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ETHANOL REGULATION OF GLUCOCORTICOID RESPONSIVE GENES
A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy at Virginia Commonwealth University.

by

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Virginia Commonwealth University
Richmond, Virginia
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“Let me tell you the secret that has led me to my goal. My strength lies solely in my tenacity.”

Louis Pasteur

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List of Abbreviations

2-Arachidonoylglycerol (2-AG)

Adeno-associated virus Serotype 2 (AAV-2)

Adrenocorticotropin hormone (ACTH)

Adrenalectomized (ADX)

Anandamide (AEA)

Analysis of Variance (ANOVA)

Blood Ethanol Concentration (BEC)

Cannabinoid receptor (CB1)

Corticosterone (Rosser et al.)

Corticotrophin Releasing Hormone (CRH)

Delta-9-tetrahydrocannabinol (THC)

Dexamethasone Suppression Test (DST)

Dopamine (DA)

Fatty acid amide hydrolase (FAAH)

FK506 binding protein 5 (Fkbp5)

Gamma-Aminobutyric acid (GABA)

G protein-coupled inwardly-rectifying potassium channel (GIRKs)

G-protein-coupled receptor (GPCR)

G-protein-coupled receptor 6 (Gpr6)

Glucocorticoid Receptor (GR)

Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)

Glucocorticoid Response Element (GRE)

Hypothalamic Pituitary Adrenal (HPA)

Knockout (KO)

Loss of Righting Reflex (LORR)

Median eminence (ME)

Monoacylglycerol lipase (MAG-L)

Magnetic Resonance Imaging (MRI)

N-methyl-D-aspartate (NMDA)

Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (Nr3c1)

Nucleus Accumbens (NAC)

Opioid receptor, mu 1 (Oprm1)

Paraventricular nucleus (PV)

Proopiomelanocortin (POMC)

Pre-frontal cortex (PFC)

Protein phosphatase 2, regulatory subunit B, alpha (Ppp2r2a)

Quantitative polymerase chain reaction (Q-rtPCR)

Mineralocorticoid Receptor (MR)

Mifepristone, RU-38486 (RU-486)

Serum Glucocorticoid Kinase 1 (Sgk1)

Serum Glucocorticoid Kinase 1.1 (Sgk1.1)

Single Nucleotide Polymorphism (SNP)

Ubiquitin-like domain containing CTD phosphatase (Ublcp1)

Ventral tegmental area (VTA)

Wildtype (WT)

ABSTRACT

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A dissertation submitted in partial fulfillment of the requirements for the degree of
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by

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Glucocorticoid hormones modulate acute and chronic behavioral and molecular responses to drugs of abuse including psychostimulants and opioids. Acute ethanol activates the hypothalamic pituitary adrenal (HPA) axis causing the release of adrenal glucocorticoid hormones, but following chronic ethanol the HPA axis is dysregulated in both humans and rodents. Thus, there is growing evidence that glucocorticoids might also modulate behavioral and molecular responses to ethanol.

Previous microarray studies in the Miles' laboratory have shown that the well-known glucocorticoid responsive gene, Serum and Glucocorticoid-regulated Kinase 1, *Sgk1*, is prominently up regulated by acute ethanol (2 g/kg) in the prefrontal cortex (PFC) of DBA/2J mice. Functionally, *Sgk1* is an important focal point of intracellular signaling cross-talk through which the cell surface receptors, nuclear receptors, and cellular stress pathways converge to control many cellular processes including receptor or ion channel trafficking, cell proliferation and/or apoptotic responses. In the aforementioned microarray studies, *Sgk1* was accompanied by a highly correlated group of genes, many of which are also known to respond to glucocorticoids. This suggests that stress-related

signaling events might play an important role in ethanol regulation of the *Sgk1* gene network. Prior work by others showed that Sgk1 plays an important role modulating synaptic plasticity occurring in memory. Based on these findings, it is hypothesized that glucocorticoids and glucocorticoid responsive genes are responsible for modulating acute and chronic cellular and behavioral responses to ethanol including locomotor activation and ethanol sensitization. In particular, because Sgk1 is regulated by ethanol, has a well-established role in learning and memory and is responsive to glucocorticoid signaling we hypothesize that Sgk1 is involved in modulating acute and chronic cellular and behavioral responses to ethanol including ethanol sensitization.

Our results indicate that the induction of glucocorticoid responsive genes may play a role in regulating acute behavioral and cellular responses to ethanol.

Adrenalectomized (ADX) and mifepristone (RU-486) both impaired acute ethanol (2 g/kg) induced locomotor activation in DBA/2J mice without affecting basal locomotor activity. ADX mice showed microarray gene expression changes in the PFC that significantly overlapped with acute ethanol-responsive gene sets derived by our prior microarray studies. Additionally, acute ethanol regulates Sgk1 transcription via glucocorticoid receptor binding to the Sgk1 promoter. Furthermore, increases in *Sgk1* may occur to compensate for decreases in SGK1 protein and phosphorylation of SGK1 and its well-known target N-myc downstream-regulated gene 1 (NDRG1) is significantly increased 15 minutes following ethanol administration. Finally, Sgk1 intensifies and prolongs the expression phase of sensitization in D2 mice.

Our studies suggest that ethanol's activation of adrenal glucocorticoid release and subsequent glucocorticoid receptor activation may partially modulate ethanol's acute

locomotor activation in male D2 mice. Furthermore, adrenal glucocorticoid basal tone regulates PFC gene expression. A significant set of acute ethanol-responsive genes are regulated by adrenal glucocorticoid basal tone suggesting that glucocorticoid regulated PFC gene expression may be an important factor modulating acute behavioral responses to ethanol. *Sgk1* is acutely regulated following ethanol administration by the glucocorticoid receptor binding to the *Sgk1* promoter. Altogether, these results suggest a critical role for the hypothalamic pituitary adrenal axis and *Sgk1* in regulating the acute and chronic cellular and behavioral responses to ethanol.

CHAPTER 1

Introduction

Concern over individuals who consume alcohol in excess goes back centuries, but it wasn't until after the 18th Amendment was repealed in 1932, ending Prohibition, that a movement developed characterizing alcoholism as a curable illness rather than a disease of the will (Jellinek, 1960, Hewitt, 1995). During the 1930s, Bill Wilson and Bob Smith founded Alcoholics Anonymous (AA) and the successes of the organization demonstrated that alcoholics could recover from alcoholism and live productive lives. By the 1950s, public health organizations were addressing alcoholism in health care settings and by the 1960s, the American Psychiatric Association declared alcoholism an illness. It became clear as perceptions on alcoholism changed that a federal organization must be established to coordinate alcohol research and in 1970, President Nixon signed into law a bill that would create the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Ultimately, this act represented a fundamental shift in the way Americans perceive alcohol and alcoholism from the idea that alcoholism resulted from moral failings or character flaws to acknowledging alcoholism as a serious, but treatable, public health problem (Hewitt, 1995).

According to the National Institutes of Health as of April, 2010, 28% of adults ages 18 and older consume alcohol at levels that put them at risk for developing alcoholism, liver disease and other problems (Services, April, 2010). There is no perfect definition of alcoholism, but most diagnoses require individuals to have been drinking heavily over an extended period of time and to have subsequently suffered multiple major life problems due to their alcohol consumption. Additionally, it is estimated that alcohol

consumption often meets or exceeds a fifth of spirits or its equivalent in wine or beer per day (Schuckit, 1987). At risk drinking increases one's chances of injuries; health problems including liver disease, heart disease, sleep disorders, depression, stroke, bleeding from the stomach, sexually transmitted diseases and several types of cancer; and drinking during pregnancy increases the risk of birth defects for the unborn child (Services, April, 2010). Despite the considerable impact of alcohol on society, the molecular mechanisms underlying neuroadaptations or toxicity from ethanol are still poorly understood (Vengeliene et al., 2008, Rivier, 1996).

It is known that ethanol is lipophilic molecule that rapidly crosses the blood-brain barrier. Until the 1980s, it was generally believed that ethanol's actions on biologic systems resulted from alterations in the fluidity of cell membranes (Goldstein and Chin, 1981). More recently, many targets of acute ethanol have been identified including the *N*-methyl-D-aspartate (NMDA) receptor, δ -opiate receptor, GABA_A, glycine, 5-hydroxytryptamine 3 (serotonin, 5-HT₃) and nicotinic acetylcholine receptors (nAChR) as well as L-type Ca²⁺ channels and G-protein-activated inwardly rectifying K⁺ channels (GIRKs). NMDA receptors are ionotropic glutamate receptors whose receptor function is dose dependently inhibited by acute ethanol (Hoffman et al., 1989, Lovinger et al., 1989, Lovinger et al., 1990, White et al., 1990). Ethanol also inhibits the δ -opiate receptor (Charness et al., 1983). In addition, alcohol potentiates 5-HT₃ (serotonin) receptor function (Lovinger, 1999) and neuronal nAChR function (Narahashi et al., 1999). Inhibitory GABA_A and glycine receptor function is enhanced by acute ethanol (Mihic, 1999). Ethanol inhibits L-type Ca²⁺ channels (Wang et al., 1994). Finally, ethanol opens

GIRKs (Kobayashi et al., 1999b, Lewohl et al., 1999). Despite the many targets of ethanol, GABA_A and NMDA receptors are considered the major targets of ethanol.

Considerable evidence suggests that ethanol's action on GABA receptors is of utmost importance in producing intoxication (Ticku, 1990, Sigel et al., 1993). γ -aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the brain (Baur et al., 2006). The GABA receptor is the major inhibitory neurotransmitter receptor, which has been a long-time focus for studies on ethanol actions (Baur et al., 2006, Wallner et al., 2003). GABA acts at two types of receptors GABA_A and GABA_B. GABA_A receptors are ligand-gated chloride channels, whereas GABA_B receptors are coupled to potassium and calcium channels via guanine nucleotide binding proteins (G proteins) (Nicoll, 1988). Although GABA_B receptors appear to mediate some of ethanol's responses (Allan and Harris, 1989, Mehta and Ticku, 1990), ethanol activation of GABA_A receptors appears to be more important. It is known that low, intoxicating concentrations of ethanol can enhance Cl⁻ flux in synaptoneurosomes (Suzdak et al., 1986b) and cultured neurons (Mehta and Ticku, 1988). In addition, ethanol-stimulated GABA_A-mediated chloride flux corresponds closely with ethanol concentrations that produce intoxication (Suzdak et al., 1986b, Allan and Harris, 1987, Mehta and Ticku, 1988, Nishio and Narahashi, 1990) and Ro15-4513, a GABA inverse agonist, reversed some of the acute intoxicating effects of ethanol in rats (Suzdak et al., 1986a, Suzdak et al., 1986b).

In 1989, Lovinger et al. first reported that ethanol (5-100 mM) acutely inhibits NMDA-activated ion currents in a concentration-dependent manner in cultured mouse hippocampal neurons (Lovinger et al., 1989). Ethanol's inhibitory actions on the channel

were further demonstrated by measuring NMDA receptor-mediated excitatory postsynaptic potentials in slice cultures from various brain regions (Wright et al., 1996, Wirkner et al., 2000, Li et al., 2002, Calton et al., 1998, Nie et al., 1994, Yin et al., 2007). The reduction in NMDA receptor activity following acute ethanol exposure is not only concentration-dependent, but it also has a rapid onset (Wirkner et al., 2000, Peoples and Stewart, 2000, Criswell et al., 2004). While the exact method by which ethanol inhibits NMDA receptor function is still unclear, single channel recordings in cultured cortical neurons revealed that ethanol decreases the open channel probability and mean open time of native NMDA receptors (Wright et al., 1996). Additionally, while acute ethanol inhibits NMDA receptors, chronic ethanol exposure increases the synaptic expression of NR2B subunit-containing NMDA receptors (Carpenter-Hyland et al., 2004, Hendricson et al., 2007). Increases in NMDA receptor number occurs presumably as an adaptive response to the prolonged reduction of NMDA receptor activity in the presence of ethanol (Kroener et al., 2012).

Even though alcohol has many targets and alcoholism is a complex disease that develops over many years and includes many cycles of withdrawal, craving, and relapse, acute behavioral responses to ethanol have predictive validity in terms of risk for high levels of ethanol intake in animal models or alcoholism in humans (Schuckit, 1994, Metten et al., 1998, Palmer et al., 2002). The behavioral effects of acute ethanol can range from loss of inhibition to sedation and even hypnosis, with increasing concentrations of alcohol (Vengeliene et al., 2008). Acute behavioral responses to ethanol have predictive validity in terms of risk for alcoholism in humans. For example, individuals who experience sedation following ethanol consumption are less likely to

become alcoholics than those who experience euphoria and disinhibition (Schuckit, 1994).

It is known that genetic predisposition is thought to confer more than 50 percent of the risk for becoming an alcoholic (Schuckit, 1987, Kalsi et al., 2009). Family studies have revealed a threefold to fourfold increased risk for alcoholism in the sons and daughters of alcoholics (Schuckit, 1987). Additionally, Schuckit et al. has demonstrated that young men with a positive family history of alcoholism show a decreased ataxic response to a test dose of ethanol. This strongly predicts the development of alcoholism 10 years later (Schuckit, 1994). In a recent study, Ramchandani et al. investigated whether the mu opioid receptor (OPRM1) 118G variant modulated striatal dopamine release in response to ethanol. The functional OPRM1 118G variant confers enhanced subjective alcohol responses in humans (Ray and Hutchison, 2007) and a functional equivalent in rhesus macaques, the 77G variant, confers enhanced alcohol-induced psychomotor stimulation (Miller et al., 2004). The authors found that 118G carriers had a markedly more vigorous striatal dopamine (DA) response to ethanol compared to subjects homozygous for the major 118A allele. The authors also created humanized mice carrying the human exon 1 of the OPRM1 gene either as the major 118A allele or with the 118G SNP. Direct microdialysis measures of the response to a rewarding dose of ethanol showed a fourfold higher peak dopamine response to the ethanol challenge in mice carrying the 118G mutation (Ramchandani et al., 2011). These studies show how genetics can influence an individual's response to ethanol. They also provide an example of a recent study in which an animal model helped to develop a better understanding of how genetics can influence a human's response to ethanol.

Dramatic increases in possible new targets for the treatment of alcoholism have resulted from more recent work using human or animal model genetics and whole genome expression profiling with microarrays. It is well known that ethanol alters gene expression patterns both acutely and chronically. The Miles' laboratory has used acute ethanol exposure and genome-wide expression profiling to identify gene networks functioning in acute behavioral responses to ethanol or affecting drinking behavior (Kerns et al., 2005a, Wolstenholme et al., 2011). For example, Kerns et al. examined gene expression patterns in the mesolimbic dopamine reward pathway of 2 inbred mouse strains, DBA/2J (D2) and C57BL/6J (C57), exhibiting contrasting acute behavioral responses to ethanol following saline versus acute ethanol administration. The authors identified 788 genes differentially expressed in control D2 versus C57 mice and 307 ethanol regulated genes differentially expressed in the nucleus accumbens (NAC), pre-frontal cortex (PFC), and ventral tegmental area (VTA) of the 2 mouse strains (Kerns et al., 2005a). These results suggest that genetic factors may play a role in mediating the divergent responses to ethanol in D2 versus C57 mice. Genes involved in glucocorticoid signaling were differentially regulated in the PFC of D2 versus C57 mice (Kerns et al., 2005a) and it is well known that acute ethanol activates the hypothalamic pituitary adrenal (HPA) axis consisting of the hypothalamus, pituitary gland and adrenal glands in both humans and rodents leading to the release of the glucocorticoids cortisol in humans and corticosterone in rodents (Koob, 2010, Rivier, 1996). This work will use animal models to examine the role of the HPA axis in regulating gene expression following ethanol administration. Additionally, animal models will be used to determine whether the induction of glucocorticoid responsive genes, Serum glucocorticoid kinase 1 (Sgk1)

particularly, might play an important role in modifying cellular and behavioral responses to ethanol.

CHAPTER 2

Background and Significance

In this chapter, we will discuss two key anatomical systems involved in ethanol reward and dependence, the **mesocorticolimbic dopamine pathway** and the **hypothalamic pituitary adrenal (HPA) axis**. These regions are important as we believe ethanol's activation of the HPA axis may play a role in regulating the expression of Serum glucocorticoid kinase 1 (Sgk1) in the PFC, part of the mesocorticolimbic dopamine pathway. We will also discuss **glucocorticoid nongenomic signaling mechanisms**, which are hypothesized to involve **endocannabinoid signaling**. We believe glucocorticoid nongenomic signaling and endocannabinoid signaling could play a role in behavioral responses to ethanol; particularly ethanol induced acute locomotor activation. We will use **Adeno-Associated Virus 2 (AAV-2)** as method of gene delivery later in this work to overexpress Sgk1 and we will discuss AAV-2 and why we chose it as our gene delivery method. Finally, we will discuss the glucocorticoid responsive, ethanol responsive gene **Sgk1** and ethanol responsive behaviors that we hypothesize Sgk1, glucocorticoids or other glucocorticoid responsive genes may regulate including ethanol induced **acute locomotor activation** and ethanol **sensitization**.

Mesocorticolimbic Dopamine Pathway

Neurochemical and behavioral studies have identified the mesocorticolimbic dopamine pathway as a key anatomical system involved with ethanol reward and dependence (Kalsi et al., 2009). The mesocorticolimbic dopamine pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAC), the ventral striatum,

the nuclei of the stria terminalis, parts of the amygdala, the hippocampus, the lateral septal nuclei, the entorhinal cortex, the mesial frontal cortex, and the anterior cingulate cortex (Kandel ER, 2000). All major drugs of abuse, including ethanol, acutely activate the mesolimbic dopamine system (Di Chiara and Imperato, 1988). For example, neuroimaging techniques have shown that ethanol enhances extracellular dopamine levels in the human ventral striatum (Boileau et al., 2003, Urban et al., 2010, Yoder et al., 2007, Ramchandani et al., 2011). Furthermore, dopamine levels in the rat NAC increase in anticipation of ethanol consumption (Katner et al., 1996, Melendez et al., 2002).

Increases in dopamine per se are not sufficient to account for the process of addiction, after all drugs of abuse increase dopamine levels in naïve as well as addicted subjects. In the case of cocaine addiction, the magnitude of drug-induced dopamine increases, and the intensity of self-reports of the drug's reinforcing properties appears smaller in addicted versus naïve subjects (Volkow et al., 1997). This may be because the function of the mesolimbic dopamine system is severely impaired upon cessation of subchronic and chronic exposure to drugs of abuse including ethanol. In support of this, alterations of brain dopamine systems occur in abstinent alcoholics including reduced dopamine synthesis (Heinz et al., 2005) and reduced numbers of dopamine D2 receptors in the striatum (Volkow et al., 1996, Volkow et al., 2002, Martinez et al., 2005). The decreased function of the mesolimbic dopamine system has been associated with enhanced drug intake, perhaps to restore baseline function of the mesolimbic dopamine system (Diana et al., 1993, Ahmed and Koob, 2005).

While less studied than the VTA and NAC, the PFC is increasingly being recognized as a region that plays central role in addiction. Frontal lobe decreases in

volume have been identified in alcoholic subjects via magnetic resonance imaging (Jernigan et al., 1991, Pfefferbaum et al., 1997). The outcome of chronic drug use may be frontal cortical cognitive dysfunction, resulting in an inability to inhibit inappropriate unconditioned or conditioned responses elicited by drugs, by related stimuli or by internal drive states (Jentsch and Taylor, 1999). Work by Volkow et. al suggests that the orbitofrontal cortex region of the PFC modulates the value of reward by regulating the magnitude of dopamine increases in the ventral striatum and that disruption of this regulation may underlie the decreased sensitivity to rewards in addicted subjects. They show that methylphenidate, a stimulant drug, induced much smaller dopamine increases in the striatum of alcoholic subjects versus control subjects. Furthermore in alcoholics, metabolism in PFC regions is not correlated with dopamine changes suggesting that the regulation of DA cell activity by prefrontal efferents is disrupted (Volkow et al., 2007). The PFC sends glutamatergic efferents to the VTA and the NAC, which modulate the function of these brain regions (Jentsch and Taylor, 1999, Carr and Sesack, 2000). These efferents play key roles in regulating the firing pattern of dopamine cells and dopamine release, respectively (Carr and Sesack, 2000, Gariano and Groves, 1988, Murase et al., 1993). Disruption of these efferent pathways may contribute to the pathology underlying addiction.

The orbitofrontal cortex and the anterior cingulate cortex are the frontal cortical areas most frequently implicated in drug addiction. These regions are involved in higher order cognitive and motivational processing and are activated in addicted subjects during intoxication, craving, and binge drug consumption, and they are deactivated during withdrawal (Goldstein and Volkow, 2002). The cingulate cortex has been linked with

conditioned emotional learning, assessments of motivational content and assigning emotional salience to internal and external stimuli (Devinsky et al., 1995), and thus its activation by dopamine may be one of the mechanisms by which dopamine modulates drive (Kiyatkin, 1995). In a study using functional magnetic resonance imaging (MRI) to test whether brain activation was detectable in regions associated with cocaine cue-induced craving, significant activation was detected in the anterior cingulate cortex in the cocaine-using group versus control subjects. Additionally, a correlation between self-reported levels of craving and activation in the anterior cingulate was found supporting the idea that the anterior cingulate cortex may be involved in drug craving (Maas et al., 1998).

Hypothalamic Pituitary Adrenal Axis

The HPA axis consisting of the hypothalamus, the anterior lobe of the pituitary gland and adrenal glands is activated following acute ethanol exposure (Koob, 2010, Rivier, 1996). The hypothalamus consists of several nuclei; the one of interest for this project is the paraventricular nucleus (PV), which releases the neurotransmitter CRH (Rivier, 1996). The axons of CRH containing neurons in the PV nucleus terminate in the median eminence (ME), which is located in close proximity to the hypophyseal portal vessels (Rivier, 1996). The pituitary is located beneath the hypothalamus and is anatomically connected to the hypothalamus by the portal vessels (Rivier, 1996). ACTH is released from the corticotrophs in the adenohypophysis of the anterior pituitary (Rivier, 1996, Dallman et al., 1985). The adrenals are located above the kidneys and are composed of the cortex, which synthesizes glucocorticoids, aldosterone and androgens

and the chromaffin tissue, which produces epinephrine and norepinephrine (Rivier, 1996).

Following ethanol exposure, neurosecretory neurons in the parvocellular subdivision of the paraventricular nucleus of the hypothalamus receive convergent impulses from several neurotransmitter systems including stimulatory signals from serotonergic and noradrenergic neurons and inhibitory signals from GABA and β -endorphin-releasing neurons that eventually lead to the synthesis and release CRH into the portal blood vessels that enter the anterior pituitary gland (Rivier, 1996, Oswald and Wand, 2004). Once CRH is released it stimulates ACTH secretion from the anterior pituitary (Oswald and Wand, 2004). Binding of CRH to the CRH 1 receptor on pituitary corticotropes stimulates the release of ACTH from corticotropes (Rivier, 1996, Heilig and Koob, 2007, Koob, 2010, Kiefer and Wiedemann, 2004). ACTH acts on the adrenal gland to stimulate the release of glucocorticoids, cortisol in humans and corticosterone in rodents (Rivier, 1996, Kiefer and Wiedemann, 2004). Cortisol and corticosterone also regulate the HPA axis through a negative feedback mechanism by acting on hypothalamic glucocorticoid receptors to decrease CRH release (Munck et al., 1984) and in the pituitary, the hormones directly inhibit ACTH release and the production of its precursor hormone proopiomelanocortin (POMC) (Rivier, 1996, Kiefer and Wiedemann, 2004, Dallman et al., 1985). Glucocorticoids enhance glucose availability, modulate immune function, maintain vascular tone and regulate gene transcription through the direct binding of homodimers or heterodimers of glucocorticoid receptors to nuclear DNA, or through protein–protein interactions with transcription factors (Falkenstein et al., 2000, Rivier, 1996).

Ethanol's acute stimulatory effect on the HPA axis in both humans and rodents is well documented, but the response of the HPA axis to repeated ethanol exposure is more variable (Ellis, 1966, Wand and Dobs, 1991b, Zgombick and Erwin, 1988). In humans, small subsets of alcoholics (<5%) develop clinical features of hypercortisolism or Cushing's syndrome (Smalls et al., 1976, Jordan et al., 1979, Rees et al., 1977). Most alcoholics do not develop Cushing's syndrome, but numerous studies have demonstrated inadequate suppression of the HPA axis following the dexamethasone suppression test (DST) (McIntyre and Oxenkrug, 1984, Burov et al., 1986, Dackis et al., 1986, Swartz and Dunner, 1982). In the most widely employed procedure for the DST in psychiatry, the long-acting synthetic steroid dexamethasone is administered and cortisol levels are measured the next day. Dexamethasone acts by mimicking feedback effects of glucocorticoids on the HPA axis. It suppresses the release of cortisol into plasma by blocking release of CRH from the hypothalamus and ACTH from the anterior pituitary (1987). It must be recognized that characterization of the HPA axis in alcoholics is complicated. HPA axis dysfunction in alcoholics could also be due to abnormalities related to ethanol-induced liver disease (Bode et al., 1978), withdrawal (Burov et al., 1986), malnutrition (Bode et al., 1978), and depression (Dackis et al., 1986). Depressed individuals frequently show hypercortisolism and their HPA axes do not suppress appropriately following the DST (Coppin et al., 1983, Wand and Dobs, 1991b). It has been suggested that approximately 25-50% of actively drinking alcoholics are also depressed (Dackis et al., 1986). One study by Wand et al., characterized the HPA axis in a group of actively drinking, nondepressed chronic alcoholics, without evidence of liver disease or withdrawal. Most alcoholics in this study showed a blunted response to acute

intervening stress, including CRH, low dose ACTH, and metyrapone, a cortisol synthesis inhibitor, blockade suggesting that alcoholics have ethanol-induced HPA axis injury and perhaps cannot respond appropriately to nonethanol-induced stress. (Wand and Dobs, 1991b). Additionally, the animal literature also suggests that chronic ethanol exposure can impair the ability of the HPA axis to respond to stress (Dave et al., 1986).

Glucocorticoid Nongenomic Signaling Mechanisms

Although it has long been recognized that steroid hormones exert their effects on neuronal function through their classical actions or their ability to modulate gene transcription in the nucleus, many glucocorticoid effects have been documented to occur in a fashion that cannot be explained by genomic regulation (de Kloet, 2000, Dallman, 2005). Such findings have prompted the hypothesis that glucocorticoids possess membrane-associated receptors through which nongenomic signaling may evoke rapid effects on physiology and behavior and it is now believed that glucocorticoids exert their actions through both genomic and nongenomic pathways (Dallman, 2005, de Kloet, 2000). In the 1990s, a high-affinity binding site for corticosterone, which seemed to meet all of the criteria for a functional membrane-associated corticosteroid receptor, was partially purified and characterized in neuronal membranes from the amphibian brain (Moore and Orchinik, 1994). The studies by Orchinik and Moore demonstrated that in the *Taricha granulosa* (a rough-skinned newt) glucocorticoid receptors were present in neuronal membranes and associated with G proteins to modulate intracellular signaling. In the newt, a clear bioassay was established in which glucocorticoids were found to dampen stimulus-induced neuronal activation of medullary neurons, which resulted in a

reduction of courtship clasping behavior, all of which occurred in a time span of 10 minutes (Moore and Orchinik, 1994). These findings stimulated similar research in rodents, the result of which was an array of mixed findings that were less conclusive than the newt studies.

The search for the mammalian membrane-bound glucocorticoid receptor had come to a halt, until a laboratory used *in vitro* electrophysiological recordings of neurons in the paraventricular nucleus of the rat hypothalamus to demonstrate that glucocorticoids rapidly suppressed glutamatergic release onto parvocellular neurons through a mechanism that involved postsynaptic activation of a membrane-bound glucocorticoid receptor (Di et al., 2003). Activation of this receptor launched a G protein signaling cascade that induced synthesis of endocannabinoid ligands, which traversed back across the synapse where they bound to presynaptic CB1 receptor localized on glutamatergic terminals and inhibited subsequent glutamate release (Di et al., 2003). A pathway was defined in which glucocorticoids elicited a nongenomic induction of endocannabinoids, which in turn was the catalyst for glucocorticoids to modulate local neuronal transmission (Hill and McEwen, 2009). This model was also applied to the newt preparation and it was shown that the ability of glucocorticoids to inhibit sensory-evoked stimulation of medullary neurons and courtship clasping was also mediated by endocannabinoids in this model (Coddington et al., 2007). These studies integrated glucocorticoid activation of a membrane-bound G protein receptor and endocannabinoid synthesis that could inhibit neurotransmitter release.

The Endocannabinoid System

The current project expanded to also investigate the nongenomic actions of glucocorticoids and this required additional knowledge of the endocannabinoid system. Δ^9 -tetrahydrocannabinol (THC), the active component of marijuana, as well as other exogenous and endogenous cannabinoids, have been demonstrated to bind to and activate two types of cannabinoid receptors that have been cloned, CB1 (Matsuda et al., 1990) and CB2 (Gerard et al., 1991). These receptors are members of the superfamily of G protein coupled receptors and exert their actions predominantly through $G_{i/o}$ proteins (Howlett, 2002, Howlett, 2005). Cannabinoid receptor activation decreases cyclic adenosine monophosphate (cAMP) production via inhibition of adenylyl cyclase and they activate mitogen-activated protein kinase (Howlett, 1984, Howlett, 2004). Cannabinoid receptors also decrease Ca^{2+} conductance (Mackie and Hille, 1992, Caulfield and Brown, 1992) and increase K^+ conductance through inwardly-rectifying K^+ channels (Mackie et al., 1995).

CB1 receptors are distributed throughout the central nervous system and the periphery; whereas CB2 receptors are mainly associated with immune cells in both the CNS and periphery (Cabral and Marciano-Cabral, 2005, Van Sickle et al., 2005, Xi et al., 2011). In particular, CB1 receptors are expressed at high densities in the hippocampus, frontal cortex, basal ganglia and cerebellum (Ong and Mackie, 1999). CB1 receptors are localized on presynaptic terminals of both GABAergic (Katona et al., 1999) and glutamatergic neurons (Huang et al., 2001, Szabo and Schlicker, 2005). It has been consistently shown that on-demand activation of CB1 receptors by their endogenous agonists, endocannabinoids (ECs), modulates the release of different neurotransmitters in many brain areas, including those involved in cognition, memory and maintenance of

mood, such as the hippocampus and the prefrontal cortex (Wilson and Nicoll, 2002, Freund et al., 2003). Depolarization-induced suppression of inhibition (DSI) occurs when cannabinoid receptor activation causes transient suppression of the inhibitory GABAergic synaptic events in a cell (Llano et al., 1991, Pitler and Alger, 1992). Conversely, when suppression of the transient stimulatory neurotransmitter (e.g. glutamate) occurs it is called depolarized-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001). Both result in cannabinoid receptor mediated hyperpolarization of a repetitively depolarized neuron, which suppresses subsequent vesicular fusion and release of glutamate or GABA.

The two best-characterized endogenous CB1 receptor ligands include AEA and 2-arachidonoylglycerol (2-AG) (Devane et al., 1992, Mechoulam et al., 1995). Both AEA and 2-AG are postsynaptically synthesized signaling molecules that are not stored in vesicles, but are generated on demand from membrane phospholipid precursors to act in a retrograde fashion on presynaptically localized CB1 receptors (Hill et al., 2010, Clapper et al., 2009). AEA is a partial agonist at CB1 and 2-AG is a full agonist (Ahern, 2003, Ahn et al., 2009, Long et al., 2009). The endogenous endocannabinoid signaling lifespan is regulated by ill-defined uptake processes and by intracellular hydrolysis of AEA by fatty acid amide hydrolase (FAAH) and 2-AG by monoacylglycerol lipase (MAG-L), respectively (Hill et al., 2010). Genetically engineered FAAH knockout (KO) mice are severely impaired in their ability to degrade AEA and exhibit 10- to 15-fold increases in brain AEA levels (Cravatt et al., 2001).

Adeno-associated Virus Serotype 2 (AAV-2)

Gene therapy consists of the introduction of nucleic acid into a patient's cells for a therapeutic purpose. Viruses can be used as gene-therapy vectors. Viruses used for gene therapy purposes include, among others, retroviruses (Miller, 1990, Miller and Rosman, 1989), adenoviruses (Smith, 1995, Berkner, 1988) and adeno-associated viruses (Smith, 1995, Miyake et al., 2012). Retroviruses are a class of enveloped viruses containing single-stranded RNA as the viral genome. Viral RNA is reverse transcribed to yield double-stranded DNA, which integrates at random into the host genome and is expressed in a wide variety of cell types over extended periods. Retroviral expression is limited to replicating cells (Smith, 1995, Vannucci et al., 2013). Adenoviruses are nonenveloped viruses containing linear double-stranded DNA that can infect both non-dividing and dividing cells. Adenoviruses display low pathogenicity and wide cellular tropism, but they are also highly immunogenic and this limits their use (Smith, 1995, Vannucci et al., 2013). Adeno-associated virus is a linear, non-enveloped, single-stranded DNA virus that requires co-infection with certain other viruses to replicate. Wild type AAV can integrate into the host chromosome in a specific region of chromosome 19 in cells in the absence of helper virus. AAV provides long-term expression in both dividing and nondividing cells, is not known to cause disease and induces mild immune responses (Smith, 1995, Vannucci et al., 2013, Daya and Berns, 2008, Terzi and Zachariou, 2008).

In our studies, we chose to use adeno-associated Virus (AAV) for gene delivery because of its long-term expression, lack of pathogenicity, and the virus' ability to infect dividing and nondividing cells (Daya and Berns, 2008, Terzi and Zachariou, 2008). Twelve AAV serotypes with unique properties have been used to produce most expression vectors. The AAV-2 serotype shows CNS and liver specific expression and

enters the cell through the heparin sulfate proteoglycan (HSPG) receptors (Miyake et al., 2012, Summerford and Samulski, 1998). Competition experiments have demonstrated that soluble heparin can block AAV-2 binding and transduction (Summerford and Samulski, 1998). Within the CNS, AAV-2 shows neuron specific expression (Daya and Berns, 2008, Terzi and Zachariou, 2008).

