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Behavioral and Molecular Analysis of Individual Variation in Ethanol Drinking

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BEHAVIORAL AND MOLECULAR ANALYSIS OF INDIVIDUAL VARIATION IN ETHANOL DRINKING

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Abbreviations

129SvJ	129x1Sv/J Inbred Mouse Strain
5-HT	5-Hydroxytrytamine (Serotonin)
5-HT1A	5-Hydroxytrytamine Receptor 1A
5-HIAA	5-hydroxyindoleacetic acid
ACTH	Adrenocorticotropin hormone
AMY	Amygdala
ANOVA	Analysis of Variance
BEC	Blood Ethanol Concentration
BDNF	Brain Derived Neurotrophic Factor
C57	C57BL/6J Inbred Mouse Strain
CBA	CBA Inbred Mouse Strain
Chr	Chromosome
CRF	Corticotrophin Releasing Factor
CRHR	Corticotrophin Releasing Hormone Receptor
CORT	Corticosterone
Cox	Cytochrome C Oxidase
DOC	Deoxycorticosterone
DA	Dopamine

Abbreviations continued

DRD4	Dopamine Receptor Type 4
DRN	Dorsal Raphe Nuclei
EASE	Expression Analysis Systematic Explorer
GABA	Gamma-aminobutyric Acid
GCOS	Gene Chip Software v4.1
Grik	Kainate, Glutamate Receptor 1
HAD	High Alcohol Drinking
HCE	Home Cage + Ethanol
HCW	Home Cage + Water
HDAC	Histone Deacetylase Complex
Hip	Hippocampus
HPA	Hypothalamic-pituitary adrenal
Hsd	Hydroxysteriod dehydrogenase
IPA	Ingenuity Pathway Analysis
LAD	Low Alcohol Drinking
MAPK	Mitogen-activated Protein Kinase
mPFC	Medial Prefrontal Cortex
MAOA	Monoamine Oxidase A
NAc	Nucleus Accumbens

Nduf	NADH dehydrogenase
NMDA	N-methyl-D-aspartate
NP rats	Non-alcohol preferring Rats
NPY	Neuropeptide Y
P rats	Alcohol Preferring Rats
PC	Principle Component
PFC	Prefrontal Cortex
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and Tensin Homolog
POMC	Pro-opiomelanocortin
PREG	Pregnenolone
RIA	Radioimmune Assay
RMA	Robust Multiarray Average
RM ANOVA	Repeated Measures ANOVA
RSD	Repeated Social Defeat
SERT	Serotonin Transporter
SSRI	Selective Serotonin Reuptake Inhibitor
SDE	Social Defeat + Ethanol
SDW	Social Defeat + Water
T-MeV	TIGR Multi Experiment Viewer
TSA	Trichostatin A
VTA	Ventral Tegmental Area

Abstract

BEHAVIORAL AND MOLECULAR ANALYSIS OF INDIVIDUAL VARIATION IN ETHANOL DRINKING

By Jennifer T. Wolstenholme, PhD

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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A majority of Americans regularly consume alcohol, but the risk factors leading to excessive drinking and alcohol abuse are unevenly distributed throughout the population. Genetic differences can account for only 40-60% of this variability. While variations in ethanol preference drinking in rodent models have been reported, the neurobiological factors underlying these behaviors are still not completely understood. Thus, these studies were designed to determine behavioral and molecular factors associated with the initiation

of ethanol drinking preference in an inbred mouse model. We harnessed the power of inter-individual variation of ethanol drinking within an inbred mouse strain to essentially eliminate genetic variability and focus on environmental factors. Our studies have characterized robust, persistent individual variability in ethanol intake in C57 mice using a two-bottle choice paradigm. Ethanol intake differences were not due to litter effects or differences in taste preference. Social rank nor basal anxiety phenotypes could account for ethanol preference.

Based on the shared co-morbidity of anxiety and alcoholism, and that alcoholics report anxiety and stress reduction as major motivational factors for drinking, we used an ethologically-relevant social defeat model to investigate stress-influences on ethanol drinking. We found that social defeat has bidirectional effects on ethanol drinking. Mice with a low predilection for ethanol tend to increase drinking following social stress while high preference mice decrease drinking. Even though social defeat produced a measurable physiological response in mice, defeat stress did not alter anxiety measures in the light-dark box. Thus, the current findings did not fully support the tension-reduction hypothesis of alcoholism.

In order to determine the molecular factors underlying these differences in ethanol preference drinking, we employed genome-wide expression profiling to identify gene networks altered in ethanol-preferring and ethanol-avoiding mice. Genes involved in synaptic vesicle release, glutamate and BDNF signaling were differentially altered in drinking mice. Following stress-influenced ethanol drinking, expression profiling identified transcripts involved in dopamine signaling, the extra-hypothalamic stress

response and alterations in steroid and glucocorticoid synthesis. Most importantly, these expression studies and behavioral analysis following histone deacetylase inhibition may be the first to implicate epigenetic factors involving chromatin acetylation and/or methylation as contributing to environmental modulation of ethanol intake.

CHAPTER 1 Introduction

Alcohol is by far the most widely used drug of abuse in the United States with more than half of the population reporting drinking alcohol in the recent past (Administration 2005). However, the abusive potential of alcohol use has been reaching a global health epidemic. Alcohol abuse and alcoholism are an enormous social and economic burden costing over $186 billion dollars in 1998 and were responsible for more than 3.5% of all deaths in the Unites States (Mokdad et al. 2004). Even though a majority of Americans are exposed to alcohol, its consumption is not evenly distributed throughout the population. Nearly 64% of adults actively drink alcohol, but strikingly 20% of this population consumes over 80% of all the alcohol sold (Dawson 2000). Despite the fact that a majority of Americans regularly drink alcohol, only 7% will go on to develop alcoholism or alcohol dependence (Administration 2005). Thus, the risk factors for excessive alcohol use must be unevenly distributed in individuals. Research directed at predicting which individuals are at a high risk for alcohol abuse disorders is a matter of urgent public health concern for the development of both therapeutic interventions and prevention.

Alcoholism is a chronic relapsing disorder characterized by a lifetime of cycles of intense ethanol intoxication alternating with periods of abstinence resulting in withdrawal

syndromes. It is characterized by continuous or periodic impaired control over drinking, preoccupation with the drug alcohol, use of alcohol despite adverse consequences, and distortions in thinking, most notably denial. Alcohol abuse is defined by the 4th edition Diagnostic and Statistical Manual of Mental Disorders as continued substance use regardless of physical hazards or recurrent use-related legal problems, with a failure to fulfill major work or personal obligations. Some alcohol abusers may progress to dependence which can be characterized by alcohol craving, loss of control, tolerance and signs of physical withdrawal after cessation.

Stressors, anxiety state and the anxiolytic properties of ethanol play important roles in the predisposition of ethanol intake, initiation of ethanol abuse, dependence and relapse drinking. Indeed, the belief that alcohol consumption is stress reducing is a long-established hypothesis (Conger 1956). Alcoholics often report anxiety or stress reduction as a major motivational factor for drinking (Conger 1956; Pohorecky 1981; Newlin et al. 1990). Persons diagnosed with alcohol dependence or people who have exceeded daily drinking limits have a high rate of depression, social phobia, generalized anxiety, anti-social personality disorder and obsessive-compulsive personality disorder (NIAAA 2000). Additionally, persons with a history of stress and mood disorders have approximately 3 times the risk for developing an alcohol-related disorder (Regier et al. 1990).

