α-THP may be limited by rapid metabolism (Purdy et al., 1990a). Therefore, longer acting synthetic GABAergic neuroactive steroids such as ganaxolone may be more practical from a treatment standpoint. Ganaxolone has been shown to produce biphasic effects on operant ethanol self-administration in P rats, but the dose of ganaxolone that reduced ethanol responding also produced sedation (Besheer et al., 2010a). In mice, low (5 mg/kg) to mid (10mg/kg) dose ganaxolone has been shown to alter homecage ethanol consumption during a 24 hour exposure similar to low dose 3α,5α-THP, but over an extended duration (Ramaker et al., 2011). Furthermore, a 10 mg/kg dose of ganaxolone decreased consumption using a 2 hour limited access paradigm, but 5 and 10 mg/kg doses of ganaxolone did not significantly change operant ethanol self-administration (Ramaker et al., 2012). Higher doses of ganaxolone that may more consistently reduce ethanol drinking in mice hasn't been tested, but there are concerns that 3α,5α-THP and ganaxolone may produce sedation at effective doses, thus, limiting therapeutic value. However, the synthetic GABAergic neuroactive steroid 3α,5β-20-oxo-pregnane-3-carboxylic acid (PCA) has been shown to dose dependently reduce operant ethanol self-administration in Wistar rats at doses that do not produce sedation (O'Dell et al., 2005).

The biphasic nature of 3α,5α-THP and ganaxolone modulation of ethanol consumption is not clear, but could result from low doses increasing the reinforcing effect of ethanol and high doses decreasing ethanol's reinforcing effects. Previous studies have shown that 3α,5α-THP can be reinforcing. For example, rats were shown to preferentially consume a 3α,5α-THP solution over water in a two-bottle choice procedure (Sinnott et al., 2002). Multiple studies also have shown that 3α,5α-THP has ethanol-like

discriminative stimulus (i.e. 3α,5α-THP feels like ethanol) properties (Grant et al., 1996; Bowen et al., 1999; Hodge et al., 2001). In DBA/2J mice, systemic administration of 3α,5α-THP has been shown to produce conditioned place preference (Finn et al., 1997). In rats, however, ICV administration of 3α,5α-THP has been shown to produce a conditioned place aversion (Beauchamp et al., 2000). Interestingly, a similar high dose of ICV 3α,5α-THP was shown to reduce dopamine release in the NAc and mPFC (Motzo et al., 1996). Furthermore, low dose ICV 3α,5α-THP administration increases dopamine release in the NAc (Rouge-Pont et al., 2002). Therefore, it is possible that 3α,5α-THP-mediated biphasic changes in dopamine release may underlie the observed biphasic changes in ethanol reinforcement and consumption.

Increasing levels of GABAergic neuroactive steroids may have therapeutic value for treating individuals suffering from alcohol use disorders. The preclinical studies mentioned here have shown 3α,5α-THP reduces ethanol reinforcement and consumption. Furthermore, human alcoholics have reduced blood plasma levels of 3α,5α-THP during alcohol withdrawal (Romeo et al., 1996). Therefore, restoring levels of 3α,5α-THP during abstinence and withdrawal may be therapeutic. GABAergic neuroactive steroids also may alleviate withdrawal symptoms by reducing anxiety and CNS excitability, which are characteristic of alcohol withdrawal.

RATIONALE FOR AIMS OF STUDIES

A large body of evidence has shown that ethanol produces pharmacologically relevant increases of 3α,5α-THP in the brain (for review see, Morrow, 2007). Specifically, it has been shown that ethanol increases 3α,5α-THP in the cerebral cortex and hippocampus. These increases of 3α,5α-THP are thought to contribute to the

behavioral and neurophysiological effects of ethanol in rats. Importantly, 3α,5α-THP contributes to the subjective effects of alcohol in humans (Pierucci-Lagha et al., 2005) and levels of 3α,5α-THP are reduced in the blood of human alcoholics during withdrawal. Administration of 3α,5α-THP also can reduce ethanol reinforcement and consumption in rodent models of excessive alcohol consumption. Therefore, understanding how ethanol regulates 3α,5α-THP levels in the brain is important for understanding ethanol action and developing new treatments for alcohol use disorders. Although these studies are focused on ethanol-related effects, results and approaches from these experiments may be applied to other neuropsychiatric disorders where neuroactive steroids may be altered and/or changing levels of neuroactive steroids may be therapeutic.

AIM I RATIONALE

It has been known for more than 10 years that ethanol increases levels of 3α,5α-THP in the blood plasma, whole cerebral cortex, and whole hippocampus of rats (Barbaccia et al., 1999; VanDoren et al., 2000). Elevations of 3α,5α-THP contribute to the pharmacological effects of ethanol, presumably via actions at $GABA_A$ receptors across many brain regions. However, due to limitations in available 3α,5α-THP assays, it is not known if ethanol alters levels of 3α,5α-THP in other brain regions. Therefore, we optimized an immunohistochemical assay for measuring cellular levels of 3α,5α-THP in the rat brain. Preliminary results investigating ethanol-induced changes in cellular 3α,5α-THP levels showed that ethanol produced increases, decreases, or no change in 3α,5α-THP dependent on the brain region or cellular population evaluated. These results are intriguing and suggest that ethanol alters local synthesis and/or metabolism of 3α,5α-THP

in the brain. Previous *in vivo* results have shown that ethanol-induced increases of 3α,5α-THP in the cerebral cortex are dependent on adrenal gland activation since adrenalectomy prevents 3α,5α-THP elevations. However, there is *in vitro* evidence for local brain synthesis of 3α,5α-THP in hippocampal slices, in the absence of adrenal influence. Therefore, aim I focused on examining the effects of ethanol on cellular 3α,5α-THP expression in several brain regions implicated in alcohol use disorders, and to determine the role of the adrenal glands in these effects.

AIM II RATIONALE

Systemic administration of the endogenous neuroactive steroids pregnenolone and 3α,5α-THP reduces ethanol reinforcement and consumption in rodents. Systemic 3α,5α-THP administration also reduces escalation of cocaine self-administration (Aker et al., 2010). It is not known where in the brain neuroactive steroids act to reduce drug self-administration, but ICV administration of 3α,5α-THP produces biphasic effects on ethanol consumption as well as dopamine release in mesolimbic circuitry. Therefore, we hypothesized that increased 3α,5α-THP in mesolimbic regions would reduce ethanol self-administration. The therapeutic potential of 3α,5α-THP, however, may be limited by rapid metabolism as well as nonspecific effects such as sedation. Therefore, we developed a recombinant adeno-associated serotype 2 (rAAV2) vector to overexpress the P450scc enzyme and drive neurosteroidogenesis locally within neurons of the NAc or VTA of P rats self-administering ethanol. The AAV2 vector was chosen because it selectively infects neurons, produces minimal immune response, and allows us to examine long-term effects on ethanol self-administration. Transduction of P450scc will only be effective in cells that contain other requisite biosynthetic enzymes for production

of the neuroactive steroids. The selectively bred P rat fulfills the requirement of an animal model of alcoholism (Lester and Freed, 1973) since they readily consume ethanol in amounts that produce physiologically significant blood alcohol concentration, maintain ethanol preference in the presence of palatable solutions, and develop ethanol tolerance and dependence. We predicted that behavioral effects of increasing neurosteroidogenesis with the rAAV2-P450scc vector would be associated with increased $3\alpha,5\alpha$-THP due to its potency at $GABA_A$ receptors, involvement in ethanol action, and ability to modulate ethanol self-administration.

CHAPTER 2. **EFFECTS OF ETHANOL ON 3α,5α-THP IMMUNOHISTOCHEMISTRY IN THE RAT BRAIN**

INTRODUCTION

Neuroactive steroids are endogenous neuromodulators capable of altering neuronal activity. Synthesis of neuroactive steroids occurs in the adrenal glands, gonads, and *de novo* in the brain. The 5α-reduced pregnane steroids, (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC), are positive allosteric modulators of γ-aminobutyric acid type A ($GABA_A$) receptors. $GABA_A$ receptors are the primary inhibitory receptor family in the brain and mediate many of the behavioral effects of ethanol. Both 3α,5α-THP and 3α,5α-THDOC enhance neuronal inhibition at a known binding site on $GABA_A$ receptor α-subunits (Hosie et al., 2006), and have corresponding behavioral effects similar to ethanol. These GABAergic neuroactive steroids are very potent positive modulators of $GABA_A$ receptors, which produce pharmacologically relevant effects at nanomolar concentrations (Morrow et al., 1987).

A large body of evidence suggests increased levels of 3α,5α-THP and 3α,5α-THDOC following ethanol administration contribute to both electrophysiological and behavioral effects of ethanol in rodents. In rats, ethanol activates the hypothalamic-pituitary-adrenal (HPA) axis (Boyd et al., 2010) leading to physiologically significant

increases of 3α,5α-THP and 3α,5α-THDOC in the blood plasma, cerebral cortex, and hippocampus (Barbaccia et al., 1999; VanDoren et al., 2000; Porcu et al., 2009). Adrenalectomy or inhibition of 5α-reduced steroid synthesis with the 5α-reductase (5α-R) inhibitor finasteride reduces some of the behavioral effects of ethanol, including the hypnotic (Khisti et al., 2003), anxiolytic-like (Hirani et al., 2005), anticonvulsant (VanDoren et al., 2000), and anti-depressant-like (Hirani et al., 2002) effects in rats. Furthermore, finasteride reduces some of the subjective effects of alcohol in healthy men (Pierucci-Lagha et al., 2005). Finasteride also blocks ethanol inhibition of neuron firing in the medial septum (VanDoren et al., 2000), hippocampus (Tokunaga et al., 2003b), hippocampal slice (Sanna et al., 2004), and long-term potentiation (LTP) in the hippocampal slice preparation (Tokuda et al., 2011). Taken together, these findings suggest ethanol-induced elevations of 3α,5α-THP and 3α,5α-THDOC contribute to many of the physiological and behavioral effects of ethanol. However, outside of the cerebral cortex and hippocampus, it is not known if ethanol increases levels of 3α,5α-THP in other brain regions that contribute to ethanol's myriad of pharmacological effects.

GABAergic neuroactive steroids are involved in stress responses; therefore, steroid concentrations may be altered in stress related neurocircuitry following ethanol administration. GABAergic neuroactive steroids are increased in the plasma and cerebral cortex following environmental stress (Purdy et al., 1991a; Barbaccia, 1996), including ethanol administration. GABAergic neuroactive steroids contribute to negative feedback on the HPA axis at the level of the hypothalamus in rats (Owens et al., 1992; Patchev et al., 1994; Patchev et al., 1996), but not in C57BL/6J mice (Sarkar et al., 2011). Furthermore, recent evidence in C57BL/6J mice suggests GABAergic neuroactive

steroids are involved in initiation of the stress response (Sarkar et al., 2011). In the current study, we investigated ethanol-induced changes of 3α,5α-THP in the paraventricular nucleus (PVN) of the hypothalamus, the bed nucleus of the stria terminalis (BNST), and amygdala due to the involvement of these regions in stress, emotion, and ethanol responses (Armario, 2010; Cui et al., 2012; Koob, 2013).

Ethanol and 3α,5α-THP both impair learning and memory performance in a similar manner (Matthews et al., 2002), which may be due to modulation of activity of specific cellular populations in the hippocampus (Matthews et al., 2002; Tokunaga et al., 2003b; Tokuda et al., 2011). *In vitro*, ethanol increases 3α,5α-THP immunoreactivity in hippocampal pyramidal cells (Tokuda et al., 2011), and finasteride prevents ethanol's inhibitory effect on these cells in anesthetized rats (Tokunaga et al., 2003b). Therefore, we examined the cellular layer specificity of ethanol effects on 3α,5α-THP levels in the hippocampal formation.

Systemic and intracerebroventricular administration of exogenous 3α,5α-THP or systemic administration of the longer acting 3α,5α-THP analogue, ganaxolone, alter ethanol consumption in rodents. Both 3α,5α-THP and ganaxolone have been shown to produce biphasic effects on ethanol consumption, with low doses increasing consumption and high doses decreasing consumption (Janak et al., 1998; Morrow et al., 2001; Ford et al., 2005; Ford et al., 2007; Besheer et al., 2010a). The mechanisms by which 3α,5α-THP affects ethanol consumption are not clear, but these effects could be due to modulation of dopamine release in mesocorticolimbic circuitry (Motzo et al., 1996; Rouge-Pont et al., 2002). Therefore, we examined the effects of ethanol on 3α,5α-THP immunoreactivity in

the ventral tegmental area (VTA), nucleus accumbens (NAc), striatum, and medial prefrontal cortex (mPFC).

Previous studies measuring ethanol-induced changes of 3α,5α-THP in the brain have used radioimmunoassay (RIA) or gas chromatography-mass spectroscopy (GC-MS), which lack the sensitivity to determine levels in discrete cell layers or brain regions with low levels of 3α,5α-THP. Although ethanol increases levels of 3α,5α-THP in the cerebral cortex and hippocampus, it is unknown whether ethanol alters levels of 3α,5α-THP in other brain regions. Immunohistochemistry (IHC) using an anti-3α,5α-THP primary antibody was used to examine ethanol-induced changes in cellular 3α,5α-THP levels. This technique has been used to determine the cellular distribution of 3α,5α-THP in the rat brain (Saalmann et al., 2007) and displays the sensitivity to detect changes in immunoreactivity that are associated with corresponding changes in electrophysiological measurements (Saalmann et al., 2006; Tokuda et al., 2011). We examined ethanol-induced changes in cellular 3α,5α-THP expression across multiple brain regions that are implicated in alcohol use disorders. This approach provides brain region specificity of neuroactive steroid measurements that has not previously been reported following ethanol administration, and may reveal novel mechanisms of ethanol action.

MATERIALS AND METHODS

Subjects

Adult male Wistar rats (~250 g/7-8 per group) were purchased from Harlan Laboratories (Indianapolis, IN, USA). The animals were housed in Plexiglass cages (2 to 4 per cage) with food and water available *ad libitum*. The colony room was maintained on a normal 12 hr light-dark cycle (light onset at 0700 hr) and at a constant temperature of 22 ± 2°C and relative humitity of 65%. The animals were allowed 1 week to acclimate to the colony room. Following acclimation, the animals were habituated to handling and intraperitoneal (i.p.) saline injections for 5 days. Experiments were conducted between 0800 and 1300 hr to minimize potential circadian fluctuation in neuroactive steroid levels.

Ethanol (2g/kg, 20% v/v in saline) or saline was administered by i.p. injection 60 minutes before transcardial perfusion. Animal care and handling procedures followed National Institutes of Health Guidelines under University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee approved protocols.

Antibody specificity tests

The specificity of the affinity purified 3α,5α-THP antibody was measured by RIA. Standards of 3α,5α-THP, 3α,5α-THDOC, (3α,5β)-3-hydroxypregnan-20-one (3α,5β-THP), pregnenolone, progesterone and (3α)-3-hydroxy-4-pregnen-20-one (3α-HP) were diluted in 95% ethanol at an initial concentration of 0.1 mg/ml. The compounds were serially diluted to a range of 0.0049 - 40.0 ng/ml, no more than 24 hours before the assay. RIAs were repeated three times for each compound, following previously described methods (Janis et al., 1998). Briefly, 5 µl of each concentration of the tested

compounds were mixed with 10,000 CPM of [^{3}H]-3α,5α-THP and a 1:500 dilution of the affinity purified 3α,5α-THP antiserum. Unbound [^{3}H]- 3α,5α-THP was removed by centrifugation after adding dextran-coated charcoal. The supernatant was mixed with Ecoscint H (National Diagnostics) and [^{3}H]-3α,5α-THP was measured in a scintillation counter. The resulting curves were analyzed using a one-site competition model (Prism, GraphPad Software, La Jolla, CA, USA) for EC_{50} values. We observed cross reactivity with 3α, hydroxy-4-pregnen-20-one (3α-HP; 41±0.14%), (3α,5β)-3-hydroxypregnan-20-one (3α,5β-THP; 22±0.43%), progesterone (14±1.95%), 3α,5α-THDOC (11±0.29%), and pregnenolone (9±1.61%) as expected from previous reports using a different antibody preparation (Janis et al., 1998; VanDoren et al., 2000; Khisti et al., 2003; Boyd et al., 2010).

Immunohistochemistry

Fifty minutes after 2g/kg ethanol or saline injection the animals were anesthetized with pentobarbital (100mg/kg, i.p.; Professional Compounding Centers of America, Houston, TX, USA) and transcardially perfused approximately 1hr following ethanol or saline injection with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Tissue was post-fixed in 4% paraformaldehyde for 24hr at 4°C, sectioned coronally on a vibrating microtome at 40μm, and stored at -30°C until further processing.

Immunohistochemical assays were performed using a procedure modified from (Saalmann et al., 2007). No detergents or organic solvents were used to prevent the possibility of neuroactive steroid leeching. Free floating brain sections (3-4 sections/animal/brain region) were rinsed in PBS, followed by incubation in 1% hydrogen peroxide to block endogenous peroxidase activity, and then blocked using 10%

rabbit serum in PBS. Next, the tissue was incubated in sheep affinity purified anti-3α,5α-THP antiserum (targeted against 3α,5α-THP carboxymethyl ether coupled to bovine serum albumin; purchased from Dr. R.H. Purdy) at a 1:2500 dilution for 48 hr at 4°C. Following rinsing in PBS, tissue was incubated in a rabbit anti-sheep biotinylated secondary antibody (1:200; Vector laboratories, Burlingame, CA, USA) for 1 hr. After rinsing in PBS, avidin biotin amplification was performed with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB; Polysciences, Inc., Warrington, PA, USA and Sigma-Aldrich, St. Louis, MO, USA) using the manufacturers' recommended procedures.

Immunohistochemical analysis

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA, USA) and images were captured with a digital camera (Regita model, QImaging, Burnaby, BC). Image analysis software (Bioquant Life Sciences version 8.00.20; Bioquant Life Sciences, Nashville, TN, USA) that utilizes linear integrated optical density was used for comparing relative changes in immunoreactivity between groups. The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition. Immunoreactive positive pixel count measurements were calculated from a circumscribed field (e.g., brain region), divided by the area of the region in square millimeters, and expressed as positive pixels/mm^2. Data were acquired from 3-4 sections/animal/brain region, and data were averaged within a brain region for an individual animal to obtain one value per subject. Inter-rater reliability was determined by calculating the intraclass-correlation coefficient for two raters blind to the

experimental conditions. The polymorph dentate gyrus (DG), NAc, and mPFC were chosen randomly from 6 brain regions for analysis and values of $r = 0.85$ ($p < 0.001$), $r = 0.78$ ($p < 0.01$), and $r = 0.77$ ($p < 0.005$) were obtained, respectively. Intraclass-correlation coefficient was calculated using MATLAB (MathWorks, Natick, MA, USA). Immunoreactivity was measured separately for each brain region and statistically analyzed using Student's t-test (Prism, GraphPad Software, La Jolla, CA, USA) to compare the ethanol versus saline group within each brain region.

Brain region analyses were performed using histological coordinates as follows: mPFC (+3.00 to +2.20 AP), amygdala [central nucleus (CeA); lateral amygdala; basolateral amygdala; -2.56 to -3.14 AP], NAc (+ 1.70 to + 1.00 AP), dorsomedial striatum [DMS (+1.70 to + 1.00 AP)], VTA (-5.20 to -6.04 AP), hippocampus [pyramidal cell layer of CA1 (-2.56 to -3.14 AP), polymorph (-2.56 to -3.14 AP) and granule (-2.56 to -3.14 AP) cell layers of the DG, BNST (-0.26 to -0.40 AP), hypothalamus [PVN, (-1.60 to -2.12 AP)]. All analyses were based on coordinates relative to bregma in the Rat Brain Atlas (Paxinos and Watson, 1998).

RESULTS

Ethanol-induced increases of cellular 3α,5α-THP

Previous studies using RIAs have shown ethanol-induced increases in 3α,5α-THP levels in the rat cortex and hippocampus (Barbaccia et al., 1999; VanDoren et al., 2000). Therefore, we first examined these regions to determine if the elevations displayed sub-region or cellular layer specificity. Ethanol administration (2g/kg, i.p.) increased 3α,5α-THP immunoreactivity in the mPFC [24±6%; $t(13)=2.996$, $p < 0.01$]. The effects of ethanol appear uniform across the cortical cell layers (Fig. 2.1A). In hippocampus, ethanol increased 3α,5α-THP immunoreactivity in the pyramidal cells of the CA1 region [32±12%; $t(14)=2.401$, $p < 0.05$] (Fig. 2.1B), and the polymorph cell layer of the DG [52±5%; $t(14)=5.288$, $p < 0.001$] (Fig. 2.2A), but had no effect on cellular 3α,5α-THP in the granule cell layer of the DG (Fig. 2.2B), indicating cellular layer specificity in the response to ethanol.

Ethanol also increased 3α,5α-THP immunoreactivity in brain regions involved in stress responses. We examined ethanol effects on cellular 3α,5α-THP levels in the PVN due to its role in modulating HPA axis activity. We also investigated ethanol's effects on cellular 3α,5α-THP in the BNST due to its role as an interface between stress and reward circuitry and involvement in ethanol responses (Cui et al., 2012). Ethanol administration (2g/kg, i.p.) increased 3α,5α-THP immunoreactivity in both the PVN [36±6%; $t(14)=3.406$, $p < 0.01$] (Fig. 2.3A) and BNST [44±15%; $t(13)=2.346$, $p < 0.05$] (Fig. 2.3B).

Ethanol-induced decreases of cellular 3α,5α-THP in regions associated with ethanol reinforcement and consumption

Multiple studies suggest that 3α,5α-THP alters ethanol consumption (Morrow et al., 2001; Ford et al., 2005; Ford et al., 2007) and reinforcement (Janak et al., 1998; Janak and Michael Gill, 2003), so we examined the effects of ethanol administration (2g/kg, i.p.) in regions that are known to contribute to these phenomena. Ethanol administration did not alter 3α,5α-THP immunoreactivity in the VTA. Interestingly, ethanol administration decreased 3α,5α-THP immunoreactivity in the NAc [-22±5%; $t(12)=3.214$, $p < 0.01$] (Fig. 2.4A) but had no effect on 3α,5α-THP in the DMS (Fig. 2.4 B) or VTA (Fig. 2.4C). The ethanol-induced reduction of 3α,5α-THP in the NAc was only detected in the NAc "shore" (core-shell border), as no changes in 3α,5α-THP were found in the NAc core or the NAc shell (data not shown), indicating sub-region specificity of ethanol effects on 3α,5α-THP levels in the ventral striatum. Ethanol administration reduced 3α,5α-THP immunoreactivity in the CeA [-21±3%; $t(14)=2.508$, $p < 0.05$] (Fig. 2.5A), but there was no change in 3α,5α-THP in the lateral (Fig. 2.5B) or basolateral amygdala (Fig. 2.5C). Therefore, ethanol produces sub-region specific effects on 3α,5α-THP in amygdala regions as well.

DISCUSSION

The goal of the present study was to use IHC to examine ethanol-induced changes in cellular 3α,5α-THP expression in multiple brain regions implicated in alcohol use disorders. Our findings support previous evidence that ethanol increases 3α,5α-THP concentrations in the rat cerebral cortex and hippocampus using RIA or GC-MS. We extend previous findings by showing that ethanol increases cellular 3α,5α-THP in the mPFC, CA1 pyramidal cell layer, and polymorph cell layer of the DG but not in granule cells of the DG. Therefore, ethanol-induced increases of cellular 3α,5α-THP in the hippocampus are isolated to specific cellular populations. We also show that ethanol increases cellular 3α,5α-THP levels in the BNST and the PVN of the hypothalamus. Interestingly, acute ethanol administration reduced cellular 3α,5α-THP levels in the NAc "shore" (core-shell border) and the CeA. To our knowledge, this is the first example of acute ethanol reducing 3α,5α-THP levels in the rat brain or periphery. We also determined that ethanol does not alter 3α,5α-THP levels in the VTA, DMS, or the lateral or basolateral amygdala. Therefore, ethanol produces divergent brain region and cellular layer specific changes in 3α,5α-THP concentrations.

The immunohistochemical technique used in the present study provides brain region and cell layer specificity of ethanol-induced changes in 3α,5α-THP. A similar IHC assay was used to demonstrate that 3α,5α-THP is located primarily in GABAergic and glutamatergic projecting principle neurons, but not in interneurons or glia (Saalmann et al., 2007). The present results indicate similar relative intensity of basal staining across brain, with strong labeling of cortical and hippocampal cell layers as well as the striatum. There are some important differences between measurements of 3α,5α-THP with IHC

compared to RIA measurements. IHC measures cellular levels of 3α,5α-THP but RIA measures absolute levels of neuroactive steroids. Therefore, if there are significant pools of extraneuronal 3α,5α-THP present in brain tissue, one might predict IHC measurements would differ from results obtained by RIA. Our current IHC results in the mPFC, CA1 pyramidal, and DG polymorph cell layer show that ethanol increased relative 3α,5α-THP immunoreactivity by 24±6%, 32±12%, and 52±5% , respectively. However, RIA studies measuring ethanol-induced increases of 3α,5α-THP in whole cerebral cortex and hippocampus have found larger increases of 3α,5α-THP using similar doses. For example, increases of 5-17 fold (Barbaccia et al., 1999), ~3 fold (VanDoren et al., 2000; Khisti et al., 2004), and ~2 fold (Boyd et al., 2010) have been observed in the cerebral cortex, while studies examining whole hippocampus have found 4-11 (Barbaccia et al., 1999) and ~6 fold (Khisti et al., 2004) increases in 3α,5α-THP concentrations. The discrepancies in magnitude of ethanol-induced increases of 3α,5α-THP could be due to relatively small increases of 3α,5α-THP in the mPFC when compared to the whole cortex, and similarly, small relative increases of 3α,5α-THP in the CA1 pyramidal cells and the polymorph cell layer of the DG compared to whole hippocampus. It is important to note we are using an affinity purified 3α,5α-THP antiserum that appears to be more specific in detecting 3α,5α-THP. The only other study examining ethanol's effects on 3α,5α-THP using the affinity purified 3α,5α-THP antiserum used IHC with immunofluorescent detection and found over a 200% increase in fluorescent intensity in CA1 pyramidal cells (Tokuda et al., 2011) compared to a 32±12% increase in immunoreactivity in the present study using DAB detection. Although ethanol increased

3α,5α-THP using both fluorescent and DAB visualization, the magnitude of 3α,5α-THP changes may differ due to differences in immunohistochemical methodology.