Studies in our lab (Bhandari et al., 2012) and others have successfully used rAAV to over-express various proteins in the brain and alter behavioral responses. For example, Homer proteins are known to be important in calcium signaling events, glutamate receptor signaling/trafficking, and synaptic remodeling (Szumlinski et al., 2004). Homer2 knockout mice show a phenotype similar to that of animals withdrawn from repeated cocaine administration and infusion of Homer2b-AAV into the NAC of Homer2 knockout animals reverses this phenotype (Szumlinski et al., 2004). Furthermore, over expression of Δ FosB in the orbitofrontal cortex (OFC) sensitizes rats to the locomotor stimulant actions of cocaine (Winstanley et al., 2009). Animals over-expressing Δ FosB through the use of Δ FosB-AAV in the OFC appear pre-sensitized showing enhanced locomotor responses to acute cocaine which are indistinguishable from rats receiving chronic cocaine treatment (Winstanley et al., 2009).

Serum Glucocorticoid Kinase I (Sgk1)

Sgk1 is a glucocorticoid responsive gene involved in synaptic plasticity and learning and memory that is known to regulate the function of ion channels, play an important role in intracellular cross-talk, and allow the convergence of cell surface receptors, nuclear receptors, and cellular stress pathways (Firestone et al., 2003, Lee et

al., 2006, Ma et al., 2006, Tsai et al., 2002). Sgk1 was identified in 1993 in a screen of glucocorticoid responsive genes in mamillary tumor cell lines (Webster et al., 1993). Since that time it has been identified as being transcriptionally controlled by a wide variety of additional hormones and regulators including the increase of cytosolic Ca^{2+} activity and NO, transforming growth factor β , interleukin 6, thrombin, endothelin, cell shrinkage, and Rett syndrome, to name a few (Lang et al., 2010, Meng et al., 2005, BelAiba et al., 2006, Wolf et al., 2006, Chen et al., 2009, Nuber et al., 2005). It is also known that Sgk1 availability and function are regulated transcriptionally, post-transcriptionally and via post-translation modifications. It is phosphorylated and activated as a downstream action of both the PI 3-kinase pathway and the MAPK/ERK signaling pathways (Lee et al., 2006, Firestone et al., 2003). Activation of SGK1 after exposure to serum triggers entry of SGK1 into the nucleus, whereas activation of SGK1 by glucocorticoids enhances cytosolic localization of the kinase (Firestone et al., 2003). Additionally, the phosphorylation and activation of Sgk1 by PDK1 may stimulate its entry into the nucleus (Park et al., 1999).

It is now known that a single Sgk1 mRNA produces 4 different protein isoforms with different N-termini due to alternative sites of initiation of translation (Arteaga et al., 2007). The long isoforms, 49-kDa, 47-kDa, are more abundant, localize to the ER membrane, and are rapidly degraded (Arteaga et al., 2007). The short isoforms, 45-kDa and 42-kDa, are expressed at low basal levels, have decreased protein turnover and localize to the cytoplasm and nucleus (Arteaga et al., 2007). The isoforms have distinct functions as, for example, the 49-kDa isoform stimulates the epithelial sodium channel (ENaC) and the 42-kDa isoform phosphorylates glycogen synthase kinase-3 β (GSK3 β)

(Arteaga et al., 2007). Sgk1 is expressed in the human pancreas, liver, heart, lung, skeletal muscle, placenta, kidney and brain (Waldegger et al., 1997). Two additional mammalian isoforms of Sgk have been identified, termed Sgk2 and Sgk3, and their catalytic domains share 80% amino acid sequence identity with one another and with Sgk1 (Kobayashi et al., 1999a).

In addition to having additional isoforms and alternative sites of initiation of translation, alternative splicing produces two distinct isoforms of Sgk1 in the brain, Sgk1 and Sgk1.1, that are driven by distinct promoters and differ in N-terminal exons (Arteaga et al., 2008). Sgk1.1 is a brain-specific Sgk1 isoform that modulates the Acid-sensing ion channel 1 (ASIC1) and the δ -ENaC and is more stable than Sgk1 due to the absence of a proteasomal degradation signal in its N-terminus (Arteaga et al., 2008, Raikwar et al., 2008, Wesch et al., 2010). Additionally, Sgk1.1 was recently found to regulate M-current, which plays a central role in neuronal excitability (Miranda et al., 2013). Sgk1's function has been most thoroughly characterized in the aldosterone-sensitive distal nephron (ASDN) where it is induced by aldosterone and modulates transcellular sodium reabsorption by activating the sodium/potassium ATPase and enhancing transport of ENaC channels to the cell surface (Vallon et al., 2005).

In addition to regulating ion transport in the ASDN, Sgk1 plays an important role in modulating synaptic plasticity in the brain and spinal cord. It is well documented that Sgk1 is involved in memory consolidation of spatial learning (Lee et al., 2006, Tsai et al., 2002, Lee and Rivier, 1997) as well as neuronal plasticity and long-term potentiation in hippocampal neurons (Ma et al., 2006). Sgk1 was also identified by microarray analysis and confirmed to be involved in synaptic plasticity in the spinal cord during induction

and maintenance of inflammatory pain states (Geranton et al., 2007).

More recent studies have demonstrated a significant role for Sgk1 in the central nervous system, particularly the PFC. Sgk1 transcription is enhanced with neurological disorders (Wang et al., 2010) and neurodegenerative diseases (Schoenebeck et al., 2005). Wang et al. showed that SGK1 expression was enhanced in the temporal neocortex of patients with drug-refractory epilepsy and was also highly expressed in the rat brain during different phases of the epileptic process. Additionally, SGK1 expression was related with the elevation of the glutamate transporter EAAT3, and EAAT3 expression decreased following SGK1 knockdown (Wang et al., 2010). In neuronal disease, SGK1 regulates glutamate transporters (Bohmer et al., 2004, Schniepp et al., 2004) and up-regulates glutamate receptors (Liu et al., 2010). One group of researchers showed that corticosterone increased alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)-mediated synaptic transmission and AMPAR membrane trafficking in PFC pyramidal neurons. This mechanism was dependent on SGK phosphorylation of the guanosine nucleotide dissociation inhibitor (GDI) at Ser-213. The GDI then formed a complex with Rab4 facilitating a cycle of Rab4 mediated recycling of AMPARs to the synaptic membrane (Liu et al., 2010). Additional studies from this group showed that acute stress induced a potentiation of glutamatergic transmission in the PFC through an SGK1/3-induced increase in the delivery of NMDARs and AMPARs to the synaptic membrane. More specifically, acute stress activated glucocorticoid receptors, which regulated Sgk1/3 expression, SGK1/3 activation of Rab4 increased the trafficking and function of NMDARs and AMPARs, and this process leads to potentiated synaptic transmission (Yuen et al., 2011). Finally, Miyata et al. showed that a chronic stress

paradigm that elevated plasma corticosterone levels similar to those found in depressed individuals lead to activation of the phosphatidylinositol 3-kinase (PI3K)-3-phosphoinositide-dependent protein kinase (PDK1), Sgk1, and Ndr1 pathway with increases in both *Sgk1* mRNA and SGK1 phosphorylation (Miyata et al., 2011).

The Miles' laboratory previously showed that *Sgk1*, along with several other glucocorticoid responsive genes, was upregulated in PFC of D2 mice following acute ethanol exposure (Kerns et al., 2005a). Other investigators have also shown Sgk1 induction in the brain following acute ethanol (Treadwell and Singh, 2004, Piechota et al., 2010b), morphine (Piechota et al., 2010b), heroin (Piechota et al., 2010b), methamphetamine (Piechota et al., 2010b) or amphetamine (Gonzalez-Nicolini and McGinty, 2002). Similar to findings in Kerns et al., Piechota et al. identified a group of genes (including *Fkbp5* and *Sgk1*) that increased following ethanol and opioid administration that are controlled, in part, by the release of steroid hormones. After showing Sgk1 induction following acute ethanol, morphine, heroin, and methamphetamine, Piechota et al. showed that knockdown of Sgk1 resulted in alterations to dendritic spines in mice, possibly reflecting an altered potential for plastic changes (Piechota et al., 2010b).

Locomotor Activation

Drug-induced locomotor stimulation is a common response to many drugs of abuse, including ethanol, in rodents and is known to involve the mesocorticolimbic dopamine pathway (Boehm et al., 2002). It is known that 1 to 2 g/kg doses of ethanol stimulate locomotor activity in D2 mice and this stimulant response can be blocked by dopamine receptor antagonists (Boehm et al., 2002, King et al., 2002). It has been

suggested that ethanol's locomotor stimulating effects in rodents serve as a model of human ethanol-induced euphoria and arousal (Boehm et al., 2002, Palmer et al., 2002, Phillips et al., 2002) or the increased locomotion may represent heightened anxiety over the perception of ethanol's physiological effects (Boehm et al., 2002). The idea that ethanol's locomotor activating effects serve as a model of human ethanol-induced euphoria is interesting as Stewart, De Wit, and Eikelboom proposed that euphoric effects of drugs actually show sensitization rather than tolerance with repeated administration, whereas dysphoric effects tolerate (Stewart et al., 1984). Additionally, King et al. examined the acute subjective and objective effects of ethanol in heavy drinkers versus light drinkers and found that elevated initial sensitivity to the positive stimulant effects of alcohol is associated with greater alcohol consumption in humans and may be a predictor of the likelihood of an individual to develop alcoholism (King et al., 2002).

Mouse lines that differ in sensitivity to ethanol's locomotor activating effects have been produced via selective breeding and can be used to evaluate traits associated with the risk for high levels of alcohol consumption (Beckstead and Phillips, 2009). Although they exhibit little difference in basal locomotor activity, following ethanol treatment FAST-1 and 2 mice are more sensitive to ethanol's locomotor stimulating properties and SLOW-1 and 2 mice are insensitive to ethanol's locomotor activating properties—although it could also be said that the SLOW lines reflect greater sensitivity to ethanol-induced sedation (Boehm et al., 2002). FAST mice, those more sensitive to the stimulant effects of ethanol, consume more ethanol than SLOW mice (Beckstead and Phillips, 2009). FAST AND SLOW mice show differences in HPA axis sensitivity (Boehm et al., 2002). In a dose-response and time-course analysis of corticosterone

(Rosser et al.) levels in SLOW and FAST mice, saline treated SLOW mice returned to near basal CORT levels more quickly than saline treated FAST mice and SLOW mice showed significantly higher serum CORT levels at 45 and 90 minutes following 2 g/kg ethanol administration than 2 g/kg ethanol treated FAST mice (Boehm et al., 2002). FAST and SLOW mice show differences in dopamine levels in the mesolimbic dopamine pathways as FAST mice exhibit greater dopamine levels in the NAC than SLOW mice following both ethanol and cocaine treatment (Meyer et al., 2009). The two mouse lines also show differences in dopaminergic neuron spontaneous firing in the ventral midbrain—the spontaneous firing rate being higher basally in FAST mice and significantly increased in FAST mice following acute ethanol administration (Beckstead and Phillips, 2009). It is suspected that the difference in dopaminergic neuron firing is due to differences in nonselective cation conductance as blocking nonselective cation conductance can eliminate the difference in firing rate between FAST and SLOW mice (Beckstead and Phillips, 2009). There were also differences in GABAergic input to dopamine neurons—the amplitude of GABA_A inhibitory postsynaptic currents being significantly larger in midbrain dopamine neurons obtained from SLOW mice, but there was no difference in GABA_A transmission between ethanol treated dopamine neurons obtained from FAST and SLOW mice (Beckstead and Phillips, 2009). Thus, studies of ethanol induced acute locomotor activation can have predictive validity in terms of the liability for high levels of ethanol intake in animal models.

Sensitization

Behavioral sensitization, or the augmentation of the locomotor activating effects of a drug with repeated exposure, appears to be a common denominator across different

classes of addictive drugs, involves the mesocorticolimbic dopamine pathway and is a long-lasting change in the behavioral response to drugs (Lessov and Phillips, 2003, Phillips et al., 1997a). Behavioral sensitization may be relevant to drug addiction because progressive enhancements of the incentive qualities of the drug, and associated stimuli, may mediate compulsive drug-seeking and drug-taking behavior (Phillips and Fibiger, 1990, Robinson and Berridge, 1993, Taylor and Horger, 1999). Both rodents and humans have been shown to sensitize to the locomotor activating properties of ethanol (Masur and Boerngen, 1980, Newlin and Thomson, 1991). While studies with human volunteers in which an abused drug was administered more than once have been rare, Newlin and Thomson found that sons of alcoholics tended to show chronic sensitization in finger pulse amplitude across sessions with alcohol, particularly in the early stages of the response to the drug (Newlin and Thomson, 1991). In contrast, low-risk participants, who reported no parental history of alcoholism, tended to demonstrate chronic tolerance to alcohol (Newlin and Thomson, 1991). Newlin and Thomson's model in which high-risk individuals derive greater reward and euphoria from alcohol than do low-risk individuals in the rising alcohol curve is different from that described by Schuckit earlier in which high-risk participants have a blunted response to alcohol compared with low-risk individuals (Newlin and Thomson, 1991, Schuckit, 1994).

In animal models, sensitization can be separated into two phases, induction and expression (Harrison and Nobrega, 2009b). The induction of behavioral sensitization includes the sequence of cellular events that leads to enduring changes in neural function (Harrison and Nobrega, 2009b). Expression refers to the enduring neural alterations that occur following repeated drug administration (Harrison and Nobrega, 2009b).

Sensitization in animal models has been observed for psychostimulants, opioids, ethanol and stimulants such as nicotine suggesting that drugs of abuse may exert their effects through shared neural mechanisms (Lessov and Phillips, 2003). Furthering the suggestion of common mechanisms among abused drugs is cross-sensitization, wherein pretreatment with one drug results in the sensitized response to another. Cross-sensitization has been observed between ethanol and other drugs of abuse including morphine and cocaine (Lessov and Phillips, 2003).

Sensitization is known to involve drug induced changes in mesocorticolimbic dopaminergic projections and sensitization to the effects of psychostimulant drugs has been studied in the greatest detail (Roberts et al., 1995, Phillips et al., 1997a, Morice et al., 2010). Dopaminergic system involvement in the sensitized response is clear as studies indicate that mice lacking the dopamine transporter (*DAT* knockout mice) do not sensitize to the locomotor activating effects of cocaine or d-amphetamine (Mead et al., 2002, Spielwoy et al., 2001). Furthermore, mice heterozygous for the *vesicular monoamine transporter 2 (VMAT2)* that is responsible for transporting monoamines, particularly neurotransmitters such as dopamine from the cytosol into vesicles, show a profound sensitivity to amphetamine following an initial acute treatment with the drug and they do not sensitize to the locomotor activating effects of the drug (Wang et al., 1997, Uhl et al., 2000). D1 and D3 receptor knockout mice do not show a sensitized response to the locomotor activating effects of ethanol (Harrison and Nobrega, 2009a). *DAT* knockouts show increased acute ethanol evoked activity compared to WT and heterozygous littermates and do show a sensitized response following repeated ethanol administration (Morice et al., 2010). Mouse genetic background plays an important role

in sensitization studies and it should be mentioned that all knockouts and heterozygotes were on a C57 background except for *DAT* knockouts used in ethanol studies in which case both D2 and C57 *DAT* knockout mice were generated (Wang et al., 1997, Morice et al., 2010, Spielowoy et al., 2001, Takahashi et al., 1997, Uhl et al., 2000, Fukushima et al., 2007, Harrison and Nobrega, 2009a). D2 mice, not C57, are the strain of mouse best known to sensitize to the locomotor activating effects of ethanol and ethanol-induced sensitization was potentiated in *DAT* knockout mice on a D2 background (Phillips et al., 1997a). It is also important to note that results in knockout animals could be due to compensatory mechanisms or altered developmental events known to occur in these animals.

Studies investigating the mechanisms mediating ethanol sensitization not involving knockout animals have yielded conflicting and diverse results. For example, cross-sensitization was observed between the dopamine uptake inhibitor GBR 12909 and ethanol, but not the weaker dopamine uptake inhibitor bupropion (Broadbent et al., 2005). The D2/D3 receptor agonist quinpirole did not produce cross-sensitization with ethanol in D2 mice, but ethanol-sensitized genetically heterogeneous Swiss mice showed increased dopamine D2 receptor binding in the caudate-putamen compared to saline-treated and ethanol-treated non sensitized Swiss control mice (Broadbent et al., 2005, Souza-Formigoni et al., 1999). The GABA_A agonist, THIP, had no effect on the development of ethanol induced locomotor sensitization in D2 mice, whereas the GABA_B agonist, baclofen, blocked the development of ethanol induced locomotor sensitization (Broadbent et al., 2003). In male D2 and Swiss albino mice, the uncompetitive NMDA receptor antagonist MK-801 blocked the expression of ethanol-induced sensitization

(Broadbent et al., 2003, Kotlinska et al., 2006). However, in D2 mice Broadbent et al. showed that MK-801 reduced the stimulant effects of ethanol suggesting it is possible that MK-801's ability to block the expression of sensitization may represent a non-specific suppression of locomotor activity (Broadbent et al., 2003). In male D2 mice, the NR2B selective uncompetitive NMDA antagonist ifenodil did not effect the expression of sensitization and the non-NMDA glutamate receptor antagonists DNQX and GYKI 52466 decreased or blocked the expression of ethanol sensitization, respectively (Kotlinska et al., 2006). But, GYKI 52466 also reduced the locomotor activity of control saline treated animals at the same doses at which it blocked the expression of ethanol sensitization (Broadbent et al., 2003). Again, the differing genotypes of mice used in the above studies could account for some of the differences observed, but overall the mechanism behind ethanol sensitization appears complex and remains illusive.

It has been suggested that HPA axis activation is a common pathway by which abused drugs induce neuroadaptations leading to sensitization (Roberts et al., 1995, Pastor et al., 2008). The HPA axis may play a role in the sensitized response as glucocorticoid receptor antagonists or adrenalectomy block the induction, respectively, of ethanol or psychostimulant locomotor sensitization and stress can substitute for drug administration in cross sensitization studies (Roberts et al., 1995, Deroche et al., 1995). We were unable to replicate the findings of Roberts et al. in which glucocorticoid receptor antagonists block the induction of ethanol sensitization in our own recent studies, but we do recognize this could be due to methodological differences (Costin et al., 2012). Glucocorticoid receptor antagonists do not block the expression of behavioral sensitization to ethanol (Pastor et al., 2008).

It is hypothesized that extrahypothalamic central CRH/ CRH 1 may play a large role in the expression of ethanol sensitization. Extrahypothalamic CRH refers to CRH that acts outside of the HPA axis to control autonomic and behavioral responses (Koob, 2010). CRH-like immunoreactivity is present in the neocortex, extended amygdala, medial septum, hypothalamus, thalamus, cerebellum and ventral tegmental area (Koob, 2010). CRH1 and CRH1+2 receptor double knockout mice do not show psychomotor sensitization to ethanol, however CRH2 knockout mice do show a sensitized response to ethanol (Pastor et al., 2008). In one study by Pastor et al., CRH1 antagonists attenuated the induction and prevented the expression of ethanol induced sensitization (Pastor et al., 2008). However, in an earlier study by Fee et al. CRH1 antagonists did not attenuate the induction of ethanol sensitization, but did block the expression of ethanol mediated sensitization (Fee et al., 2007). It is suggested that the different findings in these two works can be attributed to different doses of the CRH1 antagonist CP-154,526 administered as Pastor et al. administered 30 mg/kg of the CRH1 receptor antagonist and Fee et al. only administered 10 mg/kg CRH1 receptor antagonist in studies examining the effects of CP-154, 526 on the initiation of ethanol sensitization (Fee et al., 2007, Pastor et al., 2008). Doses up to 10 mg/kg may not be adequate for full CRH1 receptor occupancy and the 30 mg/kg dose of CP-154,526 did not effect locomotor activation in Pastor et al. (Pastor et al., 2008).

Behavioral sensitization to drugs of abuse may involve learning processes as the initiation and expression of behavioral sensitization can be largely influenced by contextual cues surrounding drug administration. Some authors have even suggested that neuroadaptations underlying behavioral sensitization may be closely related to those

mediating learning and memory processes (Trujillo and Akil, 1995). In mice, ethanol-induced locomotor sensitization is potentiated by repeated pairing of ethanol injections and the testing chamber (Quadros et al., 2003). Other reports also document that the expression of sensitization can be context-specific (Pert et al., 1990, Badiani et al., 1995a, Badiani et al., 2000). In discussing the experimental design for such studies, there are typically three groups of animals, animals in the Paired group, animals in the Unpaired group and Control animals. The animals in the Paired group are transported from their home cage to a unique test environment, where they receive drug treatments. Animals in the Paired group usually also receive an injection of saline in their home cage. Animals the Unpaired group receive saline in the test environment and the drug later, in their home cage. Control animals receive saline in both the test environment and at home. On test day, all animals receive a challenge injection of the drug in the test environment. In many studies of this kind, sensitization is expressed in the Paired group, but not the Unpaired group. Thus, sensitization is said to be context-specific because it is expressed only in the group that previously experienced the drug in the test environment (Robinson et al., 1998).

Thesis Objectives

We hypothesize that glucocorticoids and glucocorticoid responsive genes are responsible for modulating acute and chronic cellular and behavioral responses to ethanol including locomotor activation and ethanol sensitization. In particular, because Sgk1 is regulated by ethanol, has a well-established role in learning and memory and is responsive to glucocorticoid signaling we hypothesize that Sgk1 is involved in modulating acute and

chronic cellular and behavioral responses to ethanol, specifically ethanol sensitization.

This hypothesis will be tested in the following specific aims:

- 1) **Determine the role of HPA axis signaling in regulating ethanol related behaviors.**
- 2) **Examine the role of the endocannabinoid system in regulating the HPA axis following ethanol administration.**
- 3) **Investigate the role of HPA axis signaling in ethanol regulation of Sgk1 in the PFC. Further characterize ethanol regulation of *Sgk1* mRNA and SGK1 protein in the PFC of DBA/2J mice.**
- 4) **Explore the role Sgk1 may play in ethanol induced locomotor activation and sensitization through the viral overexpression of Sgk1 in the PFC of DBA/2J mice.**

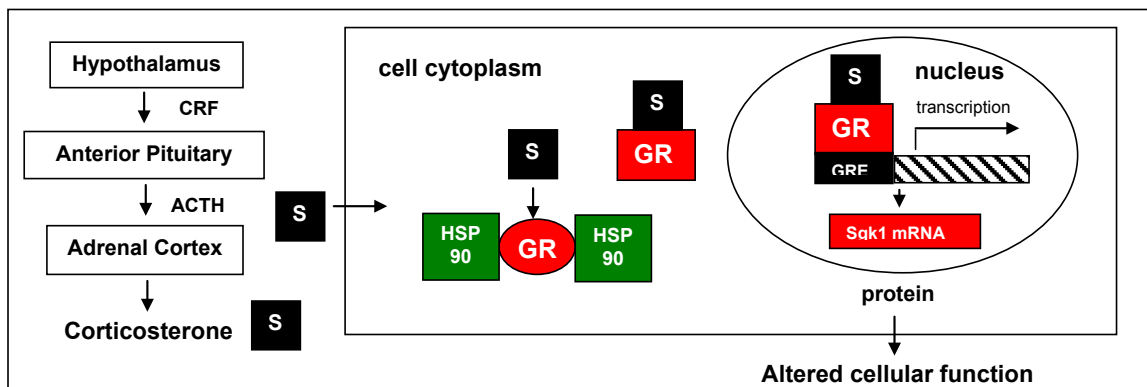


Figure 1: Above we present a simplified model of part of our hypothesis. Ethanol activates the HPA axis leading to the release of corticosterone (S) in mice. Corticosterone acts at the glucocorticoid receptor (GR) which resides in the cytoplasm of the cell bound to chaperone proteins (HSP90) in its inactive state. Steroid hormones bind to the glucocorticoid receptor causing a change in receptor conformation (as indicated by the change in the receptor shape above), induce GR nuclear translocation and activate transcription of target genes containing a Glucocorticoid Response Element (GRE). *Sgk1* is a glucocorticoid responsive gene with a GRE in its promoter. We hypothesize that *Sgk1* upregulation in the prefrontal cortex may be a downstream effect of HPA axis activation by ethanol and may ultimately modulate behavioral responses to ethanol.

CHAPTER 3

Role of Adrenal Glucocorticoid Signaling in Prefrontal Cortex Gene Expression and Acute Behavioral Responses to Ethanol

INTRODUCTION

Ethanol and other drugs of abuse acutely activate the hypothalamic pituitary adrenal (HPA) axis leading to glucocorticoid release (Piechota et al., 2010b), and alcoholics show a blunted HPA axis while drinking and upon withdrawal (Costa et al., 1996, Wand and Dobs, 1991a). Glucocorticoid hormones are the final step in activation of the HPA axis and are known to function in the biological response to stress and circadian activity (Marinelli et al., 1997, De Kloet et al., 1998). Not only do glucocorticoids regulate the stress response, but their feedback regulation helps to terminate HPA activation (De Kloet et al., 1998).

Drug-induced locomotor stimulation is a common acute response to drugs of abuse, including ethanol, in rodents (Boehm et al., 2002, Phillips et al., 1992). It is suggested that ethanol's locomotor stimulation in rodents serves as a model of human ethanol-induced euphoria and arousal (Boehm et al., 2002, Palmer et al., 2002, Phillips et al., 1992). Behavioral sensitization, a long-lasting augmentation of the locomotor activating effects of a drug with repeated exposure, also occurs across different classes of addictive drugs, and represents a form of neural plasticity affecting chronic behavioral responses to ethanol and other drugs (Phillips et al., 1997a). Sensitization has been observed for psychostimulants, opioids, and ethanol, suggesting that drugs of abuse may exert effects on neural plasticity through shared mechanisms (Lesso and Phillips, 2003).

It has been suggested that HPA axis activation is a common pathway by which abused drugs induce neuroadaptations leading to sensitization (Deroche et al., 1995, Roberts et al., 1995, Piechota et al., 2010b). Studies have shown that the HPA axis plays a role in the sensitized response as stress can substitute for drug administration in cross sensitization studies and adrenalectomy blocks the induction of psychostimulant locomotor sensitization (Roberts et al., 1995, Deroche et al., 1995). However, not all studies with adrenalectomized (ADX) animals have reached the same conclusion. Badiani et al. found that ADX does not alter amphetamine sensitization (Badiani et al., 1995b) and Prasad et al. showed that corticosterone does not mediate long term cocaine sensitization (Prasad et al., 1998).

Our laboratory and others have used genome-wide expression profiling to identify gene networks functioning in acute and chronic behavioral responses to ethanol (Kerns et al., 2005a, Treadwell and Singh, 2004, Wolstenholme et al., 2011). We previously identified a group of genes prominently regulated by acute ethanol in prefrontal cortex (PFC) of DBA2/J (D2) mice, but not C57BL/6 mice (Kerns et al., 2005b). Contained in this group were well-characterized glucocorticoid responsive genes such as *Fkbp5* and *Sgkl*. D2 mice show a robust locomotor activation response following acute ethanol administration, and reliably show locomotor sensitization upon repeated ethanol (Phillips et al., 1997a), whereas C57 mice do neither. Taken together, these results and prior work by Phillips and colleagues (Phillips et al., 1997b) suggests that glucocorticoid signaling might play a role in modulating acute ethanol locomotor activation and the initiation of locomotor sensitization. Here we have further studied the relationship between glucocorticoid signaling and acute ethanol locomotor activation. Our results show that

glucocorticoid signaling and glucocorticoid-responsive genes may represent an important endophenotype modulating the acute response to ethanol.

MATERIALS AND METHODS

Animals

Mice were maintained in a temperature controlled room ($23\pm 1^{\circ}\text{C}$) with 12 h light/dark cycles and free access to standard chow (Harlan Teklad #7912, Madison, WI) and water. Cages and bedding (Harlan Sani-chips, #7090A, Harlan, Teklad, Madison, WI) were changed weekly. All tests were carried out between 0900 and 1200 h. Procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the NIH Guide for the Care and Use of Laboratory Animals. All mice were DBA2/J mice from Jackson Laboratories (Bar Harbor, Maine) purchased at 10-13 weeks of age. ADX and SHAM mice were adrenalectomized or SHAM treated at Jackson and delivered following recovery from surgery. ADX mice were supplemented with 1% w/v saline solution in their drinking water. All mice were allowed to habituate to the animal facility for at least 1 week prior to testing.

Adrenalectomy

All adrenalectomies and SHAM procedures were conducted using aseptic and atraumatic surgical techniques at Jackson laboratories and were approved by the institution's Animal Care and Use Committee. Surgeries were performed using the lateral abdominal approach. Left and right adrenal glands were removed through separate incisions. The animals were anesthetized using tribromoethanol and carprofen was administered for

analgesia and the surgical site was prepared. The animals were placed in right lateral recumbency and a 5-8mm incision was made parallel and ventral to the spine and midway between the last rib and iliac crest. The underlying muscle was opened and the adrenal gland was located cranial to the left kidney. The adrenal gland was grasped with ring forceps and exteriorized. The adrenal and adjacent adipose tissues were excised. The incisions in the abdominal wall and skin were closed separately. Bupivacaine was applied topically to the incision site for local analgesia. Skin closure material was removed prior to shipment. The same procedure was followed for excision of the right adrenal gland except the skin incision was made immediately caudal to the last rib. This adjustment was necessary to accommodate for the more cranial location of the right adrenal gland relative to the left in the abdomen.

Drugs

All drugs were administered intraperitoneally (i.p.). Saline solutions were 0.9% w/v sterile saline. Ethanol solutions were prepared from 200-proof absolute anhydrous ethanol (Pharmco-Aaper brand, Brookfield, CT). Ethanol was administered at 20% v/v in 0.9% saline. RU-486 (Sigma, St. Louis, MO), was dissolved by sonication in 20% w/v beta-cyclodextrin (Sigma, St. Louis, MO).

Behavioral Testing

Mice were habituated to injections with saline in their home cage for 2 days prior to experiments and allowed a 1-hour acclimation period to the behavioral room prior to testing. Locomotor activity was measured immediately following injection with either saline or ethanol during a 10-minute session in locomotor activity chambers (Med-Associates, model ENV-515; St. Albans, VT). Loss-of-righting reflex (LORR) assays

were done following 3.8 g/kg ethanol administration. After ethanol injection, mice were returned to their home cage until they exhibited LORR as defined by the inability to right themselves three times in 30 seconds after placement in the supine position in a V-shaped trough. The duration of LORR was calculated by subtracting time of onset of LORR from the time at recovery from LORR. Anxiety testing was done using the light-dark transition model (Crawley and Goodwin, 1980). Five behavioral experiments were performed:

Exp. 1 -- Glucocorticoid receptor blockade by RU-486 and acute locomotor

responses to ethanol: Six groups of mice (n = 7-9) were treated as follows: vehicle + saline (VS), vehicle + 2 g/kg ethanol (VE), 20 mg/kg RU + saline (RU20 S), 20 mg/kg RU + 2 g/kg ethanol (RU20 E), 35 mg/kg RU + saline (RU35 S), and 35 mg/kg RU + 2 g/kg ethanol (RU35 E). On test day 3, RU-486 was administered 30 minutes prior to ethanol or saline. Control mice received vehicle followed by saline or ethanol.

Measurement of locomotor activity was performed as described above.

Exp. 2 -- Ethanol acute locomotor activation and sensitization following ADX: ADX and control animals (n=14-30) received one of three treatments: saline-saline (SS), saline-ethanol (SE) or ethanol-ethanol (EE) (**Table 1**). Animals were tested on day 3 for acute responses to either saline (SS, SE) or ethanol (EE). The dose of ethanol used for test days (days 3 and 14) was 2.0 g/kg. On days 4-13, animals received daily injections in their home cages of either saline (SS, SE) or 2.5 g/kg ethanol (EE). On test day 14 the SS group received saline, the SE group received ethanol and the EE group received ethanol as on day 3.

Exp. 3 -- Effects of RU-486 on initiation of ethanol sensitization: Four groups of D2 mice (n = 9) were treated according to the sensitization protocol and either vehicle or RU-486 pretreatment was administered prior to drug: vehicle + saline, RU + saline, vehicle + ethanol and RU + ethanol. On test day 3, vehicle or 25 mg/kg of RU-486 was administered 30 minutes prior to 2.0 g/kg ethanol or saline. Days 4-13 mice received vehicle + saline (VS) or 2.5 g/kg ethanol (VE), or 25 mg/kg RU-486 + saline (RS) or ethanol (RE). On day 14, only vehicle pretreatment was administered for all groups and locomotor activation was measured as described above in response to saline (VS, RS) or 2 g/kg ethanol (VE, RE).

Exp. 4 -- Anxiolytic-like response to ethanol following ADX: ADX and SHAM mice were tested in the light-dark transition model of anxiety. Testing took place in locomotor activity chambers described above with a black plastic partition inserted, dividing the chamber in half. SHAM and ADX mice (n=12-14) were restrained in a conical tube for 15 minutes, removed, injected with either saline or ethanol and placed into activity chambers facing the dark compartment.

Exp. 5 -- The Effect of ADX or RU-486 on Ethanol-Induced Loss of Righting Reflex (LORR): Mice (n = 6-7) were either adrenalectomized or injected with 35 mg/kg RU-486 prior to injection with ethanol and LORR measurement. Treatment groups include: ADX, 3.8 g/kg ethanol; SHAM, 3.8 g/kg ethanol; RU-486, 3.8 g/kg ethanol; VEH, 3.8 g/kg ethanol. RU-486 and VEH were administered 30 minutes prior to ethanol.

Radioimmunoassay (RIA)

Four hours following ethanol or saline administration in behavioral Exp. 1, trunk blood was collected from individual mice. Serum was isolated by centrifugation at 2500 x g for

15 minutes and stored at -80°C until RIA. A RIA containing I¹²⁵ labeled corticosterone (MP Biomedicals, Cleveland, Ohio) was performed according to the manufacturer's instructions.

Brain Micropunch Dissection

Four hours following ethanol or saline administration in Exp. 1 at the time of blood collection, brain tissue was also collected. In a separate experiment, ADX and SHAM D2 mice (n = 4-5) were habituated to handling for two days, on day 3 they were sacrificed and brains were extracted. Collection of brain tissue occurred exactly as described in Kerns et al. (Kerns et al., 2005a). The medial prefrontal cortex (PFC) dissection contained tissue from the dorsal and ventral anterior cingulate and some secondary motor cortex.

RNA Isolation and Microarray Hybridization

RNA was isolated from PFC tissue samples using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration and quality was assessed by Experion automated electrophoresis (BioRad, Hercules, CA). 100ng total RNA were transcribed into double-stranded cDNA using the GeneChip® 3' IVT Express Kit (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was synthesized from cDNA, purified and fragmented according to the manufacturer's instructions. Labeled cRNA from individual animals (n=3/group) was hybridized to a single microarray. Samples were analyzed with oligonucleotide arrays (Affymetrix Mouse Genome 430A 2.0 array) with array hybridization and scanning performed according to the manufacturer.