Due to the need for more successful therapies, a variety of research efforts have focused on understanding individual differences in the susceptibility to ethanol abuse and dependence. While several aspects of the response to ethanol need to be considered to fully comprehend these individual differences, the acute response or initial sensitivity to ethanol

appears to account for a large portion of variability in the susceptibility to alcoholism (Schuckit et al. 1996; Schuckit 1999). These studies are designed to determine behavioral and molecular factors associated with the initiation and maintenance of ethanol drinking in a mouse model. We have harnessed the power of robust inter-individual variation of ethanol drinking within an inbred mouse strain to essentially clamp the genetic variability and determine environmental effects which contribute to variations in ethanol drinking. Based on the long established tension-reduction hypothesis leading to alcohol abuse, we also used an ethologically-relevant mouse model of social stress to investigate the effects of social stress on ethanol drinking and anxiety-like behaviors. Social stress causes long lasting signaling alterations in the brain which influence ethanol drinking. Additionally, the neurobiological factors underlying ethanol drinking variability and stress-influenced drinking are not completely understood so we employed whole genome molecular profiling to identify gene networks altered in ethanol preferring and ethanol avoiding mice. The ultimate goal of these studies is to 1) characterize the inter-individual variation of ethanol drinking behavior in C57 mice; 2) to study the behavioral differences in anxiety or in response to social stress on the modulation of drinking behaviors and 3) to determine the molecular factors that may contribute to the observed phenotypes. Ultimately these studies should contribute novel insight into the underlying molecular mechanisms involved in the proclivity to consume ethanol and possible identify novel potential therapeutic targets for excessive ethanol consumption.

CHAPTER 2 Background and Significance

Individual differences in ethanol drinking

The risk to develop alcohol abuse disorders from social drinking is not evenly distributed through the population, where less than 10% of social drinkers progress towards alcohol abuse. Approximately half of this risk can be attributed to genetic factors (Cloninger 1987; Gordis et al. 1990; Enoch et al. 2001; Radel et al. 2001). Further compounding this are the considerable differences in how an individual perceives both pleasurable and anxiety-provoking stimuli which may be a primary contributing factor to the risk towards excessive alcohol consumption.

It is well documented that environmental influences such as stress or exposure to conditional stimuli can modify ethanol drinking or cause recidivism in abstinent alcoholics. Evidence for environmental impact on the development of alcoholism can be found in studies on craving and social conditioning, in which an environment becomes associated with a rewarding substance. Alcoholics, following completion of a treatment program, often report increased craving and relapse drinking after being exposed to a familiar drug-taking environment or drug-related paraphernalia (Franken 2003). Indeed, images and words associated with drugs or alcohol are often used in neuroimaging studies and have been shown to increase brain activity in regions associated with craving in addicts and were correlated to self-reported levels of craving (Goldstein et al. 2002;

Connolly et al. 2009). Physical environment is not the only type of environmental factor modulating risk for alcohol abuse. Social stressors, specifically isolation (Parker et al. 1974; Schenk et al. 1990; Wolffgramm et al. 1991) and overcrowding (Hannon et al. 1976), increase ethanol self-administration in rodent models. Social stress through subordination or repeated social defeat also modifies ethanol consumption (Blanchard et al. 1987; Hilakivi-Clarke et al. 1992; van Erp et al. 2001). A few recent studies have investigated individual variation of ethanol drinking in cynomolgus monkeys and shown a correlation between responsiveness of the hypothalamus-pituitary-adrenal (HPA) axis and ethanol consumption (Porcu et al. 2006; Porcu et al. 2006). Understanding the molecular mechanisms underlying such environmental influences on ethanol behaviors would augment the current understanding of non-genetic risk factors for alcohol abuse.

C57BL/6 (C57) inbred mice have been widely used as a model for studying alcohol abuse related behaviors and the genetic basis of alcohol abuse since these mice voluntarily consume large volumes of unadulterated ethanol (McClearn 1972; Goodrick 1978; Belknap et al. 1993; Bachmanov et al. 1996; Gill et al. 1996; Middaugh et al. 1999). Early researchers had challenged the utility of this strain as a model organism for high ethanol consumption due to the dissimilarity between humans and mice for their motivation to drink. Inability to discriminate based on taste and extreme ethanol metabolism rates were cited as the major disparity. For example, they suggested that mice consume ethanol for its caloric value and do not reach pharmacologically relevant blood ethanol levels (Dole et al. 1985; McMillen et al. 1998). However, studies by Middaugh have shown that B6 mice indeed consume ethanol for its postingestive pharmacological effects, i.e. hedonic value

(Middaugh et al. 2000). C57 mice voluntarily consume various concentrations of ethanol to produce ethanol intake in the range of 8-16 g/kg in a 24 hour period, and will exceed the pharmacologically relevant blood ethanol concentration (BEC) level of 100 mg/dL (Bachmanov et al. 1996; Phillips et al. 1998; Middaugh et al. 1999). Deficiencies in taste discrimination alone cannot account for the high ethanol drinking in C57 mice. In fact, C57 mice prefer ethanol concentrations of 6-12% over water and can discriminate ethanol concentrations as low as 3% (Middaugh et al. 2000; Becker et al. 2004). C57 mice will also work (i.e. lever press) for an ethanol reward as demonstrated by operant self-administration studies (Grahame et al. 1997; Kelley et al. 1997). These mice also display preference for an area previously paired with ethanol suggesting that, indeed, C57 mice experience conditioned rewarding effects of ethanol (Nocjar et al. 1999; Cunningham et al. 2006). Additionally, our laboratory and others have demonstrated ethanol craving in two substrains of C57 mice using the alcohol deprivation effect, a temporary increase in ethanol drinking following a period of forced abstinence. Thus, C57 mice are one of the few well-accepted model organisms for excessive ethanol consumption modeling several (but not all) components of alcohol abuse.

Intriguingly, a few prior reports have documented remarkable degrees of stable, individual variation in 2-bottle choice drinking behavior in rodents and monkeys. Several of these have shown that individual variation in drinking behavior can occur within a single inbred strain (Dole et al. 1988; Little et al. 1999; O'Callaghan et al. 2002). This removes factors such as genetic differences in taste or ethanol reward as causal for the variation in drinking behavior. Early reports on 24-hour ethanol preference in B6 mice

have shown that each animal's individual variation accounted for the major part of the non-genetic variance in drinking behavior (Dole et al. 1988). C57BL/6J mice also display persistent individual variation in a model for high intoxication under short-term ethanol access (Rhodes et al. 2005). Although the authors did not further investigate this variation, they have suggested that subtle environmental differences during rearing or normal animal caretaking and handling may be responsible for individual ethanol preference. In a typically high alcohol preferring sub-strain, C57/BL10 mice, Little *et al.* have shown a bimodal distribution of ethanol drinking patterns where ethanol preference was not correlated with gender or ethanol metabolism, and could not be altered by simple environmental disturbances (O'Callaghan et al. 2002). Selective breeding did not show a simple genetic link to this variation (Little et al. 1999), as low preferring mice bred to other low preferring mice also produced offspring with a bimodal distribution of ethanol preference. Individual variability in ethanol drinking patterns in cynomolgus monkeys also persists, where intake during the induction phase was highly predictive of ethanol drinking behavior over the next 12 months (Grant et al. 2008).