The 3α,5α-THP antisera used in the present study was found to cross-react with 3α-HP (41±0.14%) and 3α,5β-THP (22±0.43%). This is not a major limitation since both of these steroids have similar GABAergic activities as 3α,5α-THP. Furthermore, there is no evidence that 3α-HP (Griffin and Mellon, 2001) or 3α,5β-THP are present in the brain in appreciable concentrations. In addition, ethanol does not elevate 3α,5β-THP levels in rat plasma (Porcu et al., 2010). Therefore, 3α,5α-THP is the most likely endogenous antigen immunolabelled using this approach. A potential limitation of the study is that only one dose of ethanol was examined. In the cerebral cortex, a threshold dose of ethanol (1.3-1.5 g/kg) is required to produce an increase of 3α,5α-THP and increasingly higher doses produce less of an increase of 3α,5α-THP (VanDoren et al., 2000; Boyd et al., 2010). However, since only one dose was used it is not known if a similar threshold dose is needed to produce increases or decreases of 3α,5α-THP in the cortical and subcortical brain regions examined in the present study. Similarly, it is not clear if ethanol produces a biphasic effect on 3α,5α-THP levels in these brain regions.

The diverse effects of ethanol administration on cellular 3α,5α-THP levels suggest that ethanol may alter local synthesis and/or metabolism of 3α,5α-THP in the rat brain. These results suggest ethanol-induced changes of cellular 3α,5α-THP in the brain are not due exclusively to adrenal gland derived elevations of neuroactive steroids or steroid precursors, which would be expected to increase 3α,5α-THP throughout the brain. However, we observed no change in 3α,5α-THP levels after acute ethanol administration in the granule cell layer of the DG and the DMS, which both display dense labeling of

3α,5α-THP (Saalmann et al., 2007) as well as the biosynthetic enzymes 5α-R and 3α-hydroxysteroid dehydrogenase (3α-HSD) needed for 3α,5α-THP synthesis (Agis-Balboa et al., 2006). Furthermore, there was a reduction of cellular 3α,5α-THP in the NAc and the CeA. Based on these findings our data suggest there are unknown mechanisms contributing to ethanol's effects on 3α,5α-THP concentrations. In the cerebral cortex, ethanol-induced elevations of 3α,5α-THP are dependent on the pituitary-adrenal axis (Khisti et al., 2003; O'Dell et al., 2004; Boyd et al., 2010). *In vitro*, however, there is evidence for ethanol-induced brain synthesis of 3α,5α-THP in hippocampal slices (Sanna et al., 2004; Tokuda et al., 2011). Thus, it is not known if ethanol alters brain synthesis of 3α,5α-THP *in vivo* independent of circulating steroids. Therefore, it will be important to determine the role of circulating steroids in the current observations, which may provide insight into this important question.

The most striking finding in the present study is that ethanol produces brain region and cellular layer specific changes in 3α,5α-THP concentrations. These data suggest ethanol alters local synthesis of 3α,5α-THP in the rat brain. A possible mechanism that may explain these findings is ethanol may change local expression and/or activity of steroidogenic enzymes. Steroidogenesis is initiated by cholesterol transport to cytochrome P450 side chain cleavage (P450scc), resulting in conversion of cholesterol to pregnenolone. Previous work from our laboratory has shown that *de novo* adrenal synthesis of the cholesterol transporter, steroidogenic acute regulatory protein (StAR), is necessary for ethanol-induced increases of 3α,5α-THP in the cerebral cortex (Boyd et al., 2010). Acute ethanol administration increases StAR and P450scc mRNA in the rat frontal cortex and hypothalamus, and StAR mRNA is also increased in the hippocampus (Kim et

al., 2003). The synthesis of 3α,5α-THP from progesterone is accomplished by the sequential actions of 5α-R and 3α-HSD. Acute ethanol administration increases 5α-R type 1 (5α-RI) and 3α-HSD mRNA in the frontal cortex and 3α-HSD mRNA in the hypothalamus, but no change in transcript expression of either enzyme were detected in the hippocampus (Kim et al., 2003). Unfortunately, there are no data examining the effects of acute ethanol on expression of these enzymes in the NAc or CeA where we observed reductions in 3α,5α-THP. However, chronic intermittent ethanol administration reduces 3α,5α-THP levels in the hippocampus, which is associated with concurrent decreases in 5α-RI and 3α-HSD mRNA expression (Cagetti et al., 2004). Taken together, ethanol-induced changes in steroidogenic enzyme expression may underlie the divergent changes in 3α,5α-THP levels observed in the rat brain. Additionally, ethanol may directly or indirectly change the activity of steroidogenic enzymes, which could alter steroid concentrations. Ultimately, ethanol may alter the expression and/or activity of enzymes involved in steroid synthesis and metabolism, resulting in local changes in neurosteroid levels that are dependent on which enzymes are expressed in a particular cell.

Another possible explanation for the reduction in cellular 3α,5α-THP levels observed in the NAc and CeA is extracellular redistribution of 3α,5α-THP. Neuroactive steroids have been proposed to act on membrane bound receptors by a paracrine or autocrine mechanism (Herd et al., 2007), or by lateral diffusion through the cell membrane to access transmembrane neuroactive steroid binding sites on $GABA_A$ receptors (Akk et al., 2007). Although controversial, we cannot rule out the possibility that ethanol leads to a "release" or redistribution of 3α,5α-THP into the extracellular space, perhaps in a brain region specific manner.

Significance of ethanol-induced changes in cellular expression of 3α,5α-THP

Evidence suggests ethanol-induced increases of 3α,5α-THP contribute to the behavioral effects of ethanol in rodents and some of the subjective effects of alcohol in humans. Contribution of 3α,5α-THP to the pharmacological effects of ethanol is likely due to potentiation of $GABA_A$ receptors across multiple brain regions. The localization of 3α,5α-THP and the biosynthetic enzymes 5α-R and 3α-HSD in principle output neurons suggests a major role of 3α,5α-THP may be to modulate neural circuitry (Agis-Balboa et al., 2006; Saalmann et al., 2007). The neurophysiological effects of 3α,5α-THP have been best characterized in hippocampal pyramidal cells. *In vivo*, ethanol (1.5g/kg) reduces spontaneous activity of hippocampal pyramidal cells in anesthetized rats, which is prevented by finasteride pre-administration (Tokunaga et al., 2003b). *In vitro*, ethanol (50, 100 mM) increases 3α,5α-THP in isolated hippocampal tissue and amplitude of $GABA_A$ receptor mediated spontaneous and evoked IPSCs in CA1 pyramidal cells (Sanna et al., 2004), the latter of which is prevented by pretreatment with finasteride. Ethanol (60 mM) also inhibits LTP in CA1 pyramidal cells, with concomitant increases in 3α,5α-THP immunostaining (Tokuda et al., 2011). Furthermore, pretreatment with finasteride blocks ethanol's inhibition on LTP, suggesting that ethanol's ability to inhibit LTP is mediated by 3α,5α-THP. In the present study, ethanol increased 3α,5α-THP in the polymorphic cell layer but not the granule cell layer of the DG. Although we did not distinguish which polymorphic cells exhibit increased 3α,5α-THP, the most prominent cell type in this layer is the mossy cell. Mossy cells in the polymorphic layer project to the molecular and granule cell layers of the DG (Amaral et al., 2007), the latter of which constitutes the only projection from the DG to the hippocampus. Considering the major

role the hippocampus and DG play in learning and memory, 3α,5α-THP induction by ethanol may contribute to ethanol's effects on memory and cognition.

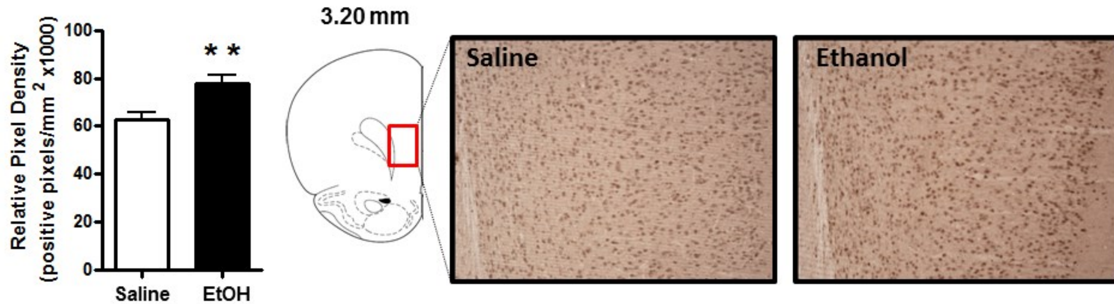
Ethanol-induced changes in brain concentrations of 3α,5α-THP may also modulate neuronal activity in other brain regions examined in the present study. *In vivo* evidence suggests ethanol-induced increases of 3α,5α-THP modulate neuronal activity outside of the hippocampus as well. For example, finasteride prevents ethanol (1.5g/kg) inhibition of spontaneous firing of medial septal/diagonal band of Broca neurons (VanDoren et al., 2000). In addition to hippocampal pyramidal cells, we observed increases of cellular 3α,5α-THP in the polymorph DG, mPFC, BNST, and PVN of the hypothalamus. The physiological consequences of 3α,5α-THP increases may alter neuronal activity and synaptic plasticity in these regions and related circuitry. Clearly, studies are needed to examine how increases of 3α,5α-THP in these brain regions may contribute to the physiological and behavioral effects of ethanol.

Ethanol-induced reductions of cellular 3α,5α-THP were observed in the NAc and CeA. These observations are particularly interesting because these two brain regions are strongly associated with ethanol reinforcement and consumption (McBride, 2002; Gonzales et al., 2004). A reduction in cellular 3α,5α-THP would presumably reduce the amount of $GABA_A$ receptor mediated neuronal inhibition, which may increase firing of mesolimbic dopamine neurons and CeA circuitry involved in ethanol reinforcement. In the NAc, reductions of 3α,5α-THP were isolated to the "shore" (core-shell border), as no changes in 3α,5α-THP were detected exclusively in the shell or core. This is an interesting finding since ethanol associated cues and operant self-administration of ethanol both increase dopamine release in the "shore" following operant training

(Howard et al., 2009), but in the core or shell only ethanol associated cues increase dopamine release following operant training. It is important to note, however, that acute systemic ethanol administration increases dopamine release in the NAc core and shell (Imperato and Di Chiara, 1986; Yim et al., 2000) where ethanol did not alter 3α,5α-THP levels. Administration of 3α,5α-THP produces biphasic effects on ethanol consumption (Janak et al., 1998; Ford et al., 2005; Ford et al., 2007; Finn et al., 2010) and dopamine release in the NAc (Motzo et al., 1996; Rouge-Pont et al., 2002). Furthermore, 3α,5α-THP modulates ethanol's effects on mPFC dopamine content (Dazzi et al., 2002). Therefore, investigating possible interactions of 3α,5α-THP and dopaminergic activity in the mesocorticolimbic system may be valuable in the effort to reduce ethanol consumption via neuroactive steroid modulation.

The current data show that ethanol produces divergent brain region and cell layer specific changes in cellular 3α,5α-THP concentrations. These divergent effects suggest that ethanol induces local regulation of 3α,5α-THP levels. These changes in 3α,5α-THP levels likely contribute to the neurophysiological and behavioral effects of ethanol in rats. Examining the mechanisms by which ethanol alters levels of 3α,5α-THP across the brain may lead to new therapeutic strategies for treating alcohol use disorders.

FIGURES AND TABLES



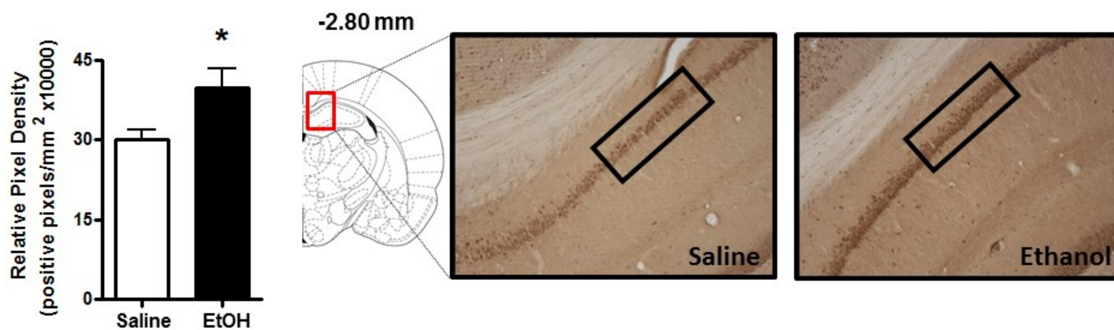


Figure 2.1: Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the mPFC and pyramidal cell layer of the CA1 hippocampus. (A) Ethanol administration increased 3α,5α-THP immunoreactivity in the mPFC compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the mPFC (3.20 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration. (B) Ethanol administration increased 3α,5α-THP immunoreactivity in the pyramidal cell layer of the CA1 hippocampus compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in CA1 pyramidal cells (highlighted in rectangle, -2.80 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. * $p < 0.05$, ** $p < 0.01$ compared to saline administration. Medial prefrontal cortex (mPFC), ethanol (EtOH), Cornu Ammonis area 1 (CA1).

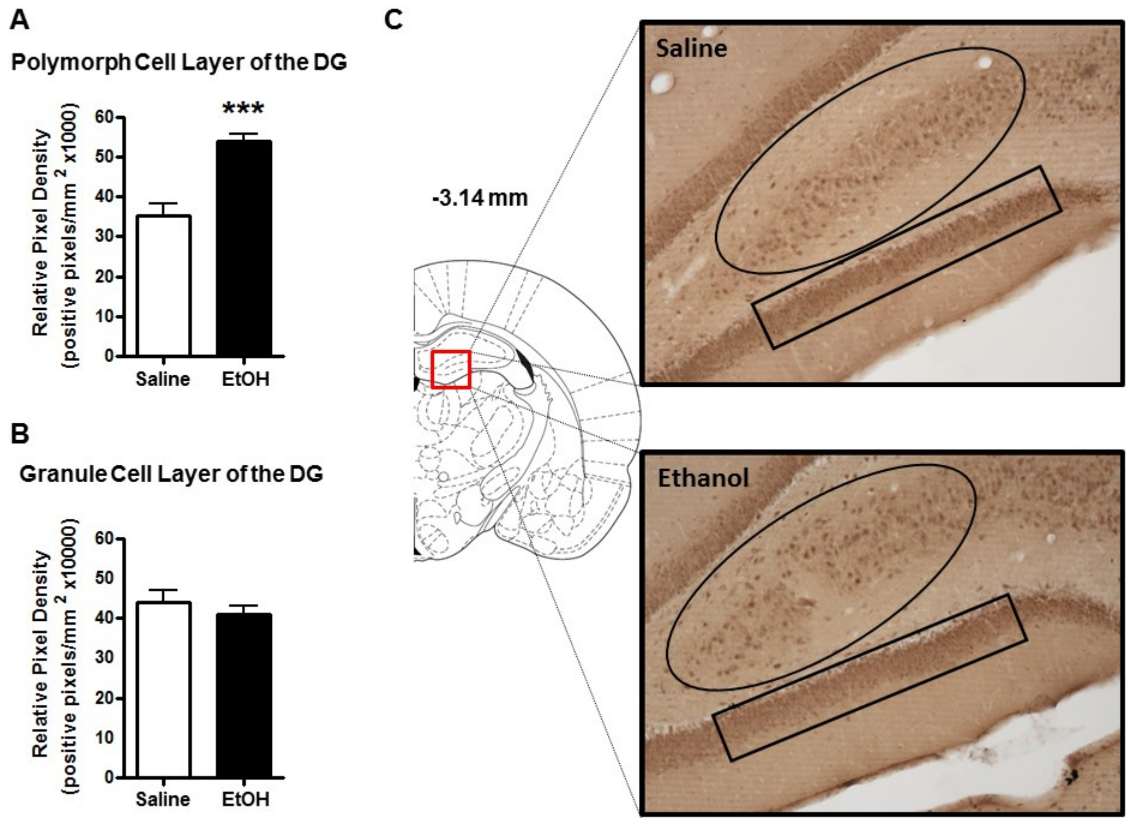
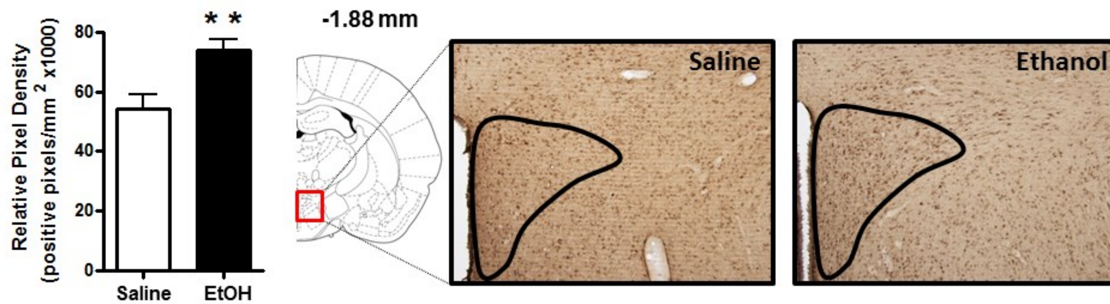


Figure 2.2: Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the polymorph and granule cell layers of the DG. (A) Ethanol administration increased 3α,5α-THP immunoreactivity in the polymorph cell layer compared to saline controls. (B) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the granule cell layer compared to saline controls. (C) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the polymorph cell layer (oval) and granule cell layer (rectangle) of the DG (-3.14 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. *** $p < 0.001$ compared to saline administration. Dentate gyrus (DG), ethanol (EtOH).



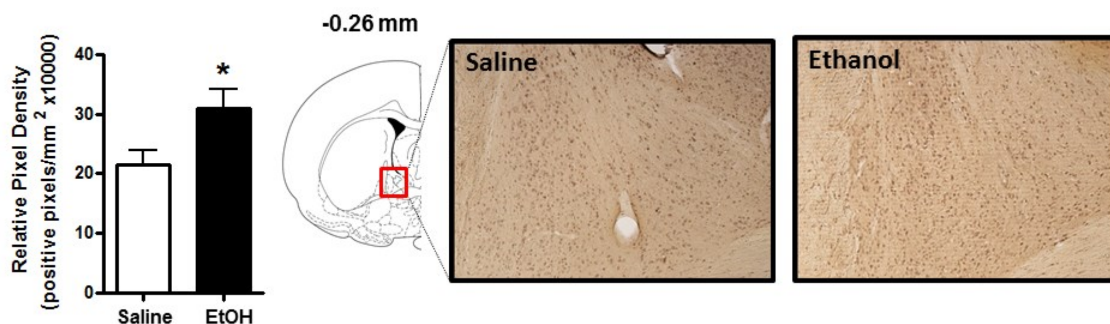


Figure 2.3: Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the PVN of the hypothalamus and BNST. (A) Ethanol administration increased 3α,5α-THP immunoreactivity in the PVN compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the PVN (-1.88 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. (B) Ethanol administration also increased 3α,5α-THP immunoreactivity in the BNST compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in BNST (-0.26 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. * $p < 0.05$, ** $p < 0.01$ compared to saline administration. Paraventricular nucleus (PVN), bed nucleus of the stria terminalis (BNST), ethanol (EtOH).

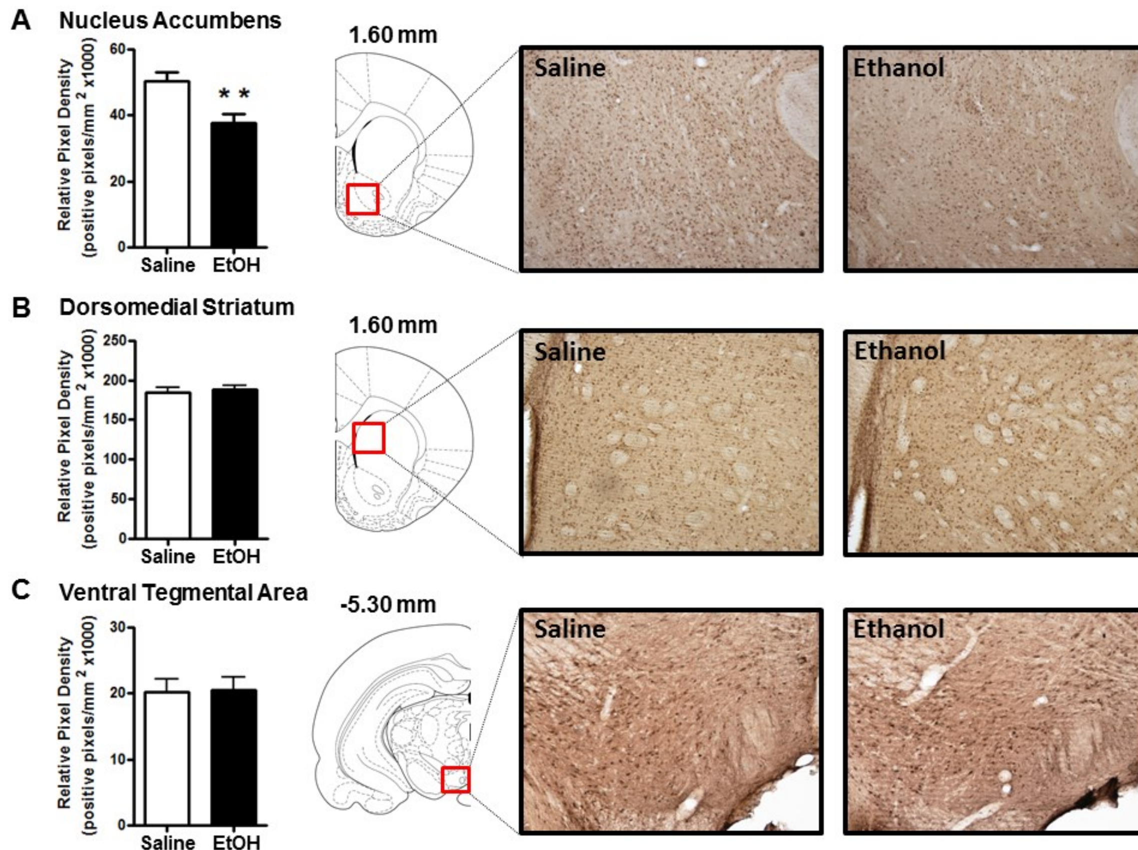


Figure 2.4: Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in NAc "shore" (core/shell border), DMS, and VTA. (A) Ethanol administration reduced 3α,5α-THP immunoreactivity in the NAc "shore" compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in NAc "shore" (1.60 mm relative to bregma) following saline (n=7) or ethanol (n=7) administration. (B) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the DMS compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in DMS (1.60 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration. (C) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the VTA compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the VTA (-5.30 mm relative to bregma) following saline (n=7) or ethanol (n=7) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. ** $p < 0.01$ compared to saline

administration. Nucleus accumbens (NAc), dorsomedial striatum (DMS), ventral tegmental area (VTA), ethanol (EtOH).

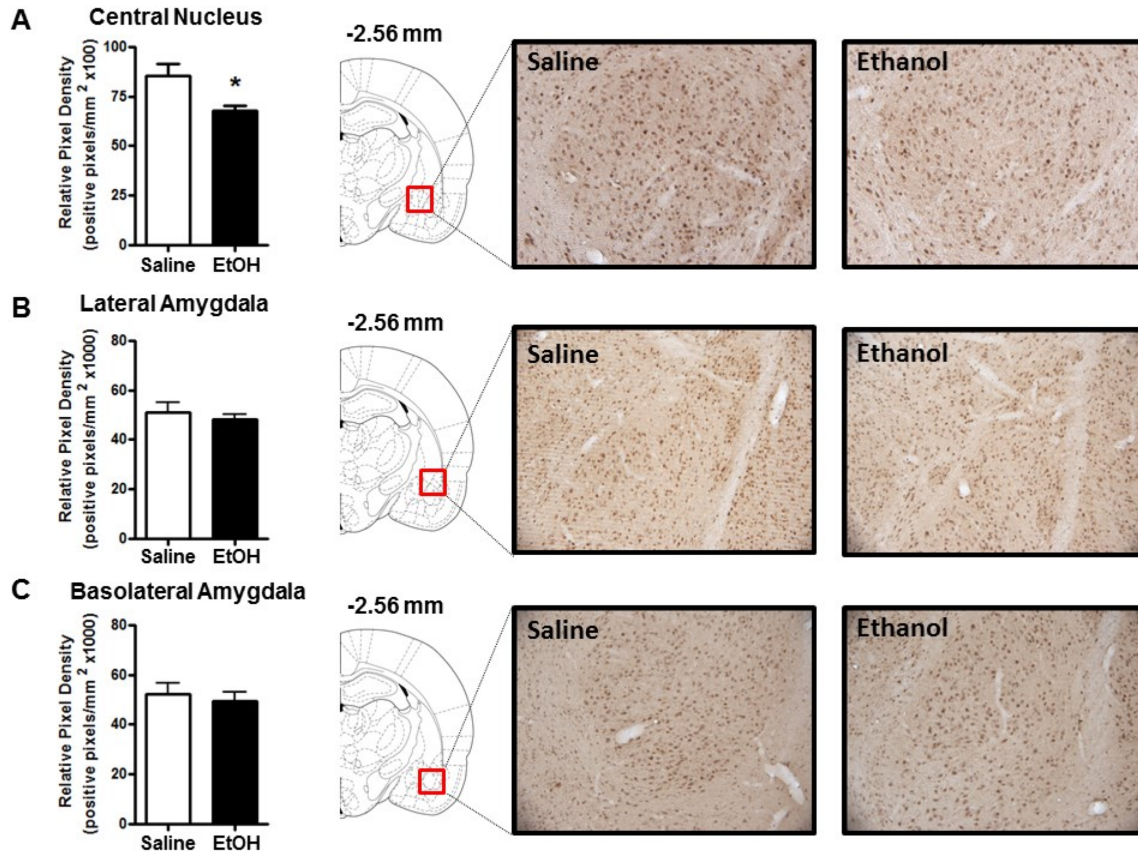


Figure 2.5: Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in CeA, lateral amygdala, and basolateral amygdala. (A) Ethanol administration reduced 3α,5α-THP immunoreactivity in the CeA compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in CeA (-2.56 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. (B) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the lateral amygdala compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the lateral amygdala (-2.56 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration. (C) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the basolateral amygdala compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the basolateral amygdala (-2.56 mm relative to bregma) following saline (n=7) or ethanol (n=7) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM.

* $p < 0.05$ compared to saline administration. Central nucleus of the amygdala (CeA), ethanol (EtOH).