Microarray and Bioinformatics Analysis

Microarray data were processed using GeneChip Operating Software v4.1 (GCOS, Affymetrix) and normalized to a mean total hybridization intensity of 190. Array quality was assessed by accepting arrays with a scaling factor < 3 , 3'-5' actin ratio < 2 , and by examining chip validity and linearity. Chip-chip correlations all exceeded 0.98 and percent present calls by the Affymetrix GCOS software were greater than 58% for all arrays. Probesets with RMA expression values < 3.5 consistently across all microarrays were filtered to reduce variance from low expressing genes. Differential gene expression between ADX and SHAM treated mice was assessed using the R (R Development Core Team, 2011) implementation of the S-score algorithm (Kennedy et al., 2006). For a single gene, an $|S\text{-score}| > 2$ corresponds to a $p\text{-value} < 0.05$. We used the Benjamini-Hochberg false discovery rate (FDR) method to calculate q-values for all S-scores. The differential expression of genes with q-values < 0.05 were considered statistically significant. Additional bioinformatics analyses were performed using Toppgene (<http://toppgene.cchmc.org/>) with a Bonferroni correction and p-value cutoff of 0.05. The networks were generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com).

Quantitative Real Time Polymerase Chain Reaction (Q-rtRCR)

cDNA was generated from 1 mg total RNA by reverse transcription with the iScript CDNA kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Q-rtPCR was performed using the iCycler iQTM system (Bio-Rad) according to the manufacturer's instructions for SYBR Green I-based detection. Quantification of gene expression levels was determined based on the threshold cycle for each well using the provided software and all results were normalized to multiple reference genes using

Genorm as described in Vandesompele et al. (Vandesompele et al., 2002). Primers used were as follows, *Fkbp5* (Forward-*GCCGACTGTGTGTGTAATGC* and Reverse-*CACAATACGCACTTGGGAGA*), *Gpr6* (Forward-*CCTCATCTTACACTTCGTGTTCC* and Reverse-*GGTCCACTGTGATAGCAAGCA*), *Gapdh* (Forward-*TTCCAGTATGACTCCACTCACGG* and Reverse-*TGAAGACACCAGTAGACTCCACGAC*), *Ppp2r2a* (Forward-*ATCTCTCACCCCTTGCCCTTT* and Reverse-*CCCATTTTGTGTGCTTTCGT*), *Ublcp1* (Forward-*ATGACAGGGACAGGACAAGC* and Reverse-*TACAATGACACCCGACTGGA*), and *Ndufv1* (Forward- *GACCGTGCTAATGGACTTCG* and Reverse-*GGCATCTCCCTTCACAAATC*).

Blood Ethanol Concentrations

ADX and SHAM D2 animals were treated with 2 g/kg ethanol and harvested 10 min., 1 hour, 2 hours and 4 hours following ethanol administration (n = 9-10/group; 78 mice total). D2 animals were pretreated with vehicle (VEH) or 35 mg/kg RU-486 30 minutes prior to being treated with 2 g/kg ethanol and harvested 10 min., 1 hour, 2 hours and 4 hours following ethanol administration (n = 5-6/group; 48 mice total). Blood ethanol concentrations were measured by capillary column headspace gas chromatography.

Corticosterone Time Course

D2 mice were treated with saline or ethanol and harvested 5 minutes, 10 minutes, 1 hour and 2 hours following ethanol administration (n = 5-6/group; 52 mice total) and a RIA was performed to measure corticosterone levels.

Statistics

Data were expressed as mean \pm SEM and analyzed parametrically. Data were analyzed with analysis of variance (ANOVA) or Kruskal-Wallis One Way Analysis of Variance on Ranks (jump counts) using appropriate between-and within subject factors. All post hoc comparisons were made using Student Newman-Keul's test or Dunn's test (rank transformed, uneven sample number analysis). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Ethanol-induced acute locomotor response following RU-486.

The effect of two different doses of RU-486 on ethanol-induced locomotor activation was tested in Exp. 1. A one-way ANOVA showed a significant effect of treatment ($F_{5,42} = 20.88, p < 0.01$). Post-hoc analysis identified no difference between VS, RU20 S or RU35 S groups showing that RU-486 had no effect on basal locomotor activity (**Fig. 2A**). A significant increase in locomotor activity compared to saline controls was found with VE, RU20 E and RU35 E treatments, confirming locomotor activation following acute ethanol. VE and RU20 E groups showed significantly greater locomotor activity compared to RU35 E treated animals indicating that higher doses of RU-486 blunted the locomotor activating effect of ethanol while having no effect on basal activity. In examining corticosterone levels in mice following RU treatment, a one-way ANOVA showed a significant effect of treatment ($F_{5,26} = 13.42, p < 0.01$) and post-hoc analysis revealed significant increases in corticosterone levels in animals treated with R20 E, R35 S and R35 E compared to those treated with VS, VE and R20 S (**Fig. 2B**).

Acute locomotor activation and sensitization following adrenalectomy.

Acute and repeated ethanol exposure was studied in a sensitization protocol (**Table 1**) with SHAM and ADX treated D2 mice. A mixed model repeated measures ANOVA (phenotype x treatment x time) showed an overall effect of time ($F_{1,112} = 212.14, p < 0.01$) indicating that both SHAM and ADX treated mice sensitized to the locomotor activating effects of ethanol (**Fig. 3A.**). A two-way ANOVA (phenotype x treatment) evaluating animals on just day 3 of sensitization studies showed a significant effect of phenotype ($F_{1,115} = 6.20, p < 0.01$), treatment ($F_{1,115} = 167.61, p < 0.01$) and a significant interaction ($F_{1,115} = 4.87, p < 0.03$). Both ADX and control animals showed locomotor activation following ethanol treatment. Post-hoc analysis identified significant differences between ethanol treatment in ADX vs. SHAM, with ADX animals exhibiting a blunted locomotor activation response. This suggests that ADX, like treatment with RU-486, blunted the ethanol-induced acute locomotor activation response while having no effect on basal locomotor activity. A two-way ANOVA (phenotype x treatment) evaluating animals on day 14 of sensitization studies showed a significant effect of phenotype ($F_{1,112} = 5.40, p < 0.05$) and treatment ($F_{1,112} = 153.45, p < 0.01$), but no significant interaction. Post-hoc analysis showed that while both SHAM and ADX animals sensitized to the locomotor activating effects of ethanol, chronically ethanol treated ADX mice showed a blunted locomotor activation response on day 14 of sensitization, similar to day 3. These results suggest that ADX blunted the locomotor activating properties of ethanol in naïve and sensitized animals, but had no effect on sensitization *per se*.

SHAM and ADX mice treated with repeated ethanol also sensitized to jump counts (**Fig. 3B.**). Both groups of mice showed significant increases in jump counts on day 14 of sensitization studies compared to day 3. Acute ethanol (day 3 or day 14) completely abrogated jumping, whereas chronically ethanol treated mice showed jump counts similar to saline controls.

Initiation of ethanol sensitization following RU-486 treatment.

To determine whether direct glucocorticoid receptor blockade altered the initiation of ethanol sensitization, we pre-treated mice with RU-486 (25 mg/kg) or vehicle prior to saline or ethanol injections during the initiation phase of sensitization (**Table 1**). From our earlier acute studies (**Fig. 2**), we hypothesized 25 mg/kg RU-486 was a dose of drug that would antagonize the GR without producing the effects on acute locomotor activity seen at 35 mg/kg in Fig. 1. A mixed model repeated measures ANOVA showed a significant effect of treatment ($F_{3,32} = 182.02, p < 0.01$), day ($F_{1,32} = 47.98, p < 0.01$) and a significant interaction (treatment x day) ($F_{3,32} = 20.42, p < 0.01$) (**Fig. 4A.**). Post-hoc analysis revealed that both vehicle and RU pre-treated mice showed significant increases in locomotor activation following ethanol treatment on day 3 of sensitization studies. Both groups of mice also showed significant increases in locomotor activation on day 14 of sensitization studies compared to day 3, indicating both groups of mice sensitized to the locomotor activating effects of ethanol. There were no differences between RU and VEH pre-treated animals on day 14 of sensitization studies, indicating that RU-486 had no effect on the initiation of the sensitized response. Like SHAM and ADX treated animals, RU pre-treated animals also sensitized to jump counts (**Fig. 4B.**). Both groups

of mice showed significant increases in jump counts on day 14 compared to day 3.

Ethanol-induced anxiolysis and LORR responses to ADX or RU-486.

To determine if glucocorticoid receptor blockade or adrenalectomy affected acute responses to ethanol other than locomotor activity, we tested anxiolytic-like responses to ethanol (2g/kg) or incoordinating responses to ethanol (3.8 g/kg). A two-way ANOVA (phenotype x treatment) revealed an overall effect of treatment for % distance traveled in the light ($F_{1,49} = 17.80, p < 0.01$) and % time spent in the light ($F_{1,49} = 24.46, p < 0.01$), but no differences between phenotypes and no interaction (**Figs. 5A-B**). Post-hoc analysis showed that both ethanol-treated ADX and SHAM animals spent a significantly greater percentage of time and traveled a larger distance in the light compared to saline treated control animals. A student's t-test between ADX vs. SHAM mice and RU-486 vs. VEH pre-treated mice revealed no significant differences in the time taken to recover the righting reflex (**Fig. 5C, D**).

Microarray analysis of ADX-induced changes in PFC gene expression.

The data presented above suggests that glucocorticoid signaling modulates acute locomotor activation by ethanol. To initiate studies on the brain mechanisms that might underlie this action, we performed a study of gene expression in the PFC regulated by ADX. We reasoned that ADX-responsive genes might be involved in the mechanism of glucocorticoid modulation of acute ethanol locomotor activation. We chose the PFC for expression studies due to the known feedback interaction between the PFC and dopamine signaling (Durstewitz et al., 2000); because the PFC is well characterized as a

glucocorticoid-responsive brain region (Mizoguchi et al., 2003); and our prior microarray studies showed induction of a number of glucocorticoid-responsive genes by acute ethanol in the PFC (Kerns et al., 2005a).

Microarray analysis of the PFC identified 269 probesets (255 genes) with significant basal differences between SHAM and ADX mice. These included 75 probesets up-regulated and 194 down-regulated by adrenalectomy. Q-rtPCR was used to validate select genes from the microarray results. G protein-coupled receptor 6 (*Gpr6*) was significantly increased in ADX mice (**Fig. 6A**) while FK506 binding protein 5 (*Fkbp5*) expression was decreased by adrenalectomy (**Fig. 6B**) (student's t-test, $p < 0.05$). Bioinformatics functional over-representation analysis revealed significant differences between SHAM and ADX animals in gene groups involved in nerve impulse transmission and genes regulated by the synthetic glucocorticoid, dexamethasone (**Table 2**). Of particular interest, the entire ADX regulated set of genes had a highly significant overlap ($p=1.8 \times 10^{-8}$) with those identified in our prior microarray studies on acute ethanol with D2 mice (Kerns et al., 2005a). These overlapping genes included: heat shock 70kDa protein (*Hspa8*), potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (*Kcnma1*), phosphatase and tensin homolog (*Pten*), proteolipid protein 1 (*Plp1*), early growth response 2 (*Egr2*), early growth response 1 (*Egr1*), and *Fkbp5* (**Table 2, ***). Of note, all these genes showed significant decreased expression in the PFC of ADX mice, except for *Plp1*, which had increased expression. Acute ethanol increased the expression of all these genes in our prior studies. Thus, except for *Plp1*, there was the expected inverse relationship between our prior microarray results on ethanol responses and results from ADX mice. These results not only

demonstrate that ADX alters PFC gene expression, but that there is a potentially causal relationship between ethanol-evoked glucocorticoid release and at least a subset of acute ethanol-responsive genes in the PFC.

To provide further initial evidence for the role of glucocorticoids in acute ethanol-responsive PFC gene expression, we determined whether RU-486 would block acute ethanol induction of *Fkbp5*, one of the genes mentioned above as overlapping between our microarray studies on acute ethanol and ADX, and which functions as a critical regulator of glucocorticoid receptor function (Sinars et al., 2003). Q-rtPCR analysis of *Fkbp5* mRNA levels in PFC four hours following ethanol or saline administration (+/- RU-486) was done with animals from behavioral Exp. 1. One-way ANOVA showed an overall significant effect of treatment ($F_{5,22} = 8.99, p < 0.01$) (**Fig. 6C.**). Post-hoc analysis showed that RU pre-treatment had no effect on basal *Fkbp5* levels. Ethanol significantly increased *Fkbp5* levels compared to VS and RU-486 at the higher dose (RU35) blocked this response. These results indicated that higher doses of RU-486 blunted both the acute locomotor activating effects of ethanol and ethanol-induced *Fkbp5* expression.

We used Ingenuity pathway analysis to identify networks overrepresented among genes identified through microarray and S-score analysis to be significantly different in SHAM vs. ADX treated D2 mice. Overrepresented networks include a network of genes involved in protein synthesis, behavior, and nervous system development and function (**Fig. 7A.**) and a network of genes involved in hereditary disorder, neurological disease and skeletal and muscular disorders (**Figs. 7B.**) These results indicate that genes regulated following ADX have functional implications for nervous system function and

behavioral responses.

Blood Ethanol Concentration (BEC)

ADX animals showed a lower peak BEC and unaltered rate of clearance following 2 g/kg ethanol. A two-way ANOVA (phenotype x time) showed an overall effect of time ($F_{3,70}=858.41$, $p < 0.001$), an overall effect of phenotype ($F_{1,70}=10.92$, $p < 0.001$) and a significant interaction ($F_{3,70}=3.02$, $p < 0.05$). Post-hoc analysis revealed significant differences between BEC 10 minutes, 1 hour, 2 hours and 4 hours following 2 g/kg ethanol administration and significant differences between BEC in ADX and SHAM animals 10 minutes, 1 hour and 2 hours following 2 g/kg ethanol administration (**Fig. 8A.**).

In animals pre-treated with 35 mg/kg RU-486 versus those pre-treated with VEH 10 min., 1 hour, 2 hours and 4 hours following 2 g/kg ethanol administration, a two-way ANOVA (treatment x time) showed an overall effect of time ($F_{3,31}=86.69$, $p < 0.001$), but no effect of treatment. Post-hoc analysis revealed significant differences between BEC in VEH and RU-486 pre-treated animals 10 minutes, 1 hour, 2 hours and 4 hours following 2 g/kg ethanol administration, but no significant differences between VEH vs. RU-486 pretreated animals at any of these time points (**Fig. 8B.**). This indicates that there were no differences in BEC between D2 animals pretreated with VEH versus those pretreated with RU-486 10 min., 1 hour, 2 hours and 4 hours following 2 g/kg ethanol administration.

Corticosterone Timecourse

A corticosterone (cort.) time course was performed to measure HPA axis activation following 2 g/kg ethanol administration. A two-way ANOVA (treatment x time) showed an overall effect of treatment ($F_{1,41} = 28.79, p < 0.001$) and time ($F_{4,41} = 4.50, p < 0.01$). Post hoc analysis revealed cort. levels were significantly elevated 5 minutes, 10 minutes, 1 hour and 2 hours following ethanol administration (**Fig. 9**). There were no differences in cort. levels between saline treated animals. Levels peaked 1 hour following ethanol administration. Despite a strong trend towards elevated levels 4 hours following ethanol administration, cort. levels between saline vs. ethanol treated animals did not differ from one another at this time indicating that corticosterone levels returned to normal 4 hours following 2 g/kg ethanol administration.

DISCUSSION

Defining the mechanisms underlying acute behavioral responses to ethanol has significant biomedical implications since acute responses to ethanol serve as a predictor of risk for alcoholism or excessive ethanol intake (Schuckit, 1994, Metten et al., 1998). Our prior genomic studies (Kerns et al., 2005a) suggest that glucocorticoid signaling in the PFC might play an important role in brain gene expression responses to acute ethanol. Here we characterized the role of glucocorticoid signaling in responses to acute ethanol, ethanol sensitization, and in regulating PFC gene expression. We found that ADX and RU-486 both impair acute ethanol induced locomotor activation, but do not alter the induction of ethanol sensitization or basal locomotor activity. Further, ADX altered expression of genes in PFC that include a significant number of previously identified ethanol responsive genes. Overall, this work points to a previously uncharacterized

mechanism modifying acute locomotor responses to ethanol and identifies an important role for glucocorticoid signaling in ethanol-responsive gene expression.

One prior study showed that ADX reduced ethanol stimulated locomotor activity in female C3H mice (Wallis et al., 1984). However, neither the effects of GR antagonists nor adrenalectomy on the acute locomotor activating effects of ethanol have been investigated in D2 mice, the strain most widely studied for motor responses to ethanol. We found that both higher doses of RU-486 and ADX blunted the acute locomotor activating effects of ethanol. In addition, acute ethanol activated the HPA axis leading to significant increases in CORT as soon as 5 minutes following ethanol administration (Fig. 9). Thus, ethanol-induced glucocorticoid release could partially modulate ethanol-induced locomotor activation. The work presented here does not differentiate whether glucocorticoids mediate a portion of ethanol-induced locomotor activation, or simply provide a permissive effect in such a response to ethanol. However, since both RU and ADX treatments only affected a portion of the ethanol locomotor activation response, this clearly suggests a permissive effect. Future work will be needed to directly test this hypothesis.

Our results are consistent with prior studies on other drugs of abuse. ADX reduces the acute psychomotor stimulant effects of cocaine (Marinelli et al., 1997, Deroche et al., 1997) and amphetamine (Cador et al., 1993) in the rat and the locomotor response to psychomotor stimulants in ADX animals can be restored by corticosterone administration (Cador et al., 1993, Marinelli et al., 1997). Discrepancies in the psychostimulant literature do exist, as a more recent study found that GR inactivation in the nervous system did not affect acute cocaine locomotor activation in mice, suggesting that the

acute locomotor effects of cocaine do not require GR signaling (Deroche-Gamonet et al., 2003). But it has been suggested that these divergent findings are due to methodological differences (Marinelli and Piazza, 2002).

Both RU-486 and ADX would affect more than just the glucocorticoid system as RU-486 is an antagonist of both the progesterone receptor (PR) and GR (Chrousos et al., 1988). Further, ADX alters the synthesis of numerous steroid hormones, in particular corticosterone and aldosterone, which both interact with the mineralocorticoid receptor (Piechota et al.). ADX also removes adrenal medullary catecholamines such as epinephrine. However, the adrenal medulla does not provide the body's only source of catecholamines and RU-486 presumably has no effect on catecholamine action and only minimal binding affinity for the MR (Ricordi et al., 1988). Thus, the combined use of both ADX and RU-486 in our studies increases the likelihood that the alterations in ethanol-induced locomotor activation with these treatments were due to GR actions. Additional evidence for a role of the GR comes from our studies of RU-486 and ethanol effects on circulating CORT levels. Acting through hypothalamic and pituitary GR, RU-486 disrupts negative feedback loops responsible for returning corticosterone levels to normal following HPA axis activation (De Kloet et al., 1998). We only found significant basal RU-486 induced increases in circulating CORT at the higher dose of drug (35 mg/kg), which was also the only dose inhibiting ethanol-induced locomotor activation.

Studies on other drugs of abuse also support GR rather than MR as a mediator of the locomotor response. Blockade of MRs by spironolactone had no significant effects on locomotion induced by systemic morphine (Marinelli et al., 1998). Still, the possibility that other steroid hormones play a role in ethanol-induced locomotor activation cannot be

ignored.

ADX or RU-486 effects on ethanol pharmacokinetics could be an alternative explanation for our results with ethanol-induced locomotor activation. Gililand and Finn showed that ADX in D2 mice produced slightly increased ethanol clearance rates following 4 g/kg ethanol administration, but had no effect on peak BEC (Gililand and Finn, 2007). We found slight decreases in peak BEC following 2 g/kg ethanol in ADX D2 mice (Fig. 8A). However, decreased ethanol locomotor activation was seen with both ADX and RU-486 treatment and RU-486 pre-treatment did not alter ethanol metabolism (Fig. 8B). We also found that ADX and RU pre-treatment had no effect on ethanol-induced LORR or anxiolysis (Fig. 5), indicating that ADX and RU selectively altered acute ethanol-induced locomotor activation. This combination of data strongly suggests that pharmacokinetic factors do not totally explain ethanol locomotor activation behavioral differences in ADX animals.

Our finding that RU-486 and ADX did not block initiation of behavioral sensitization (Figs. 3-4), were contrary to previously published findings showing that RU-486 blunted initiation of ethanol behavioral sensitization (Roberts et al., 1995). Some of the discrepancies could be due to differences in experimental design. Roberts et al. used female D2 mice, a shorter protocol with lower doses of ethanol and lower doses of RU-486 (Roberts et al., 1995). Interestingly, we found that not only did all groups of mice sensitize to the locomotor activating effects of ethanol, but they also sensitized to jump counts. To our knowledge, this is the first report of ethanol sensitization to jumping behavior.

We hypothesized that basal differences in gene expression may be partially

responsible for the observed behavioral differences between SHAM and ADX animals following acute ethanol administration. IPA analysis showed that genes identified through microarray and S-score analysis to be significantly different in SHAM vs. ADX treated D2 mice were overrepresented in networks involved in behavior and neurological disease. This result supports our hypothesis that ethanol's activation of the HPA axis may be responsible for regulating gene expression following ethanol administration and that this regulation may result in behavioral alterations and neurological alterations that may eventually lead to pathologies such as alcoholism. Our microarray studies showed prominent changes in basal gene expression of ADX animals in PFC, a brain region known to be sensitive to glucocorticoids (Mizoguchi et al., 2003), and a significant proportion of these were identified as ethanol-responsive genes in PFC in our prior studies. For example, *Fkbp5* was decreased basally in ADX mice whereas previously it was found to be induced by ethanol in D2 PFC (Kerns et al., 2005a). Of note, *Fkbp5* regulates glucocorticoid receptor sensitivity and polymorphisms in *Fkbp5* lead to a dysregulated stress response and have been found in individuals with depression, bipolar disorder and post-traumatic stress disorder (Binder, 2009).

In addition, pre-treatment with doses of RU-486 that blunted ethanol locomotor activation also diminished ethanol induced *Fkbp5* expression (Fig. 6C). This overlap between ADX, RU-486 and ethanol-sensitive gene expression is consistent with our prior hypothesis that a subset of acute ethanol-responsive genes in PFC were possibly due to involvement of GR signaling. Our results also suggest that such GR and ethanol-responsive genes in PFC may play a role in modulating aspects of basal acute sensitivity to ethanol.

Table 1: Outline of experimental design for ethanol sensitization

Phase of sensitization		Initiation		Expression
Group	Days 1-2	Day 3	Days 4-13	Day 14
	Habituation	Activity test	Conditioning	Activity Test
SS	Saline	Saline	Saline	Saline
SE	Saline	Saline	Saline	Ethanol 2.0 g/kg
EE	Saline	Ethanol 2.0 g/kg	Ethanol 2.5 g/kg	Ethanol 2.0 g/kg

Table 1. Sensitization protocol.

Table 2: Gene ontology over-representation analysis for ADX microarray results

Ontological Category	Gene Symbol	Gene Name	Entrez Gene ID	q-value; s-score
Nerve Impulse Transmission	HRH1	histamine receptor H1	3269	0.0169; 4.01
	HSPA8*	heat shock 70kDa protein 8	3312	0.0030; -4.52
	SLC6A13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	6540	< 0.001; 5.30
	NTRK2	neurotrophic tyrosine kinase, receptor, type 2	4915	0.0500; -3.62
	KCNMA1*	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	3778	0.0257; -3.87
	KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)	3791	0.0022; 4.62
	CLDN5	claudin 5	7122	< 0.001; 7.24
	SOD1	superoxide dismutase 1, soluble	6647	0.0126; -4.11
	CACNG3	calcium channel, voltage-dependent, gamma subunit 3	10368	0.0257; 3.88
	RAB11A	RAB11A, member RAS oncogene family	8766	0.0013; -4.75
	RAB14	RAB14, member RAS oncogene family	51552	0.0080; -4.26
	CNP1	2',3'-cyclic nucleotide 3' phosphodiesterase	1267	0.0482; 3.63
	CPLX2	complexin 2	10814	0.0318; -3.79
	SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	6507	0.0259; 3.87
	SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2	6506	0.0009; -4.87
	HOMER1	homer homolog 1 (Drosophila)	9456	0.0004;

				-5.03
	ATXN1	ataxin 1	6310	0.0109; -4.15
	KIF1B	kinesin family member 1B	23095	< 0.001; -6.42
	PTEN*	phosphatase and tensin homolog	5728	0.0453; -3.66
	CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	773	0.0061; -4.34
	CREB1	cAMP responsive element binding protein 1	1385	0.0075; -4.28
	SYN2	synapsin II	6854	0.0274; -3.84
	PLP1*	proteolipid protein 1	5354	0.0244; 3.89
	EGR2	early growth response 2	1959	< 0.001; -5.81
	EGR1*	early growth response 1	1958	0.0014; -4.74
	HTR2C	5-hydroxytryptamine (serotonin) receptor 2C	3358	0.0441; -3.68
	ARC	activity-regulated cytoskeleton-associated protein	23237	0.0005; -5.01
	GRIA1	glutamate receptor, ionotropic, AMPA 1	2890	0.0036; -4.48
Dexamethasone	DCN	decorin	1634	0.0001; 5.30
	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	3290	< 0.001; 5.65
	HRH1	histamine receptor H1	3269	0.0169; 4.01
	HSPA8*	heat shock 70kDa protein 8	3312	0.0030; -4.52
	KCNMA1*	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	3778	0.0257; -3.83
	KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1	3745	< 0.001; -6.35
	SOD1	superoxide dismutase 1, soluble	6647	0.0126; -4.11
	FKBP5*	FK506 binding protein 5	2289	0.0380; -4.12
	PLIN2	perilipin 2	123	0.0480; 3.64
	APCDD1	adenomatosis polyposis coli down-regulated 1	147495	0.0316; 3.80

	EPHX2	epoxide hydrolase 2, cytoplasmic	2053	0.0274; 3.84
	EDNRB	endothelin receptor type B	1910	0.0200; 3.95
	S100A9	S100 calcium binding protein A9	6280	0.0005; -5.01
	CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	781	0.0009; -4.84
	SULT1A1	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	6817	< 0.001; -7.05
	CXCL12	chemokine (C-X-C motif) ligand 12	6387	0.0365; 3.74
	TTR	transthyretin	7276	< 0.001; 9.17
	FOS	FBJ murine osteosarcoma viral oncogene homolog	2353	< 0.001; -8.72
	EGR1	early growth response 1	1958	0.0014; -4.74
	NR2C2	nuclear receptor subfamily 2, group C, member 2	7182	< 0.001; -6.11
	TPM1	tropomyosin 1 (alpha)	7168	0.0244; -3.90

Table 2. Over-represented gene ontology categories from analysis of genes with significant q-values using ToppGene (<http://toppgene.cchmc.org/>) with a Bonferroni correction and p-value cutoff of 0.05. A positive s-score indicates a gene that is significantly increased; conversely a negative s-score indicates a gene that is significantly decreased. *Represents a gene previously identified in our laboratory as an ethanol responsive gene (Kerns et al., 2005a).

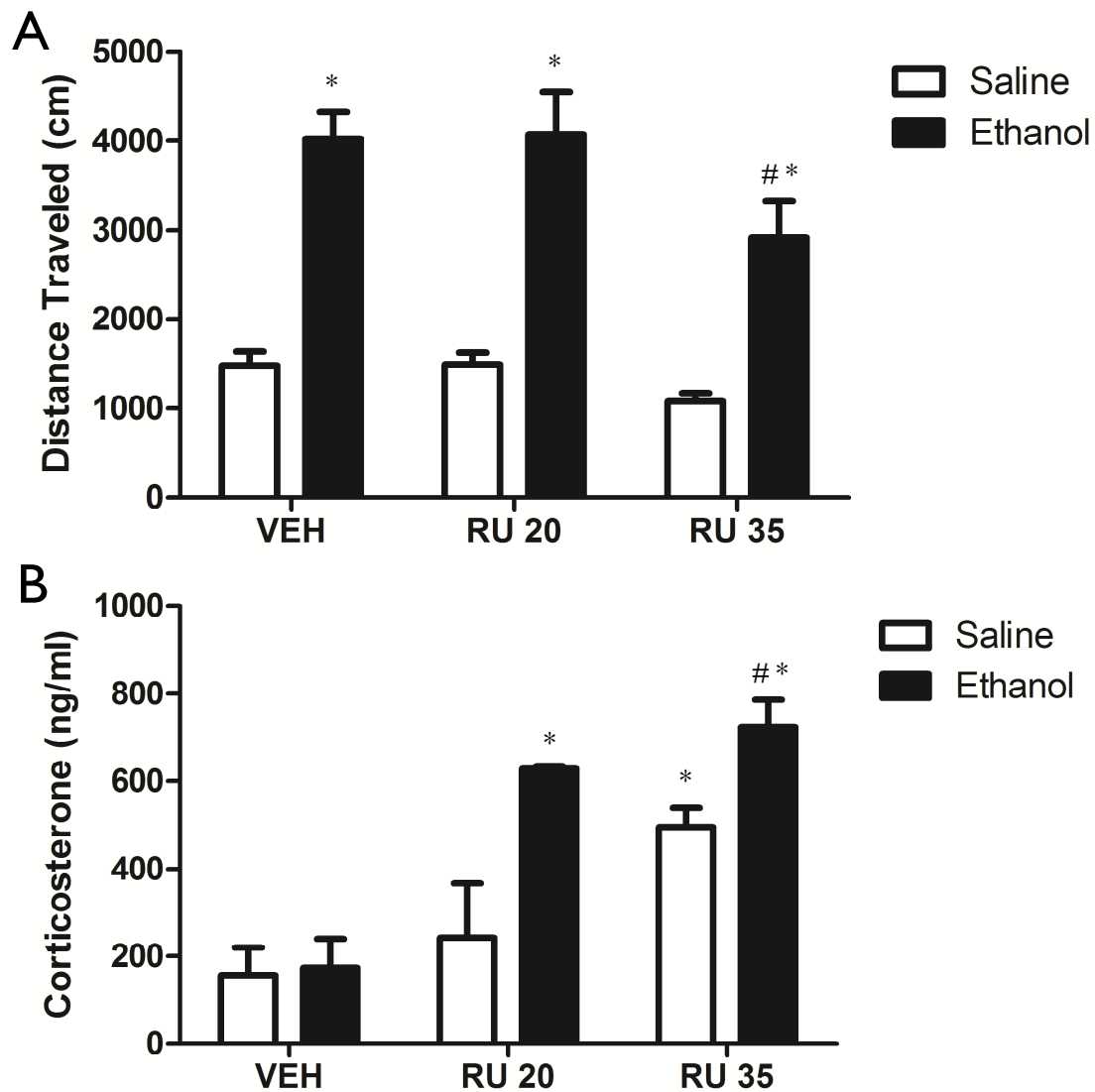


Figure 2. Antagonism of glucocorticoid receptor function by RU-486 alters acute locomotor responses to ethanol and produces compensatory changes in serum corticosterone. Panel A: RU-486 at 35 mg/kg (RU 35), but not 20 mg/kg (RU 20), blunted the acute locomotor activating effects of ethanol in D2 mice. * $p < 0.05$ vs. all saline-treated animals; # $p < 0.05$ vs. VEH ethanol and RU 20 ethanol. Panel B: Corticosterone levels were determined 4 hours after behavioral testing. Corticosterone levels were significantly elevated in animals treated with RU 35 followed by saline or animals treated with RU 20 or RU35 following by ethanol. * $p < 0.05$ vs. VEH treated groups and RU 20 S; # $p < 0.05$ vs. RU 35 S.