In addition to the increased emphasis on individual variability in ethanol drinking, research focus has been shifting towards individual variability in stress responsivity (Bartolomucci et al. 2005; Grant et al. 2008; Koolhaas 2008). Baseline deoxycorticosterone and pregnenolone response to a dexamethasone challenge correlated with the average ethanol intake over the next 12 months of drinking (Porcu et al. 2006; Porcu et al. 2006). Variations in coping styles and the related neuroendocrine stress reactivity have been posited as standards for investigating individual vulnerability in stress

response (Koolhaas 2008). Direct study of HPA axis modulators suggests that variation in the central activity of corticotrophin releasing factor (CRF) may mediate individual ethanol preference in C57BL/10 mice (O'Callaghan et al. 2005). Thus, using a model where genetic factors are strictly controlled offers considerable power for studying behavioral and molecular mechanisms of environmental modulation of ethanol drinking behavior.

Neurobiology of stress

In humans, the stress response is a multidimensional process composed of six elements: 1) the stressor event; 2) cognitive interpretation of the event coupled with its affective integration into the limbic system; 3) neurological triggering mechanisms; 4) physiological stress response; 5) target-organ activation; and 6) coping behavior (George S. Everly 2003). The first critical step following a psychosocial stressor is that the individual must perceive and interpret the event as stressful. Perception involves the primary sensory projections and association cortices (McEwen et al. 1993), while appraisal of the event relies on sensory input from the thalamus, insula and sensory association areas. The emotional arousal from the perceived threat or stress activates several limbic centers such as the locus coeruleus, septal nuclei, amygdala and hippocampus and the hypothalamus (Mac 1949; Gellhorn 1964; Gellhorn 1965; Gellhorn 1967; Redmond et al. 1979; Aggleton et al. 1992). Limbic regions interact with sub-cortical and prefrontal cortical areas, which contribute to determine the meaning and significance of the event. These brain regions are interconnected through excitatory noradrenergic and serotonergic projections (Nauta 1982).

Activation of these limbic and cortical regions set a neurological tone which is capable of triggering the stress response, see Figure 1 (George S. Everly 2003). The stress response is mediated through alterations in the hypothalamic-pituitary-adrenal (HPA) axis and through changes in serotonin, dopamine and opioid neurotransmitter signaling cascades in other brain regions (Brady et al. 1999). Activation of the opioid pathway directly leads to pain relief or analgesia (Bodnar et al. 1980) while increased dopamine release causes increased blood pressure and heart rate peripherally as well as activating the brain reward pathway. Activation of the septal-hippocampal complex (Henry 1976; Ely et al. 1977) may directly activate the hypothalamus to release corticotrophin-releasing factor (CRF) into the hypothalamic portal system (Rochefort et al. 1959). Afferent CRF neurons project from the hypothalamus, dorsal raphe and other amygdaloid nuclei to the central nucleus of the amygdala. Reciprocal CRF neurons project back to various hypothalamic and midbrain nuclei (Uryu et al. 1992; Gray 1993). CRF stimulates the release of adrenocorticotropin hormone (ACTH) and β-endorphin, an endogenous opioid peptide, from the pituitary into the systemic circulation. It is at this point when precursors to endogenous opioids are released (Rossier et al. 1980) which mediate the analgesic aspects of the stress response. ACTH at the adrenal cortex stimulates the production and release of glucocorticoids, their precursor, pregnenolone (PREG), and neuroactive steroids such as deoxycorticosterone (DOC), pregnenolone, cortisol (corticosterone in rodents), mineralocorticoids and aldosterone. Glucocorticoids induce and regulate the body's physiological response to stress such as changes in cardiovascular function and sweat gland activity. Glucocorticoids (primarily cortisol in humans and corticosterone in rodents)

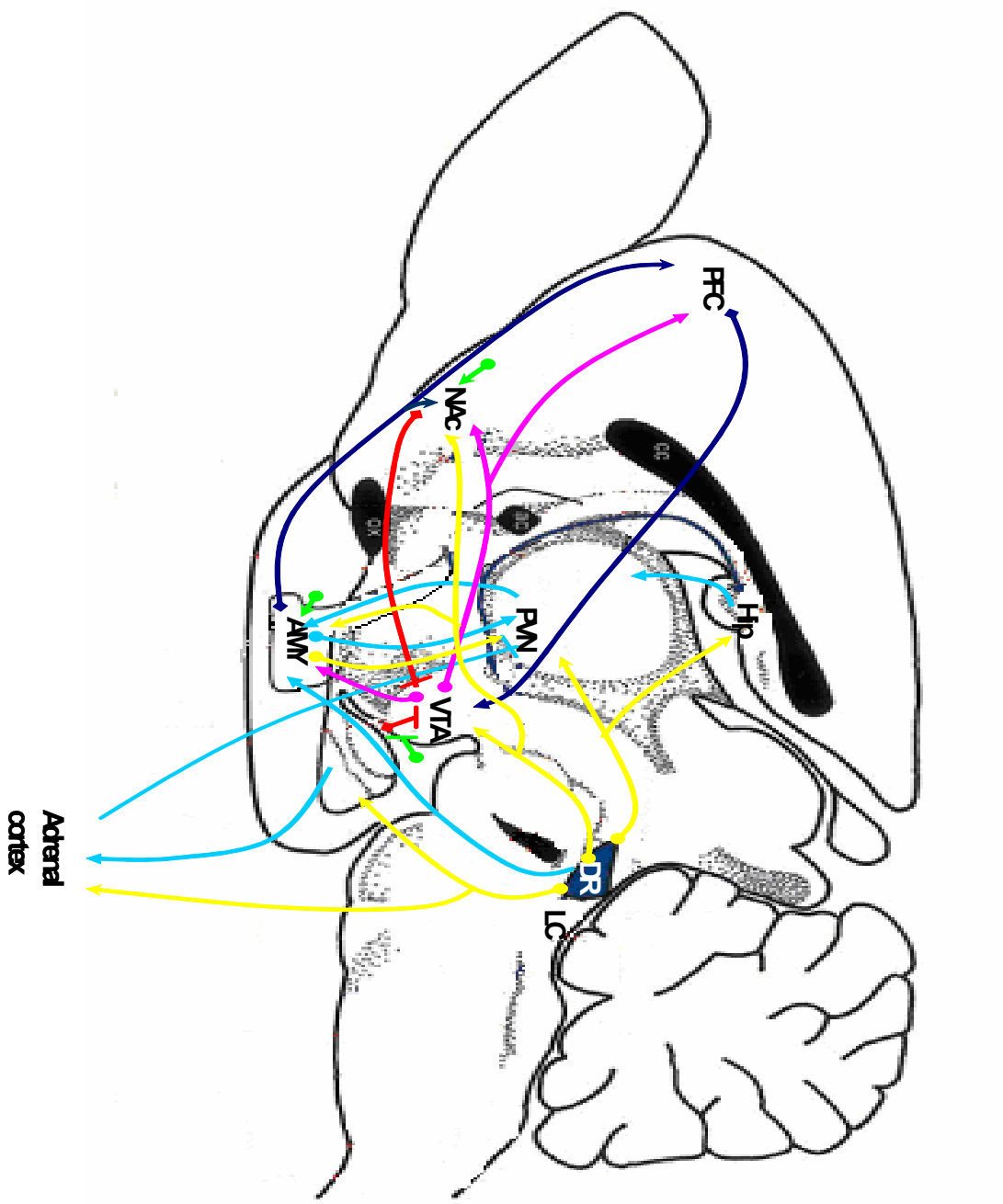

Figure 1: Schematic Diagram of Stress-responsive and Ethanol-responsive Pathways in the Brain. ox=optic chiasm, cc=corpus callosum, ac=anterior commissure. AMY=amygdala, DR=dorsal raphe, Hip=hippocampus, NAc=nucleus accumbens, PFC=prefrontal cortex, PVN=paraventricular nucleus of the hypothalamus. Light blue=CRH responsive circuit, yellow= serotonergic projection, pink=dopaminergic projection, red=GABAergic projections, dark blue=glutametergic projections, green=opioid projections.

also regulate the HPA axis through a negative feedback mechanism by acting on hypothalamic glucocorticoid receptors to decrease CRF release (Munck et al. 1984) and in the pituitary, directly inhibit ACTH release and the production of its precursor POMC (Dallman et al. 1985).