CHAPTER 3. **EFFECTS OF ADRENALECTOMY ON ETHANOL-INDUCED CHANGES IN 3α,5α-THP IMMUNOHISTOCHEMISTRY**

INTRODUCTION

Neuroactive steroids are endogenous neuromodulators capable of altering neuronal activity. Synthesis of neuroactive steroids occurs centrally in the brain and peripherally in the adrenal glands and gonads. The 5α-reduced pregnane steroids, (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC), are positive allosteric modulators of γ-aminobutyric acid type A ($GABA_A$) receptors. $GABA_A$ receptors are the primary inhibitory receptor family in the brain, which mediate many of the behavioral effects of ethanol. Both 3α,5α-THP and 3α,5α-THDOC enhance neuronal inhibition at known binding sites on $GABA_A$ receptor α-subunits (Hosie et al., 2006), and produce behavioral effects similar to those produced by ethanol. These GABAergic neuroactive steroids are very potent positive modulators of $GABA_A$ receptors, producing pharmacological effects in nanomolar concentrations (Morrow et al., 1987), and modulating GABAergic steroid levels may have therapeutic value for treating multiple psychiatric disorders (Uzunova et al., 1998; Strohle et al., 2002; Marx et al., 2006; Rasmusson et al., 2006; Morrow, 2007).

Evidence suggests ethanol increases levels of 3α,5α-THP and 3α,5α-THDOC, which contribute to ethanol's behavioral and electrophysiological effects in rodents.

Ethanol activates the hypothalamic-pituitary-adrenal (HPA) axis (Boyd et al., 2010) leading to physiologically significant increases of 3α,5α-THP and 3α,5α-THDOC in the blood plasma, cerebral cortex, and hippocampus (Barbaccia et al., 1999; VanDoren et al., 2000; Porcu et al., 2009). Adrenalectomy or inhibition of 5α-reduced steroid synthesis with the 5α-reductase (5α-R) inhibitor finasteride reduces some of the behavioral effects of ethanol, including the anticonvulsant (VanDoren et al., 2000), anti-depressant-like (Hirani et al., 2002), hypnotic (Khisti et al., 2003), and anxiolytic-like (Hirani et al., 2005) effects in rats. Finasteride also blocks ethanol inhibition of neuron firing in the medial septum (VanDoren et al., 2000), the hippocampus both *in vivo* (Tokunaga et al., 2003b) and *in vitro* (Sanna et al., 2004), and long-term potentiation (LTP) in hippocampal slice preparations (Tokuda et al., 2011). Importantly, finasteride also reduces some of the subjective effects of alcohol in healthy men (Pierucci-Lagha et al., 2005). Taken together, these findings suggest ethanol-induced elevations of 3α,5α-THP and 3α,5α-THDOC contribute to many of the physiological and behavioral effects of ethanol.

In vivo studies suggest ethanol-induced elevations of 3α,5α-THP in the cerebral cortex are dependent on the adrenal glands (Khisti et al., 2003; O'Dell et al., 2004; Porcu et al., 2004). *In vitro* studies, however, suggest ethanol produces local brain synthesis of 3α,5α-THP in hippocampal tissue from intact (Sanna et al., 2004; Tokuda et al., 2011) and adrenalectomized/gonadectomized (Follesa et al., 2006) rats. Therefore, an important question is whether ethanol can alter brain synthesis of 3α,5α-THP *in vivo*, independent of adrenal influence. The answer to this question has remained elusive due to limitations in 3α,5α-THP measurements. Most experiments measuring ethanol-induced changes in

3α,5α-THP have used radioimmunoassay (RIA) or gas chromatography—mass spectroscopy (GC-MS), which lack the sensitivity to measure 3α,5α-THP in many brain regions.

We have recently demonstrated ethanol-induced changes in immunohistochemical labeling of 3α,5α-THP in several rat brain regions that are implicated in alcohol use disorders. This technique displays the sensitivity to detect changes in 3α,5α-THP immunoreactivity associated with corresponding changes in electrophysiological measurements (Saalmann et al., 2006; Tokuda et al., 2011). Ethanol produces divergent changes in cellular 3α,5α-THP levels that are brain region and cellular population specific (Cook et al., 2013). For example, ethanol increased cellular 3α,5α-THP levels in the medial prefrontal cortex (mPFC), hippocampal CA1 pyramidal cell layer, polymorphic cell layer of the dentate gyrus (DG), bed nucleus of the stria terminalis (BNST), and the paraventricular nucleus (PVN) of the hypothalamus. In contrast, ethanol reduced cellular 3α,5α-THP levels in the nucleus accumbens (NAc) shore (core-shell border) and the central nucleus of the amygdala (CeA). Interestingly, no changes were observed in the granule cell layer of the DG, dorsomedial striatum, lateral or basolateral amygdala, or the ventral tegmental area. Taken together, these region specific effects of ethanol on cellular 3α,5α-THP suggest there may be local brain synthesis of 3α,5α-THP independent of the adrenal glands and novel mechanisms that reduce cellular 3α,5α-THP. In the present study, we tested whether ethanol-induced changes in immunohistochemical labeling of 3α,5α-THP are dependent upon the adrenal glands. We used male Wistar rats that had undergone ADX or sham surgery and measured the ability of ethanol (2g/kg, i.p.) to alter cellular 3α,5α-THP levels in brain regions/cellular populations where we had

previously observed ethanol-induced changes in 3α,5α-THP. We also investigated the effects of ethanol on 3α,5α-THP levels in perfused and non-perfused rat brain in order to determine the contribution of circulating steroids in brain measurements of 3α,5α-THP using RIA. Finally, we evaluated the effect of the pentobarbital anesthetic on 3α,5α-THP levels in the cerebral cortex and hippocampus.

MATERIALS AND METHODS

Subjects

For the ADX experiments adult male Wistar rats (~275 g/8-11 per group) that had undergone ADX or sham surgery were purchased from Harlan Laboratories (Indianapolis, IN, USA). The animals were single housed in Plexiglass cages with food and water (animals that underwent ADX received 0.9% saline instead of water) available *ad libitum*. Animals were sacrificed 7 days following surgery and approximately 24hr after arriving in the colony room. For the RIA studies male Wistar rats (5-14 per group) were purchased from Harlan Laboratories (Indianapolis, IN, USA) and stayed in the colony room for 6 weeks to reach adulthood. The animals habituated to handling and saline i.p. saline injections for 1 week prior to the experiment. The colony room was maintained on a normal 12 hr light-dark cycle (light onset at 0700 hr) and at a constant temperature of 22 ± 2°C and relative humitity of 65%. Experiments were conducted between 0800 and 1300 hr to minimize potential circadian fluctuation in neuroactive steroid levels.

Ethanol (2g/kg, 20% v/v in saline) or saline were administered by i.p. injection 60 minutes before transcardial perfusion. Animal care and handling procedures followed

National Institutes of Health Guidelines under University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee approved protocols.

Radioimmunoassay (RIA)

Prior to RIA, 3α,5α-THP was extracted from freshly frozen cerebral cortex or hippocampus. Recovery was monitored by incorporation of 4000 dpm of $[^3H]$-3α,5α-THP. Brain samples were homogenized in 2.5ml 0.3 N NaOH with a sonic dismembrator and extracted three times in 3ml aliquots of 10% (v/v) ethyl acetate in heptane. Aliquots were combined and 5ml of heptane was added to each sample. The extracts were run through solid phase silica columns, washed in pentane, and steroids of similar polarity to 3α,5α-THP were eluted off the column by 25% acetone in pentane. Eluant was dried, and steroids reconstituted in 50µl isopropanol and 150µl 50% sodium phosphate/bovine serum albumin assay buffer. Extraction efficiency was determined in 50µl the reconstituted extract by liquid scintillation spectroscopy. The remaining 150µl was used in RIA determination of 3α,5α-THP.

Sample extracts in sodium phosphate/bovine serum albumin assay buffer with incubated with sheep polyclonal 3α,5α-THP antiserum (obtained from Dr. Robert Purdy, Scripps Research Institute) and $[^3H]$-3α,5α-THP for 1.5-2hr. Unbound $[^3H]$-3α,5α-THP was removed by centrifugation after adding dextran-coated charcoal. The supernatant was mixed with Ecoscint H (National Diagnostics) and $[^3H]$-3α,5α-THP was measured in a scintillation counter. Samples were compared with a concurrently run 3α,5α-THP standard curve. The resulting curves were analyzed using a one-site competition model (Prism, GraphPad Software, La Jolla, CA, USA) for EC_{50} values.

Immunohistochemistry

Fifty-three minutes after ethanol (2g/kg) or saline injection the animals were anesthetized with pentobarbital (100mg/kg, i.p.; Professional Compounding Centers of America, Houston, TX, USA) and transcardially perfused approximately 1hr following ethanol or saline injection with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Tissue was post-fixed in 4% paraformaldehyde for 24hr at 4°C, sectioned coronally at 40µm on a vibrating microtome, and stored at -30°C. Pentobarbital does not alter 3α,5α-THP levels using this procedure, and blood perfusion does not alter brain levels of 3α,5α-THP in intact animals using RIA (unpublished data).

Immunohistochemical assays were performed using a procedure modified from (Saalmann et al., 2007) and previously described in detail (Cook et al., 2013). Briefly, no detergents or organic solvents were used to prevent the possibility of neuroactive steroid leaching. Free floating brain sections (3-4 sections/animal/brain region) were rinsed, blocked in 10% rabbit serum, and incubated in sheep affinity purified anti-3α,5α-THP antiserum (purchased from Dr. R.H. Purdy) at a 1:2500 dilution for 48 hr at 4°C. A rabbit anti-sheep biotinylated secondary antibody (1:200; Vector laboratories, Burlingame, CA, USA) was used in collaboration with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and immunoreactivity was visualized with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) using the manufacturers' recommended procedures.

Immunohistochemical analysis

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA, USA) and images were captured with a digital

camera (Regita model, QImaging, Burnaby, BC). Image analysis software (Bioquant Life Sciences version 8.00.20; Bioquant Life Sciences, Nashville, TN, USA) that utilizes linear integrated optical density was used for comparing relative changes in immunoreactivity between groups. The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition. Immunoreactive positive pixel count measurements were calculated from a defined region (e.g., brain region), divided by the area of the region in square millimeters, and expressed as positive pixels/mm^2. Data were acquired from 3-4 sections/animal/brain region, and averaged within a brain region for an individual animal to obtain one value per subject. Inter-rater reliability was determined by calculating the intraclass-correlation coefficient for two raters blind to the experimental conditions. Data from the NAc and mPFC were chosen for intraclass-correlation analysis and values of $r = 0.93$ ($p < 0.001$) and $r = 0.88$ ($p < 0.05$) were obtained, respectively. Intraclass-correlation coefficient was calculated using MATLAB (MathWorks, Natick, MA, USA). Immunoreactivity was measured separately for each brain region and statistically analyzed using two-way between subjects design ANOVA (Prism, GraphPad Software, La Jolla, CA, USA). *A priori* planned comparisons were performed to compare ethanol to saline administration in the mPFC using Student's t-test (Prism, GraphPad Software, La Jolla, CA, USA).

Brain region analyses were performed using histological coordinates as follows: mPFC (+3.00 to +2.20 AP), hippocampal CA1 pyramidal cell layer (-2.56 to -3.30 AP), DG polymorphic cell layer (-2.56 to -3.30 AP), BNST (-0.26 to -0.40 AP), PVN of the hypothalamus (-1.30 to -2.12 AP), NAc (+1.70 to +1.00 AP), and CeA (-2.12 to -2.80

AP). All analyses were based on coordinates relative to bregma in the Rat Brain Atlas (Paxinos and Watson, 1998).

RESULTS

Effects of transcardial perfusion or pentobarbital administration on 3α,5α-THP measurements in the whole cerebral cortex or hippocampus using RIA

Previous IHC results had found a discrepancy in the magnitude of ethanol-induced increases of 3α,5α-THP between IHC and RIA measurement. Using IHC we observed a 24% and 32% increase of 3α,5α-THP in the mPFC and CA1 pyramidal cells, respectively. RIA studies have shown 2-17 fold increases in the whole cortex (Barbaccia et al., 1999; VanDoren et al., 2000; Khisti et al., 2004; Boyd et al., 2010) and 4-11 fold increase in whole hippocampus (Barbaccia et al., 1999; Khisti et al., 2004). Therefore, since IHC brains are perfused, we tested whether the blood present in RIA tissue samples contribute to the discrepancy in 3α,5α-THP magnitude. As expected, there was a main effect of ethanol in the cerebral cortex [$F(1,38) = 76.78$, $p < 0.0001$] (Fig. 3.1A) and hippocampus [$F(1,33) = 107.5$, $p < 0.0001$] (Fig. 3.1B), but there was no effect of perfusion on 3α,5α-THP levels in the cortex [$F(1,38) = 0.19$, $p = 0.6644$] (Fig. 3.1A) or hippocampus [$F(1,33) = 0.92$, $p = 0.3433$] (Fig. 3.1B).

To further validate our IHC assay we tested whether the pentobarbital anesthetic alters 3α,5α-THP in whole cerebral cortex or hippocampus. There was a main effect of ethanol in the cerebral cortex [$F(1,33) = 109.5$, $p < 0.0001$] (Fig. 3.1C) and hippocampus [$F(1,27) = 151.7$, $p < 0.0001$] (Fig. 3.1D), but there was no effect of pentobarbital on 3α,5α-THP levels in the cortex [$F(1,33) = 0.73$, $p = 0.3987$] (Fig. 3.1C) or hippocampus [$F(1,27) = 0.07$, $p = 0.7886$] (Fig. 3.1D).

Ethanol-induced increases of cellular 3α,5α-THP

Recent work in our lab has shown that ethanol increases 3α,5α-THP immunoreactivity in the mPFC, CA1 pyramidal cell layer of the hippocampus, polymorphic cell layer of the DG, BNST, and PVN of the hypothalamus (Cook et al., 2013). Therefore, we tested whether ethanol-induced elevations of 3α,5α-THP in these regions are dependent upon adrenal gland activation. Ethanol administration increased 3α,5α-THP immunoreactivity in the mPFC, but this effect was moderated by adrenalectomy (Fig. 3.2A). The two-way ANOVA indicated a main effect of ethanol treatment [$F(1,34) = 8.18$, $p < 0.01$] and surgery condition [$F(1,34) = 9.41$, $p < 0.005$], but no significant interaction. Therefore, we performed an *a priori* planned comparison between the sham saline versus sham ethanol and ADX saline versus ADX ethanol groups. mPFC 3α,5α-THP immunoreactivity was significantly increased in the sham ethanol group compared to the sham saline group [25±11%; $t(15)=3.583$, $p < 0.01$], similar to previous immunohistochemical results (Cook et al., 2013). However, ethanol did not increase 3α,5α-THP in animals that received ADX [2±7%; $t(19)=0.937$, $p > 0.05$], suggesting that the adrenals contribute to the effects of ethanol in mPFC. The effects of ethanol appear uniform across the cortical cell layers (Fig. 3.2C).

In the hippocampus, ethanol increased 3α,5α-THP immunoreactivity in the CA1 pyramidal cell layer and the polymorphic cell layer of the DG independent of the adrenal glands. In the CA1 pyramidal cell layer, 3α,5α-THP immunoreactivity was increased by 49±7% in the sham ethanol versus sham saline group and by 48±11% in the ADX ethanol versus ADX saline group (main effect of ethanol treatment [$F(1,34) = 23.15$, $p < 0.0001$]) (Fig. 3.3A). In the polymorphic cell layer of the DG, 3α,5α-THP

immunoreactivity was increased by 63±10% in the sham ethanol versus sham saline group and by 74±11% in the ADX ethanol versus ADX saline group (main effect of ethanol treatment [F (1,34) = 61.16, p < 0.0001]) (Fig. 3.3D).

In the BNST and PVN of the hypothalamus, ethanol increased 3α,5α-THP immunoreactivity independent of the adrenal glands. In the BNST, 3α,5α-THP immunoreactivity was increased by 43±5% in the sham ethanol versus sham saline group and by 40±8% in the ADX ethanol versus ADX saline group (main effect of ethanol treatment [F (1,34) = 35.68, p < 0.0001]) (Fig. 3.4A). In the PVN, 3α,5α-THP immunoreactivity was increased by 38±10% in the sham ethanol versus sham saline group and by 39±7% in the ADX ethanol versus ADX saline group (main effect of ethanol treatment [F (1,34) = 27.70, p < 0.0001]) (Fig. 3.4D).

Ethanol-induced decreases of cellular 3α,5α-THP

Recently, we have shown that ethanol administration decreases 3α,5α-THP immunoreactivity in the NAc shore and the CeA. Therefore, we tested whether ethanol-induced reductions of 3α,5α-THP in these regions are dependent on adrenal gland activation. In both the NAc shore and the CeA, ethanol reduced cellular 3α,5α-THP independent of the adrenals. In the NAc shore, 3α,5α-THP immunoreactivity was decreased by 27±5% in the sham ethanol versus sham saline group and by 26±5% in the ADX ethanol versus ADX saline group (main effect of ethanol treatment [F (1,34) = 17.58, p < 0.001]) (Fig. 3.5A). In the CeA, 3α,5α-THP immunoreactivity was decreased by 39±2% in the sham ethanol versus sham saline group and by 29±7% in the ADX ethanol versus ADX saline group (main effect of ethanol treatment [F (1,34) = 28.74, p < 0.0001]) (Fig. 3.6A).

DISCUSSION

The goal of the present study was to determine the source of ethanol-induced changes in cellular 3α,5α-THP levels in the rat brain. Ethanol-induced elevations of 3α,5α-THP in the mPFC appear to be dependent on adrenal gland activation. This finding is in agreement with studies using RIA to measure 3α,5α-THP in whole cerebral cortex (Khisti et al., 2003; Porcu et al., 2004) and GC-MS data measuring 3α,5α-THP in the frontal cortex (O'Dell et al., 2004). Interestingly, ethanol-induced elevations of 3α,5α-THP in the pyramidal cell layer of the hippocampus, polymorphic cell layer of the DG, BNST, and PVN were observed following ADX. Therefore, ethanol produces local brain synthesis of 3α,5α-THP in these brain regions and cellular populations. Furthermore, the ethanol-induced reductions of cellular 3α,5α-THP in the NAc shore and CeA were also observed following ADX. Thus, ethanol reduces local cellular levels of 3α,5α-THP in these regions independent of adrenal gland activation. The results from the sham saline and sham ethanol groups in the current study replicate previous immunohistochemical findings (Cook et al., 2013). It is important to note that this previous immunohistochemical study found no change in cellular levels of 3α,5α-THP in the dorsomedial striatum, granule cell layer of the DG, ventral tegmental area, or the lateral or basolateral amygdala. Therefore, ethanol-induced changes in cellular 3α,5α-THP are bidirectional, brain region/cell population specific, and occur locally in the rat brain. Our previous IHC study had shown that the magnitude of the effect of ethanol on 3α,5α-THP levels using IHC is much smaller than what has been observed using RIA. Here, we show that circulating blood does not contribute to RIA measurements of 3α,5α-THP in the whole cerebral cortex or hippocampus. Finally, to further validate the 3α,5α-THP IHC

assay showed that the pentobarbital anesthetic does not alter 3α,5α-THP levels in whole cerebral cortex or hippocampus.

The current IHC results and previous reports using RIA or GC-MS all suggest that ethanol-induced elevations of 3α,5α-THP in the cerebral cortex are dependent on the adrenal glands or the pituitary (Boyd et al., 2010). It is not clear why there is a dependence on HPA activation to observe cerebral cortical elevations of 3α,5α-THP, but one possibility is that there is a lack of precursor in the cortex. Indeed, it has previously been shown that administration of 5α-dihydroprogesterone (5α-DHP), the immediate precursor of 3α,5α-THP, restores ethanol-induced increases of 3α,5α-THP in the cerebral cortex and corresponding effects of ethanol on loss of righting reflex following ADX (Khisti et al., 2003). It has also been shown that *de novo* adrenal synthesis of the cholesterol transporter, steroidogenic acute regulatory protein (StAR), is necessary for ethanol-induced increases of 3α,5α-THP in the cerebral cortex (Boyd et al., 2010). StAR transports cholesterol to the inner mitochondrial membrane where it is converted to pregnenolone by cytochrome P450 side chain cleavage (P450scc). Therefore, *de novo* StAR synthesis in the adrenals may be necessary to provide precursor and/or 3α,5α-THP to the cerebral cortex following ethanol administration. Previous work has shown that acute ethanol increases StAR and P450scc mRNA expression in the rat frontal cortex (Kim et al., 2003), however, no increase in StAR protein was found in whole cortex (Boyd et al., 2010). The synthesis of 3α,5α-THP is accomplished by 5α-R converting progesterone to 5α-DHP, which 3α-HSD then converts to 3α,5α-THP. Acute ethanol administration increases 5α-R type 1 (5α-RI) and 3α-HSD mRNA expression in the frontal cortex of rats (Kim et al., 2003). Taken together, it appears that ethanol-induced

increases of 3α,5α-THP in the cerebral cortex are dependent on adrenal derived precursor, which is converted to 3α,5α-THP by locally synthesized steroidogenic enzymes.

The current results in the hippocampal formation agree with the previous *in vitro* studies that have shown ethanol-induced local brain synthesis of 3α,5α-THP. For example, it was first shown using RIA that incubation with ethanol (50 or 100 mM) can increase 3α,5α-THP in hippocampal minces from intact rats (Sanna et al., 2004) and rats that had undergone ADX/gonadectomy (Follesa et al., 2006). More recently, it was shown that ethanol increases cellular 3α,5α-THP in CA1 pyramidal cells using IHC with fluorescent detection of 3α,5α-THP (Tokuda et al., 2011). In each of these *in vitro* studies, ethanol induction of 3α,5α-THP was shown to alter neuronal function using electrophysiological measures. In the current study, we show that ethanol increases 3α,5α-THP immunoreactivity in the CA1 pyramidal cell layer as well as the polymorphic cell layer of the DG, independent of adrenal gland activation. Furthermore, ethanol's ability to stimulate brain synthesis of 3α,5α-THP in the hippocampal formation is isolated to specific cellular populations, since we did not previously observe ethanol-induced changes of 3α,5α-THP in the granule cell layer of the DG (Cook et al., 2013). The CA1 pyramidal cells, polymorphic DG, and granule cell layer of the DG all exhibit dense 3α,5α-THP staining. Therefore, the presence of this very specific effect of ethanol on cellular 3α,5α-THP in the hippocampus is intriguing and may underlie neuron specific responses to ethanol in the hippocampal formation.

Ethanol also increased 3α,5α-THP immunoreactivity in the PVN of the hypothalamus and the BNST, independent of the adrenal glands. The PVN and the BNST

are involved in stress, emotion, and ethanol responses (Armario, 2010; Cui et al., 2012; Koob, 2013), and we have previously shown that ethanol increases cellular 3α,5α-THP in these regions (Cook et al., 2013). It has also been shown that GABAergic neuroactive steroids contribute to negative feedback on the HPA axis at the level of the hypothalamus in rats (Owens et al., 1992; Patchev et al., 1994; Patchev et al., 1996), and activate the stress response in the hypothalamus of C57BL/6J mice (Sarkar et al., 2011). The present data suggest that ethanol-induced changes of cellular 3α,5α-THP in the PVN and BNST are independent of adrenal activation. Other studies are clearly needed to understand the physiological and behavioral impact these local elevations of 3α,5α-THP produce in the PVN and BNST.

Ethanol-induced reductions of cellular 3α,5α-THP in the NAc and CeA were also shown to occur independent of adrenal activation. Ethanol's ability to change local cellular levels of 3α,5α-THP within the NAc and CeA is particularly interesting since these brain regions are strongly associated with alcohol reinforcement and consumption (McBride, 2002; Gonzales et al., 2004), and exogenous 3α,5α-THP has been shown to modulate ethanol self-administration. For example, several studies have shown that 3α,5α-THP alters ethanol consumption (Morrow et al., 2001; Ford et al., 2005; Ford et al., 2007) and reinforcement (Janak et al., 1998; Janak and Michael Gill, 2003). Ethanol-induced reductions of cellular 3α,5α-THP would most likely decrease $GABA_A$ receptor mediated inhibition, which may increase firing of mesolimbic dopamine neurons and CeA circuitry involved in ethanol reinforcement. We have previously shown that the reduction of 3α,5α-THP in the NAc is isolated to the NAc shore (core-shell border), as no change in cellular 3α,5α-THP is observed in the core or shell alone (Cook et al., 2013).

Previous studies have shown that 3α,5α-THP administration produces biphasic effects on dopamine release in the NAc (Motzo et al., 1996; Rouge-Pont et al., 2002), and modulates ethanol's effects on dopamine content in the mPFC (Dazzi et al., 2002). Therefore, investigating interactions between 3α,5α-THP levels and dopaminergic activity in the mesocorticolimbic system may aid in the effort to reduce ethanol consumption via neuroactive steroid modulation.

Significance on Neuronal Activity and Potential Mechanisms of Ethanol-Induced Changes in Local 3α,5α-THP Levels

A previous study using IHC determined that 3α,5α-THP is located in principle projecting GABAergic and glutamatergic neurons, but not in interneurons or glial cells (Saalmann et al., 2007). This pattern of 3α,5α-THP expression corresponds with the expression of the biosynthetic enzymes 5α-R and 3α-HSD needed for 3α,5α-THP synthesis and metabolism (Agis-Balboa et al., 2006). Therefore, it has been suggested that a major role of 3α,5α-THP may be to modulate neurocircuitry. As previously mentioned, evidence suggests that ethanol-induced increases in 3α,5α-THP contribute to the behavioral effects of ethanol in rats and the subjective effects of alcohol in humans. The contribution of 3α,5α-THP to the pharmacological effects of ethanol is likely due to potentiation of $GABA_A$ receptors, or reduced $GABA_A$ potentiation where 3α,5α-THP is reduced, across many brain regions and cellular populations. Ethanol-induced changes in 3α,5α-THP have been shown to alter neurophysiology in the hippocampus. For example, ethanol (1.5g/kg) reduces spontaneous firing of pyramidal cells in the hippocampus of anesthetized rats, which is prevented by finasteride (Tokunaga et al., 2003b). In isolated hippocampal tissue, ethanol (50, 100mM) increases 3α,5α-THP, with concurrent

increases in amplitude of spontaneous and evoked IPSCs in CA1 pyramidal cells (Sanna et al., 2004), the latter of which is prevented by finasteride. Furthermore, in hippocampal slices, ethanol (60mM) increases 3α,5α-THP immunofluorescence and inhibits LTP in CA1 pyramidal cells (Tokuda et al., 2011). Importantly, pretreatment with finasteride prevents the increase in 3α,5α-THP immunofluorescence and ethanol's ability to inhibit LTP. Thus, 3α,5α-THP appears to mediate ethanol's effects on synaptic plasticity in CA1 pyramidal cells. In the present study, ethanol increased 3α,5α-THP in the CA1 pyramidal cell layer and the polymorphic cell layer of the DG. The most abundant cells in the polymorphic DG are mossy cells, which project to the molecular and granule cell layers of the DG (Amaral et al., 2007), with the granule cells being the only projection from the DG to the hippocampus. Ethanol-induced increases in cellular 3α,5α-THP within hippocampal pyramidal cells and the polymorphic DG may contribute to ethanol's effects on memory and cognition by altering neuronal activity and synaptic plasticity.