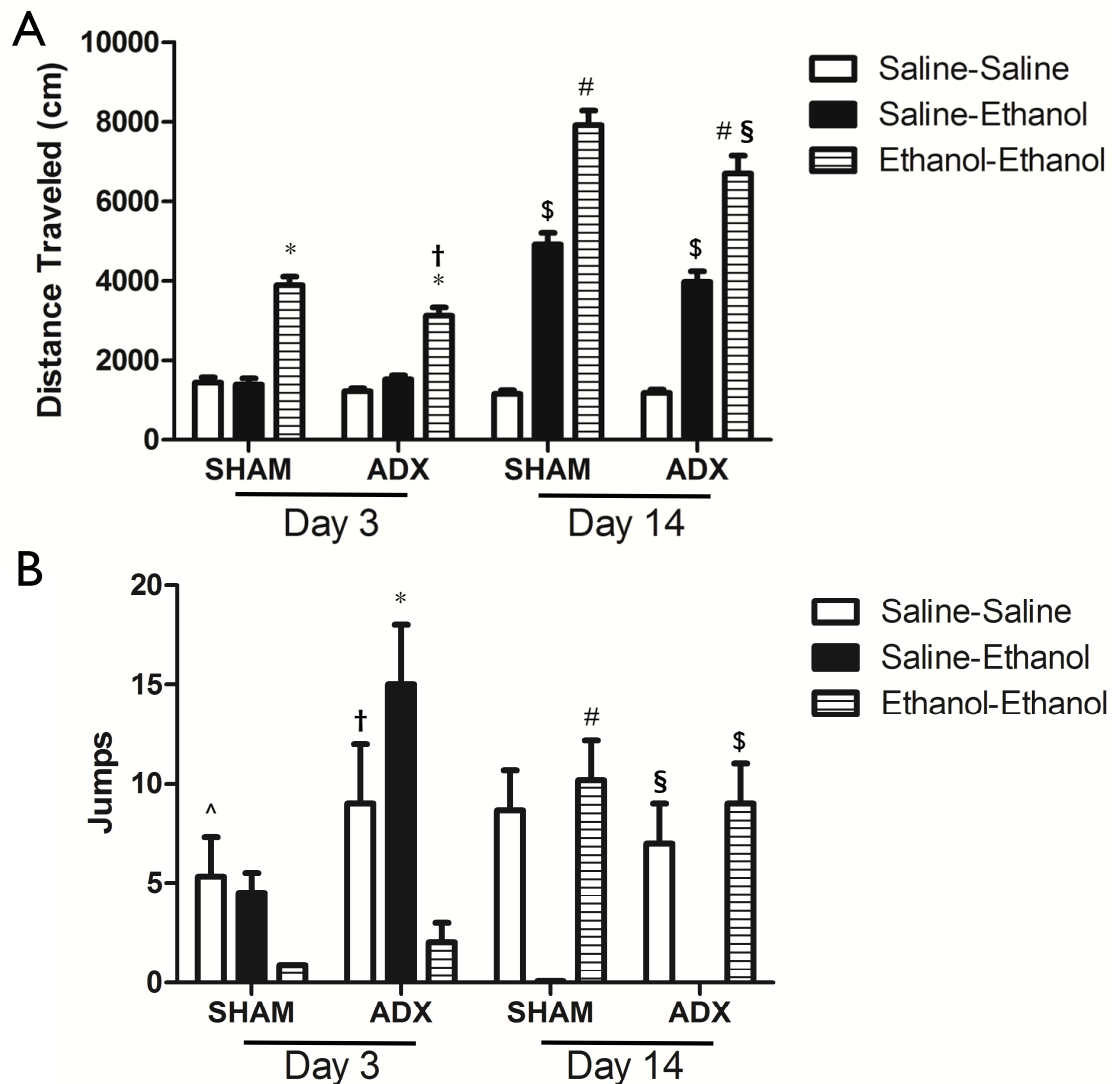


Figure 3. Adrenalectomy (ADX) alters acute ethanol locomotor activation but not ethanol sensitization. Panel A: ADX significantly blunted the acute locomotor activating response to ethanol, but not locomotor sensitization. On day 3, locomotor responses were reduced in the ADX ethanol-treated group compared to sham controls. * $p < 0.05$ vs. day 3 Saline-Saline treated mice; † $p < 0.05$ vs. Day 3 Ethanol-Ethanol treated SHAM mice; § $p < 0.05$ vs. day 14 Saline-Saline treated mice; # $p < 0.05$ vs. day 3 Ethanol-Ethanol treated mice, Saline-Saline treated mice and Saline-Ethanol treated mice; § $p < 0.05$ vs. Day 14 Ethanol-Ethanol treated SHAM mice. Panel B: SHAM and ADX mice also sensitized to Jump Counts. * $p < 0.05$ vs. SHAM Saline-Ethanol Day 14, ADX Saline-Ethanol Day 14, SHAM Ethanol-Ethanol Day 3, ADX Ethanol-Ethanol Day 3; § $p < 0.05$ vs. SHAM Saline-Ethanol Day 14, ADX Saline-Ethanol Day 14, SHAM Ethanol-Ethanol Day 3, ADX Ethanol-Ethanol Day 3; # $p < 0.05$ vs. SHAM Saline-Ethanol Day 14, ADX Saline-Ethanol Day 14, SHAM Ethanol-Ethanol Day 3, ADX Ethanol-Ethanol Day 3; § $p < 0.05$ vs. SHAM Saline-Ethanol Day 14, ADX Saline-Ethanol Day 14, SHAM Ethanol-

Ethanol Day 3; [†] $p < 0.05$ vs. SHAM Saline-Ethanol Day 14, ADX Saline-Ethanol Day 14, SHAM Ethanol-Ethanol Day 3; [^] $p < 0.05$ vs. SHAM Saline-Ethanol Day 14.

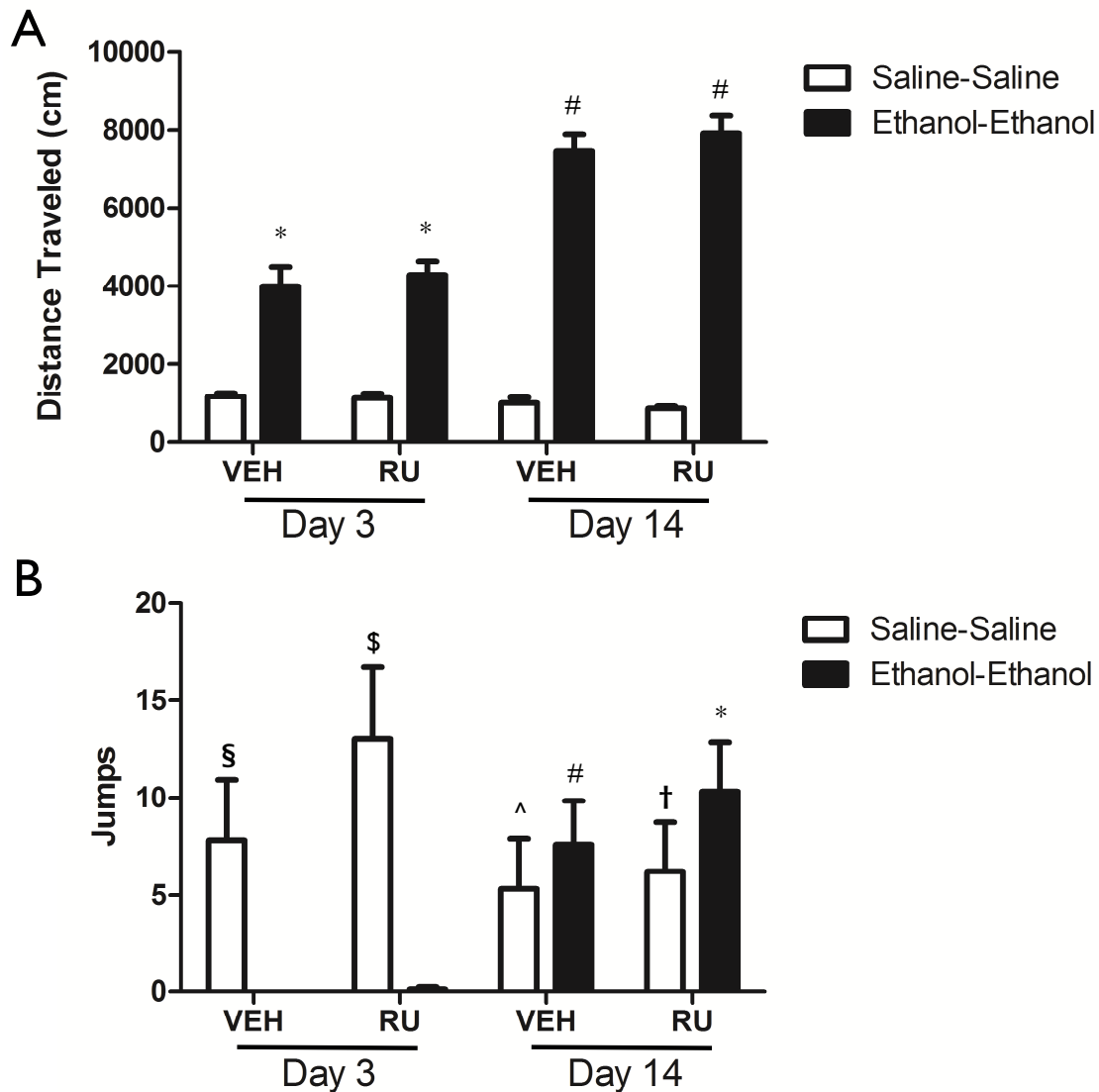


Figure 4. Pretreatment with 25 mg/kg RU-486 had no effect on the initiation of ethanol sensitization as measured via locomotor activity and jump counts. Panel A: Pretreatment with 25 mg/kg RU-486 had no effect on the initiation of ethanol sensitization as measured via locomotor activation. * $p < 0.05$ vs. Day 3 Saline-Saline treated mice; # $p < 0.05$ vs. Day 3 Ethanol-Ethanol treated mice. Panel B: VEH and RU-486 Ethanol-Ethanol pretreated mice also sensitized to Jump Counts. * $p < 0.05$ vs. VEH Ethanol-Ethanol Day 3, RU Ethanol-Ethanol Day 3; § $p < 0.05$ vs. VEH Ethanol-Ethanol Day 3 and RU Ethanol-Ethanol Day 3; # $p < 0.05$ vs. VEH Ethanol-Ethanol Day 3, RU Ethanol-Ethanol Day 3; § $p < 0.05$ vs. VEH Ethanol-Ethanol Day 3, RU Ethanol-Ethanol Day 3; † $p < 0.05$ vs. VEH Ethanol-Ethanol Day 3, RU Ethanol-Ethanol Day 3; ^ $p < 0.05$ vs. VEH Ethanol-Ethanol Day 3, RU Ethanol-Ethanol Day 3.

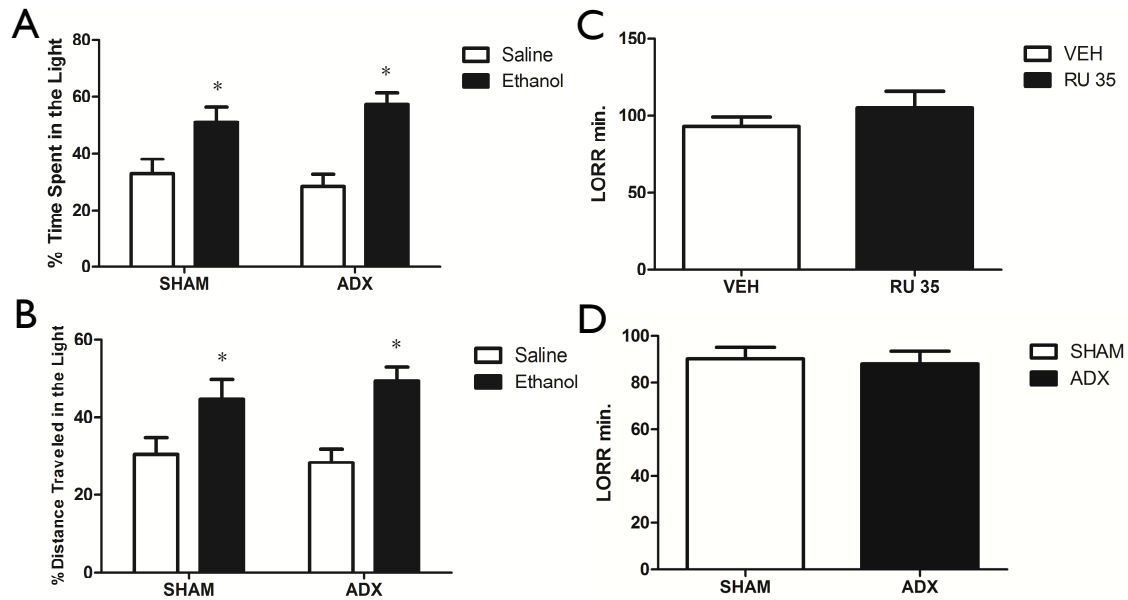


Figure 5. Acute ethanol-responsive behaviors that do not differ between VEH vs. RU or ADX vs. SHAM treated mice. Panel A: Both SHAM and ADX ethanol-treated mice spent significantly greater time in the light than their respective saline-treated counterparts. There were no differences in the % Time Spent in the Light between SHAM and ADX treated mice. Panel B: Both SHAM and ADX ethanol-treated mice traveled significantly greater distance in the light than their respective saline-treated counterparts. There were no differences in the % Distance Traveled in the Light between SHAM and ADX treated mice. Panel C: Pretreatment with 35 mg/kg RU-486 (RU 35) had no effect on ethanol-induced loss of righting reflex (LORR). Panel D: ADX had no effect on ethanol-induced loss of righting reflect (LORR). * $p < 0.05$ vs. respective saline-treated mice.

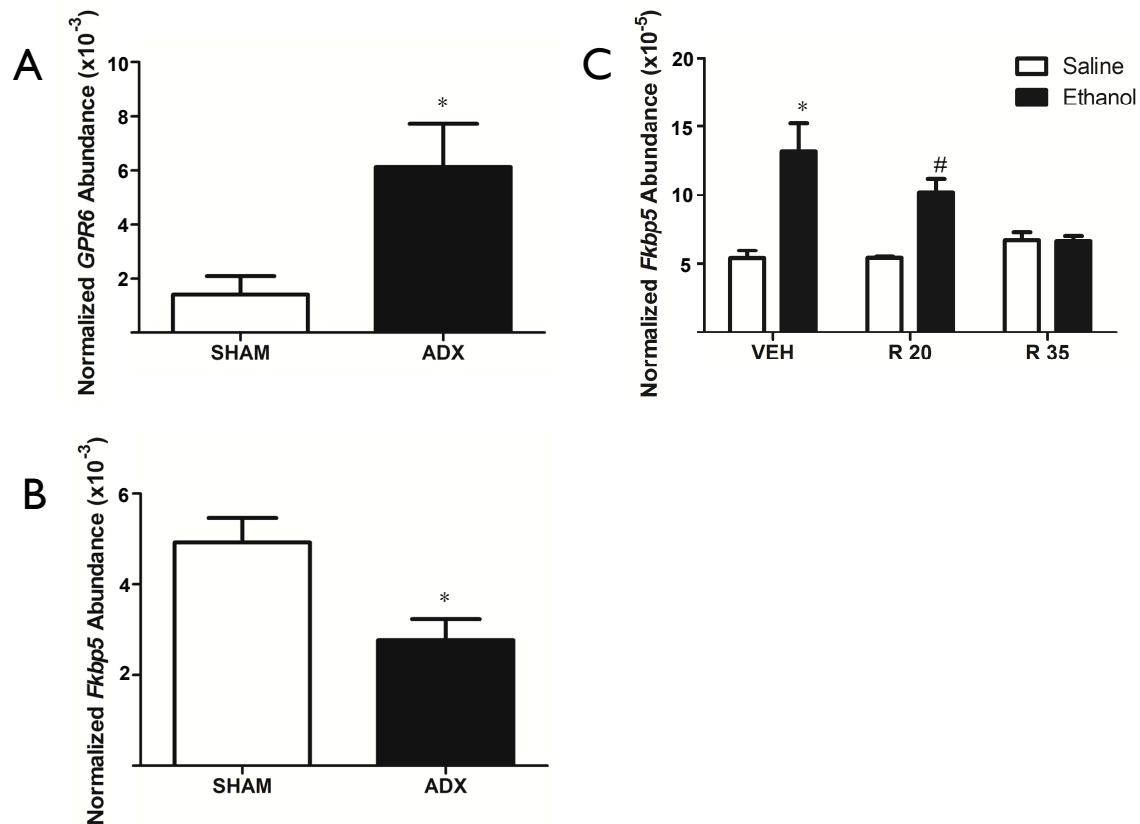
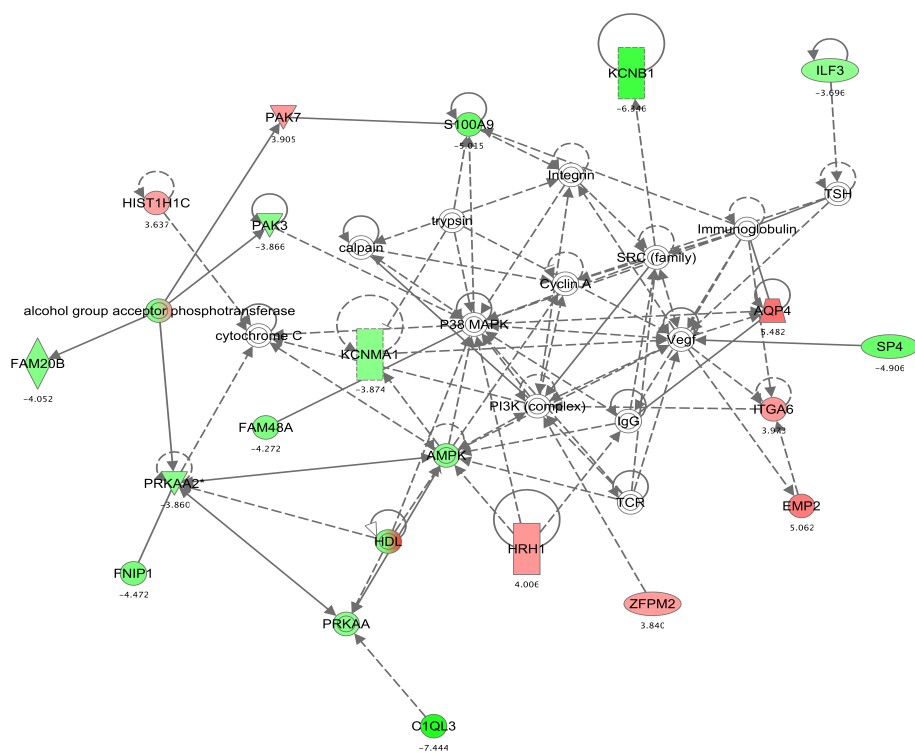


Figure 6. Genes identified through microarray and S-score analysis and confirmed to be significantly different in SHAM vs. ADX treated D2 mice and RU-486 pre-treated D2 mice. Panel A: *Gpr6* was identified and confirmed as significantly increased in ADX vs. SHAM treated mice. * $p < 0.05$ vs. SHAM treated mice. Panel B: *Fkbp5* was identified and confirmed as significantly decreased in ADX vs. SHAM treated mice. * $p < 0.05$ vs. SHAM treated mice. Panel C: Pre-treatment with higher doses of the GR antagonist RU-486 blunted ethanol-induced *Fkbp5* expression. RU pre-treatment had no effect on basal *Fkbp5* levels. * $p < 0.05$ vs. VS, RU20 S, RU35 S, RU35 E. # $p < 0.05$ vs. VS and RU20 S.

A



B

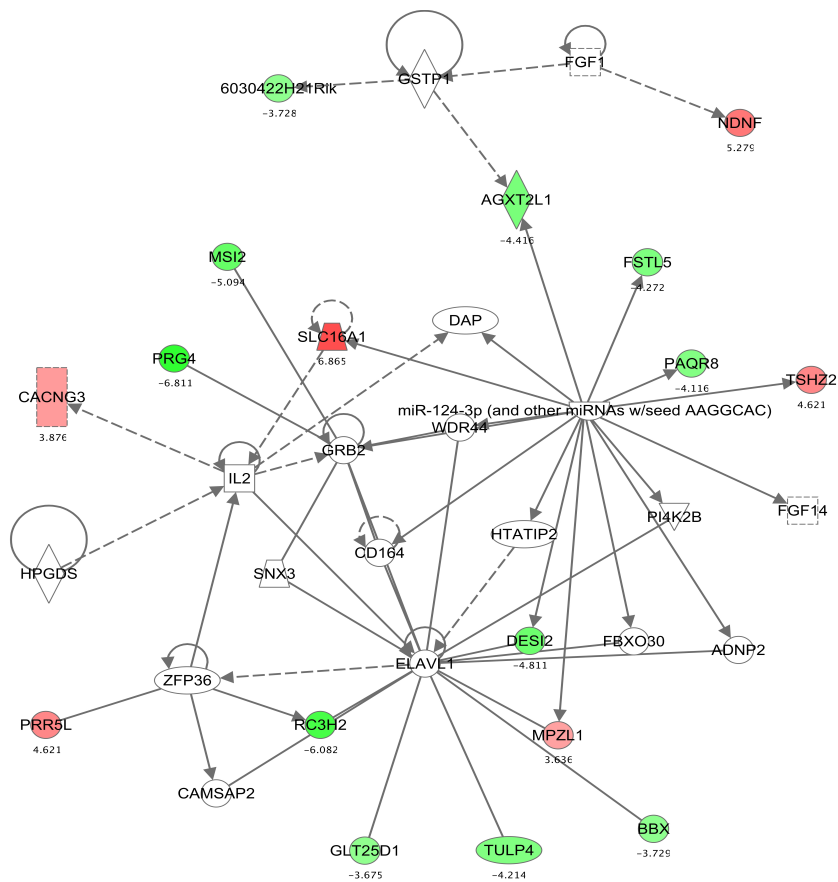


Figure 7. Networks overrepresented among genes identified through microarray and S-score analysis to be significantly different in SHAM vs. ADX treated D2 mice. Panel A: Network of genes involved in protein synthesis, behavior, and nervous system development and function. Panel B: Network of gene involved in hereditary disorder, neurological disease and skeletal and muscular disorders. Red signifies genes that increase following ADX. Green signifies genes that decrease. White signifies a molecule that is not user specified but incorporated into the network through relationships with other molecules. Respective S-scores are provided. Solid arrowheads reflect “acts on” interactions while lines without arrow indicate binding interactions only. Solid and dotted lines indicate, respectively, direct vs. indirect interactions. The molecule shapes represent their designation (ie. enzyme, kinase). For a detailed key of the main network features please see: <http://physiolgenomics.physiology.org/content/suppl/2012/10/03/physiolgenomics.00092.2012.DC1/supplegend.pdf>.

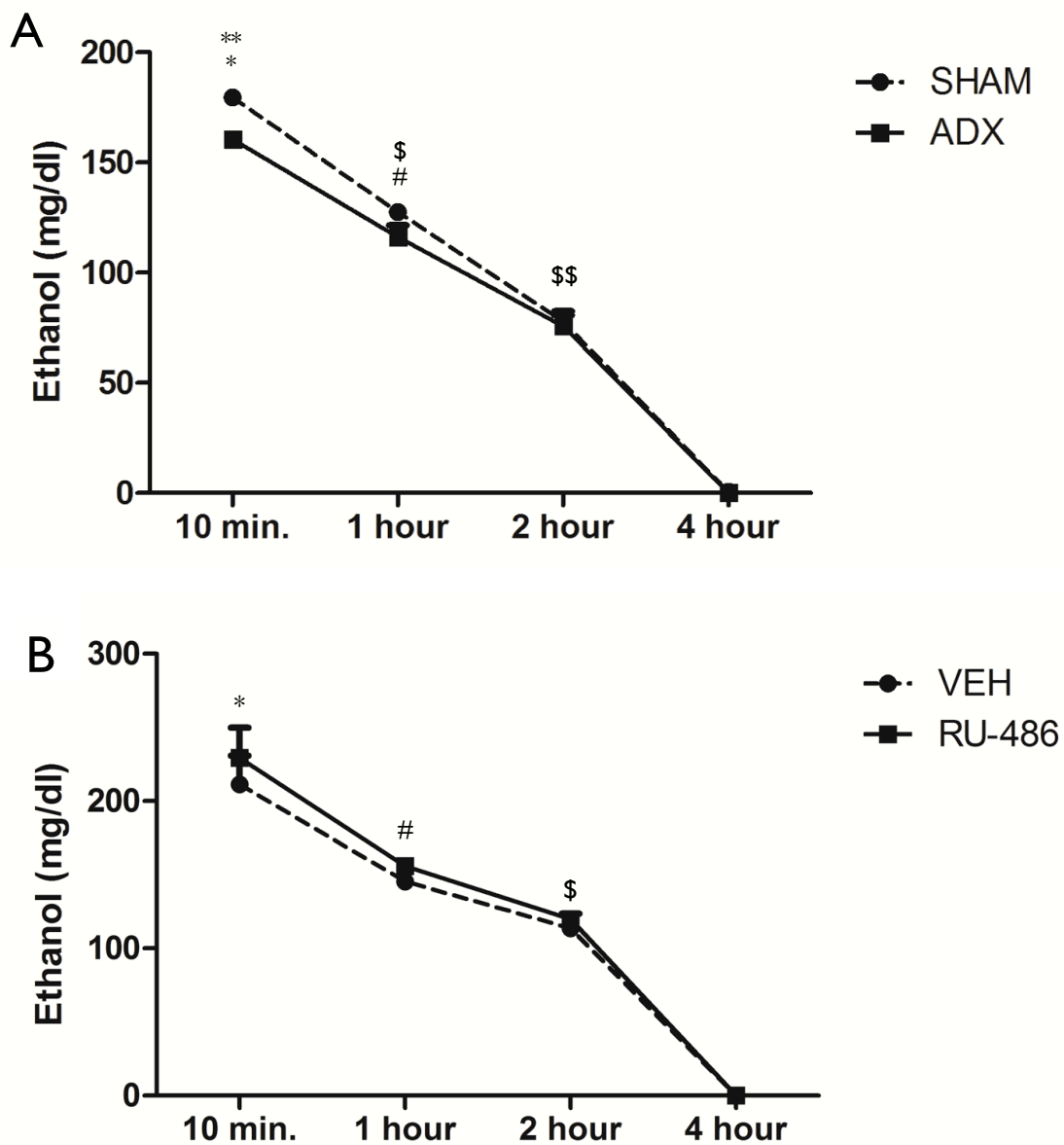


Figure 8. BEC in ADX vs. SHAM and RU-486 vs. VEH pre-treated animals. Panel A: ADX animals showed a lower peak BEC and unaltered rate of clearance following 2 g/kg ethanol. * $p < 0.05$ 10 min. SHAM vs. 10 min. ADX, # $p < 0.05$ 1 hour SHAM vs. 1 hour ADX: ** $p < 0.05$ 10 min. vs. 1 hour, 2 hours and 4 hours; \$ $p < 0.05$ 1 hour vs. 2 and 4 hours; \$\$ $p < 0.05$ 2 hours vs. 4 hours. Panel B: There were no differences in BEC between D2 animals pre-treated with 35 mg/kg RU-486 vs. those pre-treated with VEH 10 min., 1 hour, 2 hours and 4 hours following 2 g/kg ethanol administration. * $p < 0.05$ vs. 4 hour, 2 hour, and 1 hour; # $p < 0.05$ vs. 4 hour and 2 hour; \$ $p < 0.05$ vs. 4 hour.

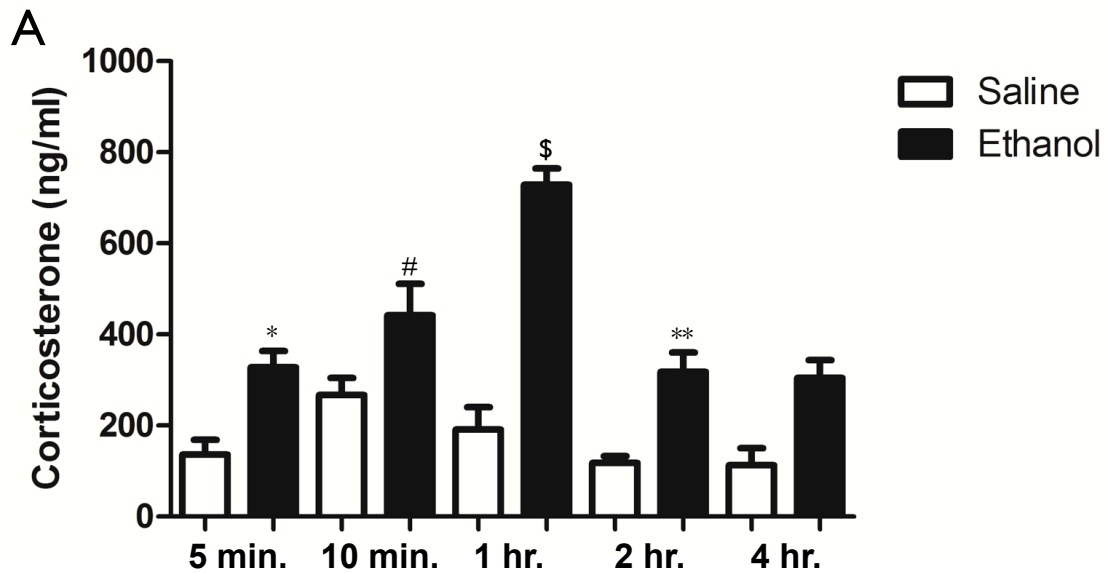


Figure 9. CORT levels were significantly elevated 5 minutes, 10 minutes, 1 hour and 2 hours following ethanol administration. There were no differences in cort. levels between saline treated animals. Levels peaked 1 hour following ethanol administration. Despite a strong trend towards elevated levels 4 hours following ethanol administration, cort. levels between saline vs. ethanol treated animals did not differ from one another at this time indicating that corticosterone levels return to normal 4 hours following 2 g/kg ethanol administration. * $p < 0.05$ vs. saline 5 min, 1 hr., 2 hr. and 4 hr.; # $p < 0.05$ vs. all saline groups; \$ $p < 0.05$ vs. all groups; ** $p < 0.05$ vs. saline 5 min., 1 hr., 2 hr. and 4 hr.

CHAPTER 4

Endocannabinoid Regulation of the Hypothalamic Pituitary Adrenal Axis following Ethanol Administration

INTRODUCTION

Previously, we hypothesized that basal differences in gene expression may be one explanation for differences in locomotor activity between D2 SHAM versus ADX mice (Costin et al., 2012). Basal differences in gene expression could facilitate different cell signaling events in D2 SHAM versus ADX mice that may lead to a blunted locomotor response to ethanol in ADX D2 mice. While our prior studies focused primarily on classical, genomic glucocorticoid actions mediated by glucocorticoids binding to intracellular receptors and causing eventual changes in gene expression, we wanted to additionally explore newly identified nongenomic glucocorticoid signaling mechanisms. Our previous findings demonstrated significant increases in corticosterone (Rosser et al.) levels as soon as five minutes following ethanol administration (Fig. 9). Via the hypothesized nongenomic pathway CORT binds to a yet-uncharacterized membrane-bound glucocorticoid receptor that activates the G_s - cAMP/PKA pathway to induce endocannabinoid synthesis (Hill and McEwen, 2009). Endocannabinoids are released into the synapse where they bind to CB1 receptors and activate signaling cascades that can alter neurotransmitter release (Hill and McEwen, 2009, Di et al., 2003).

Despite hypothesized nongenomic glucocorticoid signaling mechanisms, alternate findings exist on the relationship between cannabinoids and the HPA axis. A number of reports indicate that administration of cannabinoids stimulates the HPA axis by elevating ACTH and/or corticosterone plasma levels (Jackson and Murphy, 1997, Wenger et al.,

1997, Wenger et al., 2003). Another report indicates that the CB1 antagonist SR141716A reduces the stimulation of ACTH release induced by THC (Manzanares et al., 1999). Alternatively, it has been shown that the administration of SR141716A has been able to increase ACTH and CORT release (Patel et al., 2004, Wade et al., 2006, Navarro et al., 1997). From these varied results, it seems that factors including drug dose, environmental context, and genetic background of the subject tested may affect HPA axis activity.

The CB1 receptor is expressed in the hypothalamus and pituitary of mice, rats and humans, but the literature does not totally support its role in nongenomic glucocorticoid signaling mechanisms (Herkenham et al., 1991, Pagotto et al., 2001, Wittmann et al., 2007). It has been shown previously that basal and novelty stress-induced plasma levels of ACTH and corticosterone were higher in CB1 KO than in WT mice (Barna et al., 2004), but other laboratories have failed to reproduce this result (Wenger et al., 2003). Still other laboratories have found that CB1 KO mice had an enhanced circadian HPA axis activity peak compared to WT controls (Cota et al., 2007). Finally and interestingly, the CB1 cannabinoid receptor antagonists AM251 and AM281 blocked the negative feedback of glucocorticoids on CRH secretion in hypothalamic slice preparations (Di et al., 2003), but other labs have not been able to replicate this result (Cota et al., 2007, Barna et al., 2004). Although alternate findings exist in the literature, we hypothesized that endocannabinoid signaling may mediate a fast negative feedback loop that decreases CORT release and therefore CB1 KO animals, animals with altered endocannabinoid signaling, would have significantly elevated CORT levels compared to their WT counterparts. According to our hypothesis, FAAH KO animals would likely have unaltered or blunted CORT levels because fast feedback loops would be intact or

heightened in these animals.

To indirectly test our hypothesis, we evaluated CORT levels in FAAH and CB1 KO mice following ethanol administration. Due to our limited timeframe, we were only able to obtain KO mice on a C57 background. In our previous studies, we used D2 mice. It is well known that D2 mice show an acute locomotor activation response following ethanol administration whereas C57 mice do not (Phillips et al., 1997a). This characteristic allowed us to indirectly explore whether or not CORT plays a causal or permissive role in the locomotor activation response. For example if CORT is causal in the locomotor activation response following acute ethanol administration, we might expect CORT levels to be lower in C57 vs. D2 mice following acute ethanol. Because we wanted to ensure C57 mice would allow for the evaluation of HPA axis activation following acute ethanol administration and further evaluate the role corticosterone may play in locomotor activation, we began our studies by evaluating CORT levels in C57 versus D2 mice following acute ethanol administration. We hypothesized that both mouse strains would have significant and equal elevations in CORT following ethanol administration and therefore CORT may play a permissive rather than causal role in ethanol induced locomotor activity.

MATERIALS AND METHODS

Animals

Mice were maintained in a temperature controlled room (23±1°C) with 12 h light/dark cycles and free access to standard chow (Harlan Teklad #7912, Madison, WI) and water. Cages and bedding (Harlan Sani-chips, #7090A, Harlan, Teklad, Madison, WI) were

changed weekly. All tests were carried out between 0900 and 1200 h. Procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the NIH Guide for the Care and Use of Laboratory Animals. Mice for Expt 1. were male D2 and C57 mice from Jackson Laboratories (Bar Harbor, Maine) purchased at 7-8 weeks of age. Adult male fatty acid amide hydrolase (FAAH) (Expt. 2), CB1 knockout (KO) (Expt. 3), and wild type mice born in the NIDA Center Transgenic Colony at Virginia Commonwealth University (Richmond, VA) also served as subjects. All the genetically modified mice used in these studies were backcrossed thirteen generations onto a C57BL/6 background. The CB1 KO mice were derived from CB1 heterozygote parents (Zimmer et al., 1999) and the FAAH KO mice were derived from congenic FAAH KO parents (Varvel et al., 2007).

Drugs

All drugs were administered intraperitoneally (i.p.). Saline solutions were 0.9% w/v sterile saline. Ethanol solutions were prepared from 200-proof absolute anhydrous ethanol (Pharmco-Aaper brand, Brookfield, CT). Ethanol was administered at 20% v/v in 0.9% saline.

Expt 1.—Acute Locomotor Responses and CORT Levels in D2 and C57 Mice

Mice were habituated to saline injections in their home cage for 2 days prior to experiments and allowed a 1-hour acclimation period to the behavioral room prior to testing. Locomotor activity was measured immediately following injection with either saline or ethanol (2 g/kg) during a 10-minute session in locomotor activity chambers (Med-Associates, model ENV-515; St. Albans, VT). Mice were harvested 1 hour following saline or ethanol injections via decapitation.

Expt 2.—CORT Levels in FAAH WT and KO mice

FAAH WT and KO mice were habituated to injections with saline in their home cage for 2 days prior to experiments and allowed a 1-hour acclimation period to the behavioral room prior to injections on test day. Mice were injected with either saline or ethanol (2 g/kg) and harvested 1 hour later via decapitation.

Expt 3.—CORT Levels in CB1 WT and KO mice

CB1 WT and KO mice were habituated to injections with saline in their home cage for 2 days prior to experiments and allowed a 1-hour acclimation period to the behavioral room prior to injections on test day. Mice were injected with either saline or ethanol (2 g/kg) and harvested 1 hour later via decapitation.