Two contrasting theories exist for the role of glucocorticoids in the stress response (Sapolsky et al. 2000). In the classical viewpoint, glucocorticoids actively stimulate the stress response or act in a permissive fashion by allowing other facets of the stress response to emerge. More recently, it has been suggested that glucocorticoids suppress the stress response and prevent it from being over-activated. In the short term, activation of the HPA axis results in beneficial adaptive responses. However, prolonged or sustained activation of the HPA axis (through overproduction of stress hormones and/or failure to terminate HPA activation) results in maladaptive responses (McEwen 1998; Heuser et al. 2003).

Animal models of social stress

Acute responses to social stress in rodents share many of the characteristics that are seen in reaction to other stressful stimuli. Aggressive social conflict rapidly activates the sympathetic nervous system detected as tachycardia, hypertensive and hyperthermic responses (Fokkema et al. 1988; Meerlo et al. 1996). In fact, both the aggressive resident and the attacked intruder show elevated corticosterone and ACTH (adrenocorticotropic hormone) as well as increased heart rate and blood pressure, during the attack phase, but only the intruder shows delayed recovery to baseline (Tornatzky et al. 1993; Covington et

al. 2005) suggesting long-term and potentially dysregulated sympathetic responses in the defeated animals.

Glucocorticoid secretion by the adrenal gland is considered one of the central hallmarks to stressful events (Piazza et al. 1998) and in socially stressed animals is believed to fulfill multiple roles. Sympathetic and HPA activation follow each other in rapid succession and are part of the initial reaction to social stress (Covington et al. 2005) which activates energy metabolism and the immune response important as coping mechanisms. Glucocorticoid activation is protective and restorative in this initial phase (Sapolsky 2005). In anticipation of predictable repeated social stress, glucocorticoids activation occurs on each occasion (Pardon et al. 2004). Over time, frequent and prolonged glucocorticoid stimulation can increase the allosteric load and lead to serious pathophysiological consequences to the cardiovascular, metabolic, and immune systems as well as hippocampal-mediated cognitive functions (McEwen 1998).

Importantly, repeated exposure to novelty or startle stressors shows a rapid decline of glucocorticoid activation upon repeated exposure. This habituation does not occur when social stress is encountered intermittently in infants or adults (File 1995). In fact, repeated intermittent social stress evokes a large sympathetic and HPA response. Whereas a single social defeat increased core body temperature and three-fold increase in corticosterone, repeated defeats induced a fifteen fold corticosterone increase in subordinate animals (Keeney et al. 2001).

In addition to the neurochemical responses, social defeat stress in rodents causes a range of anxiety-provoking and depressive-like behaviors. Socially defeated animals

interact less with unfamiliar animals (Kudryavtseva et al. 1991) and readily display defensive and submissive postures (Puglisi-Allegra et al. 1988). In general, defeated animals are less active, have decreased locomotor activity and display decreased food and liquid consumption, hallmarks of anhedonia (Meerlo et al. 1996; Meerlo et al. 1996). Defeated rats avoid brightly lit open spaces showing increased anxiety-like behavior (Avgustinovich et al. 1997). Even a single episode of defeat can alter nocioception, locomotor activity and cellular activation in corticolimbic structures (Tornatzky et al. 1993; Miczek et al. 1999; Nikulina et al. 1999). Behavioral and neurobiological consequences of social defeat amplify and endure particularly if the episodes are repeatedly administered in an unpredictable, uncontrollable manner (Tornatzky et al. 1993; Yap et al. 2006). Social memory, social interaction and anticipation for a sucrose reward are all impaired or diminished even up to 3 months following the last defeat session (Von Frijtag et al. 2001).

Social defeat stress as a model for affective/depressive disorders has predictive validity. To date, both tricyclic antidepressants (imipramine, clomipramine and tianeptine) and selective serotonin reuptake inhibitor, SSRIs (citalopram and fluoxetine) attenuate several behavioral and endocrine defects engendered by repeated social defeat. Relief from anhedonia as measured by increases in preference for a sucrose solution, low locomotor activity and suppressed social contact have been restored by imipramine, citalopram or fluoxetine in mice, rats and tree shrews, as reviewed (Miczek et al. 2008). Additionally, anxiety-like behaviors induced by social defeat stress have been attenuated by anxiolytic drugs and ethanol consumption (Rodgers et al. 1993; Avgustinovich et al.

1998; Berton et al. 1999). Thus, models of repeated social defeat are an ethologically relevant form of social stress in rodents which mimic many hallmarks of stress and affective disorders in humans. These models will greatly enhance our understanding of the effects of a social stress on ethanol drinking behaviors.

Neurobiology of social stress in rodents

Early studies used postmortem tissue to demonstrate the involvement of increased dopaminergic activity on the mesolimbic areas of attacked or defeated rodents (Mos et al. 1979; Puglisi-Allegra et al. 1990). More recently, the findings were extended and confirmed by in vivo micro dialysis showing increased dopamine release in prefrontal cortex and nucleus accumbens but not striatum during a threatening attack by an aggressive resident (Tidey et al. 1996; Miczek et al. 1999). However, increased accumbal dopamine release is not specific to the experience of defeat, since aggressive residents also show a similar response when engaging in offensive threat and attack behavior (van Erp et al. 2001). Thus, it is interpreted that the dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and prefrontal cortex (PFC) may indicate a marker of the salience of the attack for both parties. Dopaminergic release from the VTA to the NAc and PFC is tightly regulated by glutamatergic feedback from the PFC and hippocampus (Fallon et al. 1978; Fallon et al. 1978; Fallon et al. 1978). Brief social defeat also leads to increased NMDA receptor binding in the hippocampal CA3 neurons of defeated rats (Krugers et al. 1993).

In addition to dopaminergic pathways, serotonergic pathways may be involved. 5-Hydroxytrytamine (5-HT) and its primary acid metabolite 5-hydroxyindoleacetic acid (5-

HIAA) were elevated in the forebrain, septum hippocampus and hypothalamus of attacked tree shrews (Miczek et al. 2008). In mice, in vivo micro dialysis revealed increased hippocampal 5-HT following repeated defeat (Keeney et al. 2006). This contrasts with decreased cortical 5-HT in aggressive rats (van Erp et al. 2001), even though both attacking and defeated animals show elevated corticosterone (Covington et al. 2005). Interestingly, the expression of genes for serotonin transporter (SERT), tryptophan hydroxylase or the 5-HT1A autoreceptor were not differentially regulated following social defeat, suggesting that non-serotonergic neurons may be the site of neuroplastic changes resulting from defeat stress at least in the dorsal raphe nucleus (Abumaria et al. 2006). In contrast to the rat data, defeated CBA/Lac mice show elevated levels of serotonin and monoamine oxidase A (MAOA) mRNA in the raphe nucleus (Filipenko et al. 2002), consistent with increased serotonin efflux in the mPFC following inescapable footshock (Bland et al. 2003). One explanation for these conflicting findings could be protocol differences between continuous versus episodic stress. Brief social defeat stress predominantly involves the VTA-medial PFC-amygdala circuit whereas continuous uncontrollable stress relies on glutamatergic modulation of the dorsal raphe nucleus corticolimbic projections (Figure 2 and reviewed in (Miczek et al. 2008). Chronic social subordination reduces 5HT1A receptor binding in hippocampus (Flugge 1995) and attenuates hypothermic and HPA response to 5-HT1A agonist challenge (Buwalda et al. 2005), which has been interpreted as evidence for 5-HT1a receptor desensitization, comparable to that seen in depressed patients (Lesch et al. 1990).