The ethanol-induced changes in 3α,5α-THP levels in other brain regions examined in the present study likely modulate neuronal activity as well. The ability of ethanol-induced elevations of 3α,5α-THP to modulate neuronal activity is not isolated to the hippocampus. Outside the hippocampus, ethanol-induced elevations of GABAergic neuroactive steroids inhibit spontaneous firing of medial septum/diagonal band of Broca neurons (VanDoren et al., 2000). In the present study we observed increases of 3α,5α-THP in the mPFC, BNST and PVN. It is not clear how elevations of 3α,5α-THP may alter neuronal activity in these regions, but we would expect an increase of inhibitory tone. We also observed ethanol-induced reductions in local cellular levels of 3α,5α-THP in the NAc shore and the CeA. Ethanol-induced reductions of cellular 3α,5α-THP would

most likely decrease $GABA_A$ receptor mediated inhibition. More studies are clearly needed to determine the extent to which these ethanol-induced changes of 3α,5α-THP contribute to the neurophysiological and behavioral effects of ethanol.

The presence of divergent local changes in cellular 3α,5α-THP suggest that ethanol may alter local synthesis and/or metabolism of 3α,5α-THP. One possibility is that ethanol alters the expression and/or activity of the cholesterol transporters and/or steroidogenic enzymes. Acute ethanol administration increases StAR mRNA in the hippocampus (Kim et al., 2003). In the hypothalamus, acute ethanol increases mRNA levels of StAR, P450scc, and 3α-HSD (Kim et al., 2003). Currently, there are no data examining ethanol's effects on steroidogenic enzymes in the BNST. Similarly, there are no data examining ethanol's effects on steroidogenic enzymes in the NAc or CeA where we observed ethanol-induced reductions in 3α,5α-THP. Chronic intermittent ethanol, however, has been shown to reduce 3α,5α-THP in the hippocampus along with concomitant decreases in 5α-R type I and 3α-HSD mRNA (Cagetti et al., 2004). Therefore, ethanol may alter levels and/or activity of steroid biosynthetic enzymes to produce divergent brain region specific changes in 3α,5α-THP. Ultimately, the changes in neuroactive steroid levels may depend on the combination of cholesterol transporter and/or synthetic enzymes expressed in a particular cell type.

An alternative possibility to explain ethanol-induced reductions of cellular 3α,5α-THP in the NAc shore and the CeA is extra-neuronal redistribution of 3α,5α-THP. GABAergic neuroactive steroids have been proposed to act on $GABA_A$ receptors via lateral diffusion through the cell membrane to access transmembrane binding sites (Akk et al., 2007), or by a paracrine or autocrine mechanism (Herd et al., 2007). Although

there is no direct evidence for active release of 3α,5α-THP, we cannot rule out the possibility that ethanol causes a release or redistribution of 3α,5α-THP into the extracellular space.

In the present study, we show for the first time *in vivo* that ethanol can change local brain levels of 3α,5α-THP independent of circulating steroids. Local changes in cellular 3α,5α-THP levels are divergent, brain region and cellular population specific, and were only observed in subcortical regions. In the mPFC, ethanol's ability to increase 3α,5α-THP is dependent on adrenal activation, perhaps due to a lack of local cholesterol transporter activity and/or precursor availability. These findings illustrate the fact that ethanol dynamically regulates 3α,5α-THP concentrations with a high degree of anatomical specificity. Furthermore, the changes observed in 3α,5α-THP levels likely contribute to the neurophysiological and behavioral effects of ethanol in rats. Understanding the mechanisms by which ethanol alters local brain levels of 3α,5α-THP may lead to new therapeutic strategies for treating alcohol use disorders.

FIGURES AND TABLES

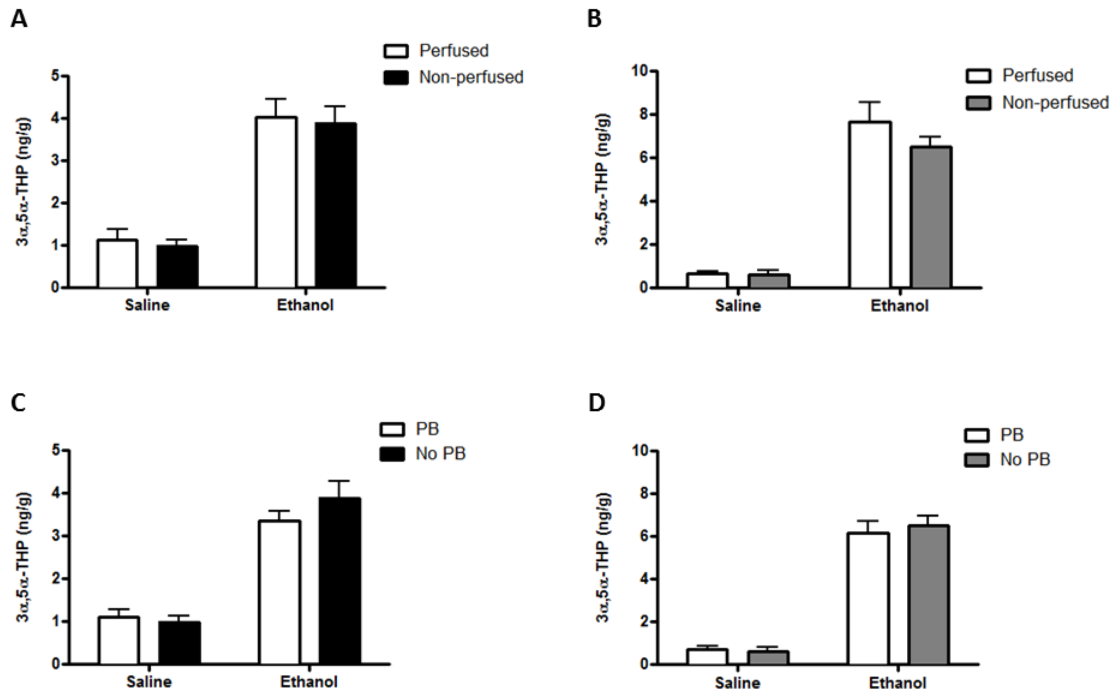


Figure 3.1: Effect of ethanol (2g/kg, i.p.) on 3α,5α-THP levels in the whole cerebral cortex or hippocampus following perfusion or pentobarbital (PB) administration. Ethanol increases 3α,5α-THP in both the (A) cerebral cortex and (B) hippocampus, which was not altered by perfusion. Ethanol increased 3α,5α-THP in both the (C) cerebral cortex and (D) hippocampus, which was not altered by PB administration. Ethanol or saline were administered 60 minutes prior to tissue collection. PB was administered 7 minutes prior to tissue collection. Data are expressed as mean nanogram 3α,5α-THP per gram of tissue ± SEM. Pentobarbital, PB.

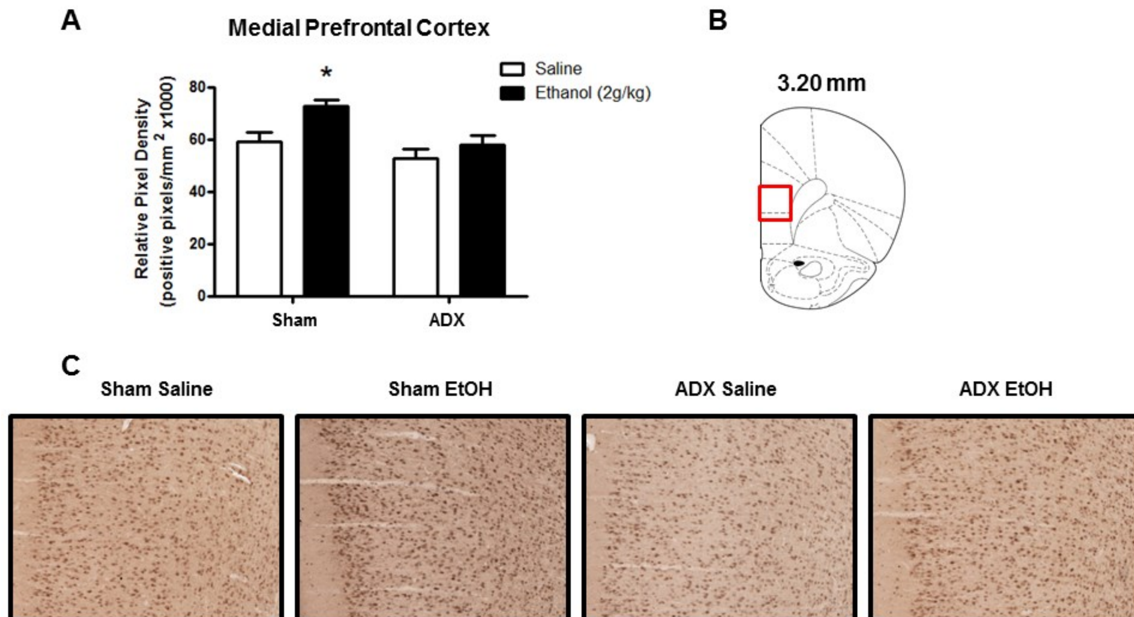


Figure 3.2: Effect of ethanol (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the mPFC of rats subjected to sham surgery or ADX. (A) Ethanol increases 3α,5α-THP immunoreactivity in the mPFC following sham surgery, but not ADX, compared to saline controls. (B) The red box indicates the location (3.20 mm relative to bregma) of representative photomicrographs. (C) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the mPFC of sham saline (n=8), sham ethanol (n=9), ADX saline (n=10), and ADX ethanol (n=11) animals. Ethanol or saline were administered 60 minutes prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. * $p < 0.01$ compared to saline administration. mPFC, medial prefrontal cortex; Sham, sham adrenalectomy surgery; ADX, adrenalectomy.

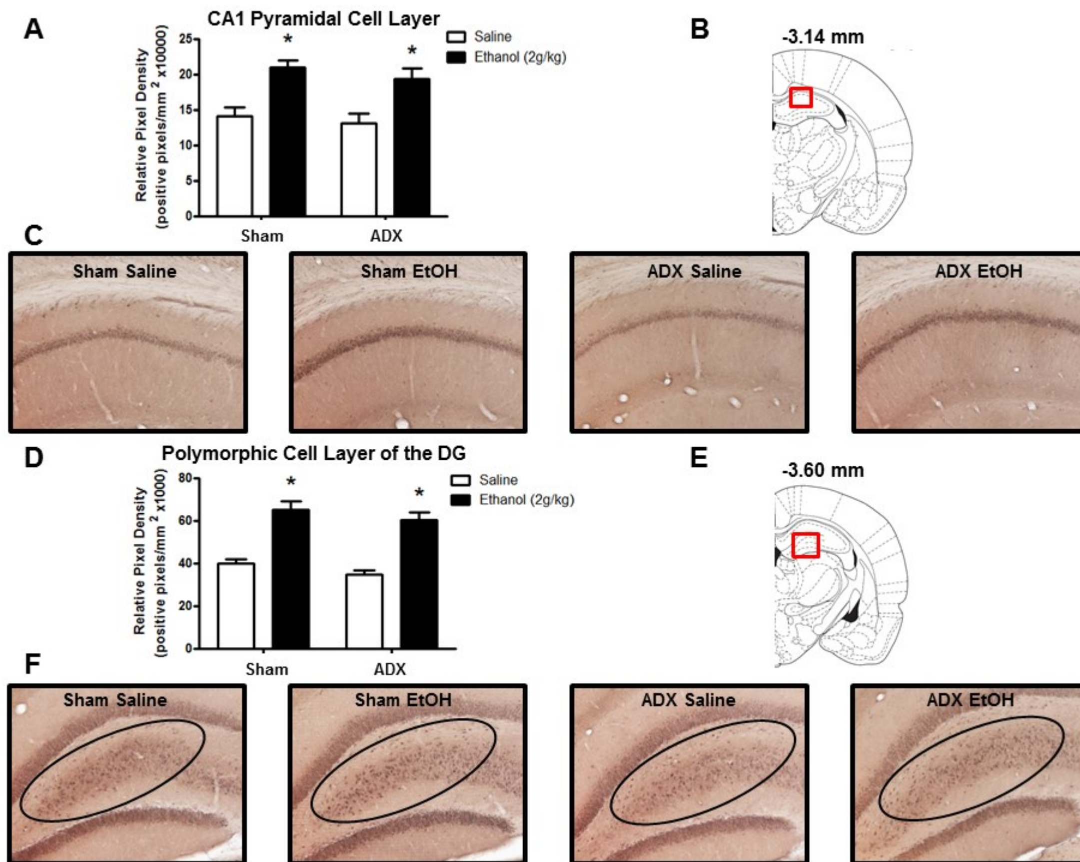


Figure 3.3: Effect of ethanol (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the CA1 hippocampus and polymorphic cell layer of the DG following sham surgery or ADX. (A) Ethanol increases 3α,5α-THP immunoreactivity in the CA1 pyramidal cell layer after sham surgery and ADX, compared to saline controls. (B) The red box indicates the location (-3.14 mm relative to bregma) of representative photomicrographs within the hippocampus. (C) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the CA1 pyramidal cell layer of sham saline (n=8), sham ethanol (n=9), ADX saline (n=10), and ADX ethanol (n=11) animals. (D) Ethanol increases 3α,5α-THP immunoreactivity in the polymorphic cell layer of the DG following sham surgery or ADX, when compared to saline controls. (E) The red box indicates the location (-3.60 mm relative to bregma) of representative photomicrographs within the DG. (F) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the polymorphic cell layer of the DG (oval). Ethanol or saline were administered 60 minutes prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ±

SEM. * indicates main effect of ethanol treatment, $p < 0.0001$. CA1, Cornu Ammonis area 1; DG, dentate gyrus; Sham, sham adrenalectomy surgery; ADX, adrenalectomy.

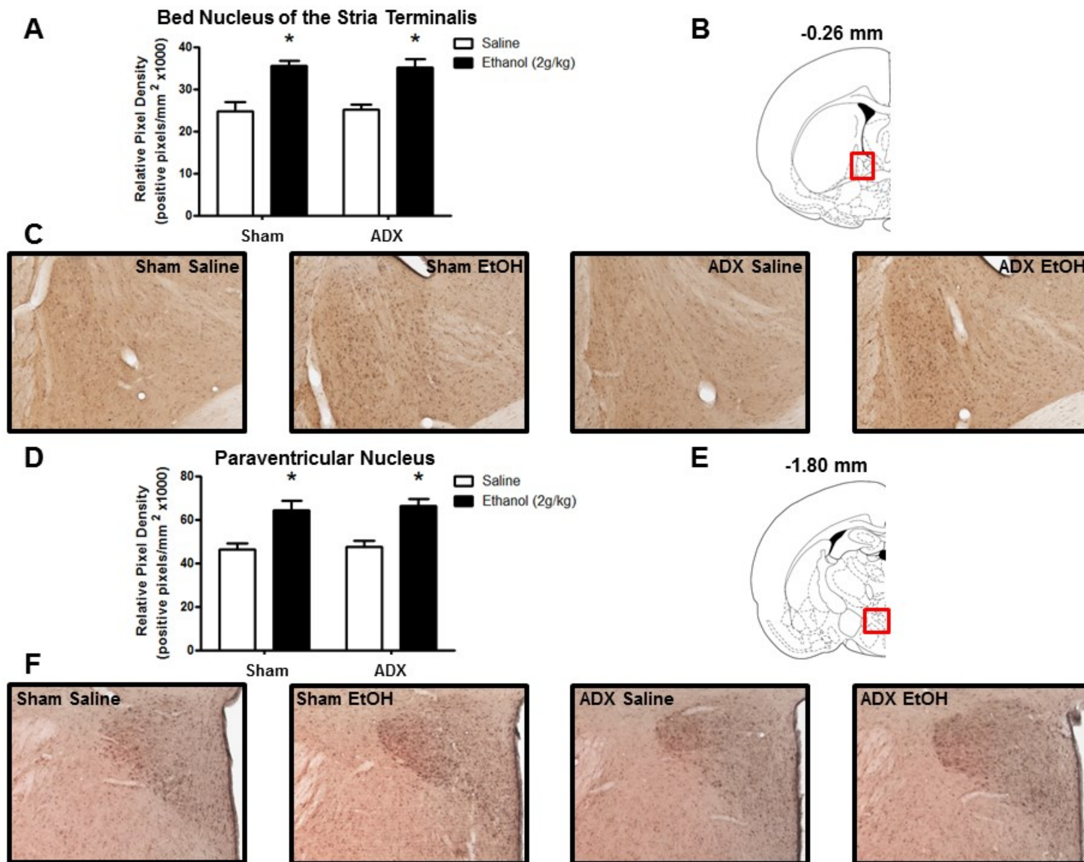


Figure 3.4: Effect of ethanol (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the BNST and PVN of the hypothalamus following sham surgery or ADX. (A) Ethanol increases 3α,5α-THP immunoreactivity in the BNST after sham surgery and ADX, when compared to saline controls. (B) The red box indicates the location (-0.26 mm relative to bregma) of representative photomicrographs within the BNST. (C) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the BNST of sham saline (n=8), sham ethanol (n=9), ADX saline (n=10), and ADX ethanol (n=11) animals. (D) Ethanol increases 3α,5α-THP immunoreactivity in the PVN of the hypothalamus after sham surgery and ADX, when compared to saline controls. (E) The red box indicates the location (-1.80 mm relative to bregma) of representative photomicrographs within the PVN. (F) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the PVN. Ethanol or saline were administered 60 minutes prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. * indicates main effect of ethanol treatment, $p < 0.0001$. BNST, bed nucleus of the stria terminalis; PVN, paraventricular nucleus; Sham, sham adrenalectomy surgery; ADX, adrenalectomy.

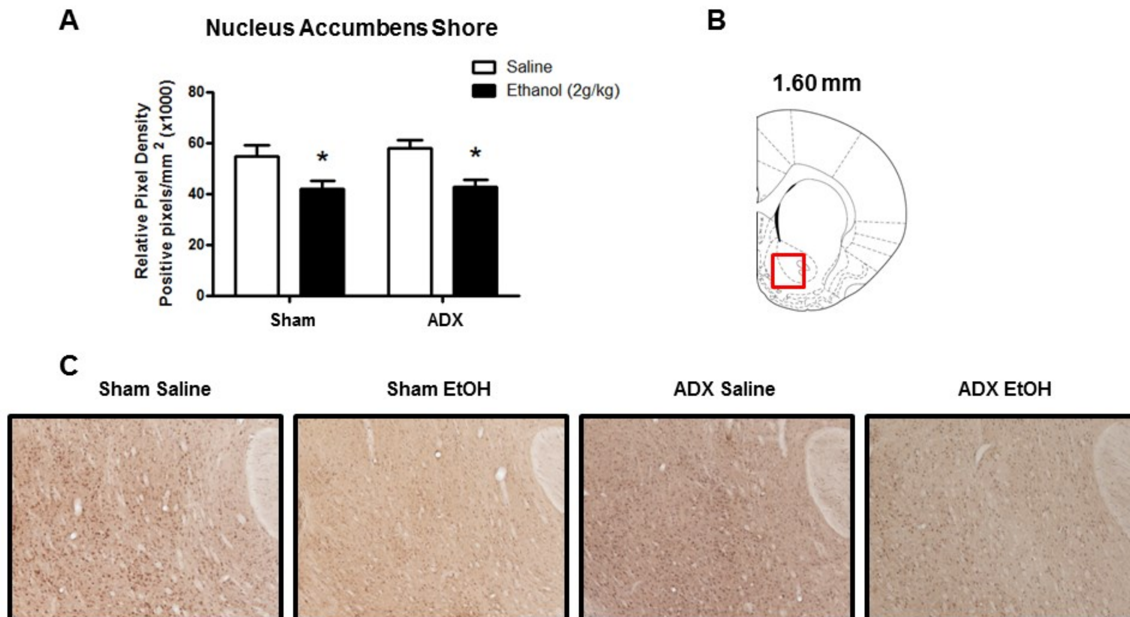


Figure 3.5: Effect of ethanol (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the NAc shore (core/shell border) after sham surgery or ADX. (A) Ethanol decreases 3α,5α-THP immunoreactivity in the NAc shore after sham surgery and ADX, when compared to saline controls. (B) The red box indicates the location (1.60 mm relative to bregma) of representative photomicrographs. (C) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the NAc shore of sham saline (n=8), sham ethanol (n=9), ADX saline (n=10), and ADX ethanol (n=11) animals. Ethanol or saline were administered 60 minutes prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. * indicates main effect of ethanol treatment, $p < 0.001$ compared to saline administration. NAc, nucleus accumbens; Sham, sham adrenalectomy surgery; ADX, adrenalectomy.

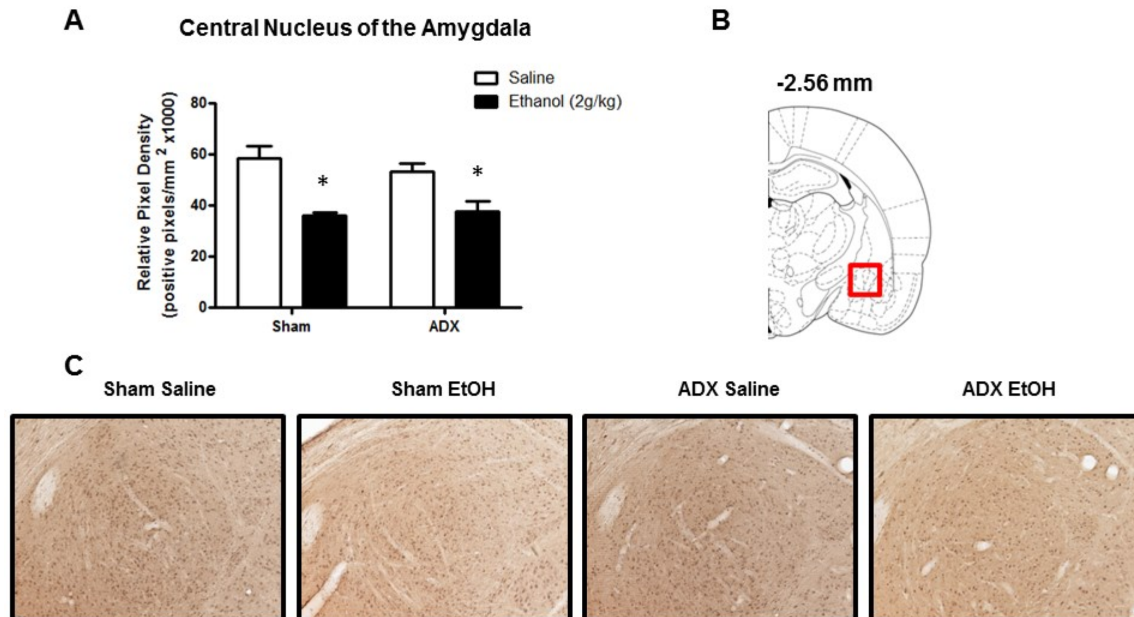


Figure 3.6: Effect of ethanol (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the CeA following sham surgery or ADX. (A) Ethanol decreases 3α,5α-THP immunoreactivity in the CeA after sham surgery and ADX, when compared to saline controls. (B) The red box indicates the location (-2.56 mm relative to bregma) of representative photomicrographs. (C) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the CeA of sham saline (n=8), sham ethanol (n=9), ADX saline (n=10), and ADX ethanol (n=11) animals. Ethanol or saline were administered 60 minutes prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. * indicates main effect of ethanol treatment, $p < 0.0001$ compared to saline administration. CeA, central nucleus of the amygdala; Sham, sham adrenalectomy surgery; ADX, adrenalectomy.

CHAPTER 4. **EFFECTS OF VIRAL VECTOR MEDIATED P450SCC OVEREXPRESSION IN THE NAC OR VTA ON OPERANT ETHANOL SELF-ADMINISTRATION**

INTRODUCTION

Neuroactive steroids are neuromodulators synthesized in the brain that modulate neuronal activity and influence motivation and emotional behaviors. The GABAergic neuroactive steroid (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) is a potent positive allosteric modulator of γ-aminobutyric acid type A ($GABA_A$) receptors. $GABA_A$ receptors are the primary inhibitory receptors in the brain and mediate many of the behavioral effects of ethanol. Ethanol-induced increases of 3α,5α-THP contribute to many of the neurophysiological and behavioral effects of ethanol in rats (VanDoren et al., 2000; Hirani et al., 2002; Khisti et al., 2003; Hirani et al., 2005) as well as subjective effects of alcohol in humans (Pierucci-Lagha et al., 2005). Furthermore, human alcoholics have reduced blood plasma levels of 3α,5α-THP during alcohol withdrawal (Romeo et al., 1996). Moreover, increasing evidence suggests that modulating GABAergic neuroactive steroid levels may have therapeutic value for treating multiple neurologic and psychiatric disorders (Marx et al., 2006; Rasmusson et al., 2006; Morrow, 2007; Rupprecht et al., 2010; Brinton, 2013).

Neuroactive steroids alter both ethanol reinforcement and ethanol consumption in rodents. For example, pregnenolone (Besheer et al., 2010a) and the synthetic GABAergic

neuroactive steroid 3α,5β-20-oxo-pregnane-3-carboxylic acid (PCA) (O'Dell et al., 2005) dose dependently reduce ethanol self-administration without producing sedation. Administration of 3α,5α-THP or the longer acting synthetic analog of 3α,5α-THP, ganaxolone, produce biphasic effects on ethanol self-administration (Janak et al., 1998; Ford et al., 2005; Besheer et al., 2010a). However, there are concerns that 3α,5α-THP and ganaxolone produce sedation (Belelli et al., 1989; Besheer et al., 2010a), limiting therapeutic effectiveness. The therapeutic potential of exogenously administered 3α,5α-THP may also be limited by rapid metabolism (Purdy et al., 1990a).