Blood collection and Radioimmunoassay (RIA)

In experiments 1-3, one hour following ethanol or saline administration, trunk blood was collected from individual mice. Serum was isolated by centrifugation at 2500 x g for 15 minutes and stored at -80°C until RIA. A RIA containing I¹²⁵ labeled corticosterone (MP Biomedicals, Cleveland, OH, United States) was performed according to the manufacturer's instructions.

Statistics

Data were expressed as mean \pm SEM and analyzed parametrically. Data were analyzed with analysis of variance (ANOVA) using appropriate between-and within subject factors. All post hoc comparisons were made using Student Newman-Keul's test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Acute locomotor responses and corticosterone levels in D2 and C57 mice

Acute locomotor responses and corticosterone levels were measured following saline and ethanol administration in D2 and C57 mice ($n = 3-4/\text{group}$). A two-way ANOVA (genotype x treatment) measuring locomotor activity in D2 versus C57 mice showed an overall effect of treatment ($F_{1,10} = 4.90, p < 0.01$), but no overall effect of genotype ($F_{1,10} = 11.60, p = 0.051$) and no genotype x treatment interaction. Because we wanted to know which saline versus ethanol treated groups of animals showed specific differences, we ran a one-way ANOVA comparing locomotor activity across treatment groups. A one-way ANOVA showed an overall effect of treatment ($F_{3,10} = 6.33, p < 0.05$). Post-hoc analysis revealed that, as expected, D2 mice showed an acute locomotor activation response following 2 g/kg ethanol, but C57 mice did not (**Fig. 10A**).

Additionally, CORT levels were significantly elevated 1 hour following 2 g/kg ethanol administration in both D2 and C57 mice. A two-way ANOVA (genotype x treatment) measuring CORT levels in C57 vs. D2 mice showed an overall effect of treatment ($F_{1,11} = 102.71, p < 0.001$), but no overall effect of genotype and no genotype x treatment interaction. Post-hoc analysis revealed that both D2 and C57 mice showed significantly elevated corticosterone levels in ethanol versus saline treated animals (**Fig. 10B**).

Corticosterone levels in FAAH WT and KO mice

Corticosterone levels were measured in FAAH WT and KO mice. A two-way ANOVA (genotype x treatment) comparing CORT levels in FAAH WT versus KO mice showed an overall effect of treatment ($F_{1,28} = 21.19, p < 0.001$), but no effect of genotype and no

genotype x treatment interaction. Post-hoc analysis revealed significant differences between all saline treated and all ethanol treated animals indicating that corticosterone levels were significantly, but not differentially, elevated in both WT animals and their KO littermates (**Fig. 11**).

Corticosterone levels in CB1 WT and KO mice

Corticosterone levels were measured in CB1 WT and KO mice. A two-way ANOVA (genotype x treatment) measuring CORT levels in CB1 WT versus KO mice showed an overall effect of treatment ($F_{1,24} = 259.40, p < 0.001$), genotype ($F_{1,24} = 6.32, p < 0.05$) and a significant treatment x genotype interaction ($F_{1,24} = 5.08, p < 0.05$). Post-hoc analysis revealed significant differences between WT saline versus ethanol treated animals and a significant difference between KO saline versus ethanol treated animals. Post-hoc analysis also revealed no significant difference between saline treated WT and KO animals, but a significant difference between ethanol treated WT and KO animals with CB1 KO animals having significantly elevated CORT levels (**Fig. 12**).

DISCUSSION

Our prior studies focused primarily on classical, genomic glucocorticoid actions mediated by glucocorticoids binding to intracellular receptors and causing eventual changes in gene expression, but because we saw alterations in locomotor activity as soon as 10 minutes following ethanol administration in ADX versus SHAM animals (Fig. 3A) we also wanted to explore nongenomic glucocorticoid signaling mechanisms. Our previous findings demonstrated significant increases in CORT levels as soon as five

minutes following ethanol administration (Fig. 9) and there are many examples of the fast actions of glucocorticoids that are too rapid to be mediated through the usual genomic mechanisms (Dallman, 2005). For example, glucocorticoids also exert rapid effects on socially aggressive behavior in rats. Male Wistar rats were significantly more aggressive in the increasing versus decreasing phase of their corticosterone fluctuation when confronting a male intruder. Additionally, males with experimentally increased plasma concentrations of corticosterone were more aggressive than counterparts with experimentally decreased plasma corticosterone concentrations (Haller et al., 2000, Haller et al., 1998).

Via the nongenomic pathway it is hypothesized that CORT binds to a yet-uncharacterized membrane-bound glucocorticoid receptor that activates the G_s -cAMP/PKA pathway to induce endocannabinoid synthesis (Hill and McEwen, 2009). Endocannabinoids are released into the synapse where they bind to CB1 receptors and activate signaling cascades that can alter neurotransmitter release (Hill and McEwen, 2009, Di et al., 2003). We hypothesized that CB1 KO animals, animals with altered endocannabinoid signaling, would have significantly elevated CORT levels compared to their WT counterparts because they lack the fast feedback regulation provided by corticosterone's actions at the CB1 receptor.

Our prior results indicated that CORT levels were significantly elevated 5 minutes following ethanol administration (Fig. 9) and ADX animals, which could no longer release CORT, had a blunted locomotor activation response following ethanol administration (Fig. 3A). Therefore we hypothesized that CORT could potentially play a role in the locomotor activating effects of ethanol. Evaluating CORT levels in C57 versus

D2 mice allowed us to indirectly explore whether or not CORT plays a causal or permissive role in the locomotor activation response. If CORT plays a causal role in the locomotor activation response, then CORT levels in D2 mice that **do** show a locomotor activation response would be expected to be greater than those in C57 mice that **do not** show a locomotor activation response following ethanol administration (Fig. 10A). CORT levels following ethanol treatment did not differ between D2 and C57 mice (Fig. 10B), our results indirectly indicate that CORT may be permissive rather than causal in the locomotor activating effects of ethanol.

This finding was not surprising because it has been shown that cocaine-induced locomotion depends on basal diurnal levels of glucocorticoids, but not on a drug-induced increase in these levels (Marinelli et al., 1997). In other words, Marienelli et al. showed the effect of adrenalectomy on cocaine-induced locomotion was dose-dependently compensated by corticosterone concentrations that were in the range of basal diurnal levels. Plasma levels of corticosterone at least eight times lower than that those induced following cocaine administration led to a full recovery of the effects of adrenalectomy on cocaine-induced locomotion (Marinelli et al., 1997). Thus, corticosterone also appears to be permissive rather than causal in the locomotor activating effects of cocaine.

To further investigate the role of CORT in mediating ethanol's effects via the nongenomic pathway, we evaluated CORT levels in FAAH and CB1 KO mice following ethanol administration. As we hypothesized, CB1 KO animals had significantly elevated CORT levels compared to their WT counterparts following ethanol administration (Fig. 12). Prior studies in other labs have produced varying results regarding CORT levels in CB1 WT versus KO animals. One report showed that CB1 KO mice display elevated

plasma corticosterone concentrations at the onset of the dark phase as compared to their WT littermates and thus we were careful to evaluate the mice at the onset of the light period when basal differences in CORT levels between WT and KO mice would not be expected (Cota et al., 2007). In addition, it has been shown previously that basal and novelty stress-induced plasma levels of ACTH and corticosterone were higher in CB1 KO than in WT mice (Barna et al., 2004), but other laboratories failed to reproduce this result (Wenger et al., 2003). While we did not evaluate ACTH levels in KO versus WT mice, there were no differences in basal CORT levels between WT and KO mice in our studies. Our results somewhat indirectly support work by Di et. al which showed that the CB1 cannabinoid receptor antagonists AM251 and AM281 blocked the negative feedback of glucocorticoids on CRH secretion in hypothalamic slice preparations (Di et al., 2003). In line with our hypothesis, FAAH KO animals had CORT levels equal to their WT counterparts because fast feedback loops would be intact in both FAAH WT and KO animals (Fig. 11). Given our current hypothesis, this would make sense as FAAH KO mice have excess levels of the endocannabinoid AEA that can act at the CB1 receptor so their fast feedback loops would not be altered following ethanol administration and HPA axis activation.

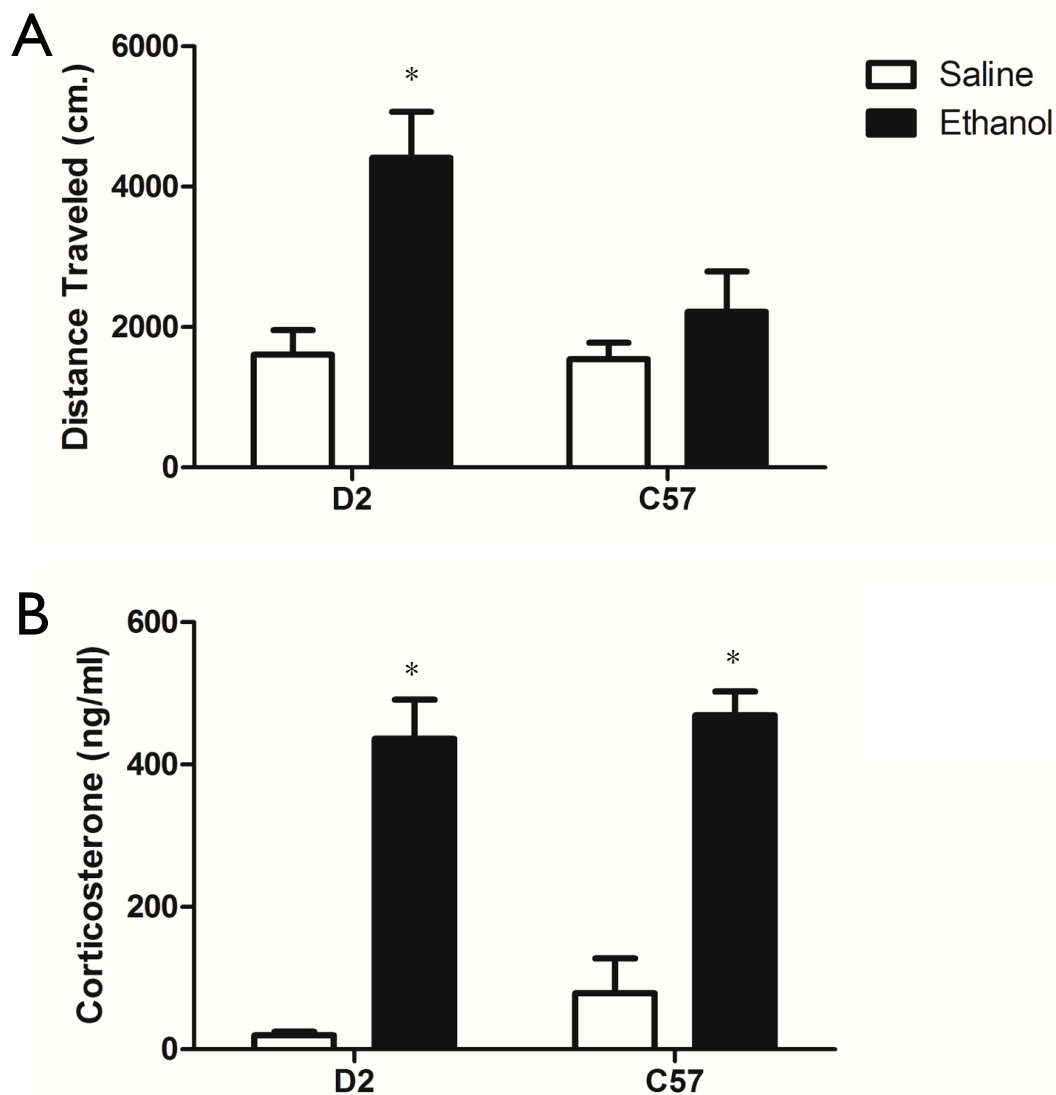


Figure 10. Acute locomotor responses (A) and corticosterone levels (B) following saline and ethanol (2 g/kg) administration in D2 and C57 mice. Panel A: D2 mice showed an acute locomotor activation response following 2 g/kg ethanol, but C57 mice did not. * $p < 0.05$ vs. D2 saline, C57 saline and C57 ethanol. Panel B: Corticosterone levels were significantly elevated 1 hour following 2 g/kg ethanol administration in both D2 and C57 mice. * $p < 0.05$ vs. D2 saline, C57 saline

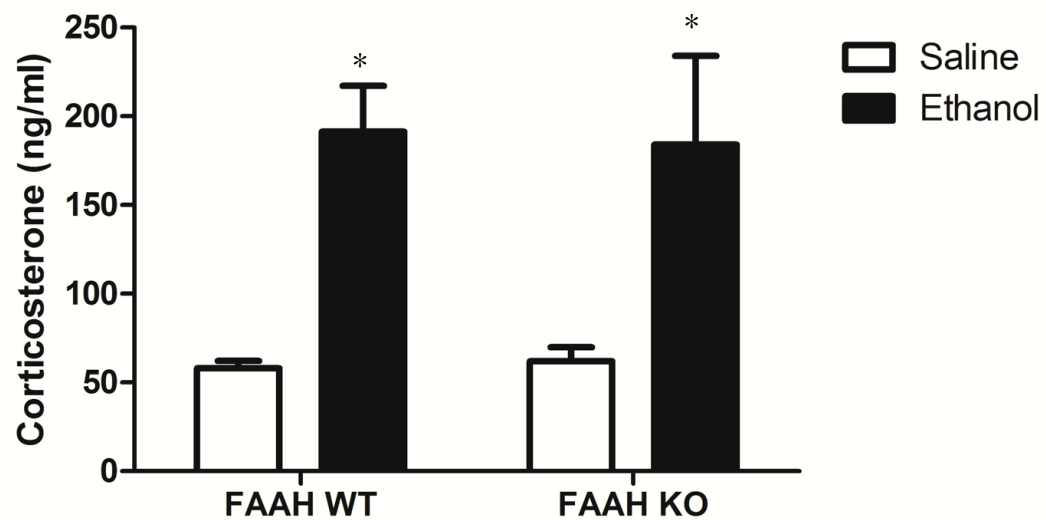


Figure 11. Corticosterone levels in FAAH WT and KO mice following saline and ethanol administration. FAAH WT and KO mice show significant increases in corticosterone levels following ethanol administration. * $p < 0.05$ vs. all saline treated animals.

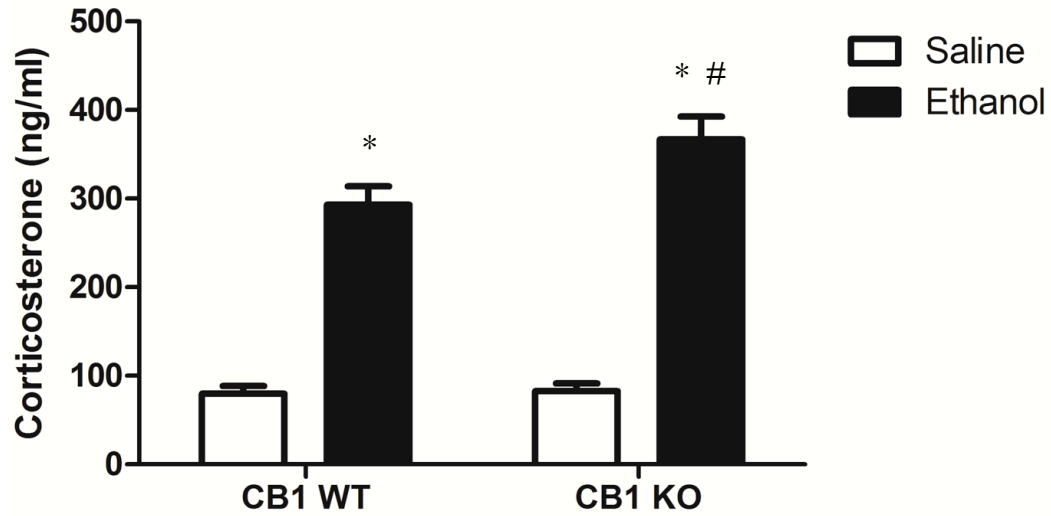


Figure 12. Corticosterone levels in CB1 WT and KO mice following saline and ethanol administration. Both CB1 WT and KO mice show significant increases in corticosterone levels following ethanol administration, but corticosterone levels in CB1 WT mice are significantly elevated following ethanol administration compared to their WT littermates. * $p < 0.05$ vs. all saline treated animals, # $p < 0.05$ vs. ethanol treated CB1 WT mice.

CHAPTER 5

Ethanol Regulation of Serum Glucocorticoid Kinase 1

INTRODUCTION

Although alcohol dependence is a complex disease that develops over many years and includes cycles of withdrawal, craving, and relapse, acute responses to ethanol have predictive validity in terms of risk for high levels of ethanol intake in animal models or alcoholism in humans (Schuckit, 1994, Metten et al., 1998). Therefore, defining the cellular mechanisms underlying acute responses to ethanol has significant biomedical implications.

Ethanol acutely activates the HPA axis leading to glucocorticoid release from the adrenal glands (Ellis, 1966). Glucocorticoid hormones are the final step in activation of the HPA axis and are known to function in the biological response to stress and circadian activity (Marinelli et al., 1997, De Kloet et al., 1998). Glucocorticoids are also well known to regulate gene expression (Webster et al., 1988). In alcohol dependence, the hypothalamic pituitary adrenal (HPA) axis is dysregulated in both humans (Costa et al., 1996, Wand and Dobs, 1991a) and rodents (Richardson et al., 2008, Rasmussen et al., 2000, Roberts et al., 1995), but the consequences of this dysregulation remain unclear.

Our laboratory and others have used genome-wide expression profiling to identify gene networks functioning in acute and chronic behavioral responses to ethanol (Kerns et al., 2005a, Treadwell and Singh, 2004, Wolstenholme et al., 2011, Costin et al., 2012, Wolen et al., 2012, Piechota et al., 2010b). We previously identified a group of genes prominently regulated by acute ethanol in the prefrontal cortex (PFC) of DBA2/J (D2) mice (Kerns et al., 2005b). Contained in this group were well-characterized

glucocorticoid responsive genes including FK506 binding protein 5 (*Fkbp5*) and Serum Glucocorticoid Kinase I (*Sgk1*) (Binder, 2009, Webster et al., 1993). *Sgk1* is a glucocorticoid responsive gene that regulates ion channel function, cell survival, and is involved in synaptic plasticity, learning and memory (Alvarez de la Rosa et al., 1999, Brunet et al., 2001, Lee et al., 2006, Ma et al., 2006, Tsai et al., 2002).

Sgk1 has multiple transcript and protein isoforms generated through alternative promoter utilization, splicing, translation and post-translational modifications (Arteaga et al., 2008, Arteaga et al., 2007). It is known that there are 5 isoforms of *Sgk1*—4 resulting from translational processing of *Sgk1* and one, *Sgk1.1*, resulting from alternative promoter utilization and splicing (Arteaga et al., 2008, Arteaga et al., 2007). Because *Sgk1* is regulated by both glucocorticoids and acute ethanol and is known to regulate ion channel function, we hypothesized that Sgk1 signaling may be an important mechanism underlying acute behavioral and cellular responses to ethanol, and that it may modulate neuronal plasticity leading to more chronic behaviors such as sensitization. We have therefore performed a detailed analysis on ethanol regulation of Sgk1 from the transcriptional to protein level. Our results indicate a complex regulation of Sgk1 transcription, protein abundance and post-translational modification following ethanol treatment. Altogether, these results suggest a critical role for the HPA axis and Sgk1 in regulating the acute and potentially chronic cellular response to ethanol.

MATERIALS AND METHODS

Animals

Mice were maintained in a temperature-controlled room ($23\pm 1^{\circ}\text{C}$) with 12 h light/dark cycles and free access to standard chow (Harlan Teklad #7912, Madison, WI, United States) and water. Cages and bedding (Harlan Sani-chips, #7090A, Harlan, Teklad, Madison, WI, United States) were changed weekly. All tests were carried out between 0900 and 1200 h. All mice were DBA2/J mice from Jackson Laboratories (Bar Harbor, ME, United States) purchased at 10-13 weeks of age and group housed 4/cage.

Adrenalectomized (ADX) and SHAM mice were treated at Jackson and delivered following recovery from surgery. ADX mice were supplemented with 1% w/v saline solution in their drinking water. All mice were allowed to habituate to the animal facility for at least 1 week prior to testing.

Adrenalectomy

All adrenalectomies and sham procedures were conducted using aseptic and atraumatic surgical techniques at Jackson laboratories and were approved by the institution's Animal Care and Use Committee. Surgeries were performed using the lateral abdominal approach. Left and right adrenal glands were removed through separate incisions. The animals were anesthetized using tribromoethanol and carprofen was administered for analgesia and the surgical site was prepared. The animals were placed in right lateral recumbency and a 5-8mm incision was made parallel and ventral to the spine and midway between the last rib and iliac crest. The underlying muscle was opened and the adrenal gland was located cranial to the left kidney. The adrenal gland was grasped with ring forceps and exteriorized. The adrenal and adjacent adipose tissues were excised. The incisions in the abdominal wall and skin were closed separately. Bupivacaine was applied topically to the incision site for local analgesia. Skin closure material was

removed prior to shipment. The same procedure was followed for excision of the right adrenal gland except the skin incision was made immediately caudal to the last rib. This adjustment was necessary to accommodate for the more cranial location of the right adrenal gland relative to the left in the abdomen.

Drugs

All drugs were administered intraperitoneally (i.p.). Saline solutions were 0.9% w/v sterile saline. Ethanol solutions were prepared from 200-proof absolute anhydrous ethanol (Pharmco-Aaper brand, Brookfield, CT, United States). Ethanol was administered at 20% v/v in 0.9% saline.

Experimental Testing

Mice were habituated to injections with saline in their home cage for 2 days prior to experimental testing. If behavioral testing was performed, mice were allowed a 1-hour acclimation period to the behavioral room prior to testing. All locomotor activity was measured immediately following injection with either saline or ethanol during a 10-minute session in locomotor activity chambers (Med-Associates, model ENV-515; St. Albans, VT, United States). The system is interfaced with Med Associates software that allows for the automatic measurement of activity using a set of 16 infrared beam sensors along the X-Y plane.

Exp. 1 – Time course and dose response analysis of *Sgk1* and *Sgk1.1* expression

following acute ethanol administration: Prior to beginning the time course experiments, four groups of mice ($n = 4$), basal, 2 hour, 4 hour and 8 hour, were evaluated to determine the effect of saline injections on *Sgk1* expression. The basal group of mice was handled for two days, and then harvested on day 3 and brain micropunch

dissections performed. The other 3 groups of mice were habituated to injections as described above and then harvested 2, 4 and 8 hours following saline injections on day 3 and brain micropunch dissections performed. For the time course experiment, six groups of mice (n = 4) were administered either saline or 4 g/kg ethanol and groups of animals, one saline treated group and one ethanol treated group, were harvested 2, 4 and 8 hours following drug administration. For the dose response experiment, three groups of mice (n = 8) were administered saline, 2 g/kg ethanol or 4 g/kg ethanol and harvested 4 hours following injection and brain micropunch dissections performed.

Exp. 2 – *Sgk1* levels following ethanol sensitization: Animals (n = 4) received one of three treatments: saline-saline (SS), saline-ethanol (SE) or ethanol-ethanol (EE) (**Table 1**). On conditioning days 3-13, animals received daily injections in their home cages of either saline (SS, SE) or 2.5 g/kg ethanol (EE). On test day 14 the SS group received saline and the SE and the EE groups received 2.0 g/kg ethanol and were placed in locomotor activity chambers. Four hours following drug administration, animals were harvested and brain micropunch dissections performed.

Exp. 3 – Corticosterone levels following ethanol sensitization: Animals (n=6) received one of four treatments: saline-saline (SS), ethanol-saline (ES), saline-ethanol (SE) or ethanol-ethanol (EE) (**Table 1**). On days 3-13, animals received daily injections in their home cages of either saline (SS, SE) or 2.5 g/kg ethanol (ES, EE). On test day 14 the SS and ES groups received saline and the SE and the EE groups received 2.0 g/kg ethanol and were placed in locomotor activity chambers. One hour following drug administration, animals were harvested and blood collection performed.

Exp. 4 – Effects of ADX on *Sgk1* induction following ethanol administration:

ADX and SHAM animals (n=11-15) received either 4 g/kg ethanol or saline. Mice were harvested four hours following ethanol or saline administration and brain micropunch dissections performed. To ensure that mice were in fact adrenalectomized, a separate group of ADX and SHAM mice (n = 4-5) were administered either saline or acute ethanol. One hour following the last drug administration, animals were harvested and trunk blood collection performed.

Exp. 5 – Glucocorticoid receptor binding to Sgk1 promoter following acute ethanol

administration: Mice (n = 8/group, 48 total) were treated with saline or 4 g/kg ethanol and harvested via cervical dislocation 1 hour following drug administration. Following cervical dislocation, the brain was removed, the olfactory bulbs were removed from the brain and a cut was made just rostral to the optic chiasm to collect the frontal pole of the brain.

Exp. 6 – Sgk1 phospho Serine (S422) and Sgk1 Levels following ethanol

administration: Mice (n = 12, 6 animals treated with saline, 6 treated with 4 g/kg ethanol) were administered saline or ethanol and harvested 15 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours following drug administration and brain micropunch dissections performed.

Brain Micropunch Dissection

At designated times following ethanol or saline administration in experiments 1, 2, 4 and 6 brain tissue was collected. Collection of brain tissue occurred exactly as described in Kerns et al. (Kerns et al., 2005a). The medial prefrontal cortex (PFC) dissection contained tissue from dorsal and ventral anterior cingulate and some secondary motor cortex.

RNA Isolation and Quantitative Real Time Polymerase Chain Reaction (Q-rtPCR)

RNA was isolated from PFC tissue samples using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. RNA concentration and quality was assessed by Experion automated electrophoresis (BioRad, Hercules, CA, United States). cDNA was generated from 1 ug total RNA by reverse transcription with the iScript CDNA kit (Bio-Rad, Hercules, CA, United States) following the manufacturer's instructions. Q-rtPCR was performed using the iCycler iQTM system (Bio-Rad, Hercules, CA, United States) according to the manufacturer's instructions for SYBR Green I-based detection. Quantification of gene expression levels was determined based on the threshold cycle for each well using the provided software and all results were normalized to multiple reference genes using Genorm as described in Vandesompele et al. (Vandesompele et al., 2002). Primers used were as follows, *Sgk1* (Forward- CGTCAAAGCCGAGGCTGCTCGAAGC and Reverse- GGTTTGGCGTGAGGGTTGGAGGAC), *Sgk1.1* (Forward- ATGCCAACATCCTGACCAA and Reverse- TGCTGGCAATCTTCTGAATAAA), *Gapdh* (Forward-TTCCAGTATGACTCCACTCACGG and Reverse- TGAAGACACCAGTAGACTCCACGAC), *Ppp2r2a* (Forward- ATCTCTCACCTTGCCCTTT and Reverse-CCCATTTTGTGTGCTTTCGT), *Ublcp1* (Forward-ATGACAGGGACAGGACAAGC and Reverse- TACAATGACACCCGACTGGA), *Ndufv1* (Forward- GACCGTGCTAATGGACTTCG and Reverse-GGCATCTCCCTTCACAAATC), and *Nr3c1* (Forward- AAGAGACAAACGAGAGTCCTTGG and Reverse- GTGTCCGGTAAAATAAGAGGCTT).

Blood collection and Radioimmunoassay (RIA)

One hour following ethanol or saline administration in Exp. 3 and Exp. 4, trunk blood was collected from individual mice. Serum was isolated by centrifugation at 2500 x g for 15 minutes and stored at -80°C until RIA. A RIA containing I¹²⁵ labeled corticosterone (MP Biomedicals, Cleveland, OH, United States) was performed according to the manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP) Assay

Frontal poles from 8 D2 mice in Exp. 5 were combined to make one individual sample and the ChIP analyses were performed using the magnetic-bead-based Chip-IT Express Enzymatic kit following the manufacturer's instructions for fresh tissue (Active Motif, Carlsbad, CA, United States). Briefly, the tissue was cross-linked with 1% formaldehyde for 10 min, sheared enzymatically and the chromatin immunoprecipitated with the indicated antibodies: rabbit anti-Glucocorticoid Receptor (Santa Cruz Biotechnology, Santa Cruz, CA, United States) and rabbit anti-immunoglobulin G (IgG) (Cell Signaling, Danvers, MA, United States). Following immunoprecipitation, the chromatin was eluted from the magnetic beads, the cross-links were reversed, and the protein was digested. Samples were then subjected to a DNA clean-up step prior to Q-rtPCR using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. The resulting DNA fragments in the range of 150 to 500 bp were analyzed by Q-rtPCR using a pair of primers (Forward-ACCCCTGCTCCCTCTAACTC and Reverse-GCGGAAATAAGTCTCTGCTCT) spanning the glucocorticoid response element (GRE) in the Sgk1 promoter region. For Q-

rtPCR, SsoAdvanced™ SYBR® Green Supermix (BioRad, Hercules, CA, United States) was used according to the manufacturer's instructions.

Western Blotting

While frozen, PFC tissue from animals in Expt. 3 was homogenized using a Dounce Tissue Homogenizer (Fisher Scientific, Waltham, MA, United States) and then suspended in Lithium Dodecyl Sulfate Loading Buffer (Invitrogen, Grand Island, NY, United States) containing Halt protease and phosphatase inhibitors (ThermoFisher Scientific, Waltham, MA, United States) and sonicated. Western blotting was performed using the XCell Surelock Mini-Cell kit (Invitrogen, Grand Island, NY, United States) according to the manufacturer's instructions. SGK1 blots were probed with rabbit anti-SGK1 (AbCam, Cambridge, MA, United States) and rabbit anti-GAPDH (AbCam, Cambridge, MA, United States). phospho-SGK1 (pSgk1) blots were probed with rabbit anti-SGK1 phospho S422 (AbCam, Cambridge, MA, United States) and goat anti-SGK1 phospho T256 (Santa Cruz Biotechnology, Santa Cruz, CA, United States), then stripped using Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, United States) and re-probed with rabbit anti-SGK1 (AbCam, Cambridge, MA, United States). phospho-N-myc downstream-regulated 1 gene 1 (pNDRG1) blots were probed with rabbit anti-NDRG1 phospho S330 (AbCam, Cambridge, MA, United States), then stripped and re-probed with rabbit anti-NDRG1 (AbCam, Cambridge, MA, United States). Blots were also probed with IRDye goat anti-rabbit 680, IRDye goat anti-rabbit 800, and IRDye donkey anti-goat 800 antibodies (Li-cor Biosciences, Lincoln, NE, United States). Western blot imaging was performed and images were quantified using

infrared imaging (Odyssey infrared imager, Li-cor Biosciences, Lincoln, NE, United States).

Statistics

Data were expressed as mean \pm SEM and analyzed parametrically. Data were analyzed with analysis of variance (ANOVA) using appropriate between-and within subject factors. All post hoc comparisons were made using Student Newman-Keul's test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Time course and dose response analysis of Sgk1 and Sgk1.1 expression following acute ethanol administration.

Because our prior microarray studies and the studies of other labs would have used probes that recognize cDNA regions common to all Sgk1 isoforms, we performed Q-rtPCR studies to determine which *Sgk1* isoform was specifically regulated by ethanol, *Sgk1* or *Sgk1.1*. Q-rtPCR was used to evaluate *Sgk1* and *Sgk1.1* levels 2, 4 and 8 hours following ethanol or saline administration. Prior to performing these studies to evaluate the effects of injection stress on Sgk1 expression, we compared *Sgk1* levels in the PFC of D2 mice basally (a 0 hour time point) to D2 mice harvested 2, 4 and 8 hours following saline injections. A two-way ANOVA (treatment x time) showed no overall effects of treatment or time and no significant treatment x time interaction indicating that injection stress did not alter *Sgk1* levels (**Fig. 13**). Because we saw no significant effects of saline injections on *Sgk1* expression, we did not include a 0 hour time point in the remainder of our studies. In evaluating *Sgk1* levels 2, 4 and 8 hours following saline and ethanol

administration, a two-way ANOVA showed an overall effect of treatment, ethanol versus saline ($F_{1,17} = 16.44, p < 0.01$), but no overall effect of time and no significant treatment x time interaction (**Fig. 14a**). To examine treatment more carefully, we used a one-way ANOVA. A one-way ANOVA showed a significant effect of treatment ($F_{5,22} = 4.51, p < 0.01$) and post-hoc analysis indicated that 4 hours following ethanol treatment *Sgk1* levels were significantly increased compared to all saline treated animals and animals treated with ethanol 8 hours prior to harvest (**Fig. 14a**). In addition, *Sgk1* levels were significantly increased in animals harvested 2 hours following 4 g/kg ethanol administration compared to those harvested 8 hours following saline administration (**Fig. 14a**). This indicates that *Sgk1* levels were significantly increased 2 and 4 hours following 4 g/kg ethanol administration and returned to basal levels 8 hours following 4 g/kg ethanol administration. A two-way ANOVA evaluating *Sgk1.1* levels showed no overall effect of treatment or time and no significant treatment x time interaction (**Fig. 14b**). A one-way ANOVA ($F_{5,18} = 5.31, p < 0.01$) indicated that animals harvested 8 hours following ethanol administration showed significantly greater levels of *Sgk1.1* than those harvested 2 and 4 hours following ethanol administration and saline administration. In addition, animals harvested 8 hours following saline treatment showed greater levels of *Sgk1.1* than those harvested 2 hours following ethanol treatment indicating that there was no effect of treatment on *Sgk1.1* expression. These results suggest that *Sgk1*, not *Sgk1.1*, is the ethanol responsive isoform of *Sgk1*.

To further evaluate which isoform of *Sgk1* was ethanol responsive, Q-rtPCR was used to evaluate *Sgk1* and *Sgk1.1* levels following an ethanol dose response assessment in which animals were administered saline, 2 g/kg and 4 g/kg ethanol. In evaluating *Sgk1*

levels, a one-way ANOVA showed a significant effect of treatment ($F_{2,18} = 50.58$, $p < 0.01$). Post-hoc analysis indicated significant differences between 4 g/kg ethanol versus saline, 2 g/kg ethanol versus saline and 4 g/kg ethanol versus 2 g/kg ethanol indicating that *Sgk1* is dose dependently increased following ethanol administration (**Fig. 14c**). Q-rtPCR was also used to evaluate *Sgk1.1* levels following saline, 2 g/kg and 4 g/kg ethanol administration (**Fig. 14d**). A one-way ANOVA showed no significant effects between the 3 groups (**Fig. 14d**). Once again this indicated that *Sgk1*, not *Sgk1.1*, is the ethanol responsive isoform.