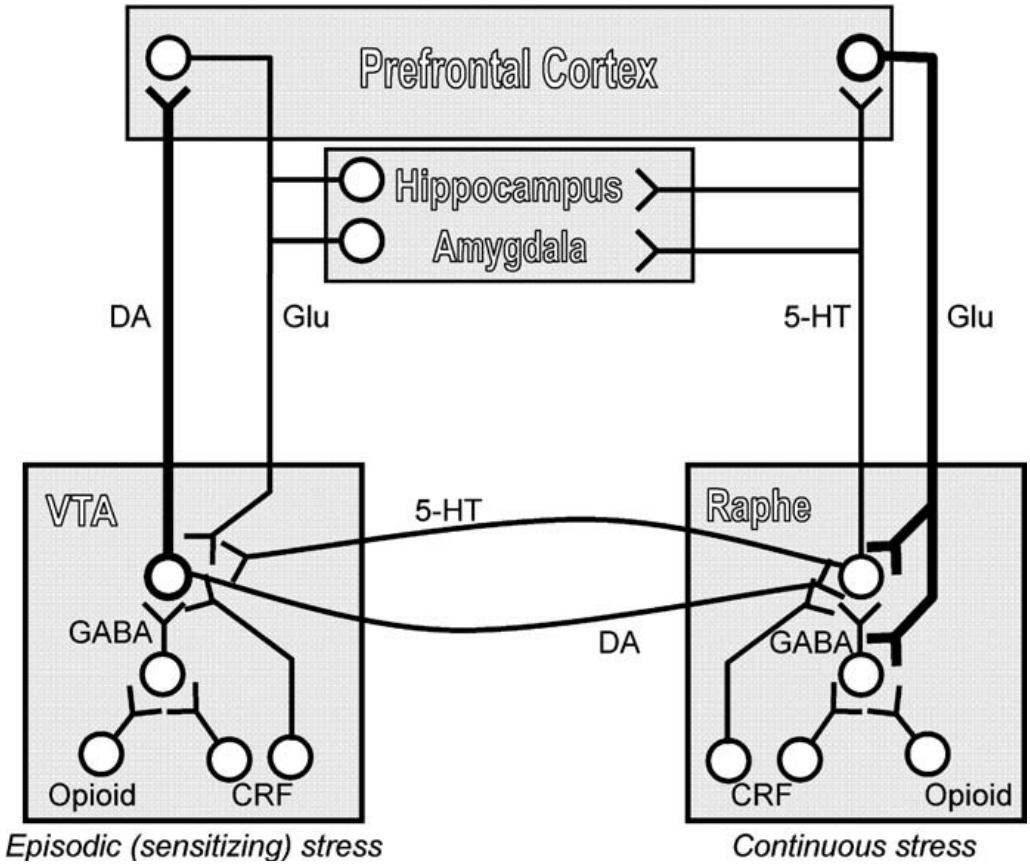

Figure 2: Neural Circuits for Brief Social Defeat Stress and Continuous Subordination Stress. The ascending DA pathway originating in the VTA and projecting to the mPFC is inhibited by GABA interneurons which in turn receive input from opioid peptides and CRH, among others. Glutamatergic feedback from PFC and amygdala modulates the DA pathway directly or by acting on GABAergic interneurons. This pathway may be rendered hyperactive as a result of brief social defeat episodes. By contrast, continuous uncontrollable subordination stress activates the serotonergic DRN cells that project to the forebrain, including the PFC. Glutamatergic feedback from PFC and limbic forebrain modulates the ascending 5-HT projections and it has been proposed that this feedback prevents dysregulation of the 5-HT system. Adapted from Miczek, Yap and Covington review (Miczek et al. 2008).

In addition to alteration in neurotransmitter levels, social defeat stress causes morphological and molecular changes in the hippocampus. Hippocampal volume and remodeling of the apical dendritic tree decreases in defeated tree shrews (Fuchs et al. 1995) and rats (Kole et al. 2004). Importantly, these changes may have clinical significance since imaging studies reveal decreased left hippocampal volume in depressed patients (Bremner et al. 2000). A possible mechanism for the hippocampal changes is reduced neurogenesis in the dentate gyrus as reduced cell proliferation in defeated mice has been reported (Yap et al. 2006). This could be especially important since glutamatergic feedback from the hippocampus modulates dopaminergic cells in the VTA (Vorel et al. 2001).

Acute social defeat stress in mice, hamsters and rats is associated with increased neuronal activity along the core of the neuroaxis where increased c-fos expression is used as a marker for neuronal activity. Acute social defeat induces c-Fos expression in the prefrontal cortex, lateral septum, medial and central amygdala, hypothalamic nuclei and several brain stem nuclei including the central and periaqueductal grey, locus coeruleus, dorsal raphe nucleus, ventral tegmental area and the nucleus of the solitary tract (Martinez et al. 1998; Miczek et al. 1999; Nikulina et al. 1999). After repeated social defeat experiences, c-fos increases remain in the anterior and ventromedial hypothalamus, medial amygdala, central grey, dorsal and medial raphe nuclei (Martinez et al. 1998). These changes could reflect neuroadaptations resulting from repeated social defeat and may mediate behavioral changes relevant to increased drug taking and affective disorders. As evidence to support this hypothesis, when challenged with a moderate amphetamine dose,

c-fos expression remains elevated in the VTA and PFC more than two months after four social defeat episodes (Covington et al. 2005; Nikulina et al. 2005). Additionally, glutamatergic feedback from several regions activated by social defeat stress (PFC, amygdala and hippocampus) feedback to the dopaminergic cells in the ventral tegmental area and are hypothesized to play an important role in the neuroadaptations that lead to intense drug taking behavior (Vanderschuren et al. 2000).

Neural pathways influencing stress and ethanol drinking

Stress and drug abuse are thought to interact on the basis of the connected inter-circuitry between the HPA axis, extended amygdala and mesocorticolimbic pathway incorporating the HPA axis, the dopamine reward pathway, and the serotonin and opioid systems. GABAergic, glutamatergic, neuropeptide Y (NPY) and the extra-hypothalamic CRF systems also play a role in the development of alcoholism, see Figure 1. Stressful experiences activate the HPA axis and can sensitize these circuits leading to increased salience of ethanol's effects. In addition, ethanol acts as an anxiolytic agent through these same pathways to reduce the negative aspects of stress. The mesocorticolimbic pathway is comprised of dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and the prefrontal cortex (PFC) and has been coined the "reward pathway" since multiple drugs of abuse activate this pathway (Fallon et al. 1978; 1978; 1978). Reciprocal glutamatergic neurons project from PFC to VTA, providing excitatory control of VTA and ultimately dopamine release in NAc. A stress-responsive dopaminergic projection from VTA to basolateral amygdala (AMY) interacts with PFC to indirectly modify dopamine release in the NAc (Stevenson et al. 2003). Following chronic