Since the mesolimbic pathway is strongly implicated in ethanol reinforcement and consumption (Koob, 1992; McBride et al., 1999; Gonzales et al., 2004), and intracerebroventricular (ICV) administration of 3α,5α-THP alters mesolimbic dopamine release (Motzo et al., 1996; Rouge-Pont et al., 2002), we explored the possibility that neuroactive steroids alter ethanol self-administration via actions in these regions. To optimize therapeutic potential and produce long-term increases of neuroactive steroids, we used viral vector-mediated gene delivery to increase neuroactive steroid production in the nucleus accumbens (NAc) or the ventral tegmental area (VTA). The synthesis of neuroactive steroids requires cholesterol conversion to pregnenolone by the mitochondrial cytochrome P450 side chain cleavage (P450scc) enzyme, the rate-limiting enzymatic reaction in steroid synthesis. Therefore, we constructed a recombinant adeno-associated serotype 2 (rAAV2) vector that overexpresses P450scc *in vivo*, which in the appropriate cell-type should lead to long-term elevations of neuroactive steroids that potentially increase $GABA_A$ receptor mediated neuronal inhibition. Not only did the viral

vector manipulation result in chronically elevated P450scc expression, pregnenolone synthesis, and levels of 3α,5α-THP, but regionally specific P450scc overexpression significantly influenced long-term operant ethanol self-administration. We also used confocal imaging to determine cell-type specific localization of 3α,5α-THP in the VTA.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (~275 g) were obtained from Harlan Laboratories (Indianapolis, IN, USA) and used for characterizing viral vectors (n=16 for *in vivo* confirmation of P450scc overexpression; n=8 to examine effects of vector infusion on 3α,5α-THP immunoreactivity in the NAc). The effects of rAAV2-P450scc transduction in the NAc on operant ethanol self-administration were assessed using adult male alcohol-preferring P rats bred in-house at the University of North Carolina at Chapel Hill. This stock of P rats was derived from breeders of the selected line of P rats provided by Indiana University (courtesy of Dr. T.K. Li). The effects of rAAV2-P450scc transduction in the VTA on operant ethanol self-administration and the double labeling experiments were performed using 2 cohorts of adult male P rats obtained from Indiana University. Baseline operant ethanol self-administration was comparable in all groups of P rats used for these studies. Animals were single housed in Plexiglass cages with food and water available *ad libitum* unless otherwise stated. The colony room was maintained on a normal 12 hr light-dark cycle (light onset at 0700 hr) and procedures followed National Institutes of Health Guidelines under University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee approved protocols.

Apparatus

Self-administration chambers

Operant ethanol self-administration was performed in conditioning chambers measuring 30.5 x 24.1 cm (Med Associates, Georgia, VT, USA) located inside sound-attenuating cubicles. Cubicles were equipped with an exhaust fan for ventilation, which also masks external sounds. Both the left and right walls of the chambers contain a lever and liquid receptacle (i.e. 2 per chamber). The appropriate number of lever press responses simultaneously activates a stimulus light over the lever and a syringe pump (Med Associates) that delivers 0.1ml of liquid solution into a receptacle over 1.66 sec duration. Lever responses during reinforcer delivery were counted, but did not result in programmed consequences. The operant chambers were connected to a computer programmed to control sessions and record the resulting data.

Locomotor chambers

Clear Plexiglas chambers (43.2 x 43.2 cm, Med Associates) with 16 photobeams per axis were used to measure locomotor activity. Total horizontal distance traveled (cm) was determined by the number of photobeam breaks during 30 minutes. Thigmotaxis was determined by measuring the percent of time spent in the center of the chamber compared to the perimeter of the apparatus. Photobeam breaks were collected via computer interface in 2 minute time intervals using Activity Monitor locomotor activity software (Med Associates).

Operant self-administration

Training

The day prior to initial training, animals were water derived for approximately 24 hr. Immediately following water deprivation, animals were placed in the operant chambers for a 16 hr lever press training session where 0.1ml of sucrose (10%, w/v) or water were concurrently available contingent on lever press responses. Lever press responses were initially maintained on a concurrent fixed-ratio 1 (CONC FR1 FR1) schedule of reinforcement and were gradually increased to CONC FR2 FR2 following delivery of 4 reinforcers, followed by a CONC FR4 FR4 schedule after delivery of 10 reinforcers. Reinforcer presentations were paired with a light cue located above each lever. Following the 16 hr training session animals were returned to their homecage for a period of 24 hr with *ad libitum* water access thereafter.

Sucrose fading, operant baseline sessions, operant self-administration testing

To facilitate lever pressing for ethanol a modified sucrose fading procedure (Samson, 1986) was used as previously described (Hodge et al., 1993; Besheer et al., 2010b). Briefly, ethanol was gradually added to the 10% (w/v) sucrose solution and sucrose was faded out until only 15% (v/v) ethanol maintained lever responding. The sequence of sucrose/ethanol solutions were as follows: 10% sucrose/2% ethanol (10S/2E), 10S/5E, 10S/10E, 5S/10E, 5S/15E, 2S/15E, 0S/15E, with two sessions conducted at each concentration. Following sucrose fading, animals experienced a minimum of 28 baseline operant self-administration sessions with 15% ethanol versus water. Baseline and testing sessions were 30 minutes on an FR4 schedule of reinforcement. One week following surgery, animals that received rAAV infusion in the

NAc underwent 14 days of operant testing sessions. Animals that received rAAV infusion in the VTA underwent 21 days of operant sessions. Ethanol (95%, w/v, Pharmco-AAPER, Shelbyville, KY) was diluted in distilled water to 15% (v/v).

P450scc AAV Plasmid Construction and Virus Packaging

An AAV vector plasmid containing P450scc was created similarly as described elsewhere (Choi et al., 2006; McCown, 2006). In brief, the P450scc sequence was amplified from mRNA isolated from the rat olfactory bulbs and converted to cDNA using a SuperScript II reverse transcriptase kit (Life Technologies, Durham, NC, USA). The primers were designed to incorporate AgeI and NotI restriction sites in the 5' and 3' portion of the sequences, respectively. The sequences of the primers were as follows: (a) 5'-ACCGGTATGCTGGCAAAAGGTCTT-3', and (b) 5'-GCGGCCGCTCATACAGTGTCGCCTTTTCTG-3'. The amplified PCR product was ligated into a TOPO TA vector as an intermediate step. Digestion of this intermediate vector with AgeI and NotI resulted in a P450scc cDNA fragment that was subsequently ligated into an AAV plasmid generously donate by Dr. Thomas McCown (University of North Carolina) containing a chicken β-actin promoter 5' to the AgeI site to drive P450scc gene expression. Inverted terminal repeats (ITRs) flanked the β-actin promoter and P450scc cDNA sequence (Fig. 4.1B). This P450scc clone was sequenced to verify the integrity of the start and stop codons as well as the cDNA sequence. A single point mutation was noted that resulted in a non-polar valine to the smaller non-polar alanine at amino acid position 151. This mutation was further determined to be inconsequential as verified by increased mRNA and protein expression by reverse-transcriptase PCR and western blot, respectively, in transient transfected fibroblasts as well as assessment of

function via conversion of 22-hydroxycholesterol to pregnenolone via RIA. Subsequently, the recombinant AAV2- P450scc (rAAV2-P450scc) viral vector was packaged by the University of North Carolina (UNC) Viral Vector Core (7.5 x 10^{11} viral particles/ml). AAV serotype 2 was used due to its previous success in infecting neuronal cells, long-term gene expression and minimal immune response. Control rAAV2-GFP vectors were also obtained from the UNC Vector Core (5 x 10^{11} - 1 x 10^{12} viral particles/ml).

Cell culture

Mouse L(tk-) fibroblast cells were used as described elsewhere (Harris et al., 1995). Briefly, cells were grown in flasks previously coated with poly-l-lysine using Dulbecco's modified Eagle's medium (DMEM) with high glucose (Life Technologies, Durham, NC, USA) along with 10% fetal bovine serum and penicillin-streptomycin. Cells were maintained in a 5% CO_2 humidified incubator. To test vector construct, cells were transiently transfected using Lipofectamine (Invitrogen).

P450scc mRNA and Protein Analysis

Cell and Tissue preparation

Cell culture samples were washed with ice-cold phosphate buffered saline (PBS), scraped and pelleted down by low-speed centrifugation. Rats received either rAAV2-P450scc or rAAV2-GFP infusions into the NAc or NAc shell and were sacrificed 1 (NAc shell) or 4 weeks (NAc) later. Brains were rapidly removed, flash frozen using isopentane, and stored at -80 C^{o} until processed.

mRNA analysis

Total RNA was isolated from microdissected tissue using Trizol and converted to cDNA using SuperScript II (Invitrogen, Durham, NC, USA) with random hexamers. cDNA was amplified using the primer pair listed above under plasmid construction with a three step PCR program. Samples were subsequently separated using a 1.0% Tris/EDTA gel. cDNA samples were re-amplified using primers specific to β-actin (Whitman et al., 2013) for normalization. Gel images were acquired using a Fotodyne imaging cabinet (Fotodyne Inc., Hartland, WI, USA), and quantified using NIH Image 1.57. Data were analyzed using Student's t-test.

Western blot analysis

Microdissected NAc shell tissue was homogenized in a whole cell lysate buffer containing 1% sodium dodecyl sulfate (SDS), 1mM EDTA, and 10mM Tris. Protein concentrations were determined by a bicinchoninic acid protein assay. Samples were subsequently denatured and separated using SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Invitrogen, Durham, NC, USA). Membranes were probed with an antibody against P450scc (Millipore, Billerica, MA, USA). Bands were visualized using enhanced chemiluminescence (GE Healthcare, Amersham, UK) under nonsaturating conditions. Blots were then re-exposed to an actin specific antibody (Millipore, Billerica, MA, USA) for normalization. Densitometric analysis was conducted using NIH Image 1.57, and data were analyzed using Student's t-test.

Surgery and viral vector infusion

Animals were anesthetized with isoflourane and placed into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The rAAV2-P450scc or control rAAV2-GFP vectors were infused bilaterally with a syringe pump (Harvard Apparatus, Holliston, MA, USA) and 10µl gastight syringes (Hamilton Company, Reno, NV, USA), connected with polyethylene tubing to needles made from stainless steel 30 gauge tubing (Smallparts Inc. Logansport, IN, USA). Needles and tubing were coated with 10% pluronic F-68 solution (Sigma-Aldrich, St. Louis, MO, USA) in order to minimize stainless steel binding of the virus. Virus was infused at a rate of 0.2µl/min and a total volume of 2-3µl/ hemisphere. Infusion coordinates relative to bregma were based on the rat brain atlas (Paxinos and Watson, 1998) as follows: NAc shell (AP + 1.6, ML ± 0.7, DV -7.5), NAc (AP + 1.6, ML ± 1.5, DV -7.5), VTA (AP - 5.5, ML ± 2.0, DV -8.3 with 10º angle). Animals were given 1 week to recover from surgery before resuming operant self-administration. Animals lacking an expected P450scc mRNA/protein increase or with needle placements outside the injection target were excluded from statistical analysis.

Immunohistochemistry (IHC)

Tissue preparation

Animals were anesthetized with pentobarbital (100mg/kg, i.p.; Professional Compounding Centers of America, Houston, TX, USA) and transcardially perfused with PBS followed by 4% paraformaldehyde. Tissue was post-fixed in 4% paraformaldehyde for 24hr at 4°C, sectioned coronally at 40µm on a vibrating microtome, and stored at -30°C until further processing.

3α,5α-THP IHC

3α,5α-THP immunohistochemical assays were performed as previously described in detail (Cook et al., 2013). Briefly, No detergents or organic solvents were used to prevent the possibility of neuroactive steroid leeching. Free floating brain sections (3-4 sections/animal/brain region) were rinsed, blocked, and incubated (48 hr at 4°C) in sheep affinity purified 3α,5α-THP antiserum (purchased from Dr. R.H. Purdy) at a dilution of 1:2500 for 3,3'-diaminobenzidine (DAB) or 1:500 for fluorescent detection. A biotinylated secondary antibody (1:200; Vector laboratories, Burlingame, CA, USA) was used in conjunction with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for DAB detection, using the manufacturers' recommended procedures. An Alexa Fluor 488 secondary antibody was used for fluorescent detection (Life Technologies, Durham, NC, USA).

3α,5α-THP IHC Cell Counts

3α,5α-THP immunoreactivity with DAB detection was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA, USA) and images were captured with a digital camera (Regita model, QImaging, Burnaby, BC). Image analysis software (Bioquant Life Sciences version 8.00.20; Bioquant Life Sciences, Nashville, TN, USA) that utilizes linear integrated optical density was used for determining positive cell counts. The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition. Positive cell count measurements were calculated from a defined region (e.g., brain region), divided by the area of the region in square millimeters, and expressed as cell counts/mm^2. Data were acquired from 4 sections/animal/brain region, and averaged

within a brain region for an individual animal to obtain one value per subject. Immunoreactivity was analyzed using Student's t-test (Prism, GraphPad Software, La Jolla, CA, USA). Brain region analyses were performed using histological coordinates as follows: mPFC (+3.00 to +2.20 AP), NAc (+ 1.70 to + 1.00 AP), and VTA (-5.20 to -6.04 AP). All analyses were based on coordinates relative to bregma in the Rat Brain Atlas (Paxinos and Watson, 1998).

GFP and glial fibrillary acid protein (GFAP) epifluorescence

Enhanced GFP (eGFP) immunofluorescence was used to determine the infection efficiency of the rAAV2 vectors. Sections were mounted on microscope slides, rinsed, blocked and incubated with eGFP primary antibody [(1:1000) Millipore, Billerica, MA, USA] applied directly to slides. Following rinses, the slides were incubated with an Alexa Fluor 488 secondary antibody (Life Technologies, Durham, NC, USA) for 1 hour at room temperature. Then, slides were rinsed and incubated with a fluorescent red nissl stain (Life Technologies) to identify neuroanatomical landmarks. GFAP immunofluorescence was used to determine needle placement of virus injections in the VTA of P rats. Briefly, free-floating sections were rinsed, blocked, incubated in GFAP primary antibody [(1:3000) Dako, Glostrup, Denmark] overnight at 4º C. Sections were then rinsed and incubated with an Alexa Fluor 594 secondary antibody (Life Technologies). Immunofluorescence was visualized with a Leica DMIRB inverted microscope and images were captured with a Q-Imaging MicroPublisher camera and computer software (Surrey, British Columbia, Canada).

Double immunofluorescent labeling and confocal microscopy

Free floating sections were rinsed, blocked, and incubated in primary antibody for cell-type specific markers: TH [(1:1000), Immunostar, Hudson, WI, USA], NeuN [(1:200), clone 60, Millipore, Billerica, MA, USA], or GFAP (1:3000) for 24 hours at 4º C. Next, sections were rinsed, blocked, and incubated with 3α,5α-THP primary antibody for 48 hours at 4º C. Then, sections were rinsed and incubated with secondary antibody (Alexa Fluor 488 for 3α,5α-THP visualization and Alexa Fluor 594 for cell-type specific markers, Life Technologies). Immunofluorescence was visualized using a Leica SP2 laser scanning confocal microscope and computer software (Buffalo Grove, IL, USA). Cell-type markers and 3α,5α-THP immunofluorescence were imaged sequentially to prevent fluorophore bleed-through.

Radioimmunoassay (RIA)

Pregnenolone concentrations were measured using a procedure previously described in detail (Porcu et al., 2006), modified for use with cell media. Briefly, pregnenolone was extracted from cell media (spiked with 1000 counts per minute of [^{3}H] pregnenolone for recovery) with 3 x 3ml of ethyl acetate, centrifuged at 3000g for 7 minutes, and the resulting extracts were dried down. The samples were reconstituted in 2 ml assay buffer, and 0.5 ml aliquots were removed for recovery and sample measurement. Pregnenolone antibody (MP Biomedicals, Orangeburg, NY) was added to samples along with pregnenolone standards. Values were determined using a one-site competition equation and corrected for extraction efficiency.

RESULTS

The P450scc construct increases P450scc and the neuroactive steroid pregnenolone

Overexpression of P450scc increases the rate limiting enzyme in steroid synthesis, thereby driving neuroactive steroid expression (Fig. 4.1A). In order to achieve this goal *in vivo*, P450scc gene expression was driven by a ubiquitous chicken β-actin (CBA) promoter in the context of an AAV vector (Fig. 4.1B). To confirm that the P450scc construct results in increases of P450scc and steroidogenesis *in vitro,* we transiently transfected mouse L(tk-) fibroblast cells and measured P450scc protein levels as well as pregnenolone levels in the cell media. Transfection of fibroblasts with the P450scc construct resulted in a 10-fold increase in P450scc protein expression at 48 hr post-transfection [$t(4) = 11.74$, $p < 0.001$] (Fig. 4.1C), as well as an increase in extracellular pregnenolone levels [$t(12) = 8.360$, $p < 0.0001$] (Fig. 4.1D).

Infusion of rAAV2-P450scc in the NAc shell increases P450scc

To confirm that rAAV2-P450scc increases P450scc *in vivo*, we infused the rAAV2-P450scc or rAAV2-GFP vectors (2 or 4μl) into the NAc shell of Wistar rats and measured mRNA and protein levels 1 week following surgery. The infection efficiency of the rAAV2-GFP vector (2μl) and location of transduction within the NAc shell (Fig. 4.2A) was determined 1 week post-infusion. The rAAV2-P450scc vector (4μl) increased P450scc mRNA by 573±122% [$t(6) = 4.532$, $p < 0.005$] (Fig. 4.2B) and protein levels by 84±9% (2μl) [$t(6) = 6.847$, $p < 0.005$] or 172±69% (4μl) [$t(6) = 2.479$, $p < 0.05$] (Fig. 4.2C).

Overexpression of P450scc in the NAc does not alter 3α,5α-THP or operant ethanol self-administration

To determine if P450scc overexpression in the NAc alters ethanol reinforcement, we infused the rAAV2-P450scc or rAAV2-GFP vectors (3μl) into the NAc of P rats trained to self-administer ethanol. We have previously shown that ethanol decreases levels of cellular 3α,5α-THP in the NAc "shore" (core-shell border) (Cook et al., 2013), and others have shown that dopamine is released in the NAc shore during operant self-administration of ethanol (Howard et al., 2009). Therefore, we aimed our viral infusions (3μl) at the NAc shore, which is also centralized within the NAc and thereby limits viral infection of surrounding brain regions (Fig. 4.3A). P450scc overexpression in the NAc did not alter operant ethanol responding (rAAV2-P450scc treatment [$F(1,156) = 2.297$, $p = 0.1555$]) (Fig. 4.3B) or ethanol intake (rAAV2-P450scc treatment [$F(1,156) = 2.186$, $p = 0.1651$]) during the 14 days of testing. Furthermore, there was no difference in water responding [$F(1,156) = 0.0048$, $p = 0.9457$] (Fig. 4.3B) or general locomotor activity [$t(12) = 1.813$, $p = 0.0948$] (Fig. 4.3C) between the rAAV-P450scc and rAAV-GFP groups. The rAAV-P450scc vector did, however, increase P450scc mRNA levels in the NAc 4 weeks following infusion [$t(12) = 6.690$, $p < 0.0001$] (Fig. 4.3D). To determine if rAAV2-P450scc infusion in the NAc increases 3α,5α-THP, we infused the rAAV2-P450scc or control vector into the NAc of a separate group of Wistar rats and performed 3α,5α-THP IHC at 1 week following infusion. IHC analysis revealed that rAAV2-P450scc did not increase 3α,5α-THP in the NAc [$t(6) = 0.1893$, $p = 0.86$] (Fig. 3E-F).

Overexpression of P450scc in the VTA reduces long-term operant ethanol self-administration

To test whether P450scc overexpression in the VTA alters ethanol reinforcement, we infused the rAAV2-P450scc or rAAV2-GFP vectors (2μl) into the VTA of P rats trained to self-administer ethanol. rAAV2-P450scc infusion in the VTA reduced ethanol responding by 20% (main effect of rAAV2-P450scc treatment [$F(1,540) = 13.67$, $p < 0.005$]; main effect of time [$F(20,540) = 2.785$, $p < 0.0001$]) (Fig. 4.4B) and ethanol intake by 14% (main effect of rAAV2-P450scc treatment [$F(1,540) = 8.174$, $p < 0.01$]; main effect of time [$F(20,540) = 3.092$, $p < 0.0001$]) (Fig. 4.4C) over the 21 days of testing. Viral infusion did not affect water responding [$F(1,540) = 0.4870$, $p = 0.4912$] or general locomotor activity [$t(27) = 0.5534$, $p = 0.5845$] (Fig. 4.4D). There was a main effect of time on water responding [$F(20,540) = 1.643$, $p < 0.05$] due to higher water responding following surgery, which over the 3 weeks stabilized at a lower level for both groups. The reduction in ethanol reinforcement and consumption was associated with an increase in 3α,5α-THP positive cells in the VTA of animals that received rAAV2-P450scc infusion [$t(27) = 3.104$, $p < 0.005$] (Fig. 4.4E-F). Since viral infusions may increase 3α,5α-THP in projection sites of the VTA, we also measured 3α,5α-THP immunoreactivity in the NAc and mPFC. There was no difference in 3α,5α-THP positive cells in the NAc [$t(13) = 1.108$, $p < 0.28$] or mPFC [$t(13) = 0.7394$, $p < 0.47$] of animals that received rAAV2-P450scc infusion in the VTA (data not shown).

3α,5α-THP co-localizes with TH and NeuN in the VTA

To identify cell-types in which 3α,5α-THP is localized in the VTA, we used scanning laser confocal microscopy and double labeled with NeuN (neuronal marker),

GFAP (astrocyte marker), or TH (putative dopamine cell marker). We found that in the VTA 3α,5α-THP is located in neurons (Fig. 4.6A), but does not appear to be present in astrocytes (Fig. 4.6B). 3α,5α-THP was located in all TH positive neurons examined (294 cells) as well as TH negative neurons (see arrows) (Fig. 4.6C).

DISCUSSION

In the present study we developed a viral vector to overexpress P450scc, and drive long-term neuroactive steroid production in neurons that contain the necessary biosynthetic enzymes. We showed that P450scc overexpression in the NAc does not alter ethanol self-administration or local 3α,5α-THP levels. However, rAAV2-P450scc infusion in the VTA produced persistent reductions in ethanol responding and consumption over the 21 days of test sessions. The effects of rAAV2-P450scc transduction in the VTA were specific to ethanol responding since there was no effect on water responding or motor activity. Reduced ethanol self-administration was associated with a 36% increase in 3α,5α-THP positive cells in the VTA. However, 3α,5α-THP immunoreactivity was not altered downstream in the NAc or the mPFC. Taken together, it appears that rAAV2-P450scc infusion in the VTA produces long-term reductions in ethanol reinforcement and consumption by increasing GABAergic neuroactive steroids, including 3α,5α-THP locally within the VTA. Further investigation revealed that 3α,5α-THP is localized to both TH positive and TH negative VTA neurons, but does not appear to be present in cells labeled with GFAP.

The ability of rAAV2-P450scc transduction of VTA neurons to modulate ethanol drinking is likely due to modulation of neural circuitry via $GABA_A$ receptor-mediated neuronal inhibition. The available data suggests that increasing 3α,5α-THP within a cell reduces the excitability of that particular cell (Saalmann et al., 2006; Akk et al., 2007; Tokuda et al., 2010; Tokuda et al., 2011). Therefore, rAAV2-P450scc transduction of a cell most likely produces a presynaptic inhibitory effect. However, it is not clear how 3α,5α-THP accesses the neuroactive steroid transmembrane binding sites on $GABA_A$

receptors, but it has been proposed to be via intracellular (i.e. presynaptic) lateral diffusion through the cell membrane (Akk et al., 2007) or by a paracrine or autocrine mechanism (Herd et al., 2007), as no active release mechanism has been identified. In the present study, we ascertained that 3α,5α-THP is located in TH positive dopamine neurons in the VTA, but we cannot rule out the possibility that glutamatergic or GABAergic neurons were also transduced. It is important to note that TH positive dopamine cells in the VTA co-release glutamate in the NAc shell during optical stimulation (Stuber et al., 2010), therefore, rAAV2-P450scc transduction of TH positive neurons in the VTA may reduce glutamatergic as well as dopaminergic activity. A recently characterized group of TH positive VTA neurons that project to the lateral habenula (LHb) have been shown to produce reward when optically stimulated (Stamatakis et al., in press). Therefore, it is possible that ethanol reward was reduced by neuroactive steroid elevations in these LHb projecting cells of the VTA. Further experimentation will be needed to understand how local increases of 3α,5α-THP in the VTA alter mesolimbic activity and identify cell-types important in these effects.

It is also likely that rAAV2-P450scc transduction of VTA neurons altered their response to ethanol in the operant self-administration procedure. Since ethanol is thought to disinhibit dopamine neurons (Spanagel and Weiss, 1999), increased 3α,5α-THP in these cells may prevent the ability of ethanol to produce this effect. However, rAAV2-P450scc transduction of VTA may have also influenced 3α,5α-THP in glutamatergic afferent projection neurons from the prefrontal cortex or pedunculopontine tegmental nucleus (PPTg)/laterodorsal tegmental nucleus (LDT), for example. These neurons provide excitatory control over dopamine neurons, and if inhibited by increases of 3α,5α-

THP, a decrease in dopamine neuron activity could be a possible outcome. In contrast, if 3α,5α-THP is increased in the glutamatergic cells that synapse on the GABAergic interneurons, that inhibit dopamine neurons, we would expect an increase in dopaminergic activity. The bed nucleus of the stria teriminalis (BNST) sends both GABAergic and glutamatergic afferent projections to the VTA that predominately synapse on TH negative GABAergic neurons (Kudo et al., 2012). Optical stimulation of these projections produce opposing effects on reward and aversion (Jennings et al., 2013), with stimulation of the GABAergic projection producing reward and stimulation of the glutamatergic projection producing aversive behavior. Therefore, although it is unclear how, increases of 3α,5α-THP in the VTA from BNST afferent projections may play a role in our behavioral results. It was recently shown that medium spiny neurons of the NAc that project to the VTA synapse on non-dopamine neurons (Xia et al., 2011). Therefore, it is difficult to determine what the effect of increased 3α,5α-THP in medium spiny neurons would be on dopaminergic activity. Taken together, there are several ways that increased intracellular 3α,5α-THP could diminish ethanol-induced dopaminergic activity and/or excitability via intracellular mediated neuronal inhibition.

Alternatively, 3α,5α-THP could be "released" or diffuse to produce extracellular actions. If 3α,5α-THP is released from neurons in the VTA, the main theoretical consequence would be inhibition of interneurons. In contrast to reducing VTA dopamine cell activity, 3α,5α-THP inhibition of interneurons would likely increase dopamine activity (Tan et al., 2010). Site specific pharmacologic manipulations in the NAc and VTA suggest that reduced dopamine activity is associated with a reduction in the maintenance of operant ethanol self-administration in non-dependent rats [for review see,

(Gonzales et al., 2004)]. Therefore, it is unlikely that increased release of 3α,5α-THP was the predominant effect of rAAV2-P450scc transduction in the VTA. Finally, we cannot rule out the possibility that rAAV2-P450scc transduction of VTA involves several of these mechanisms resulting in the overall behavioral sequelae observed (Fig. 4.7). Targeting TH neurons selectively could overcome this limitation and help to determine the precise site of behavioral regulation by the enhancement of steroidogenesis in the VTA.