Sgk1 levels following ethanol sensitization

Prior microarray studies in our lab and others had identified *Sgk1* to be an acute ethanol responsive gene and we confirmed this finding via Q-rtPCR (**Fig. 14a-d**), but we also wanted to know if *Sgk1* or its isoform *Sgk1.1* was regulated following chronic ethanol administration. To answer this question, we evaluated *Sgk1* levels acutely and chronically following ethanol sensitization studies. A one-way ANOVA showed an overall effect of treatment ($F_{2,21} = 41.96$, $p < 0.01$) (**Fig. 15a**). Post-hoc analysis revealed that ethanol-ethanol (EE) treated animals showed significantly greater locomotor activity compared to saline-ethanol (SE) and saline-saline (SS) treated animals. In addition, SE treated animals showed greater locomotor activity than SS treated animals. In evaluating *Sgk1* levels in these animals, a one-way ANOVA found an overall significant effect of treatment ($F_{2,9} = 8.76$, $p < 0.01$) (**Fig. 15b**). Post-hoc analysis showed that *Sgk1* levels were significantly increased in animals treated acutely with ethanol compared to SS and EE treated animals. Interestingly, *Sgk1* levels did not differ between SS and EE treated

animals. A one-way ANOVA identified no significant differences in *Nr3c1* (**Fig. 15c**) or *Sgk1.1* (**Fig. 15d**) levels in SS, SE or EE treated animals. These results indicated that *Sgk1* is regulated by ethanol acutely, but not chronically, *Sgk1*, not *Sgk1.1* is the ethanol responsive isoform of *Sgk1*, and changes in *Nr3c1* do not appear to be responsible for the changes in *Sgk1*.

Corticosterone levels following ethanol sensitization

Because *Sgk1* levels were not regulated in animals chronically treated with ethanol, *Sgk1* is a well-known glucocorticoid responsive gene and it is known that animals and human alcoholics show a blunted HPA axis while drinking and upon withdrawal, we hypothesized that corticosterone levels may be blunted in animals chronically treated with ethanol (EE animals). We sensitized animals to ethanol and collected blood from them one hour following behavioral testing to measure corticosterone levels across SS, SE, EE and Ethanol-Saline (ES) treated animals. In evaluating the sensitized response to ethanol, a one-way ANOVA showed an overall effect of treatment ($F_{3,15} = 159.67, p < 0.01$) (**Fig. 16a**). Post-hoc analysis revealed that EE treated animals showed a significantly greater locomotor response compared to SS, SE, and ES treated animals. In addition, SE treated animals showed a greater locomotor response compared to SS and ES treated animals. In evaluating corticosterone levels in SS, SE, EE and ES mice, a one-way ANOVA showed an overall significant effect of treatment ($F_{3,15} = 47.37, p < 0.01$) (**Fig. 16b**). Post-hoc analysis revealed that SE animals showed greater corticosterone levels than SS, EE and ES indicating that corticosterone levels were blunted in animals chronically treated with ethanol.

Effects of ADX on Sgk1 induction following ethanol administration

Because *Sgk1* was not induced in EE treated animals and the corticosterone response to ethanol was also blunted in these animals, we hypothesized that *Sgk1* induction may be due to HPA axis activation following acute ethanol administration. Therefore, we hypothesized that *Sgk1* would not be induced in ADX animals, animals lacking their adrenal glands, the organ responsible for corticosterone release and the final step in HPA axis activation. A two-way ANOVA (phenotype x treatment) evaluating *Sgk1* levels in SHAM versus ADX animals indicated an overall effect of phenotype ($F_{1,22}= 7.16, p < 0.05$), treatment ($F_{1,22}= 11.05, p < 0.01$) and a significant (phenotype x treatment) interaction ($F_{1,22}= 5.31, p < 0.05$) (**Fig. 17a**). Post-hoc analysis of phenotype indicated *Sgk1* levels were significantly increased in ethanol versus saline treated SHAM animals, but not ADX animals. Post-hoc analysis of treatment indicated significant differences between ethanol, but not saline, treated SHAM versus ADX mice indicating that *Sgk1* levels were blunted in ADX mice following ethanol administration. There were no significant differences in *Sgk1* levels in SHAM versus ADX mice (**Fig. 17c**).

To ensure that animals were, in fact, adrenalectomized, we evaluated corticosterone levels in ADX vs. SHAM animals. A two-way ANOVA (phenotype x treatment) evaluating corticosterone levels in ADX animals indicated an overall effect of phenotype ($F_{1,14}= 52.22, p < 0.05$), treatment ($F_{1,14}= 60.83, p < 0.01$) and a significant (phenotype x treatment) interaction ($F_{1,14}= 63.91, p < 0.05$) (**Fig. 17b**). Post hoc analysis revealed that there were significant differences between saline versus ethanol treated SHAM animals and significant differences between ADX and SHAM ethanol treated

mice (Fig 4b.) indicating that ethanol caused corticosterone release in SHAM, but not ADX animals. Therefore, ADX mice did not experience increases in corticosterone following saline administration and the induction of *Sgk1*, but not *Sgk1.1*, may be due to HPA axis activation and glucocorticoid signaling following acute ethanol administration.

Chromatin Immunoprecipitation (ChIP) Assay

To test whether *Sgk1* induction following ethanol administration was due to glucocorticoid receptor (GR) binding to the glucocorticoid response element (GRE) in the *Sgk1* promoter, we performed a chromatin immunoprecipitation (ChIP) assay in which we isolated chromatin from ethanol and saline treated animals and conducted immunoprecipitation studies using an anti-GR antibody and a control anti-IgG antibody. A two-way ANOVA (antibody x treatment) showed an overall effect of antibody ($F_{1,8}=140.40$, $p < 0.01$), treatment ($F_{1,8}=13.13$, $p < 0.01$) and a significant (treatment x antibody) interaction ($F_{1,8}=5.85$, $p < 0.05$) (**Fig. 18**). Post-hoc analysis revealed that looking at samples in which GR immunoprecipitations were performed, the level of *Sgk1* promoter region bound to the GR in ethanol treated samples was significantly greater than that bound in saline treated samples. Looking at samples in which IgG immunoprecipitations were performed, there were no significant differences between *Sgk1* promoter region bound to the IgG antibody in ethanol versus saline treated samples. Additionally, looking at antibody effects within saline treated animals, there was a significantly greater amount of *Sgk1* promoter region bound to the GR antibody versus the IgG antibody. Looking at antibody effects within ethanol treated animals, there was a significantly greater amount of *Sgk1* promoter region bound to the GR antibody versus

the IgG antibody. Therefore, we surmise that *Sgk1* induction in PFC following acute ethanol administration is due to activation of the HPA axis and the subsequent binding of corticosterone-activated GR to the GRE of the *Sgk1* promoter.

SGK1 phospho S422, SGK1 phospho T256, NDRG1 phospho S330, and SGK1 Levels following saline and ethanol administration

Following our characterization of *Sgk1*, we also wanted to explore what was occurring at the protein level. We performed a time course analysis of SGK1 phospho S422 and SGK1 protein levels between ethanol versus saline treated animals 15 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours following 4 g/kg ethanol. We found no significant differences in SGK1 phospho S422 levels in saline versus ethanol treated animals 1 hour, 2 hours, 4 hours, 8 hours and 24 hours following drug treatment (**Fig. 19a-f**). 15 minutes following ethanol administration we found significant increases in SGK1 phospho S422 and SGK1 phospho T256 in ethanol versus saline treated animals (student's t-test, $p < 0.05$) (**Fig. 20a-d**). This indicates that SGK1 is phosphorylated at both S422 and T256 as early as 15 minutes following ethanol, but not saline administration. Because NDRG1 is a downstream target of phosphorylated, active SGK1 we also evaluated NDRG1 phospho S330 levels in ethanol vs. saline treated animals 15 minutes following ethanol administration. We found significant increases in NDRG1 phospho S330 in ethanol versus saline treated animals (student's t-test, $p < 0.05$) (**Fig. 21a-b**).

We found no significant differences in SGK1 levels between saline and ethanol treated animals 15 minutes, 1 hour, 2 hours, 4 hours and 24 hours following drug

treatment (**Fig. 22a-f**). 8 hours following ethanol administration we found significant decreases in SGK1 in ethanol versus saline treated animals (student's t-test, $p < 0.05$) (**Fig. 23a-b**). This indicates that SGK1 is significantly decreased 8 hours following ethanol administration.

DISCUSSION

Prior studies in our laboratory and others suggest that glucocorticoid signaling might play an important role in both behavioral and brain gene expression responses to acute ethanol (Kerns et al., 2005a, Costin et al., 2012, Piechota et al., 2010b). For example, using genomic studies we previously identified a significant over-representation of glucocorticoid-responsive genes, including *Sgk1*, among those responding to acute ethanol in prefrontal cortex (Kerns et al., 2005a). More recent studies from our laboratory showed that inhibition of glucocorticoid signaling by adrenalectomy or the glucocorticoid antagonist RU486, both impaired acute ethanol-induced locomotor activation, suggesting that glucocorticoid signaling may play a role in this behavioral response (Costin et al., 2012). Further, ADX altered the basal expression of genes in PFC that included a significant number of previously identified ethanol responsive genes (Costin et al., 2012). Although there are other conflicting reports about the role of glucocorticoids in ethanol behaviors, recent studies elegantly document that blockade of glucocorticoid signaling severely impairs escalated ethanol consumption in an animal model of progressive ethanol intake (Vendruscolo et al., 2012). Furthermore, HPA axis dysregulation exists in alcohol dependent patients and individuals with a familial history of alcoholism (Sorocco et al., 2006, Dai et al., 2002). Thus, disrupted HPA

axis/glucocorticoid signaling may contribute to the risk for development of alcohol dependence. In this report, we have provided mechanistic studies confirming that ethanol modulates brain (PFC) gene expression, in part, through glucocorticoid signaling and that alterations in this gene expression regulatory loop occur with chronic exposure. We propose that ethanol modulation of brain glucocorticoid-responsive gene expression acutely could contribute to the adaptive mechanisms leading to behavioral responses seen with chronic ethanol, such as sensitization, tolerance and addiction (Kerns et al., 2005a, Wolen et al., 2012, Farris and Miles, 2012).

Our prior work identified an important role for glucocorticoid signaling in ethanol-responsive gene expression (Costin et al., 2012). ADX altered the expression of genes in the PFC that included a significant number of previously identified ethanol responsive genes (Costin et al., 2012). Interestingly, *Sgk1* wasn't basally regulated in ADX mice in these studies, although we latter showed that *Sgk1* also wasn't induced following ethanol administration in ADX animals (Fig. 17a) indicating that it is, in fact, a glucocorticoid responsive, ethanol responsive gene. *Sgk1* was first identified in a screen of glucocorticoid responsive genes and since then our lab and others have consistently identified it as a glucocorticoid responsive, ethanol responsive gene (Webster et al., 1993, Piechota et al., 2010b, Kerns et al., 2005a). However, it has also been identified as being transcriptionally controlled by a wide variety of additional hormones and regulators including the increase of cytosolic Ca^{2+} activity and NO, transforming growth factor β , interleukin 6, thrombin, endothelin, cell shrinkage, and Rett syndrome, to name a few (Lang et al., 2010, Meng et al., 2005, BelAiba et al., 2006, Wolf et al., 2006, Chen et al., 2009, Nuber et al., 2005). *Sgk1* is a gene that is highly sensitive to multiple

environmental stimuli and therefore it may not have been blunted basally in ADX mice, but following ethanol administration and robust HPA axis activation, *Sgk1* induction in SHAM mice, and lack thereof in ADX mice became apparent (Fig. 17b).

Prior studies in our laboratory and others identified the glucocorticoid-regulated gene *Sgk1* as an ethanol-responsive gene (Kerns et al., 2005a, Treadwell and Singh, 2004, Piechota et al., 2010b). Other investigators have described *Sgk1* regulation following ethanol administration in whole brain (Treadwell and Singh, 2004) and in the striatum (Piechota et al., 2010b), but not in the PFC—a region known to be important in glucocorticoid signaling (Mizoguchi et al., 2003). Here we performed a rigorous analysis of *Sgk1* regulation in PFC by ethanol. Although *Sgk1* is the predominant transcript from the gene, an alternative promoter site produces the transcript *Sgk1.1* that actually codes for a more stable form of the Sgk protein (Arteaga et al., 2008). However, ethanol only regulated expression of the *Sgk1* form (Fig. 14a), which is modulated by a glucocorticoid response element (GRE) in its promoter (Arteaga et al., 2008, Webster et al., 1993). We show for the first time that ethanol regulation of *Sgk1* occurred via evoked glucocorticoid signaling since adrenalectomy blocked ethanol induction of *Sgk1* expression (Fig. 17a) and ethanol increased occupancy of glucocorticoid receptor binding to the *Sgk1* promoter in ChIP assays (Fig. 18). Ethanol regulation of *Sgk1* transcription in prefrontal cortex is thus part of a HPA-PFC regulatory loop that could have an important role in modulating ethanol behaviors. PFC is known to have high concentrations of glucocorticoid receptors and to modulate HPA activity (Mizoguchi et al., 2003). PFC is also an integral part of the mesolimbocortical dopamine pathway that is involved in the rewarding properties of ethanol and other drugs of abuse. PFC is thus thought to contribute to the known

interaction between stress and ethanol consumption.

Ethanol regulation of *Sgk1* versus *Sgk1.1* may have functional implications since the two isoforms have been described as having different downstream targets. Sgk1 stimulates K⁺ channel activity and regulates the function and availability of the epithelial sodium channel (ENaC) in addition to its regulation of other targets (Gamper et al., 2002, Wang et al., 2001, Alvarez de la Rosa et al., 1999). Sgk1.1 is a brain specific isoform of Sgk1 that has been shown to modulate the function of the acid-sensing ion channel-1, the δ ENaC and M-current (Arteaga et al., 2008, Wesch et al., 2010, Miranda et al., 2013). Interestingly, *Sgk1.1* did show significant increases in both saline and ethanol treated animals at 8 hours (Fig 14b), perhaps reflecting regulation of this isoform by mechanisms responding to our overall experimental manipulation, rather than ethanol treatment per se.

Following repeated ethanol treatment to produce locomotor sensitization, Sgk1 and serum corticosterone levels no longer responded to ethanol treatment (Figs. 15b, 16b). This provides further confirmation of a role for glucocorticoid signaling in ethanol regulation of *Sgk1* and suggests that by dampening of the HPA response to ethanol, a network of glucocorticoid-responsive genes in PFC has undergone an adaptive response to chronic ethanol exposure. This could have functional consequences for behavioral responses to chronic ethanol although this will require the proof of future studies.

Other investigators have shown a similar trend in corticosterone levels following chronic ethanol administration; as corticosterone levels are blunted in male rats administered ethanol following chronic operant self-administration, chronic intragastric ethanol administration and rats and mice following chronic i.p. injection (Richardson et al., 2008, Lee and Rivier, 1997, Spencer and McEwen, 1990, Roberts et al., 1995).

However, this is the first work relating HPA axis dampening (or “tolerance”) to changes in PFC gene expression. Although other explanations exist for lack of ethanol-responsiveness in *Sgk1* following chronic ethanol treatment, such as decreased glucocorticoid receptor (GR) expression in PFC, the dampened corticosterone response to ethanol (Fig. 16) and ChIP results showing increased GR binding to the *Sgk1* promoter with acute ethanol (Fig. 18) are strong evidence linking the diminished *Sgk1* response to corticosterone levels. Diminished GR (*Nr3c1*) expression has been seen in the rat PFC following chronic ethanol exposure (Vendruscolo et al., 2012) but our Q-rtPCR results showed no significant changes in *Nr3c1* levels in the PFC of SS, SE vs EE treated animals (Fig 15c). Figure 15c actually showed a trend for increased *Nr3c1* expression in ethanol sensitized animals, perhaps in compensation to dampened HPA tone. Although we did not measure blood ethanol concentrations (BECs) in our studies, prior studies with chronic ethanol showed corticosterone levels were blunted despite elevated BECs (Richardson et al., 2008, Lee and Rivier, 1997). Of possible mechanistic importance, Roberts et al. reported that co-treatment with the glucocorticoid antagonist RU486 blocked the decrement in corticosterone during repeated ethanol treatment for sensitization (Roberts et al., 1995). RU486 also partially blocked ethanol locomotor sensitization in those experiments, although our prior work has not replicated this finding possible due to methodological differences (Costin et al., 2012).

These studies also identified ethanol actions on *Sgk1* expression at the level of protein expression and post-translational modification. The activation of SGK1 is triggered first by the phosphorylation of S422 lying within the C-terminal hydrophobic motif of *Sgk1* followed by the phosphorylation of a threonine residue within

the T-loop of the kinase domain (Firestone et al., 2003). Mammalian target of rapamycin (mTORC) was recently identified as the kinase that phosphorylates SGK1 at Ser422, but there is debate as to whether mTOR complex 1 or 2 is responsible for this phosphorylation (Garcia-Martinez and Alessi, 2008, Hong et al., 2008). We found that phosphorylation of SGK1 at S422 and T256 is transiently increased at 15 minutes following ethanol administration (Fig 20) but that total SGK1 protein abundance is significantly decreased as early as 6 hours (data not shown) and as late as 8 hours (Fig. 23) following ethanol administration. This is somewhat paradoxical since *Sgk1* mRNA abundance is increased at 2-4 hours after ethanol treatment (Fig. 14a). The transient increase in SGK1 Ser422 phosphorylation appears to be functional in that there is concomitant phosphorylation of the known Sgk1 substrate protein, NDRG1 (Fig. 21) (Murray et al., 2004).

This study is the first description for this complex ethanol regulation of *Sgk1* mRNA, protein and phosphorylation. Similar to our findings here, Piechota et al. showed *Sgk1* mRNA is significantly increased 2 hours following ethanol, morphine, heroin and methamphetamine administration and that SGK1 protein is significantly decreased 4 hours following morphine administration in the striatum. But those investigators did not study SGK1 phosphorylation or protein regulation following ethanol administration (Piechota et al., 2010b). It is possible that ethanol triggers a complex wave of signaling events leading to: 1) SGK1 activation by phosphorylation with subsequent phosphorylation of NDRG1 and other targets; 2) increased Sgk1 transcription by HPA activation and glucocorticoid action; and 3) compensatory SGK1 protein degradation. Of note, Miyata et al. showed that a chronic stress paradigm that elevated plasma

corticosterone levels similar to those found in depressed individuals lead to activation of the phosphatidylinositol 3-kinase (PI3K)-3-phosphoinositide-dependent protein kinase (PDK1), Sgk1, and Ndrp1 pathway with increases in both *Sgk1* mRNA and SGK1 phosphorylation (Miyata et al., 2011). Thus it is possible that HPA axis activation is causal in both Sgk1 activation and increased transcription of the Sgk1 gene.

Table 3: Outline of experimental design for ethanol sensitization

Group	Days 1-2	Days 3-13	Day 14
	Habituation	Conditioning	Activity Test
SS	Saline	Saline	Saline
SE	Saline	Saline	Ethanol 2.0 g/kg
EE	Saline	Ethanol 2.5 g/kg	Ethanol 2.0 g/kg
ES	Saline	Ethanol 2.5 g/kg	Saline

Table 3. Sensitization protocol.

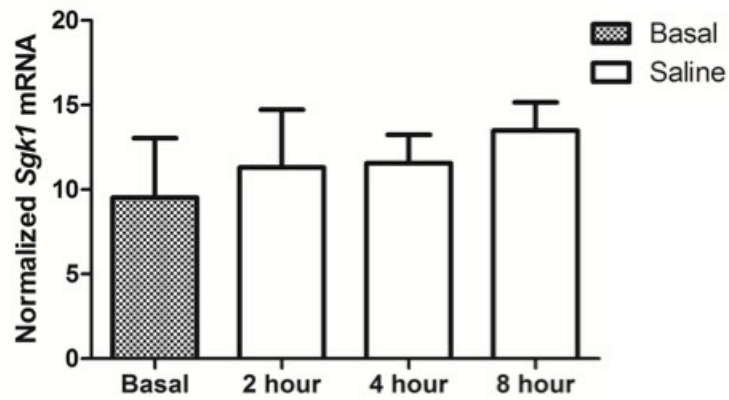


Figure 13. Sgk1 levels in the PFC of D2 mice basally (a 0 hour time point) and 2, 4 and 8 hours following saline injections. Saline injections did not significantly alter *Sgk1* levels at any time point compared to basal *Sgk1* levels.

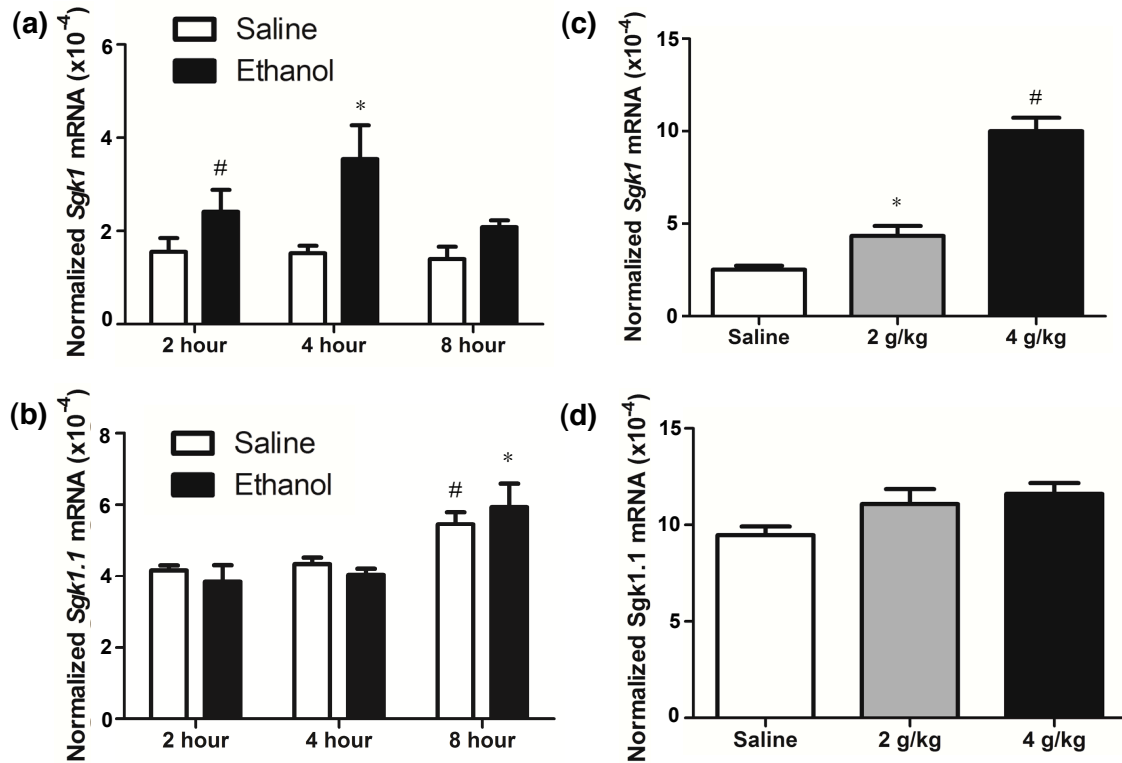


Figure 14. Acute ethanol time course and dose response analysis for *Sgk1* and *Sgk1.1* expression. Q-rtPCR analysis of *Sgk1* and *Sgk1.1*. Panels show: (a) *Sgk1* following 4 g/kg ethanol administration. * $p < 0.05$ versus all saline treated animals and 8 hour ethanol group, # $p < 0.05$ versus 8 hour saline group; (b) *Sgk1.1* levels following 4 g/kg ethanol administration. * $p < 0.05$ versus ethanol and saline animals at 2 or 4 hours, # $p < 0.05$ versus 2 hour ethanol group; (c) Ethanol dose response for *Sgk1* at 4 hours. * $p < 0.05$ versus saline treated animals, # $p < 0.05$ versus ethanol and saline treated animals (d) *Sgk1.1* ethanol dose response at 4 hours.

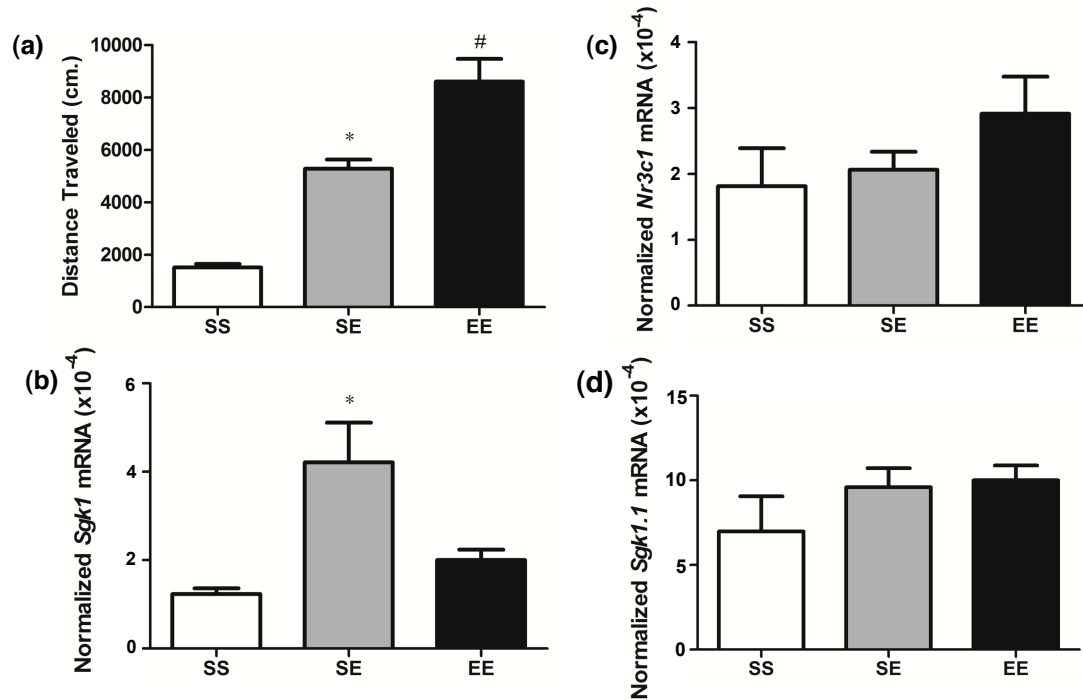


Figure 15. *Sgk1* mRNA expression following ethanol sensitization. Behavioral sensitization followed by Q-rtPCR analysis of *Sgk1* and *Nr3c1*. Panels show: (a) Total locomotor activity (cm/10min.) for saline only (SS), acute ethanol (SE) and ethanol sensitized (EE) groups. * $p < 0.05$ versus chronic saline (SS), # $p < 0.05$ versus acute ethanol (SE) (b) *Sgk1* levels in SS, SE and EE treated mice 4 hours following following saline (SS) or ethanol (SE, EE) treatment on day 14. * $p < 0.05$ versus SS and EE treated animals (c) *Nr3c1* levels in SS, SE and EE treated mice as in panel b. (d) *Sgk1.1* levels in SS, SE and EE treated mice as in panel b.

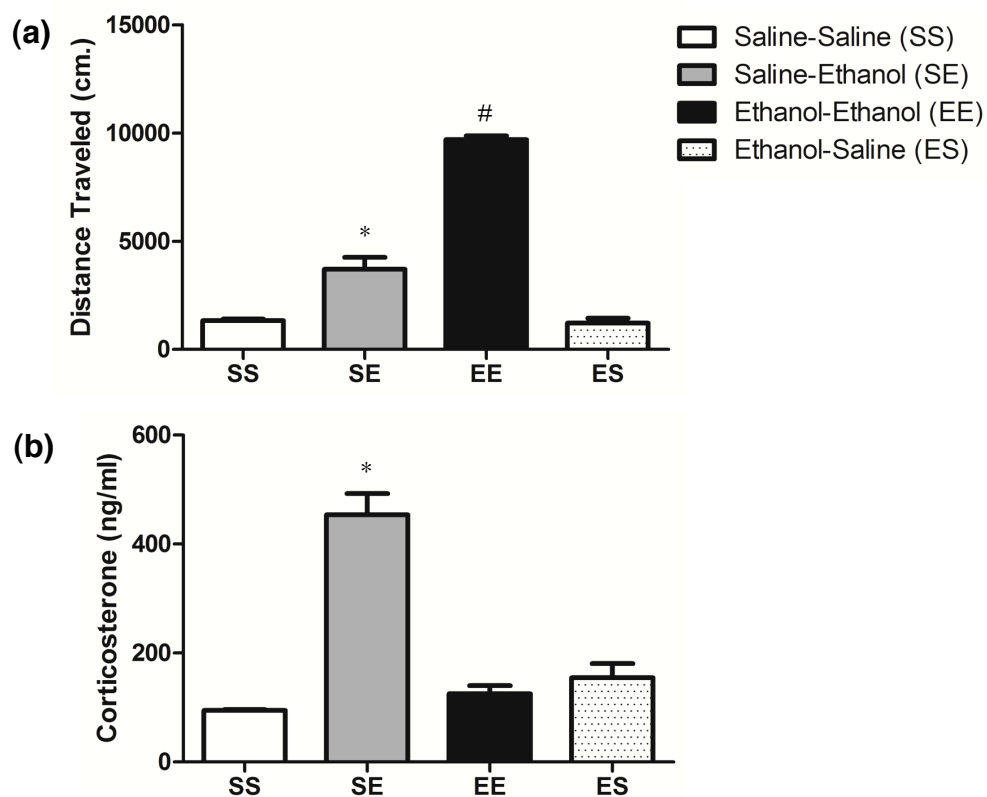


Figure 16. Serum corticosterone levels following ethanol sensitization. Behavioral sensitization followed by corticosterone quantification. Panels show: (a) Acute and sensitized locomotor response (cm/10 min.) following saline (SS, ES) or ethanol (EE, SE) administration. * $p < 0.05$ versus SS and ES groups, # $p < 0.05$ versus SE, ES and SS groups; (b) Corticosterone levels 1 hour following acute and chronic ethanol administration. * $p < 0.05$ versus SS, EE, and ES groups.

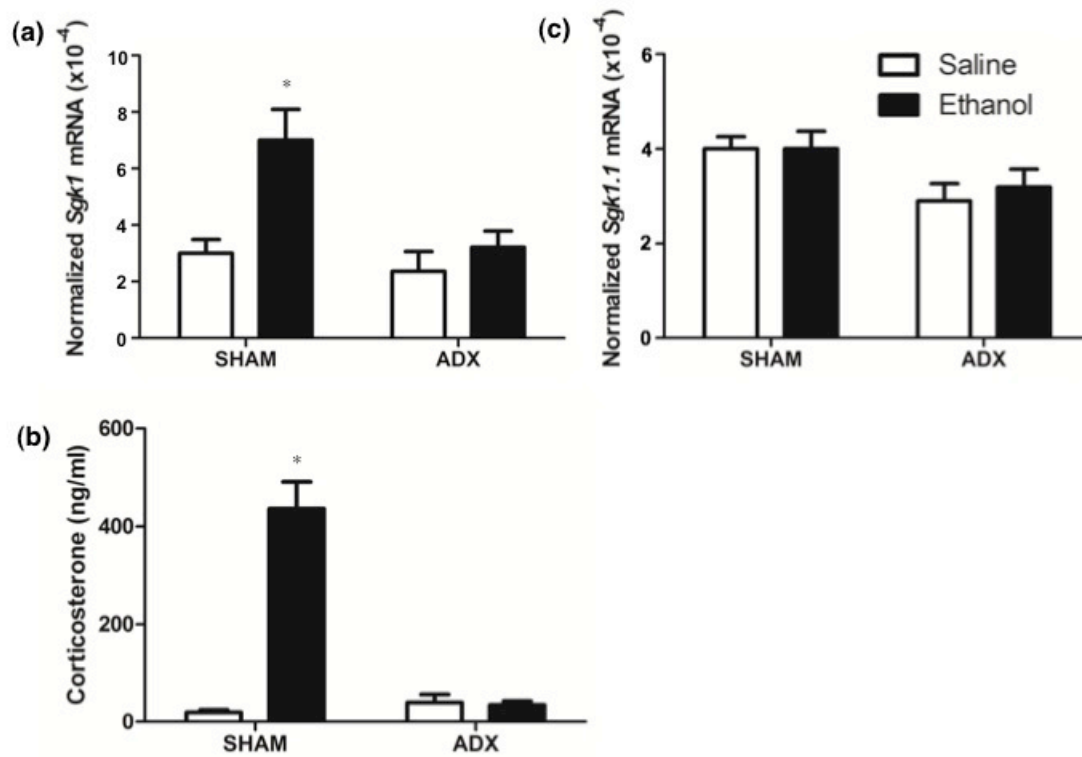


Figure 17. Effects of adrenalectomy on *Sgk1* induction following ethanol administration. (a) Q-rtPCR analysis of *Sgk1*. *Sgk1* in saline and ethanol treated SHAM versus ADX animals. * $p < 0.05$ versus saline treated SHAM animals and saline and ethanol treated ADX animals; (b) Corticosterone levels 1 hour following acute ethanol administration. * $p < 0.05$ versus saline treated SHAM animals and saline and ethanol treated ADX animals. (c) Q-rtPCR analysis of *Sgk1.1*. *Sgk1.1* in saline and ethanol treated SHAM versus ADX animals.