drug abuse, the dopamine surge into the NAc not only triggers a drug's pharmacological reward, but may also erode an individual's resolve to abstain from drug-taking behavior. In cocaine self-administering rats, dopamine spiked in the NAc in anticipation of receiving cocaine, upon presentation of a cue paired with delivery, as well as after drug delivery. Stimulation of dopamine release also initiated drug-seeking behavior, suggesting that dopamine release in the NAc may play a dual role in drug taking behavior (Phillips et al. 2003). β-endorphin neurons in the arcuate nucleus of the hypothalamus inhibit CRF release in the paraventricular nucleus of the hypothalamus (Calogero 1995) and also stimulate dopamine (DA) release in the NAc (Cowen et al. 1999). Endogenous opioid peptides are co-localized with dopaminergic neurons in several limbic regions (Maidment et al. 2002; Norton et al. 2002) and may directly or indirectly modulate activity of dopaminergic neurons. The dorsal raphe, the primary source of serotonin (5-HT), sends projections to the ventral tegmental area and nucleus accumbens (Parent et al. 1981). Acute ethanol increases release of DA and 5-HT in the NAc and the amygdala (McBride et al. 1990; Yoshimoto et al. 1992) , whereas stress from social defeat (Filipenko et al. 2002), or following inescapable footshock (Bland et al. 2003) increase 5-HT mRNA levels in the raphe nucleus and serotonin efflux in the mPFC. Together this suggests that the serotonin system may also modulate the dopaminergic reward system in response to ethanol or stress.

Behavioral effects of stress and drugs of abuse

Social stress in rodent models also modifies drug seeking behavior for a number of drugs of abuse. Four episodes of social defeat stress were sufficient to increase the rate of acquisition for cocaine self-administration in rats (Tidey et al. 1997). Intermittent social

defeat augments the locomotor activity in response to stimulant challenge with cocaine, amphetamine or morphine pointing to the sensitizing effects of social stress (Miczek et al. 1999; Covington et al. 2001; Covington et al. 2005). Defeat stress abolishes the typical circadian pattern of drug taking, seen where defeated rats also self-administer cocaine continuously during a 24 hour binge (Covington et al. 2005).

Repeated social defeat stress also induces an opioid-like analgesia which can be blocked by mu-opioid receptor antagonists (Miczek et al. 1982). The impact of social stress on opioid seeking or taking has not been adequately studied to date. One study shows decreased place preference for the morphine paired side in defeated rats (Coventry et al. 1997). But it is difficult to reconcile with other stress studies where foot shock treatment could reinstate opioid seeking and morphine induced conditioned place preference (Shaham et al. 1995; Lu et al. 2003).

Role of stress in excessive drinking and alcohol abuse

In a subset of the population, alcohol abuse becomes a problem and these individuals may become dependent. Prolonged alcohol use can decrease the functioning and responsiveness of several neurobiological pathways which become further exacerbated by repeated cycles of binge drinking and withdrawal altering the hedonic set-point leading to protracted alterations in each of these systems (Koob 2003). Stressful events (and their perception) also regulate these same pathways and can sensitize the circuits causing a hyper-excited circuit. Additionally, withdrawal from alcohol increases anxiety, negative affect and other withdrawal symptoms and through multiple withdrawal cycles, these symptoms become increasingly severe. Together with the increased sensitivity of these

neural systems and progressive withdrawal symptoms, a dependent individual may return and often increase his/her alcohol abuse. Thus, it is the underlying neurobiological pathways which have been repeatedly stressed and hyperactive which may explain why alcoholics are likely to relapse during stressful life events, even after years of abstinence.

Stress and anxiety are commonly thought to play a major role in the development of alcohol abuse and relapse drinking and several lines of evidence have linked them together. Alcoholics report anxiety-reduction as a major motivational factor for drinking (Conger 1956; Pohorecky 1981; Newlin et al. 1990). In fact, 80% of alcoholic patients report alcohol drinking to reduce feelings of anxiety, depressed mood and negative emotional states (Hershon 1977; Annis et al. 1998). During periods of chronic abstinence, alcoholics can experience mood disturbances, negative affect and anxiety (Begleiter et al. 1979; Roelofs 1985) which are correlated with relapse drinking (Hershon 1977; Annis et al. 1998). However, a majority of clinical studies involve self-reports or co-occurrence of disease and it is not always clear whether the proclivity to abuse alcohol precedes the anxiety-related disorders. Thus, a variety of animal models have been employed to investigate the relationship between anxiety-like behaviors and ethanol consumption. Variability in ethanol consumption in rodent lines may be, at least in part, due an animal's basal anxiety levels suggesting a role for basal anxiety states in the predisposition towards ethanol consumption in rodent models. Likewise, rats selectively bred for ethanol preference, P rats, show lower anxiety in three different behavioral measures as compared to ethanol-nonpreferring NP rats (Stewart et al. 1993; Pandey 2003).

Ethanol can act as an anxiolytic to reduce several measures of anxiety and neurochemical markers of HPA axis activation (Sinha et al. 2000; Sher et al. 2007). Likewise, ethanol is a well-documented anxiolytic in multiple rodent models of anxiety, including the elevated plus maze (LaBuda et al. 2000; LaBuda et al. 2001; Boehm et al. 2002), the light-dark test (Costall et al. 1988; Bilkei-Gorzo et al. 1998; Boehm et al. 2002), the social interaction test (Varlinskaya et al. 2002), and the mirrored chamber test (Cao et al. 1993; Kliethermes et al. 2003). As previously discussed, ethanol and stress responsive pathways share common overlapping neurobiological substrates and brain regions.

Alcohol abuse and alcoholism show a high degree of comorbidity with anxiety-related disorders (Bibb et al. 1986; Cornelius et al. 2003). Social stressors such as early family adversity, including abuse, emotional neglect, and harsh inconsistent punishment are also risk factors for alcohol and drug abuse (Zoccolillo et al. 1999). These clinical findings have been supported by a number of studies in animals showing that social isolation or maternal separation in early life increases alcohol and drug self-administration (Meaney et al. 2002; Brake et al. 2004).Using rhesus monkeys naturally reared by their mothers or by peers (stressed condition) during their first 6 months, Higley *et al* showed stressed monkeys drank more ethanol than naturally reared monkeys as adults. When the mother-reared monkeys were stressed by social isolation, they increased drinking level to that of their stressed peers (Higley et al. 1991; Higley et al. 1993; Sinha 2001).

Social stress and ethanol drinking behaviors

Much effort has been placed into the research of ethanol consumption and social stress. In general, the results have been inconsistent at best (see Table 1). Subordinate rats