The inability of rAAV2-P450scc transduction in the NAc to alter operant responding for ethanol may be due to an inability to increase 3α,5α-THP in NAc neurons that regulate ethanol self-administration. It is surprising that rAAV2-P450scc transduction in the NAc does not increase cellular 3α,5α-THP levels. Previous studies have shown dense cellular staining of 3α,5α-THP in the dorsal and ventral striatum (Saalmann et al., 2007; Cook et al., 2013), suggesting that the necessary biosynthetic enzymes are present in NAc neurons. However, we have recently shown that acute ethanol administration reduces 3α,5α-THP immunoreactivity in the NAc "shore" (core-shell border), in contrast to many other brain regions that display an ethanol-induced increase of 3α,5α-THP (Cook et al., 2013). Therefore, regulation of 3α,5α-THP synthesis and/or metabolism may differ in the NAc, compared to other brain regions. Nonetheless, the observation that rAAV2-P450scc transduction of NAc did not alter local 3α,5α-THP levels or ethanol self-administration supports the idea that synthesis of GABAergic neuroactive steroids, including 3α,5α-THP may have been requisite for the effects of rAAV2-P450scc transduction of the VTA.

These studies underscore the importance of endogenous GABAergic neuroactive steroids in the regulation of neurotransmission across the brain, and potential for therapeutic manipulation. Since these steroids are synthesized in brain circuitry and other endocrine organs, they provide a mechanism for interactions between many organ systems, integrating effects of all the neuroendocrine axes. It is not surprising therefore that alcohol use disorders are modulated by stress, gender, and neuroimmune processes that are all affected by neuroactive steroids. Since plasma $3\alpha,5\alpha$-THP levels are reduced during withdrawal in human alcoholics (Romeo et al., 1996), this target merits further investigation.

Genetic regulation of neuroactive steroid levels has multiple therapeutic advantages over systemic administration of neurosteroids or their analogs. First, the effects of rAAV transduction persists in the central nervous system at least 2 years in rats (Klein et al., 2002), 8 years in the rhesus monkey (Hadaczek et al., 2010), and is considered a permanent episomal modification of the genetic content of the cell. This is a critical factor for biomedical research examining chronic diseases such as addictive disorders. Next, it is possible to minimize untoward side effects by induction of steroidogenesis at sites that exhibit pathological activity, avoiding sites that confer unwanted neuroactive steroid effects. Indeed, we have recently found that chronic intermittent ethanol administration reduces neuronal $3\alpha,5\alpha$-THP immunolabeling in the VTA, but not the NAc (Maldonado-Devincci et al., in preparation) and this effect is associated with increased ethanol self-administration (Lopez et al., 2008). Thus, our present data converge with other studies upon the conclusion that selective modulation of VTA neuroactive steroids modulate ethanol reinforcement and drinking. Therefore, the

combination of P450scc gene delivery with studies of brain activity could allow targeted therapy to manipulate persistent abnormal cellular activity that may underlie alcohol use disorders or other neurological and psychiatric disease.

FIGURES AND TABLES

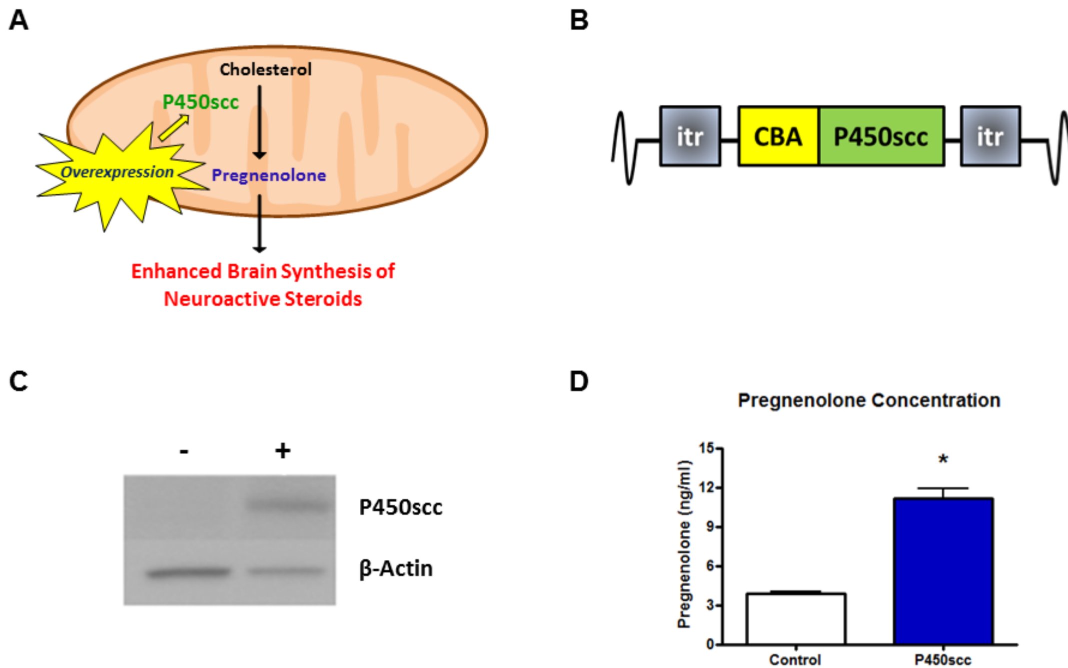


Figure 4.1: The P450scc construct increases functional P450scc expression resulting in elevated pregnenolone levels. (A) Model to enhance neuroactive steroid synthesis by overexpressing P450scc, which performs the limiting enzymatic reaction in steroidogenesis. (B) P450scc construct consisting of the P450scc gene driven by the ubiquitous chicken β-actin promoter flanked by inverted terminal repeats (itr). (C) Transient transfection of mouse (tk-) fibroblast cells increased P450scc by ~10-fold ($p < 0.001$) 48 hr post-transfection. Representative immunoblot probed for P450scc with β-actin used as a loading control, + indicates transfection with P450scc construct , - indicates control cells. (D) Pregnenolone levels were increased in the cell media ($p < 0.0001$), measured by radioimmunoassay. * indicates $p < 0.0001$ compared to control. CBA, chicken β-actin; P450scc, P450 side chain cleavage; itr, inverted terminal repeat.

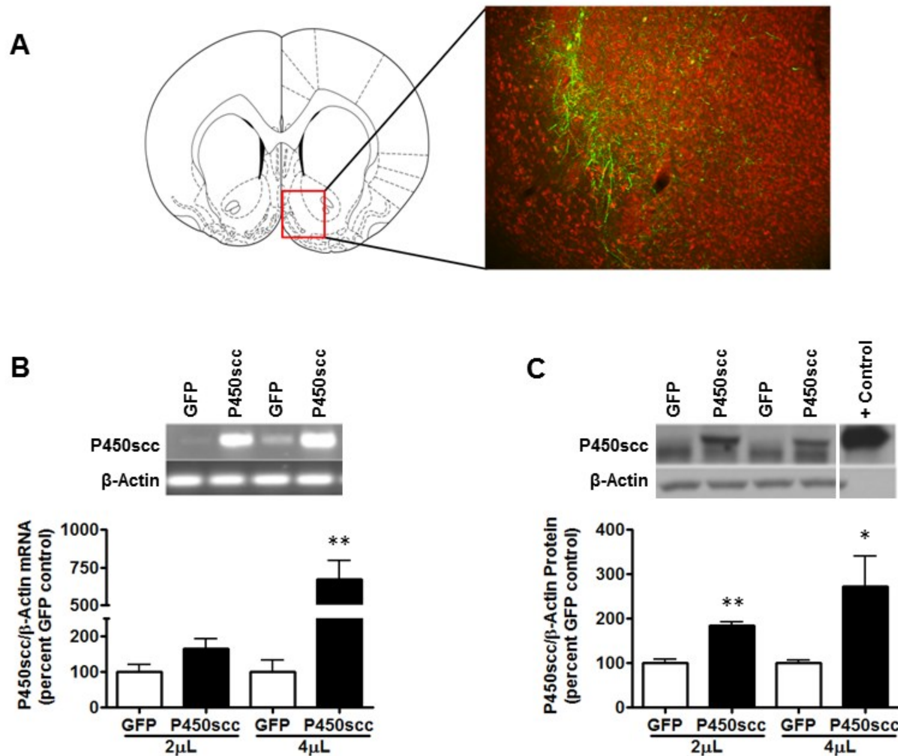


Figure 4.2: rAAV2-P450scc transduction in the NAc shell increases P450scc mRNA and protein expression dependent on the volume of virus infused. (A) Representative photomicrograph (10x) of rAAV2-GFP infection efficiency in the NAc shell 1 week after 2μL rAAV2-GFP infusion (green=GFP, red=red nissl stain). The red box indicates the location of representative photomicrograph (+1.60 mm relative to bregma) within the NAc. (B) The 4μL infusion of rAAV2-P450scc significantly increased P450scc mRNA ($p < 0.005$) at 1 week post-surgery. Representative gel showing P450scc mRNA following 4μL rAAV2-P450scc or rAAV2-GFP infusion in the NAc shell. β-actin was used as a loading control. (C) rAAV2-P450scc transduction increased protein levels of P450scc following both the 2μL ($p < 0.005$) and 4μL ($p < 0.05$) infusions at 1 week post-surgery. Representative immunoblot probed for P450scc following 4μL rAAV2-P450scc or rAAV2-GFP infusion in the NAc shell. P450scc is the uppermost band shown increased following rAAV2-P450scc transduction. β-actin was used as a loading control, but is not seen in the positive control (rat adrenal) due to the small amount of protein loaded (1μg) compared to brain samples.* indicates $p < 0.05$ compared to GFP control,

** indicates $p < 0.005$ compared to GFP control. NAc, nucleus accumbens; P450scc, P450 side chain cleavage; mRNA, messenger ribonucleic acid; rAAV2, recombinant adeno-associated virus serotype 2; GFP, green fluorescent protein.

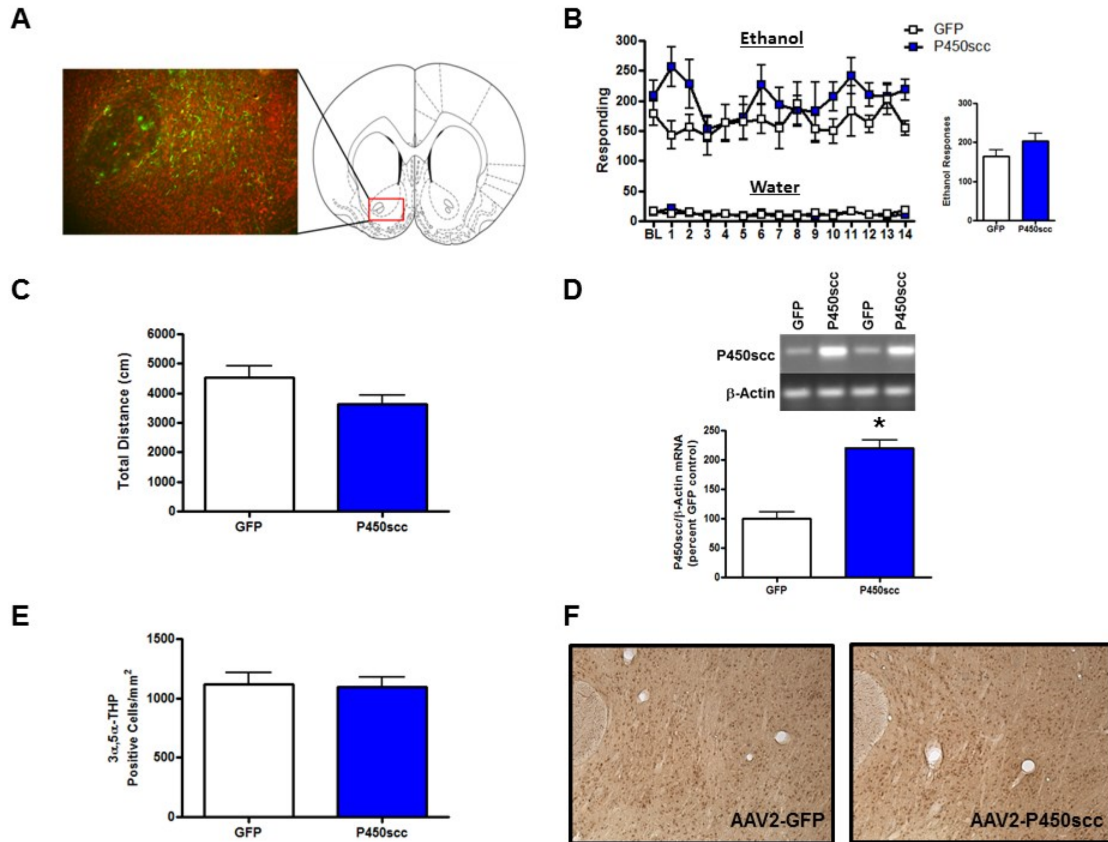


Figure 4.3: rAAV2-P450scc transduction in the NAc increases P450scc mRNA but does not alter operant ethanol self-administration or cellular 3α,5α-THP. (A) Representative photomicrograph (10x) of rAAV2-GFP infection efficiency in the NAc 1 week after 3μL rAAV2-GFP infusion (green=GFP, red=nissl stain). The red box indicates the location of representative photomicrograph (+1.60 mm relative to bregma) within the NAc. (B) rAAV2-P450scc transduction in the NAc did not alter operant ethanol responding or water responding, compared to rAAV2-GFP controls. Ethanol responding over the 14 days of test sessions is collapsed in the bar graph. (C) rAAV2-P450scc transduction in the NAc did not alter total distance traveled (cm) in the open field test. (D) The 3μL infusion of rAAV2-P450scc significantly increased P450scc mRNA in the NAc ($p < 0.0001$) at 4 weeks post-surgery. Representative gel showing P450scc mRNA level following 3μL rAAV2-P450scc or rAAV2-GFP infusion in the NAc. β-actin was used as a loading control. (E) Infusion of rAAV2-P450scc (3μL) in the NAc of Wistar rats did not alter 3α,5α-THP positive cells in the NAc at 1 week post-surgery. (F) Representative photomicrographs (10x) of cellular 3α,5α-THP immunoreactivity in the NAc 1 week

following 3μL rAAV2-GFP or rAAV2-P450scc infusion in the NAc. * indicates $p < 0.0001$ compared to GFP control. NAc, nucleus accumbens; P450scc, P450 side chain cleavage; mRNA, messenger ribonucleic acid; rAAV2, recombinant adeno-associated virus serotype 2; GFP, green fluorescent protein.

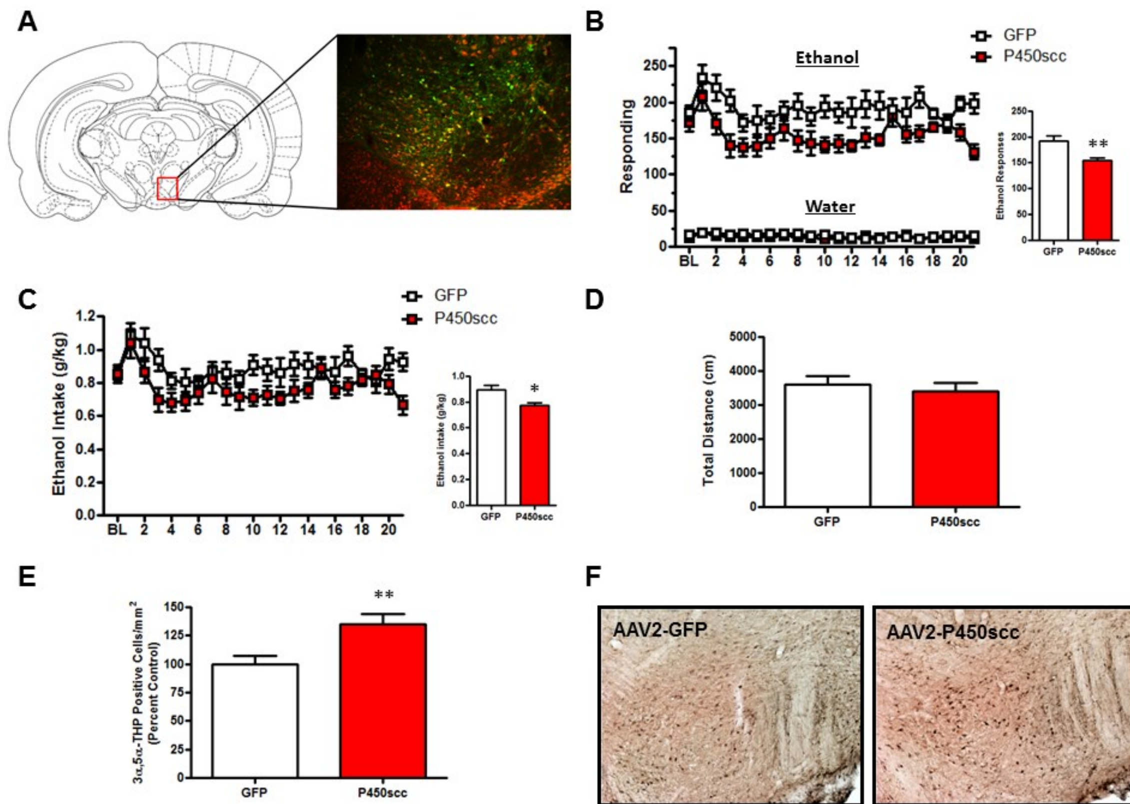


Figure 4.4: rAAV2-P450scc transduction in the VTA produces long-term reductions in operant ethanol self-administration and increases 3α,5α-THP positive cells. (A) Representative photomicrograph (10x) of rAAV2-GFP infection efficiency in the VTA 1 week after 2μL rAAV2-GFP infusion (green=GFP, red=nissl stain). The red box indicates the location of representative photomicrograph (-5.80 mm relative to bregma) within the VTA. (B) rAAV2-P450scc transduction in the VTA reduced operant ethanol ($p < 0.005$) but not water responding over the 21 days of test sessions, compared to rAAV2-GFP controls. Ethanol responding over the 21 days of test sessions is collapsed in the bar graph. (C) rAAV2-P450scc transduction in the VTA reduced ethanol intake (g/kg) ($p < 0.01$) over the 21 days of test sessions, compared to rAAV2-GFP controls. Ethanol intake (g/kg) over the 21 days of test sessions is collapsed in the bar graph. (D) rAAV2-P450scc transduction in the VTA did not alter total distance traveled (cm) in the open field test. (E) Infusion of rAAV2-P450scc (2μL) in the VTA increased 3α,5α-THP positive cells in the VTA ($p < 0.005$) at 4 weeks post-surgery. (F) Representative photomicrographs (10x) of cellular 3α,5α-THP immunoreactivity in the VTA 4 weeks

following 2μL rAAV2-GFP or rAAV2-P450scc infusion in the VTA. * indicates $p < 0.01$ and ** indicates $p < 0.005$ compared to control values. VTA, ventral tegmental area; P450scc, P450 side chain cleavage; rAAV2, recombinant adeno-associated virus serotype 2; GFP, green fluorescent protein.

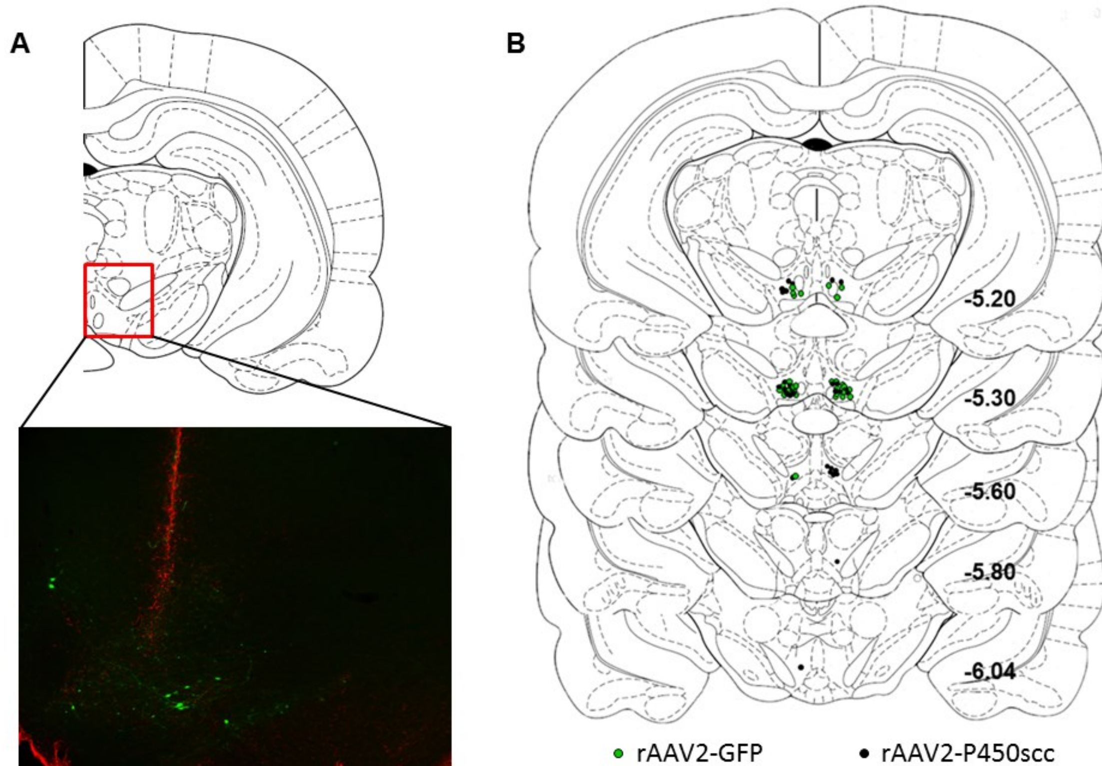


Figure 4.5: Localization of viral vector infusions was determined using GFAP immunofluorescence to visualize needle tracts. (A) Representative image showing GFAP immunofluorescence (red) at the needle tract and GFP positive cells (green) in the VTA 4 weeks following rAAV2-GFP infusion. The red box indicates the location of the representative image (-5.20 mm relative to bregma) within the VTA. (B) Location within the VTA of rAAV2-GFP (green circle) or rAAV2-P450scc (black circle) infusions, relative to bregma. GFAP, glial fibrillary acidic protein; VTA, ventral tegmental area; P450scc, P450 side chain cleavage; rAAV2, recombinant adeno-associated virus serotype 2; GFP, green fluorescent protein.

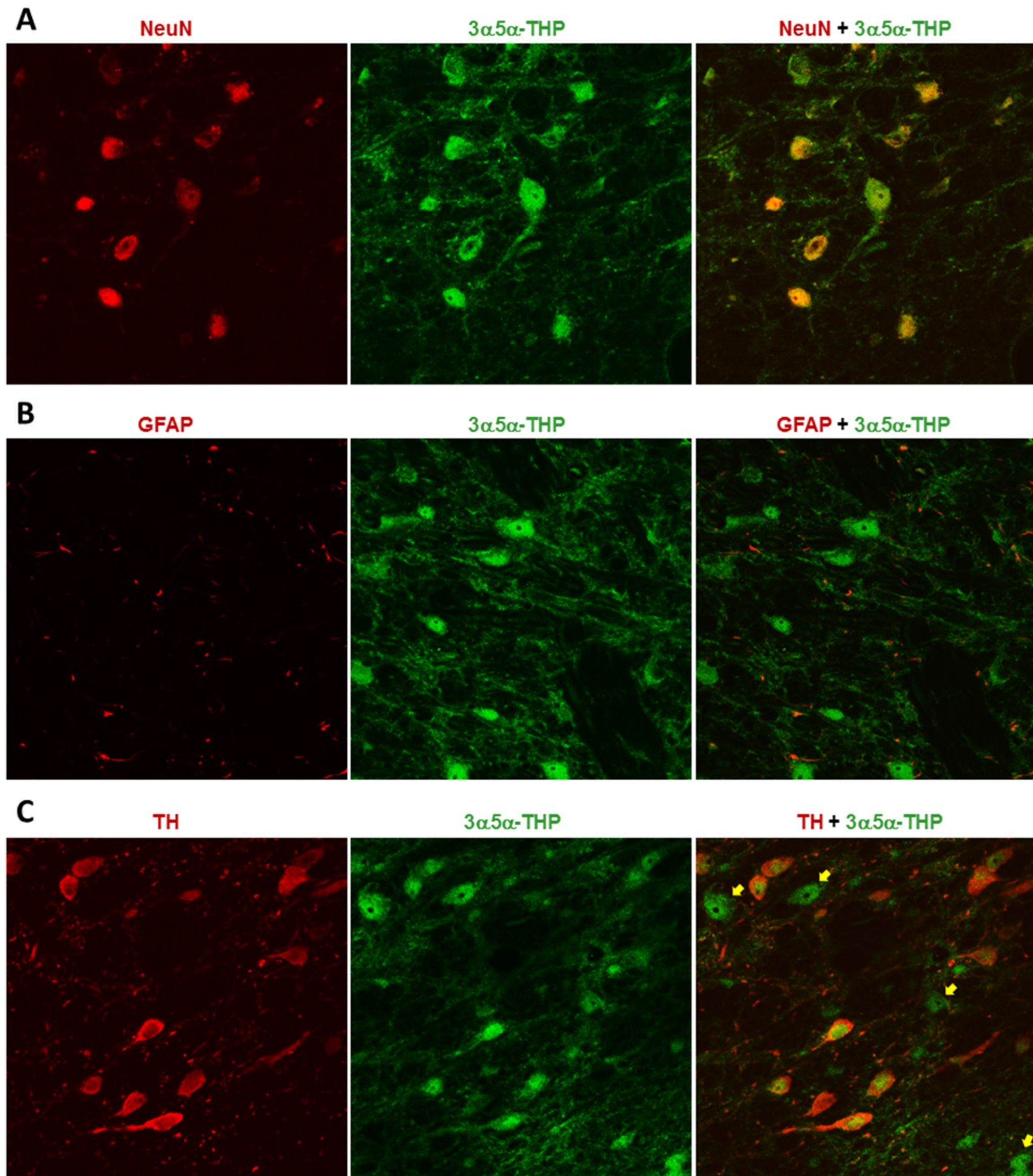


Figure 4.6: Confocal scanning microscopy revealed that 3α,5α-THP co-localizes with NeuN positive neurons, TH positive neurons, but not in GFAP positive astrocytes in the VTA of P rats. (A) 3α,5α-THP (green) co-localizes with NeuN (red) in the VTA. (B) 3α,5α-THP (green) does not co-localize with GFAP (red) in the VTA. (C) 3α,5α-THP (green) co-localized with all TH (red) positive cells examined in the VTA (294 cells).

$3\alpha,5\alpha$-THP is also located in TH negative cells in the VTA (yellow arrows). NeuN, neuronal nuclei; VTA, ventral tegmental area; GFAP, glial fibrillary acidic protein; TH, tyrosine hydroxylase.