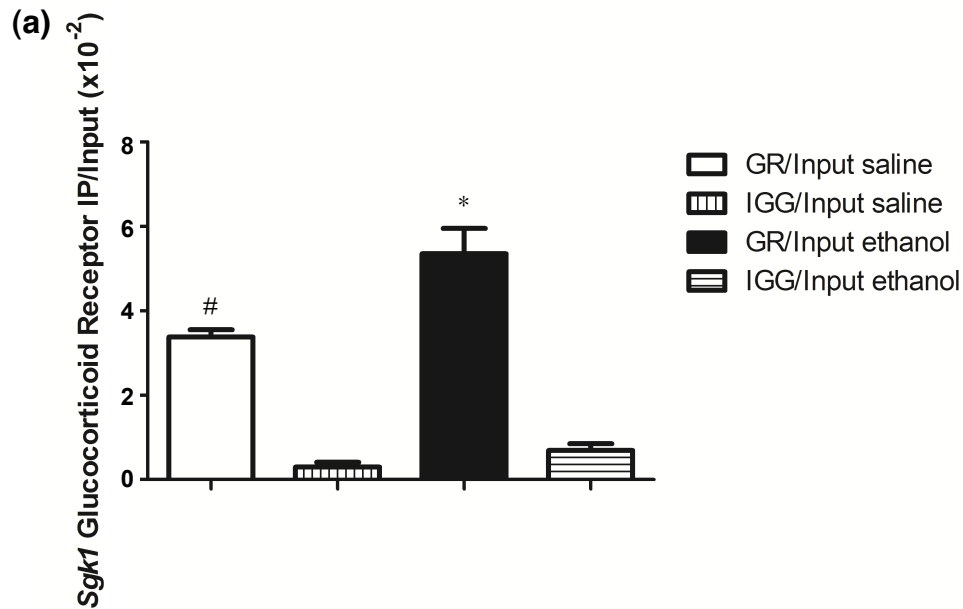


Figure 18. Chromatin immunoprecipitation (ChIP) quantification of glucocorticoid receptor (GR) bound to *Sgk1* promoter region. DBA2/J mice were treated with saline or ethanol (4 g/kg) by i.p. injection and chromatin isolated 1 hour later from a frontal pole dissection. *Sgk1* promoter DNA was quantified by PCR following immunoprecipitation by control IgG or GR antibody. * $p < 0.05$ versus GR saline treated samples and IgG saline and ethanol treated samples, # $p < 0.05$ versus IgG saline and ethanol treated samples

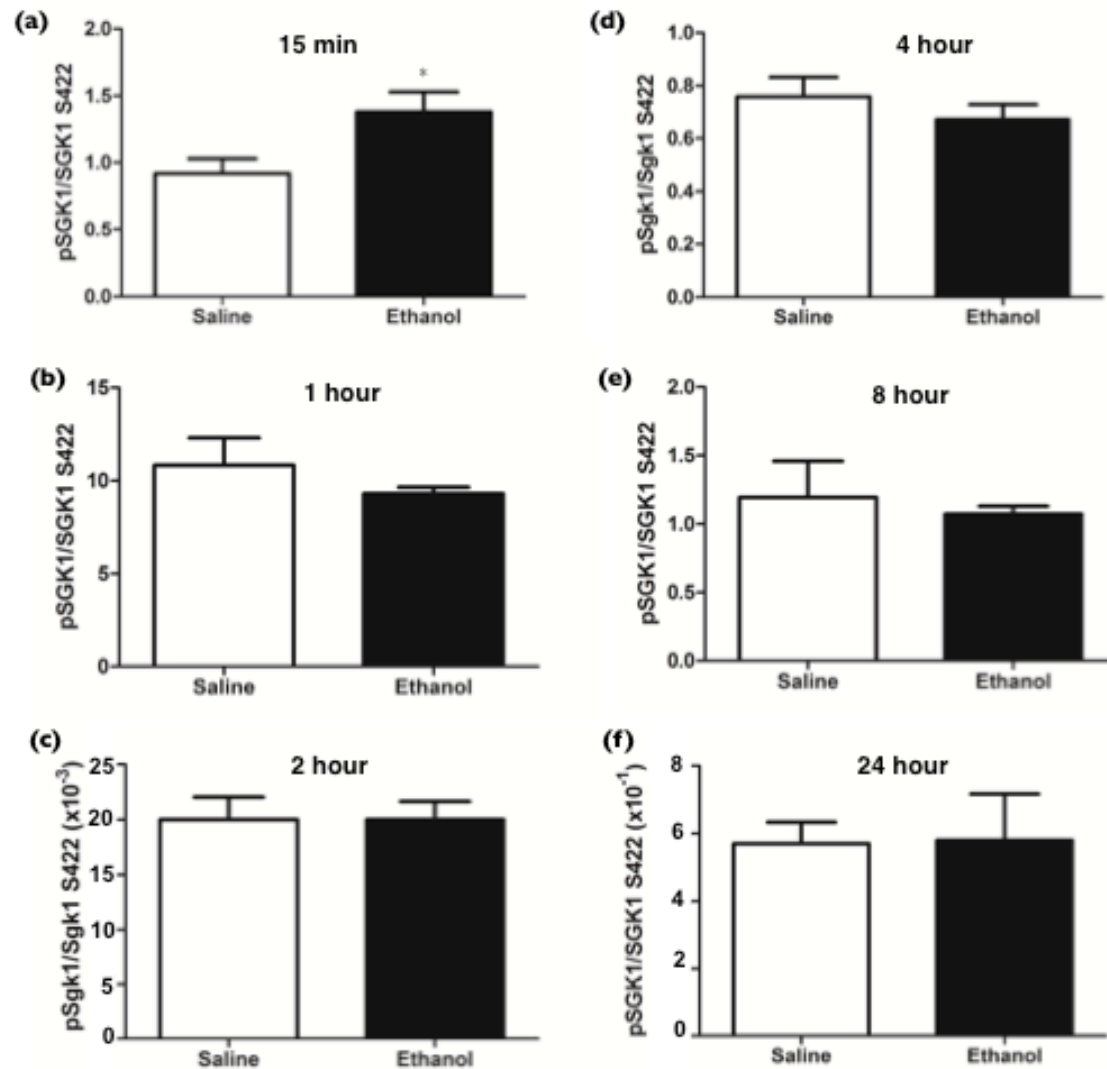


Figure 19. Time Course Western blot analysis of pSGK1 S422. pSGK1 S422 was significantly increased 15 minutes following ethanol versus saline administration (a). There were no significant changes in pSGK1 S422 levels at any other time point (b-f). * $p < 0.05$ versus saline treated animals

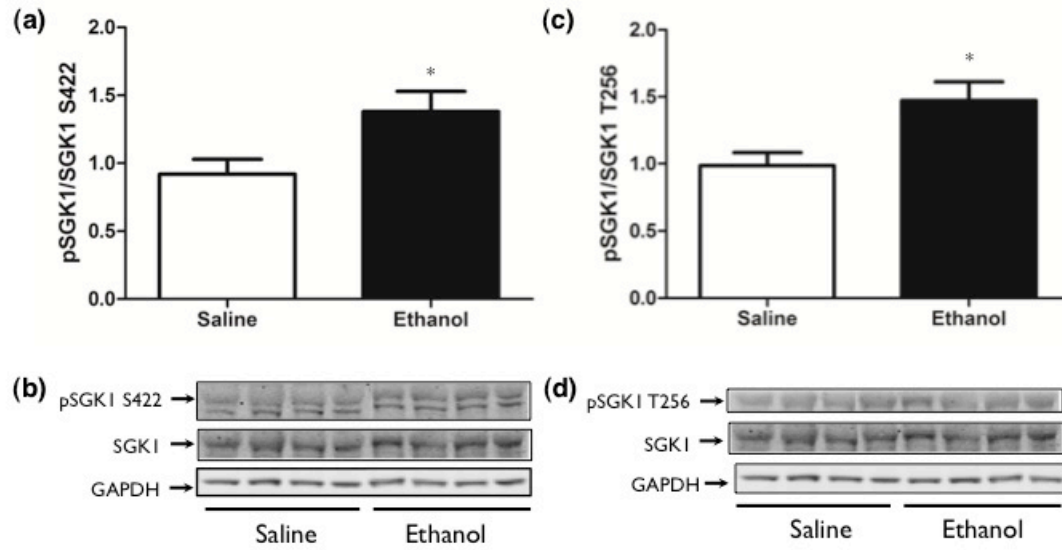


Figure 20. pSGK1 following saline and ethanol administration. Western blot analysis of pS422 SGK1 and pT256 SGK1 at 15 minutes following ethanol (4g/kg) or saline treatment. Panels show: (a) Quantification of pS422 SGK1, (b) Representative Western blot, (c) Quantification of pT256 SGK1, and (d) Representative Western blot. * $p < 0.05$ versus saline treated animals

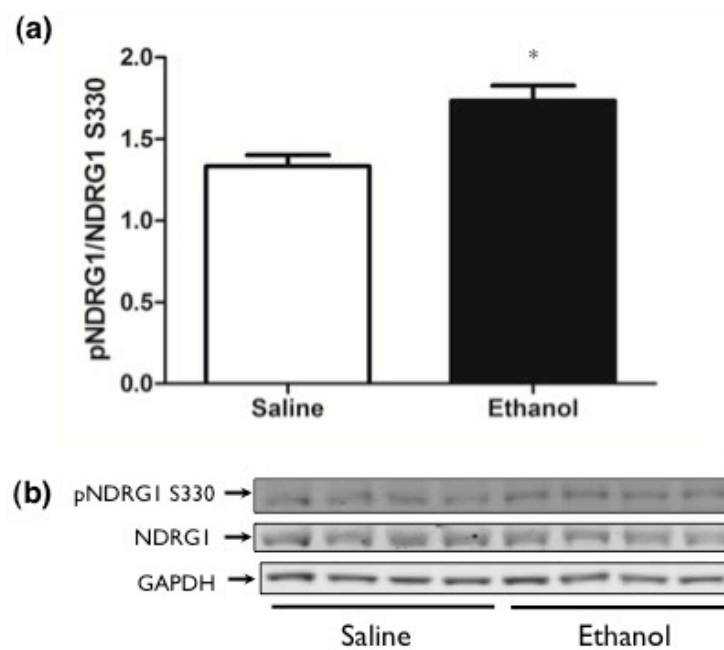


Figure 21. NDRG1 phospho S330 following ethanol administration. Western blot analysis of pS330 NDRG1 15 minutes following ethanol (4g/kg) or saline treatment. Panels show: (a) Quantification of pS330 NDRG1, (b) Representative Western blot. * $p < 0.05$ versus saline treated animals

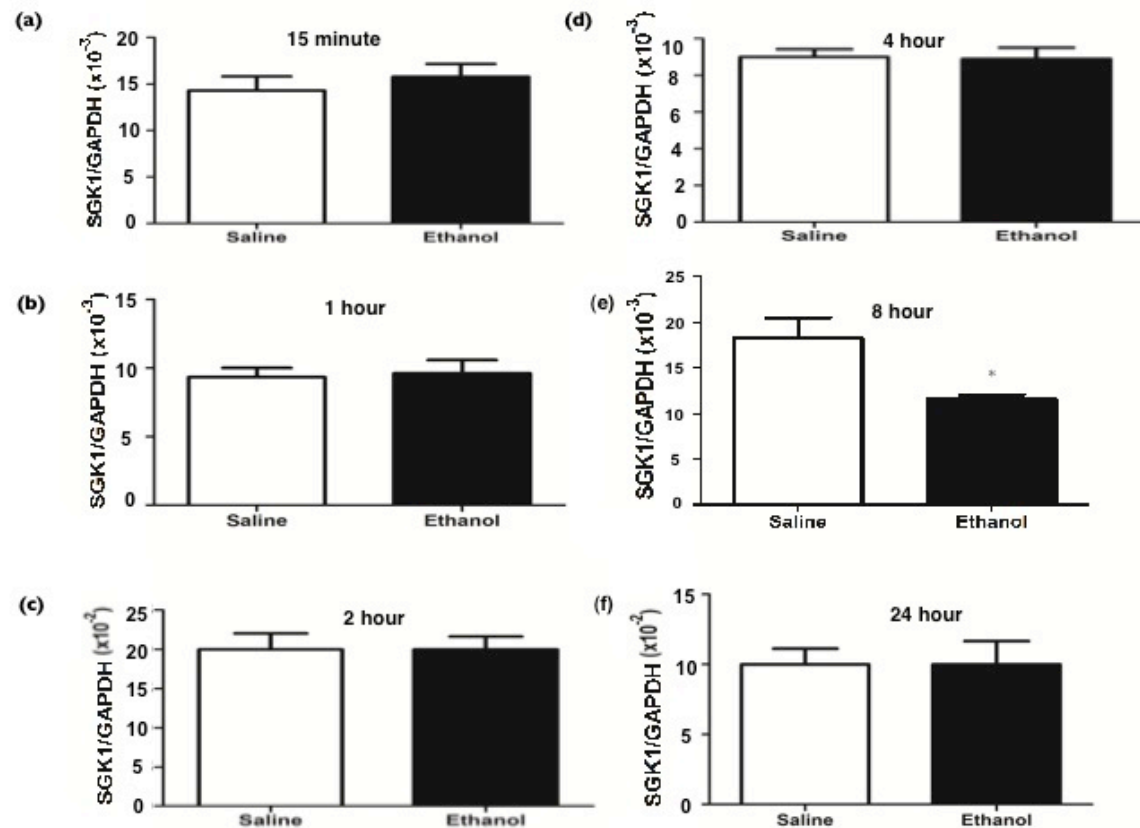


Figure 22. Time Course Western blot analysis of total SGK1. SGK1 was significantly decreased 8 hours following ethanol versus saline administration (e). There were no significant changes in SGK1 levels at any other time point (a-d, f). * $p < 0.05$ versus saline treated animals

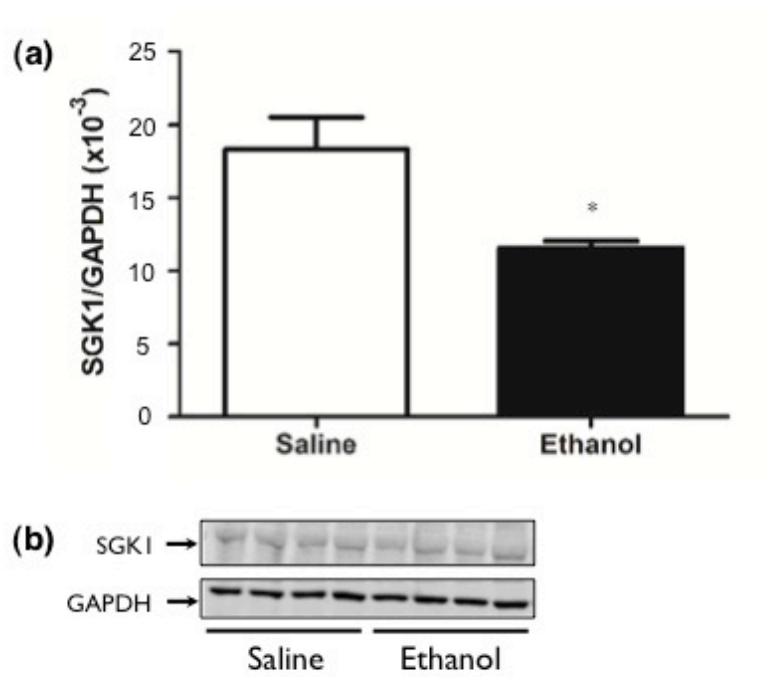


Figure 23. Total SGK1 protein following ethanol administration. Western blot analysis of SGK1 8 hours following ethanol (4g/kg) or saline treatment. Panels show: (a) Quantification of SGK1, (b) Representative Western blot. * $p < 0.05$ versus saline treated animals

CHAPTER 6

Viral Overexpression of Serum Glucocorticoid Kinase I and Behavioral Analysis

Introduction

Serum glucocorticoid kinase I (Sgk1) is a glucocorticoid responsive gene involved in synaptic plasticity and learning and memory that is known to regulate the function of ion channels and allow for the convergence of cell surface receptors, nuclear receptors, and cellular stress pathways (Lee et al., 2006, Ma et al., 2006, Tsai et al., 2002, Webster et al., 1993). Our laboratory previously showed that *Sgk1*, along with several other glucocorticoid responsive genes, was upregulated in the prefrontal cortex (PFC) of DBA/2J (D2) mice following acute ethanol exposure (Kerns et al., 2005a). Other investigators have also shown Sgk1 induction in brain following acute ethanol (Treadwell and Singh, 2004, Piechota et al., 2010b), morphine (Piechota et al., 2010b), heroin (Piechota et al., 2010b), methamphetamine (Piechota et al., 2010b) or amphetamine (Gonzalez-Nicolini and McGinty, 2002).

Sgk1 was first identified as a glucocorticoid responsive gene with a glucocorticoid response element (GRE) in its promoter (Webster et al., 1993). Since then, studies have shown that Sgk1 is involved in memory consolidation of spatial learning and synaptic plasticity in the brain and spinal chord (Lee et al., 2006, Tsai et al., 2002, Lee and Rivier, 1997, Ma et al., 2006, Geranton et al., 2007). Our recent work shows that Sgk1 is regulated transcriptionally and translationally by the glucocorticoid receptor binding to the GRE in the Sgk1 promoter following ethanol's activation of the HPA axis and subsequent glucocorticoid release. In addition following ethanol administration, Sgk1 is also regulated post-translationally as 15 minutes following ethanol administration,

SGK1 phosphorylation at S422 and T256, sites that are known to activate the kinase, is significantly increased (Park et al., 1999, Kobayashi et al., 1999a, Perrotti et al., 2001). Our prior results showed a complex regulation of Sgk1 transcription, protein abundance and post-translational modifications following ethanol treatment. They suggested a critical role for the HPA axis and Sgk1 in regulating acute and chronic cellular and behavioral responses to ethanol. In this work, we further explore how Sgk1 and the Sgk1 gene network may regulate behavioral responses to ethanol, in particular ethanol sensitization.

Behavioral sensitization, or the potentiation of drug-induced behavioral responses following repeated exposure, involves learning and memory processes, is mediated by the mesocorticolimbic dopamine pathway including the PFC, and represents a long-lasting change in the behavioral response to drugs (Lessov and Phillips, 2003). Sensitization can be separated into two phases, induction and expression. The initiation of behavioral sensitization includes the sequence of cellular events that leads to enduring changes in neural function. The expression of behavioral sensitization refers to the enduring neural alterations that occur following repeated drug administration (Harrison and Nobrega, 2009b). The initiation and expression of behavioral sensitization can be influenced by contextual cues surrounding drug administration suggesting that learning and memory processes influence the development of the sensitized state (Quadros et al., 2003). Some investigators have suggested that the neuroadaptations underlying behavioral sensitization are similar to those mediating learning and memory processes and that sensitization reflects a sensitized activation of brain reward systems in the mesocorticolimbic dopamine pathway (Trujillo and Akil, 1995, Wise and Bozarth,

1987). In support of this, lesions of the PFC prevented the expression of behavioral sensitization to amphetamine or cocaine (Cador et al., 1999, Pierce et al., 1998).

The role of the PFC has been less studied in ethanol sensitization and this is not surprising as ethanol sensitization has been less studied than sensitization to other drugs of abuse including cocaine and amphetamine (Phillips et al., 1997a). It is thought that sensitization across different classes of drugs may have common mechanisms as cross-sensitization, wherein pretreatment with one drug results in the sensitized response to another, has been observed between drugs of abuse including ethanol, morphine and cocaine (Lessov and Phillips, 2003). Because of Sgk1's regulation in the PFC following ethanol administration, its known role learning and memory, and its complex regulation following acute and chronic ethanol administration, we hypothesized that Sgk1 may play an role in modulating the sensitized response to ethanol.

MATERIALS AND METHODS

Animals

Mice were maintained in a temperature-controlled room ($23\pm1^{\circ}\text{C}$) with 12 h light/dark cycles and free access to standard chow (Harlan Teklad #7912, Madison, WI, United States) and water. Cages and bedding (Harlan Sani-chips, #7090A, Harlan, Teklad, Madison, WI, United States) were changed weekly. All tests were carried out between 0900 and 1200 h. All mice were DBA2/J mice from Jackson Laboratories (Bar Harbor, ME, United States) purchased at 6-7 weeks of age and group housed 4/cage.

Drugs

All drugs were administered intraperitoneally (i.p.). Saline solutions were 0.9% w/v

sterile saline. Ethanol solutions were prepared from 200-proof absolute anhydrous ethanol (Pharmco-Aaper brand, Brookfield, CT). Ethanol was administered at 20% v/v in 0.9% saline.

Viral Preparation

All viral work was completed under biosafety level 2 conditions. FLAG-Sgk1-AAV plasmids were created by Dr. Sajida Rahman using standard cloning procedures. In brief, Sgk1 cDNA was cloned in frame with a 3X FLAG epitope tag into pAAV-IRES-hrGFP Vector (AAV Helper-Free System, Agilent). The recombinant AAV2 viral vectors were produced in 293 cells using three-plasmid cotransfection and purified following previously published methods (Zolotukhin et al., 1999) by the Vector Core Facility, Gene Therapy Center, University of North Carolina at Chapel Hill.

Stereotaxic Microinjection

24 hours prior to and 48 hours following stereotaxic microinjection, mice were provided children's Motrin (30 mg/kg) in tap water for analgesia. Mice (n = 48, 24 animals microinjected with an AAV-2 virus overexpressing Sgk1, FLAG-SGK1-AAV, and 24 animals microinjected with an empty vector AAV-2 virus, IRES-AAV) were anesthetized using Isoflurane and secured in a stereotaxic apparatus (myNeuroLab). They then received bilateral stereotaxic microinjections at a 10° angle of 1µl of AAV per injection site (2 µl total) into the anterior cingulate region of the PFC (+1 mm from Bregma, ± 0.6 mm from midline and -2 mm ventral to pial surface). 0.1 µl of virus was injected per minute over a 10 minute time period and the injection needle was left in place for 10 minutes following injection to avoid the backflow of virus.

Sensitization of animals overexpressing FLAG-SGK1-AAV versus IRES-AAV

injected control animals

Following microinjection, mice were allowed 3 weeks for recovery and viral expression and then tested for locomotor activation and sensitization. All locomotor activity was measured immediately following injection with either saline or ethanol, during a 10-minute session in locomotor activity chambers (Med-Associates, model ENV-515; St. Albans, VT, United States). All mice were allowed a 1-hour acclimation period to the behavioral room prior to testing. Mice were divided into 4 groups: saline treated IRES and Sgk1 mice and ethanol treated IRES and Sgk1 mice, $n = 7-12$ per group. Saline treated mice received only saline injections and ethanol treated mice received only ethanol injections. On days 1 and 2, all mice were habituated to saline injections and immediately placed in locomotor boxes for 10 minutes following injections. On test day 3, 2 g/kg ethanol or saline was administered to mice and they were placed in activity chambers immediately following drug administration for 10 minutes to record the animal's acute response to ethanol. On days 4-13 mice received daily injections of either 2.5 g/kg ethanol or saline in their home cages. On test day 14, animals are once again given 2 g/kg ethanol or saline and locomotor activity documented to record the expression phase of sensitization. Following test day 14, 3 abstinence periods of 7 days are followed by activity testing on days 21, 28 and 35 identical to day 14 to record enduring neural alterations, which characterize the sensitized state (**Table 4**, summary of experimental design).

Immunohistochemistry

Following behavioral testing, viral expression and placement were examined. Animals were be deeply anesthetized with sodium pentobarbital (180 mg/kg i.p.) and perfused

transcardially with normal saline followed by 4% paraformaldehyde. Their brains were removed and post-fixed overnight in 4% paraformaldehyde, cryoprotected in 30% w/v sucrose until they sank, and frozen using dry ice cooled 2-methylbutane. 25 micron-thick coronal sections were cut using a cryostat and viral placement was measured using immunohistochemistry through 3,3'-Diaminobenzidine (DAB) anti-FLAG (Cell Signaling) staining. DAB staining was performed using Vector's DAB Substrate Kit (Vector Labs) per the manufacturer's instructions.

Statistics

Data were expressed as mean \pm SEM and analyzed parametrically. Data were analyzed with analysis of variance (ANOVA) using appropriate between-and within subject factors. All post hoc comparisons were made using Student Newman-Keul's test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Sensitization of animals overexpressing FLAG-SGK1-AAV versus IRES-AAV injected control animals

A three-way mixed model repeated measures ANOVA (day x treatment x genotype) showed a significant effect of day ($F_{3,106} = 61.0, p < 0.001$) and treatment ($F_{1,38} = 800.7, p < 0.001$) as well as a significant day x treatment interaction ($F_{3,106} = 57.9, p < 0.001$). Additionally, a three-way mixed model repeated measures ANOVA (day x treatment x genotype) comparing only the expression phase of sensitization where ethanol was no longer being chronically administered showed a significant effect of day ($F_{2,57} = 25.6, p < 0.001$), treatment ($F_{1,38} = 538.0, p < 0.001$), a genotype x treatment

interaction ($F_{1,38} = 5.1, p < 0.05$) as well as a significant day x treatment interaction ($F_{2,57} = 39.8, p < 0.001$) (**Fig. 24**). Looking more closely at individual test days, 3, 14, 21, 28, and 35, there was an overall effect of treatment on test days 3, 14 and 28 ($F_{1,38} = 160.3, p < 0.001$; $F_{1,38} = 538.9, p < 0.001$; and $F_{1,38} = 654.6, p < 0.001$, respectively). All animals showed significantly increased locomotor activation responses following ethanol treatment within their respective genotypes on days 3, 14, and 28. On test day 21, there was an overall effect of treatment ($F_{1,38} = 522.8, p < 0.001$) and genotype ($F_{1,38} = 4.6, p < 0.05$) and a significant genotype x treatment interaction ($F_{1,38} = 4.3, p < 0.05$). Post-hoc analysis showed that FLAG-SGK1-AAV versus IRES-AAV injected animals showed a significantly increased locomotor activation response following ethanol administration within their respective genotype. Further and more importantly, post-hoc analysis also identified significant differences between ethanol treated FLAG-SGK1-AAV versus IRES-AAV injected animals, $p = 0.008$, indicating that mice overexpressing FLAG-SGK1-AAV showed a significantly greater locomotor activation response following ethanol administration compared to IRES-AAV animals. On test day 35, there was an overall effect of treatment ($F_{1,38} = 98.7, p < 0.001$) and a significant genotype x treatment interaction ($F_{1,38} = 4.7, p < 0.05$). Post-hoc analysis showed that FLAG-SGK1-AAV versus IRES-AAV injected animals showed a significant locomotor activation response following ethanol administration within their respective genotype. More importantly, post-hoc analysis for day 35 also identified significant differences between ethanol treated FLAG-SGK1-AAV versus IRES-AAV injected animals, $p = 0.037$, indicating that once again mice overexpressing FLAG-SGK1-AAV showed a significantly greater locomotor activation response following ethanol administration compared to IRES-AAV

animals.

Because we were also interested in testing the duration of the sensitized response, we also evaluated FLAG-SGK1-AAV versus IRES-AAV injected control animals independently to measure the duration of the sensitized response. A two-way mixed model ANOVA (day x treatment) evaluating the duration of the sensitized response in IRES-AAV mice showed an overall effect of treatment ($F_{1,18} = 363.4, p < 0.001$), day ($F_{3,55} = 35.3, p < 0.001$) and a significant treatment x day interaction ($F_{3,55} = 41.8, p < 0.001$). Post-hoc analysis revealed that all mice showed significant increases in locomotor activity following ethanol administration compared to saline treated animals on every day tested, days 3, 14, 21, 28 and 35. Post-hoc analysis also showed significant differences in locomotor activity between days 3 versus days 14, 21, and 28 indicating that mice showed a robust, sensitized response on days 14, 21 and 28. Locomotor activity was not significantly different on days 14, 21 and 28, but locomotor activity significantly decreased on day 35 to day 3 levels indicating that mice were no longer sensitized on this day.

A two-way mixed model ANOVA (day x treatment) evaluating the duration of the sensitized response in FLAG-SGK1-AAV mice showed an overall effect of treatment ($F_{1,20} = 446.5, p < 0.001$), day ($F_{2,44} = 29.5, p < 0.001$) and a significant treatment x day interaction ($F_{2,44} = 23.7, p < 0.001$). Post-hoc analysis showed that all mice showed significant increases in locomotor activity following ethanol administration compared to saline treated animals on every day tested, days 3, 14, 21, 28 and 35. Post-hoc analysis also showed significant differences in locomotor activity between days 3 versus days 14, 21, 28 and 35 indicating that mice showed a robust, sensitized response on days 14, 21,

28 and 35. Locomotor activity was not significantly different on days 21 and 28, but it did show a significant decline on these days compared to day 14. Although mice remained sensitized on day 35, as indicated by their significantly increased locomotor response on day 35 versus day 3, locomotor activity significantly decreased on day 35 compared to days 14, 21 and 28.

Immunohistochemistry

Viral placement and expression was verified in mice microinjected with FLAG-SGK1-AAV (**Fig. 25**). Mice microinjected with IRES-AAV did not show positive anti-FLAG staining in the PFC whereas those injected with FLAG-SGK1-AAV did (**Fig 25a-b**). Additionally, viral expression in mice microinjected with FLAG-SGK1-AAV appeared specific as there was no positive staining in the most rostral brain regions, staining increased in the PFC moving caudally and then decreased (**Fig 25c-f**).

DISCUSSION

Prior work in our lab showed a complex regulation of Sgk1 transcription, protein abundance and post-translational modifications following ethanol treatment and suggested a critical role for the HPA axis and Sgk1 in regulating acute and chronic cellular and behavioral responses to ethanol (Costin et al., 2012). In this work, we further explore the role of Sgk1 in modulating acute and chronic cellular and behavioral responses to ethanol including locomotor activation and sensitization. Here we suggest that Sgk1 and the Sgk1 gene network may mediate some of the changes in neuronal plasticity that are known to occur following repeated ethanol administration and

eventually lead to the maintenance of the sensitized response.

Ethanol sensitization remains less well characterized than sensitization to the classic psychomotor stimulants such as cocaine, amphetamine and morphine. It is known that sensitization to cocaine, amphetamine and morphine persists as long as 3 months (Shuster et al., 1977), 1 year (Paulson et al., 1991), and 8 months (Babbini et al., 1975) respectively. Lessov and Phillips have shown that ethanol sensitization may last for up to 29 days in outbred female mice (Lessov and Phillips, 1998). Fish et al. found that ethanol sensitization persisted for at least 58 days following cessation of ethanol administration in outbred CFW mice (Fish et al., 2002). Boehm et al. showed that the expression of locomotor sensitization to ethanol in female D2 mice persisted for at least 14 days following cessation of ethanol administration and up to 28 days when repeated ethanol exposure was associated with a specific context (Boehm et al., 2008).

We found that ethanol sensitization persisted 14 days post-ethanol administration in animals overexpressing IRES-AAV, but it persisted 21 days following the cessation of ethanol administration in mice overexpressing FLAG-SGK1-AAV. Our findings that IRES-AAV mice remained sensitized 14 days following the cessation of ethanol administration were similar to those of Boehm et al.. Our sensitization protocol mirrors the protocol used by Boehm et al. to determine the duration of locomotor stimulation without context pairing, except we did not move animals to a separate room for their daily home cage injections during the induction phase of sensitization and we tested animals in the locomotor chambers for 10 minutes rather than 15 minutes. In addition to their context learning and duration of sensitization experiments, Boehm et al. completed a separate experiment in which they had a separate group of mice that underwent

sensitization, but did not receive repeated ethanol injections on post-sensitization days 7, 14 and 21. Rather, animals were divided into separate groups and tested for the duration of ethanol sensitization only once on their respective test day, ie. day 7, 14 or 21 following the cessation of ethanol administration. This group of mice controlled for the effect of repeated ethanol injections on post-sensitization test days which Boehm et al. hypothesized may re-sensitize mice to ethanol. However, Boehm et al. showed that both animals repeatedly administered ethanol over 7 day intervals and those that were not subject to repeated ethanol administrations showed the same duration of sensitization, 14 days following cessation of ethanol administration (Boehm et al., 2008). The only major difference between our experiments and those of Boehm et al. was they used female rather than male D2 mice (Boehm et al., 2008). Interestingly, in addition to being the first study examining locomotor activation and sensitization in mice overexpressing FLAG-SGK1-AAV, it seems our study may also be the first examining the duration of the ethanol sensitized response in male D2 mice.

It is known that locomotor sensitization is thought to occur due to alterations in the neural mechanisms mediating the acute locomotor stimulant response (Ron and Jurd, 2005). These mechanisms become increasingly sensitive to the drug and remain sensitive for extended time periods following the cessation of drug administration. Such long-lasting neuroadaptations may explain how addicted individuals relapse after long periods of abstinence. Still, it isn't clear what mechanisms mediate the sensitized response, particularly ethanol sensitization, and to date only three studies have examined the duration of ethanol sensitization. These studies do not thoroughly examine the mechanism behind the duration of ethanol sensitization (Boehm et al., 2008, Lessov and

Phillips, 1998, Fish et al., 2002). Boehm et al. does suggest contextual pairing can prolong the sensitized response and Fish et al. suggests that sensitization increases aggression in certain mouse populations. The fact that the mechanisms behind the duration of ethanol sensitization have not been explored adds a novel aspect to our studies. We show that mice overexpressing FLAG-SGK1-AAV have significantly increased locomotor activity on day 21 of the experimental protocol, or 7 days following ethanol cessation, compared to IRES-AAV mice. Additionally, FLAG-SGK1-AAV mice show a significantly greater locomotor response compared to IRES-AAV mice on day 35 of sensitization studies, 21 days following ethanol cessation. These findings indicate that overexpressing FLAG-SGK1-AAV may increase the intensity and duration of the sensitized response meaning that Sgk1 may play an important role in the expression phase of the sensitized response. We can use Sgk1 as a starting point to begin to decipher some of the mechanisms behind the expression phase of ethanol sensitization.

One way in which SGK1 may alter the sensitized response is by regulating glutamate receptors. Yuen et al. showed that acute stress induced a potentiation of glutamatergic transmission in the PFC through an SGK1/3-induced increase in the delivery of NMDARs and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors) to the synaptic membrane. More specifically, acute stress activates glucocorticoid receptors which regulate Sgk1/3 expression, SGK1/3 activation of Rab4 increases the trafficking and function of NMDARs and AMPARs, NMDARs and AMPARs are recycled between early endosomes and the plasma membrane, and this process leads to potentiated synaptic transmission (Yuen et al., 2011). Glutamatergic transmission may play a role in mediating the sensitized response, although there have

been alternate findings in the literature. In male D2 and Swiss albino mice, the uncompetitive NMDA receptor antagonist MK-801 blocked the expression of ethanol-induced sensitization (Broadbent et al., 2003, Kotlinska et al., 2006, Camarini et al., 2000). However, in D2 mice Broadbent et al. showed that MK-801 reduced the stimulant effects of ethanol suggesting it is possible that MK-801's ability to block the expression of sensitization may represent a non-specific suppression of locomotor activity (Kotlinska et al., 2006). In male D2 mice, the NR2B selective uncompetitive NMDA antagonist ifenodil did not effect the expression of sensitization and the non-NMDA glutamate receptor antagonists DNQX and GYKI 52466 decreased or blocked the expression of ethanol sensitization, respectively (Kotlinska et al., 2006). But, GYKI 52466 also reduced the locomotor activity of control saline treated animals at the same doses at which it blocked the expression of ethanol sensitization (Broadbent et al., 2003). Still, if NMDA antagonists do block the expression of the sensitized response, perhaps increasing glutamatergic transmission would potentiate the sensitized response. To indirectly determine if FLAG-SGK1-AAV may be extending the duration of the sensitized response through alterations in glutamate receptor levels, we can compare glutamate receptor levels in the brains of FLAG-SGK1-AAV versus IRES-AAV mice. Yuen et al. showed increased surface NMDAR and AMPAR clusters in the PFC following stress.