Table 1: Effects of stress on ethanol drinking in rodent models

Stress model	Effect on ethanol drinking	Strain	Reference
cold immobilization	dependent on baseline intake	Wistar rats	Rockman *et al.* 1987
footshock	decrease	Sprague-Dawley rats	Brunell & Spear 2005
footshock	decrease	Sprague-Dawley rats	Fidler & LoLordo1996
footshock	dependent on baseline intake	Sprague-Dawley rats	Volpicelli *et al.* 1990
footshock	increase	Wistar rats	Funk *et al.* 2004
footshock	increase	Wistar, P and HAD rats	Vengeliene *et al.* 2003
footshock	increase	Wistar rats	Le *et al.* 1998
footshock	increase in C57, no effect in DBA or A/J	C57, DBA and A/J mice	Matthews *et al.* 2008
restraint stress	increase in P rats, no effect in HAD rats	P and HAD rats	Chester *et al.* 2004
restraint stress	dependent on baseline intake	Wistar rats	Rockman *et al.* 1986
restraint stress	increase	Wistar rats	Lynch *et al.* 1999
restraint stress after withdrawal	increase	P rats	Breese *et al.* 2004
restraint stress after withdrawal	increase	P rats	Overstreet *et al.* 2007
restraint stress after withdrawal	no effect	WSC-1 mice	Tambour *et al.* 2008
social defeat	decrease	Wistar rats	Funk *et al.* 2005
social defeat	decrease	Long Evans rats	Van Erp *et al.* 2001
social defeat	decrease	Long Evans rats	Van Erp *et al.* 2001b
social defeat	dependent on baseline intake	C57BL/10 mice	Croft *et al.* 2005
social defeat	increase	NIH swiss mice	Hilakivi-Clarke & Lister 1992
social defeat and subordination stress	increase	Long Evans rats	Blanchard *et al.* 1987
social defeat or swim stress	increase in CRH-/- mice only	Crhr1 -/- and 129xCD1 F2	Sillaber *et al.* 2002
social stress	increase	Long Evans rats	Blanchard *et al.* 1992
social stress	increase	Fawn hooded rats	Ellison 1981
swim stress	decrease in DBA and Balb/c, no effect in C57	C57BL/6J, DBA and Balb/c mice	Boyce-Rustay *et al.* 2008
swim stress	increase in Wistar, no effect in P and HAD rats	Wistar, P and HAD rats	Vengeliene *et al.* 2003
swim stress	no effect	C57BL/6J mice	Boyce-Rustay *et al.* 2007

A summary of ethanol drinking behavior following a variety of stressors in outbred, inbred and selected strains

and monkeys tend to drink more ethanol than dominant animals (Ellison 1981; Blanchard et al. 1987). Mice that lack a functional CRH1 receptor, consume significant amounts of ethanol after only three episodes of social defeat stress. This increased ethanol drinking began three weeks following the last defeat episode and persisted for at least six months (Sillaber et al. 2002). Defeated wild-type rats drank less ethanol in their home cage after intermittent social defeat or continuous subordination (van Erp et al. 2001; van Erp et al. 2001). However, once the social stress was discontinued, increased ethanol consumption can be seen (Volpicelli et al. 1990). Intriguingly, once drinking has been extinguished, a cue associated with the experience of social defeat facilitates ethanol-seeking, but social defeat itself does not (Funk et al. 2005).

Although some clinical and animal studies point towards a positive relationship between stress and ethanol drinking, the tension-reduction hypothesis for alcohol-use has not been uniformly supported. Many clinical studies are inherently biased by self-reporting where it may be difficult for subjects to distinguish between events which resulted from alcohol use and relapse and events which precipitated alcohol use (Brady et al. 1999). Additionally, studies on the direct effects of stress on alcohol use and craving use contrived laboratory situations which also have their own inherent limitations. In animal models, the type, strength and frequency of the stressor and timing of ethanol presentation affect the drinking outcome (see Table 1) not to mention the genetic influence from the background strain. In two lines selectively bred for alcohol preference, 10 days of unpredictable restraint stress moderately decreased ethanol intake in preferring P and HAD1 rats during the stress period. Five days following the stress application, ethanol

intake increased in P rats but not HAD1 rats (Chester et al. 2004). Footshock-induced stress decreased drinking in Sprague-Dawley rats (Brunell et al. 2005), but increased ethanol intake in P and HAD rats (Vengeliene et al. 2003). On the other hand, swim stress increased drinking in Wistar rats, but decreased intake in the alcohol preferring P and HAD lines (Vengeliene et al. 2003). In studies where stress increases ethanol drinking, intake generally decreases during the stress application, but increases in the days following termination of the stress (Yavich et al. 2000; Sillaber et al. 2002; Croft et al. 2005). Importantly, where investigated, it appears that stress-influenced ethanol drinking depends on an individual's baseline response as low preferring animals tend to increase drinking following stress (Rockman et al. 1986; Rockman et al. 1987; Volpicelli et al. 1990; Croft et al. 2005).

Application of DNA microarrays to ethanol-related behaviors

Chronic drug exposure causes long term cellular and molecular changes in the brain believed to play a critical role in development of drug addiction (Nestler et al. 1997). Changes in gene expression have been implicated as crucial molecular events underlying experience dependent plasticity following long term behavioral responses to ethanol and other drugs of abuse (Miles et al. 1995; Nestler et al. 1997; Nestler 2001; Rhodes et al. 2005). Although a wide variety of ethanol-induced changes in gene expression have been documented throughout the brain, as reviewed (Worst et al. 2005), the precise mechanism underlying these changes remains unknown. One hypothesized mechanism is mediated through intracellular signaling cascades following drug perturbation of synaptic transmission (Nestler 2001). Therefore, once ethanol acts on its respective extracellular

targets, numerous intracellular signaling pathways are initiated through second messengers such as protein kinases and phosphatases, thereby eliciting corresponding physiological responses. These second messengers also simultaneously provoke drug-induced changes in gene expression by altering the activity of transcription factors (Nestler et al. 2001).

Single gene expression studies have advanced the understanding of mechanisms involved in drug abuse and have shown great promise in testing potential therapeutic targets, even though these studies have only shown limited utility in understanding the inter-related neuroadaptive changes following chronic abuse. The development of high-density DNA microarrays has enabled the unbiased and massively parallel analysis of thousands of genes simultaneously (Schena et al. 1995; Lockhart et al. 1996). Subsequent expression analysis coupled with bioinformatics techniques allow identification of gene expression profiles with potentially inter-related functions. Using microarrays, Hughes et al. first demonstrated that a large database of expression profiles can be used to identify pharmacological mechanisms for drugs with previously unknown action, and can also suggest the function of uncharacterized genes (Hughes et al. 2000). Several laboratories have used microarrays to identify and localize genes related to ethanol and its consumption (Thibault et al. 2000; Hassan et al. 2003; Saito et al. 2004; Kerns et al. 2005). Additional research using this technology has been employed to study molecular mechanisms involved in anxiety-like behaviors (Kromer et al. 2005; Weaver et al. 2006). In an elegant study, Nestler *et al.* identified expression patters that were associated with social stress and showed that deletion of the brain-derived neurotrophic factor (BDNF) gene, using viral-vector microinjection, blocked transcriptional responses to stress (Berton et al. 2006).

CHAPTER 3 Characterization of Individual Variation of Ethanol Drinking in C57 mice

Introduction

Over 121 million Americans drink alcohol, while less than 10% of the population drinks excessively (Administration 2005). Even though a majority of Americans are exposed to alcohol, its consumption is not evenly distributed throughout the population. Nearly 64% of adults actively drink alcohol, but strikingly 20% of this population consumes over 80% of all the alcohol sold (Dawson 2000). Extensive studies in humans have suggested that genetic factors account for about 40-60% of the risk for alcoholism (Cloninger 1987; Gordis et al. 1990; Enoch et al. 2001; Radel et al. 2001). These facts highlight the importance of identifying those factors which may influence the variability in drinking behaviors. Work in humans and animal models over the last 20 years has documented genetic intervals (Phillips et al. 1994; Crabbe 2002; Lovinger et al. 2005) or individual genes (Shirley et al. 2004; Fehr et al. 2005) contributing to variation in behavioral responses to ethanol.

Despite such progress on identifying genetic influences in alcoholism, little work at the molecular level has been done to identify mechanisms that mediate environmental influences on ethanol behaviors or alcoholism. It is well documented that environmental

influences such as stress or exposure to conditional stimuli can modify ethanol drinking or cause recidivism in abstinent alcoholics. A few recent studies have investigated individual variation of ethanol drinking in cynomolgus monkeys and shown a correlation between responsiveness of the HPA axis and ethanol consumption (Porcu et al. 2006; Porcu et al. 2006). Understanding the molecular mechanisms underlying such environmental influences on ethanol behaviors would augment the genetic progress mentioned above.