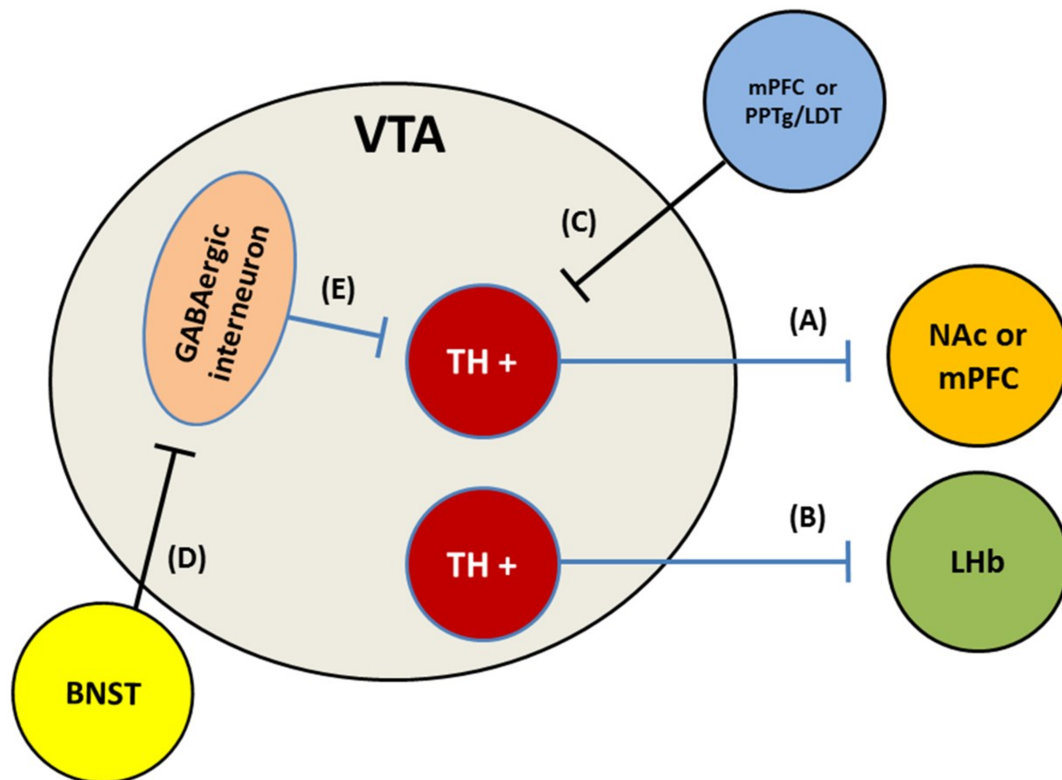


Figure 4.7: Simplified schematic of potential mechanisms of rAAV2-P450scc transduction-induced effects on VTA neurons believed to regulate ethanol reinforcement and consumption or in which optical stimulation is rewarding. Transduction of TH+ neurons may reduce activity in cells that project to (A) NAc, mPFC and/or (B) LHb or both sites. Transduction of afferent projections from (C) PFC, PPTg/LDT may decrease activation of TH+ projection neurons. Transduction of (D) BNST afferent projections onto GABAergic neurons have the potential to influence rewarding and/or aversive behavior. Transduction of inhibitory interneurons in the VTA is not expected to have any effect since biosynthetic enzymatic machinery is absent in these cells, but (E) increased extracellular levels of 3α,5α-THP may act via GABAergic interneurons to increase activity of TH+ neurons. VTA, ventral tegmental area; TH, tyrosine hydroxylase; NAc, nucleus accumbens; LHb, lateral habenula; PPTg/LDT, pedunculopontine tegmental nucleus/laterodorsal tegmental nucleus;. BNST, bed nucleus of the stria terminalis.

CHAPTER 5. **GENERAL DISCUSSION**

Alcohol use disorders continue to be pervasive in society and costly at many different levels. It is not just the individual that suffers but their families are damaged, children are born with fetal alcohol spectrum disorder, and far too many accidents and traffic fatalities are caused by alcohol disorders. Therefore, it is in everyone's interest to develop efficacious treatments for alcohol use disorders. Determining specific effects of alcohol on the brain is critical to identify potential biological targets for therapeutic exploitation. A large body of data from basic research in whole animals (for review see, Morrow, 2007), *in vitro* physiology (Sanna et al., 2004; Tokuda et al., 2011), and human studies suggest (Pierucci-Lagha et al., 2005) that GABAergic neuroactive steroids contribute to the pharmacological effects of alcohol. It has been well documented that in rats ethanol increases levels of GABAergic neuroactive steroids. Since 3α,5α-THP and 3α,5α-THDOC are very potent, producing pharmacological effects in nM concentrations, ethanol-induced increases of 3α,5α-THP and 3α,5α-THDOC can produce dramatic effects on brain activity. Furthermore, GABAergic neuroactive steroids can decrease ethanol consumption and alleviate withdrawal symptoms, making them attractive candidates as potential treatments for alcohol use disorders. Measures of steroids in brain tissue present significant challenges. Since steroids are not protein, standard laboratory measurements of protein or mRNA cannot be used. To further complicate things, steroids are lipid soluble, and the brain consists of a milieu of lipids. RIA and GC-MS measurements have

been developed to measure many steroids in blood plasma, but brain measurements are usually limited to the cerebral cortex and a few other regions highly enriched in steroids like the hippocampus and the olfactory bulb. Therefore, we have not been able to measure 3α,5α-THP in limbic/extended amygdala brain regions implicated in drug addiction disorders. Gain or loss of function studies are also difficult due to the fact that steroids do not come from genes, and there is not an antagonist for the neuroactive steroid binding sites on $GABA_A$ receptors. Therefore, the focus of this dissertation was to use innovative approaches to (1) examine ethanol-induced changes in 3α,5α-THP across the rat brain and determine if changes in 3α,5α-THP were due to peripheral or local actions (2) to use a viral vector to produce local brain changes in neuroactive steroids and measure effects on operant ethanol self-administration.

We showed that ethanol (2g/kg) produced divergent, brain region specific, changes in cellular levels of 3α,5α-THP. Many regions including the granule cells of the DG and the DMS that are densely labeled with 3α,5α-THP did not display a change in 3α,5α-THP following ethanol administration. Therefore, the ethanol-induced changes in 3α,5α-THP can be quite specific to particular cellular populations. For example, we observed an increase of 3α,5α-THP in the CA1 pyramidal cell layer, polymorphic cell layer of the DG, but not the granule cells of the DG. Since a reduction in 3α,5α-THP has not been reported previously in rats, the reductions of 3α,5α-THP in the NAc and the amygdala were surprising. Furthermore, cellular reductions of 3α,5α-THP were subregion specific, and isolated to the NAc "shore" (core-shell border) and CeA. Taken together, this work is in agreement with previous RIA and GC-MS experiments measuring 3α,5α-THP in the whole cerebral cortex and hippocampus, and extends previous results by

showing that ethanol can increase or decrease cellular 3α,5α-THP in a brain region dependent manner. The presence of ethanol-induced reductions of cellular 3α,5α-THP are particularly intriguing. The mechanism(s) that underlie this reduction are unknown, but may involve regulation of 3α,5α-THP metabolism or diffusion/release from neurons.

The predominate theory in the field at the time of this study was that *in vivo* ethanol activates the HPA axis leading to release of 3α,5α-THP and neuroactive steroid precursor from the adrenal glands into the blood plasma, resulting in an increase of 3α,5α-THP in the brain. The increase of 3α,5α-THP in the cerebral cortex has been shown multiple times to be dependent on the adrenal glands (Khisti et al., 2003; O'Dell et al., 2004; Porcu et al., 2004). Furthermore, our lab has shown that the increase of 3α,5α-THP in the cerebral cortex is dependent on *de novo* StAR production in the adrenal glands as well as ACTH release from the pituitary gland (Boyd et al., 2010). Because of this body of work and limitations in steroid measurements, assumptions were made about how ethanol increased 3α,5α-THP in the rat brain. Specifically, it appeared that ethanol-induced increases of 3α,5α-THP in the brain were due primarily to adrenal sources of 3α,5α-THP and/or 3α,5α-THP precursor since 5α-DHP administration restores ethanol-induced increases of 3α,5α-THP in the cerebral cortex following ADX. This is in stark contrast to *in vitro* experiments showing that ethanol bath application increases 3α,5α-THP in hippocampal slices (Sanna et al., 2004; Tokuda et al., 2011). The presence of divergent changes or the lack of change in cellular 3α,5α-THP following ethanol ruled out the possibility that there is a global increase of 3α,5α-THP in the brain. Furthermore, the presence of divergent changes in 3α,5α-THP suggests that ethanol alters local

synthesis and/or metabolism of 3α,5α-THP *in vivo*, similar to the observation in hippocampal slices.

Evidence for ethanol regulation of local 3α,5α-THP synthesis also suggested that these effects might be independent of adrenal synthesis of 3α,5α-THP or its precursors. Since ethanol appeared to alter local levels of 3α,5α-THP, we tested this possibility using animals that had undergone ADX or sham surgery. We examined the effects of ADX on ethanol-induced changes in 3α,5α-THP only in the brain regions where we had previously observed an effect of ethanol. We showed that in the mPFC ethanol induced increases of 3α,5α-THP appear to be dependent on the adrenal glands since ethanol failed to elevate 3α,5α-THP following ADX. This effect is probably due to a lack of local precursor (Khisti et al., 2003), which under normal conditions is converted to 3α,5α-THP with increases of locally synthesized 3α-HSD and 5α-RI in the frontal cortex (Kim et al., 2003).

In subcortical brain regions ethanol-induced increases (CA1 pyramidal cell layer, polymorphic DG, BNST, and PVN) and decreases (NAc shore and CeA) of 3α,5α-THP independent of adrenal gland activation. Therefore, we showed for the first time that ethanol alters local levels of 3α,5α-THP *in vivo*, which is likely due to local changes in brain synthesis and/or metabolism. The ability of ethanol to alter local levels of 3α,5α-THP is not clear, but may be due to local brain region specific changes in levels of steroidogenic enzymes. There is evidence that ethanol increases levels of steroidogenic enzymes involved in 3α,5α-THP synthesis and the cholesterol transporter StAR mRNA in a brain region dependent manner (Kim et al., 2003). Following chronic ethanol there is also evidence for decreases in transcript levels of the GABAergic steroidogenic enzymes

3α-HSD and 5α-RI, which is associated concurrent reduction of 3α,5α-THP in the hippocampus (Cagetti et al., 2004). Therefore, it is possible reductions in steroidogenic enzymes may underlie the observed ethanol-induced reductions in 3α,5α-THP. Ethanol can also change the activity of GABAergic neuroactive steroid biosynthetic enzymes in mice, but changes in activity do not always predict 3α,5α-THP levels (Tanchuck et al., 2009). Nevertheless, we cannot rule out the possibility that changes in enzyme activity may explain some of the current observations. Finally, as previously mentioned we cannot rule out the possibility that a reduction of cellular 3α,5α-THP is due to extracellular release. Future studies using microdialysis to investigate ethanol-induced release of 3α,5α-THP in the NAc shore or CeA could shed light on this possibility.

Other potential mechanisms that may explain brain region specific effects on 3α,5α-THP levels include ethanol-induced increases of NMDA or locally generated acetaldehyde. For example, there is evidence in CA1 pyramidal cells that ethanol-induced increases of 3α,5α-THP are due to increased NMDA levels (Tokuda et al., 2011). This is based on the fact that the NMDA antagonist APV prevents ethanol-induced increases of cellular 3α,5α-THP using IHC, as well as ethanol depression of LTP. Furthermore, low levels of NMDA increase cellular 3α,5α-THP and depress LTP similarly to ethanol. Ethanol-induced increases of NMDA are somewhat surprising since ethanol has been shown to have antagonist properties at NMDA receptors (Lovinger et al., 1989). Interestingly, a subsequent study has shown that acetaldehyde produces similar effects on 3α,5α-THP IHC and contributes to ethanol depression of LTP (Tokuda et al., 2013). Therefore, ethanol-induced increases of 3α,5α-THP in CA1 pyramidal cells may actually be due to effects of acetaldehyde and/or paradoxical increases of NMDA.

Ethanol-induced changes in cellular 3α,5α-THP that we observed in subcortical brain regions likely contribute to behavioral and neurophysiological effects of ethanol, including synaptic plasticity. Multiple studies, both *in vivo* and *in vitro* suggest that 3α,5α-THP contributes to the neurophysiological effects of ethanol (VanDoren et al., 2000; Tokunaga et al., 2003a; Sanna et al., 2004). Evidence suggests that ethanol's depression of LTP in CA1 pyramidal cells is due to increased cellular levels of 3α,5α-THP (Tokuda et al., 2011). 3α,5α-THP administration mimics ethanol-induced deficits in hippocampal dependent spatial memory (Matthews et al., 2002). Furthermore, finasteride blocks ethanol-induced deficits on spatial memory (Morrow et al., 2003). Thus, 3α,5α-THP induction in the hippocampus may contribute to ethanol-induced cognitive impairment. One of the characteristics of alcohol exposure is a change in synaptic plasticity in several key brain regions/neural circuits (for review see, McCool, 2011), which may contribute to alcohol dependence. 3α,5α-THP is localized in such a manner to directly modulate neural circuitry. Therefore, future studies should examine the contribution of 3α,5α-THP in ethanol effects on synaptic plasticity in other brain regions.

The current 3α,5α-THP IHC studies have raised compelling new questions that may further resolve the mechanisms underlying ethanol-induced changes in 3α,5α-THP. For example, *in vitro* studies using viral vector mediated RNA interference to silence gene expression may be able to parse out the role of specific biosynthetic enzymes or cholesterol transporters. In the case of the pyramidal cells of the hippocampus we recapitulated the local action of ethanol on 3α,5α-THP levels *in vivo*, so it will be interesting to see if the novel *in vivo* results reported here can be recapitulated *in vitro*. If so, this may shed light on how ethanol-induced changes of 3α,5α-THP alter

neurophysiology in other brain regions, which ultimately may lead to studies aimed at understanding how changes in cellular 3α,5α-THP across the brain affect behavior.

In our viral vector studies we used a rAAV2 vector to increase neuroactive steroids in the NAc and the VTA. This portion of my dissertation investigates sub-cortical brain regions where neuroactive steroids may act to alter ethanol reinforcement and consumption. Both 3α,5α-THP and its synthetic analog ganaxolone can reduce ethanol self-administration in rodents. However, they also produce sedation and biphasic effects on ethanol self-administration, both of which may limit their therapeutic potential. We chose to develop the rAAV2-P450scc vector to drive neurosteroidogenesis for several reasons. First, the conversion of cholesterol to pregnenolone by P450scc is the rate-limiting enzymatic process in steroid synthesis. Using a rAAV2 vector produces long-lasting expression of the gene of interest and is essentially a permanent episomal modification of the genetic content of that cell. Therefore, this approach is particularly well suited for examining long-term chronic disorders, such as alcohol use disorders. AAV2 also has the added benefit of being approved for clinical trials in humans. Finally, region specific transduction of P450scc may limit unwanted side effects of GABAergic neuroactive steroids and act specifically to reduce ethanol reinforcement and consumption.

The results from these studies showed that rAAV2-P450scc in the VTA reduced ethanol reinforcement and consumption over the 3 weeks of testing sessions, without altering motor behavior. This long-term reduction in ethanol self-administration was associated with a 36% increase in 3α,5α-THP positive cells locally within the VTA. Furthermore, we showed that 3α,5α-THP co-localizes in neurons, including TH positive putative dopamine neurons, but not in GFAP positive astrocytes.

VTA circuitry is well established in modulating motivation and reward. The most studied component of VTA circuitry in these processes is the mesolimbic dopamine pathway from the VTA to the NAc. Dopamine signaling plays a critical role in reward, motivation, and the establishment of stimulus-reward associations (Fouriezos et al., 1978; Wise et al., 1978; Spyraki et al., 1982). Natural rewards such as food and sexual behaviors increase dopamine release in the NAc (Hernandez and Hoebel, 1988; Pfaus et al., 1990). Like most drugs of abuse, ethanol increases dopamine release in the NAc (Imperato and Di Chiara, 1986) and firing of dopamine neurons (Gessa et al., 1985). Furthermore, dopamine antagonists can reduce reinforcement of natural rewards (Spyraki et al., 1982), intracranial self-stimulation of rewarding electrical current (Fouriezos et al., 1978), and ethanol (Koob and Weiss, 1990). Due to the clear involvement of dopamine signaling in reward, the dopamine hypothesis of reward (Wise, 1978) and reinforcement (Fibiger, 1978) were formulated. Over time the role of dopamine in reward and reinforcement has evolved. Studies have shown that dopamine signaling switches from activation by rewards to being time-locked to reward predictive stimuli (Ljungberg et al., 1992; Schultz, 1998). Therefore, dopamine signaling is thought to underlie cue-reward learning. In the case of ethanol self-administration, a single trial has been shown to shift dopamine signaling toward the predictive cue (Carrillo and Gonzales, 2011). The cellular mechanism that underlies cue-reward predictive learning may be enhanced glutamatergic synaptic strength onto dopamine neurons in the VTA (Stuber et al., 2008). Furthermore, administration of ethanol and other drugs of abuse strengthen excitatory synapses on dopamine neurons in the VTA (Saal et al., 2003). Moreover, ethanol biphasically alters glutamate release in the NAc of rodents (Moghaddam and Bolinao, 1994; Yan et al.,

1998; Kapasova and Szumlinski, 2008) and blockade of metabotropic glutamate receptors in the NAc reduces ethanol reinforcement and consumption (Besheer et al., 2010b; Cozzoli et al., 2012). Evidence suggests that GABAergic agonists and antagonists in the NAc modulate ethanol self-administration (Hodge et al., 1995) and NAc dopamine release when infused in the VTA (Kalivas et al., 1990; Ikemoto et al., 1997). The effects of GABAergic drugs on ethanol self-administration and dopamine release are likely dependent on dose, cell-types inhibited, and subregion examined. Taken together, evidence suggests dopamine as well as glutamate and GABA transmission in the mesolimbic pathway contribute to reward and reinforcement.

The ability of increased cellular levels of 3α,5α-THP in the VTA to reduce ethanol reinforcement is likely due to $GABA_A$ receptor mediated inhibition of projection neurons. This is based on the fact that 3α,5α-THP and its biosynthetic enzymes are located in projecting neurons (Agis-Balboa et al., 2006; Saalmann et al., 2007), and increases of intracellular 3α,5α-THP levels result in inhibition of the cell in which they are produced (Akk et al., 2007; Tokuda et al., 2010; Tokuda et al., 2011). There are multiple ways in which increased 3α,5α-THP may reduce ethanol self-administration. Since a reduction in dopamine activity is associated with a reduction in the maintenance of operant ethanol self-administration in non-dependent rats (Gonzales et al., 2004), 3α,5α-THP inhibition of VTA dopamine activity is a likely explanation for the current results. Increased 3α,5α-THP in TH positive dopamine neurons could endogenously inhibit the activity/reduce excitability of these cells. Similarly, increased 3α,5α-THP in TH positive neurons that project to the LHb could reduce ethanol reinforcement (Stamatakis et al., in press). Furthermore, increased 3α,5α-THP in glutamateric afferent

projections onto VTA neurons could reduce dopaminergic, glutamatergic or GABAergic activity. It is not clear how increases of 3α,5α-THP in projections from striatal medium spiny neurons or BNST neurons, both of which preferentially synapse on GABAergic cells in the VTA, would affect dopaminergic activity. As previously mentioned, there is no known active release mechanism for 3α,5α-THP. However, we cannot rule out the possibility that 3α,5α-THP was released or diffused within the VTA, the result of which would differ from intracellular actions only by inhibiting GABAergic interneurons. This would theoretically result in an increase of dopaminergic activity. Although the precise mechanism is not clear, increased P450scc expression in the VTA reduces ethanol reinforcement, with increased 3α,5α-THP presumably playing a role in these effects.

Future studies may be able to clarify some of the uncertainties inherent to the current studies. A central question remaining is whether rAAV2-P450scc transduction of VTA neurons altered the activity of VTA circuitry. Future studies may be able to address this issue with electrophysiological measurements and/or measuring dopamine or glutamate release in the NAc as well as GABA release in the LHb under basal conditions as well as in response to ethanol. Ideally, these experiments would be performed *in vivo*, but *in vitro* electrophysiological studies would be useful for examining effects on synaptic plasticity, specific cell-types, or circuitry involved.

One of the limitations of our rAAV2 experiments is a lack of cellular specificity. Although rAAV2-P450scc infection will only produce increases of neuroactive steroids in cells that contain steroidogenic enzymes, it is not clear which cell-types produce the effects of VTA transduction on ethanol self-administration. We used a ubiquitous CBA promoter, which produces transduction of P450scc in all cells. To more precisely increase

neuroactive steroid levels a viral vector with a cell-type specific promoter could be used. For example, using a TH or glutamic acid decarboxylase (GAD) promoter would result in P450scc transduction only in cells that express TH or GAD. Therefore, increases of neuroactive steroids would only occur in TH or GAD positive cells. Another method that may compliment a cell-type specific promoter is the use of a vector with a reporter gene. Our viral construct did not include a reporter gene due to the size of the P450scc gene and the relatively small packaging size of AAV. The incorporation of a reporter gene would be useful for *in vitro* electrophysiological studies because cells infected by the vector could easily be identified and resulting functional changes evaluated.

Several psychiatric disorders have been associated with altered levels of 3α,5α-THP (Marx et al., 2006; Rasmusson et al., 2006; Morrow, 2007; Rupprecht et al., 2010; Brinton, 2013). Translocator protein ligands increase cholesterol transport, resulting in increased steroidogenesis and GABAergic steroids. Recently, the translocator protein agonist XBD173 has shown promise for treating anxiety disorders in humans (Rupprecht et al., 2009). Historically, studies measuring 3α,5α-THP have mainly been limited to examining blood plasma levels in humans or whole cerebral cortex/hippocampus in animal studies. The studies contained in this dissertation employed techniques that can be used to examine cellular levels of 3α,5α-THP across the brain, and identify specific abnormalities that may contribute to pathological conditions. Therefore, 3α,5α-THP IHC may be useful in both translational studies in rodents and nonhuman primates as well as reverse translational studies examining pathological 3α,5α-THP levels in post-mortem human brain. Furthermore, drugs known to change 3α,5α-THP levels that are used therapeutically, such as selective serotonin reuptake inhibitors and antipsychotics may

also exhibit regional specificity on 3α,5α-THP levels in the brain, similar to the regional specificity of the effects of ethanol. Therefore, studying the anatomical localization of changes in 3α,5α-THP may reveal unknown and more precise biological targets for the treatment of CNS disease.

REFERENCES

Agis-Balboa RC, Graziano P, Zhubi A, Maloku E, Veldic M, Costa E, 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.

Aguayo LG, Peoples RW, Yeh HH, Yevenes GE (2002) GABA(A) receptors as molecular sites of ethanol action. Direct or indirect actions? Curr Top Med Chem 2:869-885.

Akk G, Covey DF, Evers AS, Steinbach JH, Zorumski CF, Mennerick S (2007) Mechanisms of neurosteroid interactions with GABA(A) receptors. Pharmacol Ther 116:35-57.

Amaral DG, Scharfman HE, Lavenex P (2007) The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog Brain Res 163:3-22.

Ariwodola OJ, Weiner JL (2004) Ethanol potentiation of GABAergic synaptic transmission may be self-limiting: role of presynaptic $GABA_B$ receptors. J Neurosci 24:10679-10686.

Armario A (2010) Activation of the hypothalamic-pituitary-adrenal axis by addictive drugs: different pathways, common outcome. Trends Pharmacol Sci 31:318-325.

Association AP (2013) Diagnostic and Statistical Manual of Mental Disorders, 5th Edition. Boston: American Psychiatric Association.

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

Barbaccia ML, Roscetti, G., Trabucchi, M., Mostallino, M.C., Concas, A., Purdy, R.H., Biggio, G. (1996) Time-dependent changes in rat brain neuroactive steroid concentrations and $GABA_A$ receptor function after acute stress. Neuroendocrinology 63:166-172.

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

Baulieu EE (1998) Neurosteroids: a novel function of the brain. Psychoneuroendocrinology 23:963-987.

Beauchamp MH, Ormerod BK, Jhamandas K, Boegman RJ, Beninger RJ (2000) Neurosteroids and reward: allopregnanolone produces a conditioned place aversion in rats. Pharmacology, Biochemistry and Behavior 67:29-35.

Belelli D, Bolger MB, Gee KW (1989) Anticonvulsant profile of the progesterone metabolite 5α-pregnan-3α-ol-20-one. European Journal of Pharmacology 166:325-329.

Besheer J, Lindsay TG, O'Buckley TK, Hodge CW, Morrow AL (2010a) Pregnenolone and ganaxolone reduce operant ethanol self-administration in alcohol-preferring P rats. Alcohol Clin Exp Res 34:2044-2052.

Besheer J, Grondin JJ, Cannady R, Sharko AC, Faccidomo S, Hodge CW (2010b) Metabotropic glutamate receptor 5 activity in the nucleus accumbens is required for the maintenance of ethanol self-administration in a rat genetic model of high alcohol intake. Biol Psychiatry 67:812-822.

Bowen CA, Purdy RH, Grant KA (1999) An investigation of endogenous neuroactive steroid-induced modulation of ethanol's discriminative stimulus effects. Behavioral Pharmacology 10:297-311.

Boyd KN, Kumar S, O'Buckley TK, Porcu P, Morrow AL (2010) Ethanol induction of steroidogenesis in rat adrenal and brain is dependent upon pituitary ACTH release and *de novo* adrenal StAR synthesis. J Neurochem 112:784-796.

Brinton RD (2013) Neurosteroids as regenerative agents in the brain: therapeutic implications. Nat Rev Endocrinol 9:241-250.

Cagetti E, Pinna G, Guidotti A, Baicy K, 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 46:570-579.

Carrillo J, Gonzales RA (2011) A single exposure to voluntary ethanol self-administration produces adaptations in ethanol consumption and accumbal dopamine signaling. Alcohol 45:559-566.

Chiou LC, Chuang KC, Fan SH, How CH, Chen JK (2002) Does ethanol activate G-protein coupled inwardly rectifying K+ channels? Neuroreport 13:163-165.

Choi DS, Wei W, Deitchman JK, Kharazia VN, Lesscher HM, McMahon T, Wang D, Qi ZH, Sieghart W, Zhang C, Shokat KM, Mody I, Messing RO (2008) Protein kinase Cdelta regulates ethanol intoxication and enhancement of GABA-stimulated tonic current. J Neurosci 28:11890-11899.

Choi VW, Asokan A, Haberman RA, McCown TJ, Samulski RJ (2006) Production of recombinant adeno-associated viral vectors and use for in vitro and in vivo administration. Curr Protoc Neurosci Chapter 4:Unit 4 17.

Compagnone N, Bulfone A, Rubenstein J, Mellon SH (1995) Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis. Endocrinol. 136:2689-2696.

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

Cook JB, Dumitru AM, O'Buckley TK, Morrow AL (2013) Ethanol Administration Produces Divergent Changes in GABAergic Neuroactive Steroid Immunohistochemistry in the Rat Brain. Alcohol Clin Exp Res, doi: 10.1111/acer.12223 [Epub ahead of print, 26 July 2013].