Our previous studies also showed that *Sgk1* expression is blunted following chronic ethanol administration as seen in ethanol sensitization and this blunting of *Sgk1* expression may be due to HPA axis habituation (Costin unpublished data, 2013). We measured *Sgk1* levels (Fig. 15) and corticosterone levels (Fig. 16) acutely and chronically following ethanol sensitization and found that *Sgk1* and corticosterone were both

increased acutely, but blunted chronically (Costin unpublished data, 2013). Our prior studies also showed that while *Sgk1* is increased as early as 2 hours following ethanol administration (Fig. 14a), total SGK1 protein abundance is significantly decreased 8 hours following ethanol administration (Fig. 23) and we believe that the SGK1 protein degradation is a compensatory response to increases in *Sgk1*. Previously, we also explored ethanol regulation of SGK1 phosphorylation. We found that phosphorylation of SGK1 at S422 and T256, sites well-known to activate the protein, is transiently increased 15 minutes following ethanol administration (Fig. 20, Costin unpublished, 2013). It could be that increases in *Sgk1* followed by SGK1 decreases are necessary to induce some of the plastic changes leading to the sensitized state and when *Sgk1* is overexpressed, as with FLAG-SGK1-AAV, these changes show a longer duration. Our chronic corticosterone and *Sgk1* measures were taken on day 14 of sensitization studies so we do not know when HPA axis and *Sgk1* habituation occurred during the sensitization paradigm, ie. day 5 or day 12 of chronic ethanol administration, and therefore increases in *Sgk1* followed by decreases in SGK1 could be important during the initiation phase of sensitization. Since *Sgk1* is no longer regulated by ethanol during the expression phase of sensitization, we would hypothesize that phosphorylation of SGK1 rather than induction of *Sgk1* followed by decreases in SGK1 is responsible for the increased duration of the sensitized response seen in FLAG-SGK1-AAV mice. Additional studies are necessary to better characterize SGK1 and phospho-SGK1 chronically, particularly during ethanol sensitization.

Table 4: Outline of experimental design for viral ethanol sensitization

Phase of Sensitization		Induction Phase		Expression Phase
Group	Days 1-2	Days 3	Days 4-13	Days 14-35
	Habituation	Acute test	Daily injections	Expression test
SS	Saline	Saline	Saline	Saline
EE	Saline	Ethanol 2 g/kg	Ethanol 2.5 g/kg	Ethanol 2.0 g/kg
Activity test	yes	yes	no	yes

Table 1. Sensitization protocol.

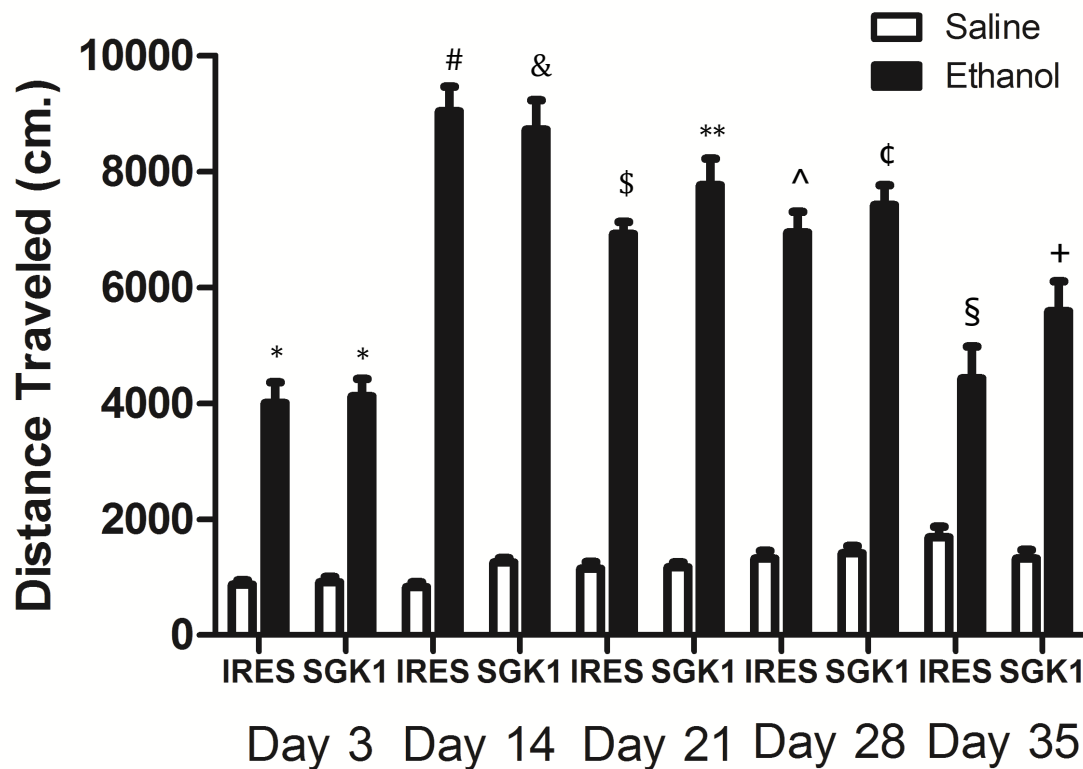


Figure 24. Ethanol sensitization following viral overexpression of Sgk1. * $p < 0.05$ vs. all saline treated animals; # $p < 0.05$ vs. all saline treated animals, day 3 IRES EtOH treated animals, and day 35 IRES EtOH treated animals; & $p < 0.05$ vs. all saline treated animals, day 3 SGK1 EtOH treated animals and day 35 SGK1 treated animals; \$ $p < 0.05$ vs. all saline treated animals, day 3 EtOH treated IRES animals, and day 35 IRES EtOH treated animals; ** $p < 0.05$ vs. all saline treated animals, day 3 EtOH treated SGK1 animals, day 21 EtOH treated IRES animals, day 14 EtOH treated SGK1 animals and day 35 EtOH treated SGK1 animals; ^ $p < 0.05$ vs. all saline treated animals, day 3 EtOH treated IRES animals, and day 35 EtOH treated IRES animals; ¢ $p < 0.05$ vs. all saline treated animals, day 3 EtOH treated SGK1 animals, day 14 EtOH treated SGK1 animals and day 35 EtOH treated SGK1 animals; § $p < 0.05$ vs. all saline treated animals; + $p < 0.05$ vs. all saline treated animals, SGK1 day 3 EtOH treated animals and IRES Day 35 EtOH treated animals

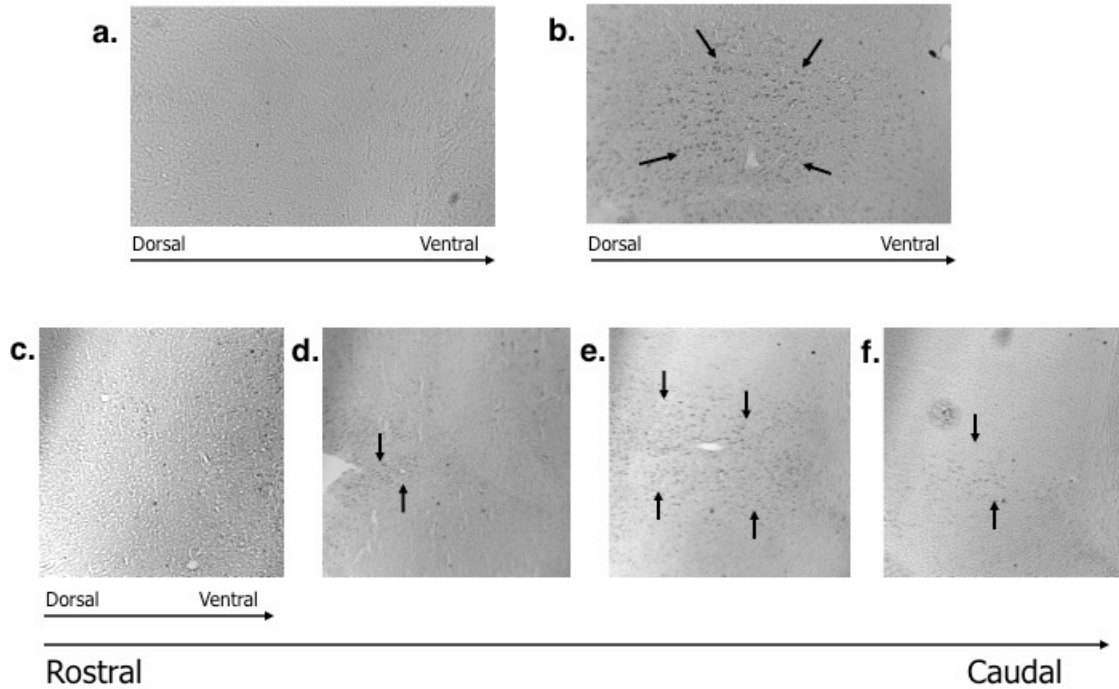


Figure 25. Viral placement and expression in mice microinjected with IRES-AAV versus those microinjected with FLAG-SGK1-AAV (a-b) and rostral to caudal movement in mice microinjected with FLAG-SGK1-AAV (c-f). Panels show: (a) anti-FLAG staining in mice expressing IRES-AAV, (b) anti-FLAG staining in mice expressing FLAG-SGK1-AAV, (c)-(f) rostral (c) to caudal (f) movement through the brain of a mouse microinjected with FLAG-SGK1-AAV. Arrows indicate positive staining.

CHAPTER 7

Concluding Remarks & Future Directions

CONCLUDING REMARKS

Overall our findings support clinical reports showing stress hypo-responsiveness in human alcoholics and provide evidence for how HPA axis tolerance can alter ethanol responses in brain stress/reward related regions such as PFC (Lovallo et al., 2000, O'Malley et al., 2002, Kiefer et al., 2011). HPA axis dysregulation exists in alcohol dependent patients and individuals with a familial history of alcoholism. Individuals who are low in sociability with a familial history of alcohol dependence show blunted cortisol responses (Sorocco et al., 2006). Individuals at high risk for alcoholism also show lower basal ACTH levels, lower stress-induced increases in plasma ACTH concentration, and delayed post-stress recovery of plasma ACTH and cortisol (Dai et al., 2002). Thus, disrupted stress responses may contribute to the risk for development of alcohol dependence, and our findings suggest that altered basal gene expression in PFC could be a mediating factor. While changes occurring at the endocrine level are characterized, less defined are molecular changes in brain stress/reward related regions that are mediated by HPA axis tolerance.

Studies of acute, sub acute and chronic ethanol administration in animal models have helped to better define ethanol's actions. Such studies have led to the development of treatments for alcohol abuse and alcoholism. Our work better defines the mechanism behind ethanol's regulation of Sgk1 both acutely and chronically. In the case of Sgk1, it seems that ethanol triggers a complex wave of signaling events leading to: 1) Sgk1 activation by phosphorylation with subsequent phosphorylation of Ndr1 and other

targets; 2) increased Sgk1 transcription by HPA activation and glucocorticoid action; and 3) compensatory Sgk1 protein degradation (Fig. 26). Additionally, our work identifies behavioral modifications induced by Sgk1 following chronic ethanol administration. Chronically, Sgk1 may alter neural mechanisms that mediate the addicted state, but additional studies are necessary to characterize this response. Future studies are needed to further characterize the mechanism behind ethanol's regulation of Sgk1, the inter-relations of glucocorticoid signaling, PFC gene expression networks, and mechanisms underlying risk for alcohol dependence.

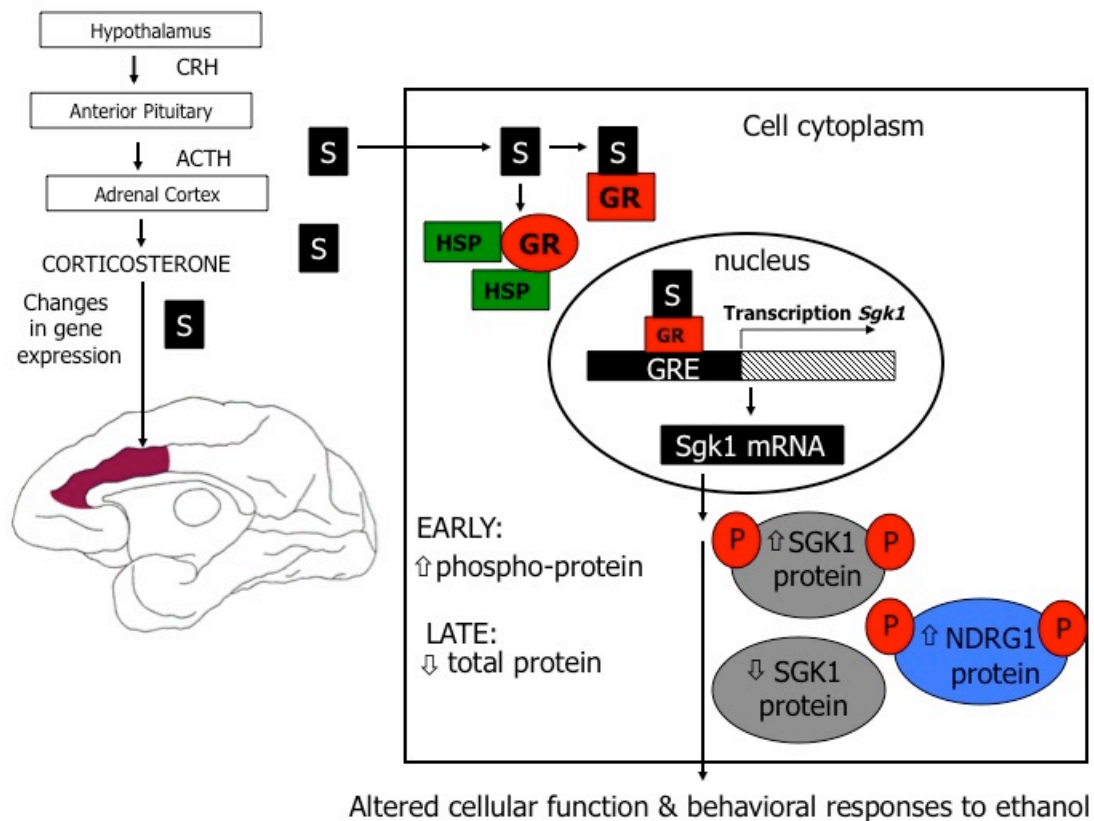


Figure 26. Brief summary of project findings. Ethanol triggers a complex wave of signaling events beginning with the activation of the HPA axis and leading to eventual changes in gene expression in the anterior cingulate region of the PFC. Sgk1 is an ethanol responsive, glucocorticoid responsive gene. Following ethanol administration a series of events occurs including: 1) SGK1 activation by phosphorylation with subsequent phosphorylation of NDRG1 and possibly other targets; 2) increased Sgk1 transcription by

HPA activation and glucocorticoid action; and 3) compensatory SGK1 protein degradation. These events are responsible for changes in cellular function that alter behavioral responses to ethanol.

FUTURE DIRECTIONS

We are currently working to verify viral placement in FLAG-SGK1-AAV and IRES-AAV mice and identify candidates in the *Sgk1* gene network that may mediate some of the behavioral differences we saw between FLAG-SGK1-AAV versus IRES-AAV mice during the expression phase of sensitization. We are completing immunohistochemistry experiments to verify FLAG-SGK1-AAV placement in the anterior cingulate region of the PFC of D2 mice. In addition, we are verifying IRES-AAV and FLAG-SGK1-AAV placement and hrGFP expression through *in vivo* imaging (Xenogen). Additionally, we are performing ethanol metabolism studies in FLAG-SGK1-AAV and IRES-AAV mice. We will also harvest IRES-AAV and FLAG-SGK1-AAV animals to possibly perform microarray analysis and Q-rtPCR verification of microarray results. Microarray experiments may help us to identify candidates in the *Sgk1* gene network that are working to alter synaptic plasticity and behavioral responses to ethanol.

Additionally, future experiments should address SGK1 and phospho-SGK1 levels chronically following ethanol sensitization. We showed that *Sgk1*, SGK1 and phospho-SGK1 levels were modified following acute ethanol administration, but we found differences in mice overexpressing FLAG-SGK1-AAV versus IRES-AAV chronically during the expression phase of ethanol sensitization and it is thus important to characterize SGK1 and phospho-SGK1 chronically. We could begin this process by measuring SGK1 and phospho-SGK1 in FLAG-SGK1-AAV and IRES-AAV treated animals. We might expect FLAG-SGK1-AAV animals to have greater SGK1 expression

compared to IRES-AAV control animals. But it would be interesting to see if overexpressing SGK1 also increases levels of phospho-SGK1 basally.

The PFC is one of the primary targets of stress hormones (Yuen et al., 2011). Miyata et al. showed that a chronic stress paradigm that elevated plasma corticosterone levels similar to those found in depressed individuals lead to activation of the phosphatidylinositol 3-kinase (PI3K)-3-phosphoinositide-dependent protein kinase (PDK1), *Sgk1*, and *Ndr1* pathway with increases in both *Sgk1* mRNA and SGK1 phosphorylation (Miyata et al., 2011). Thus, it is possible that HPA axis activation is causal in both *Sgk1* activation and increased transcription of the *Sgk1* gene. Our prior results did not explore the upstream regulation of phospho-SGK1. Future studies could address whether the HPA axis plays a role in the phosphorylation and activation of SGK1 in the PFC through evaluating phospho-SGK1 in ADX versus SHAM ethanol treated animals. Additionally, phospho-SGK1 could also be evaluated following RU-486 versus vehicle administration. We would hypothesize that mechanisms other than HPA axis activation may be responsible for ethanol's activation of SGK1. Earlier we hypothesized that the phosphorylation of SGK1 may be responsible for mediating the differences seen in FLAG-SGK1-AAV and IRES-AAV mice as we know that *Sgk1* regulation following ethanol administration is no longer present early in the expression phase of sensitization. We also know the HPA axis habituates following repeated ethanol administration. It appears that SGK1 does, however, play a role in regulating the duration and extent of the sensitized response and therefore an alternative mechanism other than the HPA axis may be responsible for this regulation. It might also be interesting to perform a more detailed characterization of *Sgk1* and corticosterone during the initiation phase of the sensitized

response to learn at what point during the initiation of sensitization do *Sgk1* and corticosterone levels no longer increase following acute ethanol administration. These findings should correspond, ie. *Sgk1* should decrease as corticosterone decreases, if *Sgk1* is in fact regulated by ethanol's activation of the HPA axis.

In the future, we could repeat the viral overexpression experiment, but lower the test day ethanol doses to 1.5 g/kg ethanol rather than 2 g/kg ethanol for animals receiving ethanol on activity test days (see Table 5 below for experimental details). It is well-known that D2 mice show a robust locomotor activation response following ethanol administration and it may be difficult to see differences in FLAG-SGK1-AAV versus IRES-AAV mice on test days following the administration of 2 g/kg ethanol as this dose may be masking differences in locomotor activity between FLAG-SGK1-AAV versus IRES-AAV mice. In other words, FLAG-SGK1-AAV mice could have a greater locomotor activation response than IRES-AAV following acute ethanol administration like we saw during the expression phase of sensitization, but due to a ceiling effect, we may be unable to detect differences in locomotor activity between these two groups of mice.

Table 5: Outline of experimental design for future ethanol sensitization

Phase of Sensitization		Induction Phase		Expression Phase
Group	Days 1-2	Days 3	Days 4-13	Days 14-35
	Habituation	Acute test	Daily injections	Expression test
SS	Saline	Saline	Saline	Saline
EE	Saline	Ethanol 1.5 g/kg	Ethanol 2.5 g/kg	Ethanol 1.5 g/kg
Activity test	yes	yes	no	yes

Table 5. Plan for future ethanol sensitization studies comparing locomotor activity during the initiation and expression phases of sensitization in FLAG-SGK1-AAV versus IRES-AAV mice.

Ideally, we could perform dose response experiments comparing locomotor activity in FLAG-SGK1-AAV versus IRES-AAV D2 mice both acutely and during the

expression phase of ethanol sensitization (see Table 6 below for experimental details). This set of experiments would be challenging to execute. Because we are overexpressing Sgk1 in a small proportion of the cells in one brain region and due to the sensitive and variable nature of behavior, we would need n = 12 for IRES-AAV mice and n = 12 for FLAG-SGK1-AAV. This would require performing approximately 100 stereotaxic injections. Such injections should be performed during the same time period to control for the rate of viral expression following microinjection. Because the sensitized response has been shown to be subject to alterations in corticosterone levels and corticosterone levels are known to change throughout the day according to circadian rhythms, we must be careful to execute our experiments at the same time daily. Perhaps these experiments could be staggered, but their difficult nature should not be overlooked in planning future experimental procedures.

Table 6: Outline of experimental design for future ethanol sensitization

Phase of Sensitization		Induction Phase		Expression Phase
Group	Days 1-2	Days 3	Days 4-13	Days 14-35
	Habituation	Acute test	Daily injections	Expression test
SS	Saline	Saline	Saline	Saline
EE	Saline	Ethanol 1.5 g/kg	Ethanol 2.5 g/kg	Ethanol 1.5 g/kg
EE	Saline	Ethanol 2.0 g/kg	Ethanol 2.5 g/kg	Ethanol 2.0 g/kg
EE	Saline	Ethanol 2.5 g/kg	Ethanol 2.5 g/kg	Ethanol 2.5 g/kg
Activity test	yes	yes	no	yes

Table 6. Plan for future ethanol sensitization studies comparing locomotor activity during the initiation and expression phases of sensitization in in FLAG-SGK1-AAV versus IRES-AAV mice. Varying doses of ethanol are administered to animals on days 3 and 14-35 as indicated above.

Like Boehm et al. (2008), we could also avoid administering repeated ethanol injections to animals during the expression phase of sensitization and have separate groups for testing on days 7, 14 and 21 (see Table 7 below for experimental details). Administering repeated injections to animals over 7 day intervals as we did initially may

re-sensitize the animals and may make it difficult to determine the true duration of the sensitized response. However, as mentioned above, Boehm et al. (2008) showed that both animals repeatedly administered ethanol over 7 day intervals and those that were not subject to repeated ethanol administrations showed the same duration of sensitization (Boehm et al., 2008). In fact, Boehm et al. even continued with their repeated ethanol challenge approach in further experiments after learning that it did not seem to affect the duration of the sensitized response (Boehm et al., 2008). This set of experiments would also be challenging to execute. We would need $n = 12$ for IRES-AAV mice and $n = 12$ for FLAG-SGK1-AAV. This would require performing approximately 144 stereotaxic injections. Such injections should be performed during the same time period and we must carefully execute our experiments at the same time daily. Perhaps these experiments could be staggered, but their difficult nature should not be overlooked in planning future experimental procedures.

Table 7: Out line of experimental design for future ethanol sensitization

Phase of Sensitization		Induction Phase		Expression Phase
Group	Days 1-2	Days 3	Days 4-13	Days 14-35
	Habituation	Acute test	Daily injections	Expression test
SS	Saline	Saline	Saline	Saline-7
EE	Saline	Ethanol 1.5 g/kg	Ethanol 2.5 g/kg	Ethanol 1.5 g/kg-7
SS	Saline	Saline	Saline	Saline-14
EE	Saline	Ethanol 1.5 g/kg	Ethanol 2.5 g/kg	Ethanol 1.5 g/kg-14
SS	Saline	Saline	Saline	Saline-21
EE	Saline	Ethanol 1.5 g/kg	Ethanol 2.5 g/kg	Ethanol 1.5 g/kg-21
Activity test	yes	yes	no	yes

Table 7. Plan for future ethanol sensitization studies comparing locomotor activity during the initiation and expression phases of sensitization in FLAG-SGK1-AAV versus IRES-AAV mice. “7” mice are only tested on day 7 following the cessation of ethanol administration. “14” mice are only tested on day 14 following the cessation of ethanol administration. “21” mice are only tested on day 21 following the cessation of ethanol administration.

In the future, we could also overexpress IRES-AAV and FLAG-SGK1-AAV in the

anterior cingulate region of the PFC of C57 mice and compare locomotor activation and sensitization in these mice. FLAG-SGK1-AAV prolonged the duration of the sensitized response possibly by altering plasticity in the PFC of D2 mice, a genotype of mouse best known to show an ethanol induced locomotor activation response and to sensitize to the locomotor activating effects of ethanol. It would be interesting to examine how the overexpression of FLAG-SGK1-AAV may affect locomotor activity in animals not known to show an ethanol induced locomotor activation response and sensitize to the locomotor activating effects of ethanol. This set of experiments could also help us to better decipher the mechanism through which *Sgk1* may be altering synaptic plasticity. For example, C57 mice have higher basal levels of *Sgk1*, but they do not experience the robust induction of *Sgk1* following acute ethanol administration. Perhaps increasing levels of *Sgk1* through microinjection of FLAG-SGK1-AAV would alter locomotor activity in C57 mice.

Finally, one of the reasons we originally hypothesized that *Sgk1* may mediate the sensitized response to ethanol is that *Sgk1* has a known role in memory consolidation of spatial learning and regulates neuronal plasticity in the brain and spinal cord and it is known that behavioral sensitization involves learning processes (Lee et al., 2006, Tsai et al., 2002, Lee and Rivier, 1997, Ma et al., 2006, Quadros et al., 2003). Although statistically significant, the differences between FLAG-SGK1-AAV and IRES-AAV mice in our ethanol sensitization studies were small. Perhaps we could accentuate differences between FLAG-SGK1-AAV and IRES-AAV in our sensitization studies by the pairing of ethanol injections and the testing chamber for both FLAG-SGK1-AAV and IRES-AAV mice during the initiation phase of sensitization. FLAG-SGK1-AAV may be

altering neuroadaptations underlying behavioral sensitization that may be closely related to those mediating learning and memory processes and including a contextual pairing component in our studies would enhance such differences.

Additionally, we want to further decipher the mechanism by which Sgk1 regulates cellular responses to ethanol by identifying the cell type in which SGK1 primarily acts; ie. is Sgk1 mediating its effects in neuronal cell populations, oligodendrocytes, or astrocytes? The AAV-2 serotype shows neuron-specific expression and we used it to overexpress Sgk1 (Daya and Berns, 2008, Terzi and Zachariou, 2008). Overexpressing Sgk1 neuronally seemed to alter synaptic events that prolonged and intensified the sensitized response during the expression phase of sensitization. We show that NDRG1, a downstream target of phosphorylated, active SGK1, phospho S330 levels were significantly increased in ethanol vs. saline treated animals 15 minutes following ethanol administration (Fig. 21). Miyata et al. showed that a chronic stress paradigm that elevated plasma corticosterone levels lead to activation of the PDK1, SGK1, and NDRG1 pathway in mouse oligodendrocytes, not neurons (Miyata et al., 2011). NDRG1 is a well-known SGK1-specific substrate minimally phosphorylated by other kinases (Garcia-Martinez and Alessi, 2008, Sahin et al., 2013, Murray et al., 2004). It is known that NDRG1 deficiency leads to Schwann cell dysfunction and that NDRG1 is essential for maintenance of the myelin sheaths in peripheral nerves (Okuda et al., 2004). Okuda et al. identified NDRG1 as mainly localized in oligodendrocytes in mouse cerebrum (Okuda et al., 2008). Another report demonstrated that the location of NDRG1 changed from hippocampal neurons to astrocytes during postnatal development in the rat brain (Okuda et al., 2008). Other works examining SGK1 location in human brain of aged

Alzheimer's versus control patients document the SGK1 location to be primarily neuronal (Sahin et al., 2013). Another study found neurons, oligodendrocytes, and microglial cells, but no astrocytes, were positive for SGK1 in the rat (Warntges et al., 2002).

We could determine the cell population through which SGK1 is mediating its effects by performing immunohistochemistry studies in 10-13 week old D2 mice, as that is the age of animal used in our prior studies. We could use neuronal, oligodendrocyte, and astrocyte markers in addition to NDRG1 and SGK1 staining to determine the cell type where SGK1 (Table 8) and NDRG1 (Table 9) are most prominently expressed basally, following saline injections and following ethanol administration. Ethanol administration could potentially change the distribution of SGK1 or NDRG1 so it is important that we evaluate NDRG1 and SGK1 expression both basally and following ethanol administration. Including the saline group will control for the effect of injection stress on the distribution of SGK1 and NDRG1. Please see Tables 8 & 9 below for the hypothesized staining combinations. We would be most interested in evaluating the PFC of D2 mice.

Table 8. Staining combinations for future studies

	Neuronal Marker (NeuN)	Oligodendrocyte Marker (CNPase)	Astrocyte Marker (GFAP)	NDRG1
SGK1	X			
SGK1		X		
SGK1			X	
SGK1				X

Table 8. Staining combinations for future studies determining the cell population or populations expressing SGK1. SGK1 expression would be examined basally, following saline treatment and following ethanol administration. Co-localization experiments would be performed between SGK1 and the following: NeuN, CNPase, GFAP and NDRG1. NeuN, CNPase, GFAP co-localization experiments with SGK1 would reveal the cell type where SGK1 is most prominently expressed. SGK1 and NDRG1 co-localization

experiments would verify that NDRG1 and SGK1 are co-expressed in cell populations, although it would not necessarily determine the type of cell population where the two proteins are co-expressed. All staining would have to be evaluated basally and following saline and ethanol treatment.

Table 9. Staining combinations for future studies.

	Neuronal Marker (NeuN)	Oligodendrocyte Marker (CNPase)	Astrocyte Marker (GFAP)
NDRG1	X		
NDRG1		X	
NDRG1			X

Table 9. Staining combinations for future studies determining the cell population or populations expressing NDRG1. NDRG1 expression would be examined basally, following saline treatment and following ethanol administration. NeuN, CNPase, GFAP co-localization experiments with NDRG1 would reveal the cell type where NDRG1 is most prominently expressed. Co-localization experiments would be performed between NDRG1 and the following: NeuN, CNPase, and GFAP. All staining would have to be evaluated basally and following saline and ethanol treatment.

This would be a first step to determining which cell population or populations are mediating the effects of SGK1. Since AAV-2 is well known to display neuronal tropism (Terzi and Zachariou, 2008, Daya and Berns, 2008), we know that alterations in plasticity leading to alterations in the sensitized response are occurring primarily in neuronal populations in our viral animals (Fig. 25). If overexpressing Sgk1 in neurons is not a biologically relevant experimental procedure, we would have to consider using a serotype of AAV that targets the biologically relevant cell type or types and repeat our experiments using the new serotype of AAV.

While we believe CORT levels were significantly elevated in CB1 KO mice following ethanol administration because they lack the fast feedback negative HPA axis regulation mediated by CORT's actions at the CB1 receptor, future studies will be necessary to draw this conclusion. We recognize that there is much additional work remaining in this line of experimentation. Due to our limited time and resources, we only

evaluated CORT levels at the one-hour time point, the time point following ethanol administration where CORT levels peaked in our time course evaluation of CORT (Fig. 9). We also only evaluated CORT levels following 2 g/kg ethanol administration. Future studies could evaluate CORT levels in CB1 KO versus WT animals following an ethanol time course and dose response curve. Perhaps we would see additional and larger differences in CORT levels in CB1 KO versus WT animals at time points earlier or latter than one hour or doses smaller or greater than 2 g/kg. Because results in KO animals could be due to compensatory mechanisms or altered developmental events known to occur in these animals, we should also follow up our findings with studies using CB1 receptor antagonist administration prior to ethanol administration, blood collection and RIA determination.

We did not evaluate locomotor activity in CB1 KO animals because they were on a C57 background and therefore WT animals would not show a locomotor activation response following acute ethanol administration. In order to evaluate whether nongenomic glucocorticoid signaling mechanisms were involved in the locomotor activating effects of ethanol in D2 mice, we could start by measuring the ethanol mediated locomotor activation response in D2 mice following the administration of a CB1 antagonist. To our knowledge, these studies have not yet been performed in D2 mice; but, CB1 KO mice on a CD1 background display a blunted locomotor activation response following acute ethanol administration compared to their WT counterparts (Naassila et al., 2004). CB1 KO mice on a CD1 background also displayed a significant reduction in basal levels of locomotion and cocaine-enhanced locomotion compared to their WT littermates and pharmacological blockade of CB1 receptors by SR141716, a

CB1 antagonist, inhibited locomotion in CB1 WT mice (Li et al., 2009). In CB1-R-deficient mice on a C57BL/6N background, locomotor responses to cocaine and D-amphetamine were decreased and sensitization was impaired (Corbille et al., 2007). These findings suggest that drugs causing an acute locomotor activation response in mice which also activate the HPA axis including ethanol, cocaine and amphetamine (Sarnyai et al., 2001) have diminished locomotor activating properties in CB1 KO mice. Thus, the idea that corticosterone may mediate some of the locomotor activating properties in D2 mice through nongenomic signaling mechanisms may warrant further investigation.

Our above findings are suggestive of a mechanism that remains illusive in the literature, but the fact remains that in our previous work high doses of RU-486, a glucocorticoid receptor antagonist, blunted the acute locomotor activating effects of ethanol in D2 mice. This suggests a possible role for the receptor itself in mediating ethanol's acute locomotor activating effects. The GR is subject to posttranslational modifications including phosphorylation on at least seven serine residues (Ser-113, Ser-134, Ser-141, Ser-203, Ser-211, Ser-226 and Ser-404); ubiquitination at a conserved lysine residue located in a PEST degradation motif and this modification targets the receptor for degradation by the 26S proteasome; and sumoylation at residues Lys-277, Lys-293 and Lys-703 (Ramamoorthy and Cidlowski, 2013). Perhaps future studies could examine post-translational GR modifications beginning with GR phosphorylation as GR phosphorylation has not yet been examined following ethanol administration to our knowledge. Although this project has made a strong argument for the role of the GR in regulating gene expression following ethanol administration, perhaps the receptor is regulated in more than one way following ethanol administration.

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