Cozzoli DK, Courson J, Caruana AL, Miller BW, Greentree DI, Thompson AB, Wroten MG, Zhang PW, Xiao B, Hu JH, Klugmann M, Metten P, Worley PF, Crabbe JC, Szumlinski KK (2012) Nucleus accumbens mGluR5-associated signaling regulates binge alcohol drinking under drinking-in-the-dark procedures. Alcohol Clin Exp Res 36:1623-1633.

Cui C, Noronha A, Morikawa H, Alvarez VA, Stuber GD, Szumlinski KK, Kash TL, Roberto M, Wilcox MV (2012) New insights on neurobiological mechanisms underlying alcohol addiction. Neuropharmacology 67C:223-232.

Dawson DA, Goldstein RB, Chou SP, Ruan WJ, Grant BF (2008) Age at first drink and the first incidence of adult-onset DSM-IV alcohol use disorders. Alcohol Clin Exp Res 32:2149-2160.

Dazzi L, Serra M, Seu E, Cherchi G, Pisu MG, Purdy RH, Biggio G (2002) Progesterone enhances ethanol-induced modulation of mesocortical dopamine neurons: antagonism by finasteride. J Neurochem 83:1103-1109.

De Witte P, Dahchour A, Quertemont E (1994) Acute and chronic alcohol injections increase taurine in the nucleus accumbens. Alcohol Alcohol Suppl 2:229-233.

Farb DH, Gibbs TT (1996) Steroids as modulators of amino acid receptor function. In: CNS Neurotransmitters and Neuromodulators: Neuroactive Steroids (TW Stone, ed), pp 23-36. New York: CRC Press.

Fibiger HC (1978) Drugs and reinforcement mechanisms: a critical review of the catecholamine theory. Annu Rev Pharmacol Toxicol 18:37-56.

Finn DA, Beckley EH, Kaufman KR, Ford MM (2010) Manipulation of GABAergic steroids: Sex differences in the effects on alcohol drinking- and withdrawal-related behaviors. Horm Behav 57:12-22.

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

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

Ford MM, Nickel JD, Phillips TJ, 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.

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

Fouriezos G, Hansson P, Wise RA (1978) Neuroleptic-induced attenuation of brain stimulation reward in rats. J Comp Physiol Psychol 92:661-671.

Garzo VG, Dorrington JH (1984) Aromatase activity in human granulosa cells during follicular development and the modulation by follicle-stimulating hormone and insulin. Am J Obstet Gynecol 148:657-662.

Gessa GL, Muntoni F, Collu M, Vargiu L, Mereu G (1985) Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. Brain Res 348:201-203.

Gonzales RA, Job MO, Doyon WM (2004) The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. Pharmacol Ther 103:121-146.

Grant BF, Dawson DA, Stinson FS, Chou SP, Dufour MC, 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 74:223-234.

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

Griffin LD, Mellon SH (2001) Biosynthesis of the neurosteroid 3 alpha-hydroxy-4-pregnen-20-one (3 alpha hp), a specific inhibitor of FSH release. Endocrinology 142:4617-4622.

Grobin AC, VanDoren MJ, Porrino LJ, Morrow AL (2005) Cortical 3α-hydroxy-5α-pregnan-20-one levels after acute administration of Δ9-tetrahydrocannabinol, cocaine and morphine. Psychopharmacology (Berl) 179:544-550.

Hadaczek P, Eberling JL, Pivirotto P, Bringas J, Forsayeth J, Bankiewicz KS (2010) Eight years of clinical improvement in MPTP-lesioned primates after gene therapy with AAV2-hAADC. Mol Ther 18:1458-1461.

Harper C (1998) The neuropathology of alcohol-specific brain damage, or does alcohol damage the brain? J Neuropathol Exp Neurol 57:101-110.

Harris RA, Proctor WR, McQuilkin SJ, Klein RL, Mascia MP, Whatley V, Whiting PJ, Dunwiddie TV (1995) Ethanol increases $GABA_A$ responses in cells stably transfected with receptor subunits. Alcohol: Clin.Exp.Res. 19:226-232.

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

Hernandez L, Hoebel BG (1988) Feeding and hypothalamic stimulation increase dopamine turnover in the accumbens. Physiol Behav 44:599-606.

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

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

Hodge CW, Chappelle AM, Samson HH (1995) GABAergic transmission in the nucleus accumbens is involved in the termination of ethanol self-administration in rats. Alcohol Clin Exp Res 19:1486-1493.

Hodge CW, Samson HH, Lewis RS, Erickson HL (1993) Specific decreases in ethanol- but not water-reinforced responding produced by the 5-HT3 antagonist ICS 205-930. Alcohol 10:191-196.

Hodge CW, Nannini MA, Olive MF, Kelley SP, Mehmert KK (2001) Allopregnanolone and pentobarbital infused into the nucleus accumbens substitute for the discriminative stimulus effects of ethanol. Alcohol Clin Exp Res 25:1441-1447.

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

Howard EC, Schier CJ, Wetzel JS, Gonzales RA (2009) The dopamine response in the nucleus accumbens core-shell border differs from that in the core and shell during operant ethanol self-administration. Alcohol Clin Exp Res 33:1355-1365.

Ikemoto S, Kohl RR, McBride WJ (1997) $GABA_A$ receptor blockade in the anterior ventral tegmental area increases extracellular levels of dopamine in the nucleus accumbens of rats. Journal of Neurochemistry 69:137-143.

Imperato A, Di Chiara G (1986) Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J Pharmacol Exp Ther 239:219-228.

Janak PH, Michael Gill T (2003) Comparison of the effects of allopregnanolone with direct GABAergic agonists on ethanol self-administration with and without concurrently available sucrose. Alcohol 30:1-7.

Janak PH, Redfern JEM, 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, Morrow AL (1998) Effects of chronic ethanol consumption and withdrawal on the neuroactive steroid 3α-hydroxy-5α-pregnan-20-one in male and female rats. Alcohol Clin Exp Res 22:2055-2061.

Jennings JH, Sparta DR, Stamatakis AM, Ung RL, Pleil KE, Kash TL, Stuber GD (2013) Distinct extended amygdala circuits for divergent motivational states. Nature.

Jia F, Yue M, Chandra D, Keramidas A, Goldstein PA, Homanics GE, Harrison NL (2008) Taurine is a potent activator of extrasynaptic GABA(A) receptors in the thalamus. J Neurosci 28:106-115.

Johnson BA, Seneviratne C, Wang XQ, Ait-Daoud N, Li MD (2013) Determination of Genotype Combinations That Can Predict the Outcome of the Treatment of Alcohol Dependence Using the 5-HT3 Antagonist Ondansetron. Am J Psychiatry.

June HL, Sr., Foster KL, Eiler WJ, 2nd, Goergen J, Cook JB, Johnson N, Mensah-Zoe B, Simmons JO, June HL, Jr., Yin W, Cook JM, Homanics GE (2007) Dopamine and benzodiazepine-dependent mechanisms regulate the EtOH-enhanced locomotor stimulation in the GABAA alpha1 subunit null mutant mice. Neuropsychopharmacology 32:137-152.

Kalivas PW, Duffy P, Eberhardt H (1990) Modulation of A10 dopamine neurons by gamma-aminobutyric acid agonists. J Pharmacol Exp Ther 253:858-866.

Kapasova Z, Szumlinski KK (2008) Strain differences in alcohol-induced neurochemical plasticity: a role for accumbens glutamate in alcohol intake. Alcohol Clin Exp Res 32:617-631.

Kelm MK, Criswell HE, Breese GR (2011) Ethanol-enhanced GABA release: a focus on G protein-coupled receptors. Brain Res Rev 65:113-123.

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

Khisti RT, VanDoren MJ, Matthews DB, Morrow AL (2004) Ethanol-induced elevation of 3alpha-hydroxy-5alpha-pregnan-20-one does not modulate motor incoordination in rats. Alcohol Clin Exp Res 28:1249-1256.

Kim HJ, Ha M, Park CH, Park SJ, Youn SM, Kang SS, Cho GJ, Choi WS (2003) StAR and steroidogenic enzyme transcriptional regulation in the rat brain: effects of acute alcohol administration. Mol Brain Res 115:39-49.

Klein RL, Hamby ME, Gong Y, Hirko AC, Wang S, Hughes JA, King MA, Meyer EM (2002) Dose and promoter effects of adeno-associated viral vector for green fluorescent protein expression in the rat brain. Exp Neurol 176:66-74.

Kobayashi T, Ikeda K, Kojima H, Niki H, Yano R, Yoshioka T, Kumanishi T (1999) Ethanol opens G-protein-activated inwardly rectifying K+ channels. Nat Neurosci 2:1091-1097.

Koob GF (1992) Neural mechanisms of drug reinforcement. Annals of the New York Academy of Sciences:171-190.

Koob GF (2013) Theoretical frameworks and mechanistic aspects of alcohol addiction: alcohol addiction as a reward deficit disorder. Curr Top Behav Neurosci 13:3-30.

Koob GF, Weiss F (1990) Pharmacology of drug self-administration. Alcohol 7:193-197.

Kralic JE, Wheeler M, Renzi K, Ferguson C, O'Buckley TK, Grobin AC, Morrow AL, Homanics GE (2003) Deletion of $GABA_A$ receptor $\alpha 1$ subunit-containing receptors alters responses to ethanol and other anesthetics. J Pharmacol Exp Ther 305:600-607.

Kudo T, Uchigashima M, Miyazaki T, Konno K, Yamasaki M, Yanagawa Y, Minami M, Watanabe M (2012) Three types of neurochemical projection from the bed nucleus of the stria terminalis to the ventral tegmental area in adult mice. J Neurosci 32:18035-18046.

Kumar S, Khisti RT, Morrow AL (2005) Regulation of native $GABA_A$ receptors by PKC and protein phosphatase activity. Psychopharmacology 183:241-247.

Lester D, Freed EX (1973) Criteria for an animal model of alcoholism. Pharmacol Biochem Behav 1:103-107.

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

Ljungberg T, Apicella P, Schultz W (1992) Responses of monkey dopamine neurons during learning of behavioral reactions. J Neurophysiol 67:145-163.

Lopez MF, Anderson RI, Becker HC (2008) Repeated cycles of chronic intermittent ethanol exposure increase both self-administration and the reinforcing value of ethanol in C57BL/6J mice. Alcohol Clin Exp Res 32:163A.

Lovinger DM, White G (1991) Ethanol potentiation of 5-hydroxytryptamine$_3$ receptor-mediated ion current in neuroblastoma cells and isolated adult mammalian neurons. Molecular Pharmacology 40:263.

Lovinger DM, White G, Weight FF (1989) Ethanol inhibits NMDA-activated ion currents in hippocampal neurons. Science 243:1721-1724.

Maldonado-Devincci AM, Cook JB, O'Buckley TK, Morrow DH, McKinley RE, Morrow AL (in preparation) Chronic intermittent ethanol exposure and withdrawal alters (3α,5α)-3-hydroxy-pregnan-20-one immunostaining in mesocorticolimbic regions of C57BL/6J mice.

Marszalec W, Kurata Y, Hamilton B, Carter D, Narahashi T (1994) Selective effects of alcohols on γ-aminobutyric acid A receptor subunits expressed in human embryonic kidney cells. Journal of Pharmacology and Experimental Therapeutics 269:157-163.

Marx CE, Duncan GE, Gilmore JH, Lieberman JA, Morrow AL (2000) Olanzapine increases allopregnanolone in the rat cerebral cortex. Biol Psychiatry 47:1000-1004.

Marx CE, Stevens RD, Shampine LJ, Uzunova V, Trost WT, Butterfield MI, Hamer RM, Morrow AL, Lieberman JA (2006) Neuroactive steroids are altered in schizophrenia and bipolar disorder: relevance to pathophysiology and therapeutics. Neuropsychopharmacology 31:1249-1263.

Matthews DB, Morrow AL, Tokunaga S, McDaniel JR (2002) Acute ethanol administration and acute allopregnanolone administration impair spatial memory in the Morris water task. Alcohol Clin Exp Res 26:1747-1751.

McBride WJ (2002) Central nucleus of the amygdala and the effects of alcohol and alcohol-drinking behavior in rodents. Pharmacol Biochem Behav 71:509-515.

McBride WJ, Murphy JM, Ikemoto S (1999) Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. Behav Brain Res 101:129-152.

McCool BA (2011) Ethanol modulation of synaptic plasticity. Neuropharmacology 61:1097-1108.

McCown TJ (2006) Adeno-associated virus-mediated expression and constitutive secretion of galanin suppresses limbic seizure activity in vivo. Mol Ther 14:63-68.

Moghaddam B, Bolinao ML (1994) Biphasic effect of ethanol on extracellular accumulation of glutamate in the hippocampus and the nucleus accumbens. Neurosci Lett 178:99-102.

Mokdad AH, Marks JS, Stroup DF, Gerberding JL (2004) Actual causes of death in the United States, 2000. JAMA 291:1238-1245.

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

Morrow AL, Khisti RT, Tokunaga S, McDaniel JR, Matthews DB (2003) GABAergic neuroactive steroids modulate selective ethanol actions: mechanisms and significance. In: Neurosteroid Effects in the Central Nervous System: The Role of the $GABA_A$ Receptor (SH Smith, ed), pp 219-245. Miami: CRC Press.

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

Motzo C, Porceddu ML, Maira G, Flore G, Concas A, Dazzi L, Biggio G (1996) Inhibition of basal and stress-induced dopamine release in the cerebral cortex and nucleus accumbens of freely moving rats by the neurosteroid allopregnanolone. J.Psychopharmacol. 10:266-272.

O'Dell LE, Alomary AA, Vallee M, Koob GF, Fitzgerald RL, 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 484:241-247.

O'Dell LE, Purdy RH, Covey DF, Richardson HN, Roberto M, Koob GF (2005) Epipregnanolone and a novel synthetic neuroactive steroid reduce alcohol self-administration in rats. Pharmacol Biochem Behav 81:543-550.

Olsen RW, Hanchar HJ, Meera P, Wallner M (2007) $GABA_A$ receptor subtypes: the "one glass of wine" receptors. Alcohol 41:201-209.

Omura T, Morohashi K (1995) Gene regulation of steroidogenesis. J Steroid Biochem Mol Biol 53:19-25.

Owens MJ, Ritchie JC, Nemeroff CB (1992) 5α-Pregnane-3α,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.

Patchev VK, Shoaib M, Holsboer F, 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.

Patchev VK, Hassan AHS, Holsboer F, 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.

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

Paxinos G, Watson C (1998) The Rat Brain. San Diego: Academic Press.

Penland S, Morrow AL (2004) 3α,5β-Reduced cortisol exhibits antagonist properties on cerebral cortical $GABA_A$ receptors. Eur J Pharmacol 506:129-132.

Pfaus JG, Damsma G, Nomikos GG, Wenkstern DG, Blaha CD, Phillips AG, Fibiger HC (1990) Sexual behavior enhances central dopamine transmission in the male rat. Brain Res 530:345-348.

Pierucci-Lagha A, Covault J, Feinn R, Nellissery M, Hernandez-Avila C, Oncken C, Morrow AL, Kranzler HR (2005) GABRA2 alleles moderate the subjective effects of alcohol, which are attenuated by finasteride. Neuropsychopharmacology 30:1193-1203.

Pinna G, Costa E, Guidotti A (2006) Fluoxetine and norfluoxetine stereospecifically and selectively increase brain neurosteroid content at doses that are inactive on 5-HT reuptake. Psychopharmacology (Berl) 186:362-372.

Porcu P, Rogers LSM, Morrow AL, 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, O'Buckley TK, Alward SE, Marx CE, Shampine LJ, Girdler SS, Morrow AL (2009) Simultaneous quantification of GABAergic 3α,5α/3α,5β neuroactive steroids in human and rat serum. Steroids 74:463-473.

Porcu P, O'Buckley TK, Alward SE, Song SC, Grant KA, de Wit H, Morrow AL (2010) Differential effects of ethanol on serum GABAergic 3α,5α/3α,5β neuroactive steroids in mice, rats, cynomolgus monkeys and humans. Alcohol Clin Exp Res 34:432-442.

Porcu P, Sogliano C, Ibba C, Piredda M, Tocco S, Marra C, Purdy RH, Biggio G, 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 1012:160-168.

Purdy RH, Morrow AL, Blinn JR, Paul SM (1990a) Synthesis, metabolism, and pharmacological activity of 3α-hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptoneurosomes. J Med Chem 33:1572-1581.

Purdy RH, Moore PH, Morrow AL, Paul SM (1991a) The 3α-hydroxy ring-A-reduced metabolites of progesterone and deoxycorticosterone: Natural ligands of central $GABA_A$ receptors. In: Neurosteroids and Brain Function (E Costa, SM Paul, eds), pp 95-102. New York: Raven Press.

Purdy RH, Morrow AL, Moore PH, Jr., Paul SM (1991b) 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.

Purdy RH, Moore PH, Rao PN, Hagino N, Yamaguchi T, Schmidt P, Rubinow D, Morrow AL, Paul SM (1990b) Radioimmunoassay of 3α-hydroxy-5α-pregnan-20-one in rat and human plasma. Steroids 55:290-296.

Ramaker MJ, Ford MM, Fretwell AM, Finn DA (2011) Alteration of ethanol drinking in mice via modulation of the GABA(A) receptor with ganaxolone, finasteride, and gaboxadol. Alcohol Clin Exp Res 35:1994-2007.

Ramaker MJ, Strong MN, Ford MM, Finn DA (2012) Effect of ganaxolone and THIP on operant and limited-access ethanol self-administration. Neuropharmacology 63:555-564.

Rasmusson AM, Pinna G, Paliwal P, Weisman D, Gottschalk C, Charney D, Krystal J, Guidotti A (2006) Decreased cerebrospinal fluid allopregnanolone levels in women with posttraumatic stress disorder. Biol Psychiatry 60:704-713.

Rehm J, Mathers C, Popova S, Thavorncharoensap M, Teerawattananon Y, Patra J (2009) Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. Lancet 373:2223-2233.

Roberto M, Madamba SG, Moore SD, Tallent MK, 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 100:2053-2058.

Romeo E, Brancati A, De Lorenzo A, Fucci P, Furnari C, Pompili E, Sasso GF, Spalletta G, Troisi A, Pasini A (1996) Marked decrease of plasma neuroactive steroids during alcohol withdrawal. J Pharmacol Exp Ther 247:309-322.

Rommerts FF, Brinkman AO (1981) Modulation of steroidogenic activities in testis Leydig cells. Mol Cell Endocrinol 21:15-28.

Rouge-Pont F, Mayo W, Marinelli M, Gingras M, Le Moal M, Piazza PV (2002) The neurosteroid allopregnanolone increases dopamine release and dopaminergic response to morphine in the rat nucleus accumbens. Eur J Neurosci 16:169-173.

Rupprecht R, Papadopoulos V, Rammes G, Baghai TC, Fan J, Akula N, Groyer G, Adams D, Schumacher M (2010) Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat Rev Drug Discov 9:971-988.

Rupprecht R, Rammes G, Eser D, Baghai TC, Schule C, Nothdurfter C, Troxler T, Gentsch C, Kalkman HO, Chaperon F, Uzunov V, McAllister KH, Bertaina-Anglade V, La Rochelle CD, Tuerck D, Floesser A, Kiese B, Schumacher M, Landgraf R, Holsboer F, Kucher K (2009) Translocator protein (18 kD) as target for anxiolytics without benzodiazepine-like side effects. Science 325:490-493.

Saalmann YB, Morgan IG, Calford MB (2006) Neurosteroids involved in regulating inhibition in the inferior colliculus. J Neurophysiol 96:3064-3073.

Saalmann YB, Kirkcaldie MT, Waldron S, Calford MB (2007) Cellular distribution of the GABAA receptor-modulating 3alpha-hydroxy, 5alpha-reduced pregnane steroids in the adult rat brain. J Neuroendocrinol 19:272-284.

Samson HH (1986) Initiation of ethanol reinforcement using a sucrose-substitution procedure in food- and water-sated rats. Alcohol Clin Exp Res 10:436-442.

Sanchez P, Torres JM, Olmo A, O'Valle F, Ortega E (2009) Effects of environmental stress on mRNA and protein expression levels of steroid 5alpha-Reductase isozymes in adult rat brain. Horm Behav 56:348-353.

Sanna E, Talani G, Busonero F, Pisu MG, Purdy RH, Serra M, Biggio G (2004) Brain steroidogenesis mediates ethanol modulation of $GABA_A$ receptor activity in rat hippocampus. J Neurosci 24:6521-6530.

Santhakumar V, Wallner M, Otis TS (2007) Ethanol acts directly on extrasynaptic subtypes of GABAA receptors to increase tonic inhibition. Alcohol 41:211-221.

Sarkar J, Wakefield S, MacKenzie G, Moss SJ, Maguire J (2011) Neurosteroidogenesis is required for the physiological response to stress: role of neurosteroid-sensitive GABAA receptors. J Neurosci 31:18198-18210.

Sarto-Jackson I, Sieghart W (2008) Assembly of GABA(A) receptors (Review). Mol Membr Biol 25:302-310.

Schuckit MA (2000) Genetics of the risk for alcoholism. Am J Addict 9:103-112.

Schultz W (1998) Predictive reward signal of dopamine neurons. J Neurophysiol 80:1-27.

Sinnott RS, Mark GP, Finn DA (2002) Reinforcing effects of the neurosteroid allopregnanolone in rats. Pharmacol Biochem Behav 72:923-929.

Spanagel R, Weiss F (1999) The dopamine hypothesis of reward: past and current status. Trends Neurosci 22:521-527.

Spyraki C, Fibiger HC, Phillips AG (1982) Attenuation by haloperidol of place preference conditioning using food reinforcement. Psychopharmacology (Berl) 77:379-382.

Stamatakis AM, Jennings JH, Ung RL, Blair GA, Weinberg RJ, Neve RL, Boyce F, Mattis J, Ramakrishan C, Deisseroth K, Stuber GD (in press) A unique population of ventral tegmental area neurons inhibits the lateral habenula to promote reward. Neuron.

Strohle A, Romeo E, di Michele F, Pasini A, Yassouridis A, Holsboer F, Rupprecht R (2002) GABA(A) receptor-modulating neuroactive steroid composition in patients with panic disorder before and during paroxetine treatment. Am J Psychiatry 159:145-147.

Stuber GD, Hnasko TS, Britt JP, Edwards RH, Bonci A (2010) Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. J Neurosci 30:8229-8233.

Stuber GD, Klanker M, de Ridder B, Bowers MS, Joosten RN, Feenstra MG, Bonci A (2008) Reward-predictive cues enhance excitatory synaptic strength onto midbrain dopamine neurons. Science 321:1690-1692.

Sullivan EV, Pfefferbaum A (2005) Neurocircuitry in alcoholism: a substrate of disruption and repair. Psychopharmacology (Berl) 180:583-594.

Suzdak PD, Schwartz RD, Skolnick P, Paul SM (1986) Ethanol stimulates γ-aminobutyric acid receptor-mediated chloride transport in rat brain synaptoneurosomes. Proceedings of the National Academy of Sciences of the United States of America 83:4071-4075.

Tan KR, Brown M, Labouebe G, Yvon C, Creton C, Fritschy JM, Rudolph U, Luscher C (2010) Neural bases for addictive properties of benzodiazepines. Nature 463:769-774.

Tanchuck MA, Long SL, Ford MM, Hashimoto J, Crabbe JC, Roselli CE, Wiren KM, Finn DA (2009) Selected line difference in the effects of ethanol dependence and withdrawal on allopregnanolone levels and 5alpha-reductase enzyme activity and expression. Alcohol Clin Exp Res 33:2077-2087.

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

Thorsell A (2013) The mu-opioid receptor and treatment response to naltrexone. Alcohol Alcohol 48:402-408.

Tokuda K, Izumi Y, Zorumski CF (2011) Ethanol enhances neurosteroidogenesis in hippocampal pyramidal neurons by paradoxical NMDA receptor activation. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:9905-9909.

Tokuda K, Izumi Y, zorumski CF (2013) Locally-generated acetaldehyde contributes to the effects of ethanol on neurosteroids and long-term potentiation in the hippocampus. Neurology and Clinical Neuroscience 1:138-147.

Tokuda K, O'Dell KA, Izumi Y, Zorumski CF (2010) Midazolam inhibits hippocampal long-term potentiation and learning through dual central and peripheral benzodiazepine receptor activation and neurosteroidogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:16788-16795.

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

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

Torres JM, Ortega E (2003) Alcohol intoxication increases allopregnanolone levels in female adolescent humans. Neuropsychopharmacology 28:1207-1209.

Torres JM, Ortega E (2004) Alcohol intoxication increases allopregnanolone levels in male adolescent humans. Psychopharmacology 172:352-355.

Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865-871.

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

Uzunova V, Sheline Y, Davis JM, Rasmusson A, Uzunov DP, Costa E, Guidotti A (1998) Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. Proc Natl Acad Sci U S A 95:3239-3244.

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

Whitman BA, Knapp DJ, Werner DF, Crews FT, Breese GR (2013) The Cytokine mRNA Increase Induced by Withdrawal from Chronic Ethanol in the Sterile Environment of Brain is Mediated by CRF and HMGB1 Release. Alcohol Clin Exp Res.

Wise RA (1978) Catecholamine theories of reward: a critical review. Brain Res 152:215-247.

Wise RA (2004) Dopamine, learning and motivation. Nature Reviews Neuroscience 5:483-494.

Wise RA, Spindler J, deWit H, Gerberg GJ (1978) Neuroleptic-induced "anhedonia" in rats: pimozide blocks reward quality of food. Science 201:262-264.

Xia Y, Driscoll JR, Wilbrecht L, Margolis EB, Fields HL, Hjelmstad GO (2011) Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. J Neurosci 31:7811-7816.

Yan QS, Reith ME, Yan SG, Jobe PC (1998) Effect of systemic ethanol on basal and stimulated glutamate releases in the nucleus accumbens of freely moving Sprague-Dawley rats: a microdialysis study. Neurosci Lett 258:29-32.

Ye JH, Tao L, Ren J, Schaefer R, Krnjevic K, Liu PL, Schiller DA, McArdle JJ (2001) Ethanol potentiation of glycine-induced responses in dissociated neurons of rat ventral tegmental area. J Pharmacol Exp Ther 296:77-83.

Yim HJ, Robinson DL, White ML, Jaworski JN, Randall PK, Lancaster FE, Gonzales RA (2000) Dissociation between the time course of ethanol and extracellular dopamine concentrations in the nucleus accumbens after a single intraperitoneal injection. Alcohol Clin Exp Res 24:781-788.

Yu D, Zhang L, Eisele JL, Bertrand D, Changeux JP, Weight FF (1996) Ethanol inhibition of nicotinic acetylcholine type alpha 7 receptors involves the amino-terminal domain of the receptor. Molecular Pharmacology 50:1010-1016.