C57BL/6 inbred mice have been widely used as a model for studying alcohol abuse related behaviors and the genetic basis of alcohol abuse since these mice voluntarily consume large volumes of unadulterated ethanol (McClearn 1972; Goodrick 1978; Belknap et al. 1993; Phillips et al. 1994; Bachmanov et al. 1996; Gill et al. 1996; Middaugh et al. 1999; Nocjar et al. 1999; Rhodes et al. 2005). However, a number of prior studies have documented remarkable degrees of stable, individual variation in 2-bottle choice drinking behavior in rodents and monkeys. Several of which have shown that individual variation in drinking behavior can occur within a single inbred strain (Dole et al. 1988; Little et al. 1999; O'Callaghan et al. 2002). This removes factors such as genetic differences in taste or ethanol reward as causal for the variation in drinking behavior. Studies in C57 mice suggest that non-genetic persistent individual differences in drinking behavior were the major source of variance in ethanol drinking in these animals, despite substantial environmental changes such as diet (Dole et al. 1988). Using such a model, where genetic factors are strictly controlled, offers considerable power for studying molecular mechanisms of environmental modulation of ethanol drinking behavior.

Here, we have characterized the individual variation of ethanol drinking behaviors within an inbred strain known to consume substantial amounts of ethanol. Subsequent chapters will investigate the molecular differences between these mice and investigate the influence of social stress and anxiety on ethanol intake.

Methods

Animals: Male C57BL/6NCrl mice (C57) at age 42 to 49 days of age were purchased from Charles River Laboratories (Wilmington, MA). All mice were habituated to the housing environment by group housing (5 mice/cage) for 1 week followed by individual housing for 1 week prior to beginning drinking experiments. Cages and bedding (Harlan Sani-chips, catalog #7090A, Harlan, Teklad, Madison, WI) were changed once a week during the 6 hour window when ethanol was not available to mice. Mice were housed in a temperature and light controlled room (12:12 h light-dark cycle, lights on at 0600) with free access to standard rodent chow (catalog # 7912, Harlan Teklad, Madison, WI) and water throughout the study. All 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 (NIH Publications No. 80-23, 1996).

Two-bottle (ethanol or water) choice drinking: Ethanol drinking was initiated by placing two bottles into the top of each mouse's home cage at the beginning of the dark cycle. One bottle contained 10% (w/v) ethanol (Aaper Alcohol and Chemical Co. Shelbyville, KY) in tap water and the other contained tap water. Bottles of ethanol and tap water were also placed on an empty cage to account for evaporation and this value was

subtracted from the amount of liquid consumed for each mouse to calculate corrected ethanol intake and preference ratios. The bottles were available for 18 hours/day at the beginning of the dark cycle. Mice were allowed free access to water for the remaining 6h/d from standard water bottles. Bottle position was varied in a double alternate design (i.e. L, L, R, R, etc.) to account for side preference. Mice remained undisturbed for each drinking session, after which fluid consumption was recorded to the nearest 0.1 ml. In some experiments, mice were given four consecutive days of drinking sessions followed by four days of abstinence. This cycle was repeated four times to give a total of 16 days of drinking. In other experiments, mice were given 14 consecutive days of ethanol access, as noted. Ethanol consumption was calculated as grams of ethanol per kilogram body weight per 18 hours. Percent ethanol preference was calculated by dividing the volume of ethanol consumed by the total volume of liquid (ethanol + water) consumed for daily 18h sessions.

For repeatability studies, C57 mice were housed in groups of 4/cage for 2 weeks on a reverse light cycle upon arrival to the facilities. At 7 weeks old, mice were singly housed for 7 days then started on two bottle choice drinking (10% ethanol (w/v) or tap water). Mice were given 24 hours of ethanol access beginning 4 hours into their dark cycle (1200 hours). We reversed the light cycle and increased the duration of ethanol access so that future studies looking at effects of social stress could be performed during the animal's active period. Standard mouse chow was supplied ad libitum throughout all studies. The position of ethanol and water bottles was switched every other day to avoid side preferences (i.e. L, L, R, R). Mice had continuous ethanol access for 14 days.

Blood ethanol concentration: Blood ethanol concentration was determined from ethanol drinking mice (n=13) after 14 days of access. In this experiment, mice were on a reverse light cycle (lights off at 0800). Mice were deprived of ethanol overnight to allow for a set start point to time the amount of ethanol access. Bottles of ethanol and water were placed onto the home cage of mice at 0930, 1.5 hours into their dark cycle. Mice were given 6 hours ethanol access and blood was collected from the saphenous vein from 1330 to 1500 hours. Blood samples were stored on ice then centrifuged at 1500 rpm at 4^{o}C. Blood ethanol concentration was determined in plasma samples using the AM1 Analox alcohol analyzer (Analox Instruments USA, Lunenberg, MA) following manufacturer's instructions exactly. Briefly, plasma was mixed with an alcohol oxidase enzyme mixture and ethanol and oxygen in the sample was oxidized into acetaldehyde and peroxide. Under the conditions of the assay, the rate of oxygen consumption is directly proportional to the ethanol concentration.

Ethanol Preference in Littermates: Two cohorts of male C57 littermates were ordered from Charles River Laboratories. Males were weaned at day 21 and remained housed as littermates until the beginning of the studies. 10 litters were represented with 3-5 males per litter. Mice were individually housed for 7 days then presented with 10% (w/v) ethanol in a two-bottle choice paradigm as above for 14 consecutive days.

Taste Discrimination: Taste preference for a bitter solution or sweet solution was measured using quinine or saccharin two-bottle choice paradigm similar to that described above. Male adult mice (n=16) were housed individually, tested for ethanol intake and preference for 14 days as above, then allowed to rest for 7 days with only water and food

available. Half the mice were given two bottles containing either a 0.1 mM quinine solution or tap water to measure taste preference for a bitter solution. Remaining mice were given a choice between bottles of saccharin (0.033%) or tap water to measure taste preference for a non-caloric sweet solution. Bottles were alternated every other day to avoid side preferences. Consumption of quinine and water or saccharin and water were measured daily for 3 days (18h/day) after which the other tastant was offered for 3 days in a counterbalanced design.

Results

C57BL/6 male mice from Charles River Laboratories consumed a substantial amount of ethanol, 6.47 ± 0.99 g/kg/18h, in the voluntary two-bottle choice (10% w/v ethanol or water) self-administration paradigm. Interestingly, mice showed a large degree of inter-individual variation in ethanol drinking (Figure 3A), ranging from 0.28 ± 0.14 g/kg/18h to 14.39 ± 0.47 g/kg/18h. This corresponds to almost complete ethanol abstinence (ethanol preference, 0.015±0.0074) to very high ethanol preference (0.95 ±0.035). Ethanol preference was significantly correlated to ethanol intake ($R=0.949$, $p<0.001$ Pearson Correlation) since there were minimal differences in total fluid consumed (Figure 3B). The only mouse with significantly higher fluid consumption had the lowest ethanol intake and preference. The variation in ethanol intake across individual mice was very consistent over the course of the drinking sessions (Figure 3C). Ethanol intake for the first 4 days of drinking were highly correlated with intake over the last 4 days of drinking ($R=0.676$, $p=0.0011$, day1-4 vs. day 25-28, see Table 2) and became even more tightly correlated over the course of the drinking session ($R=0.993$, $p<0.001$, day 17-20 vs. day 25-28). This

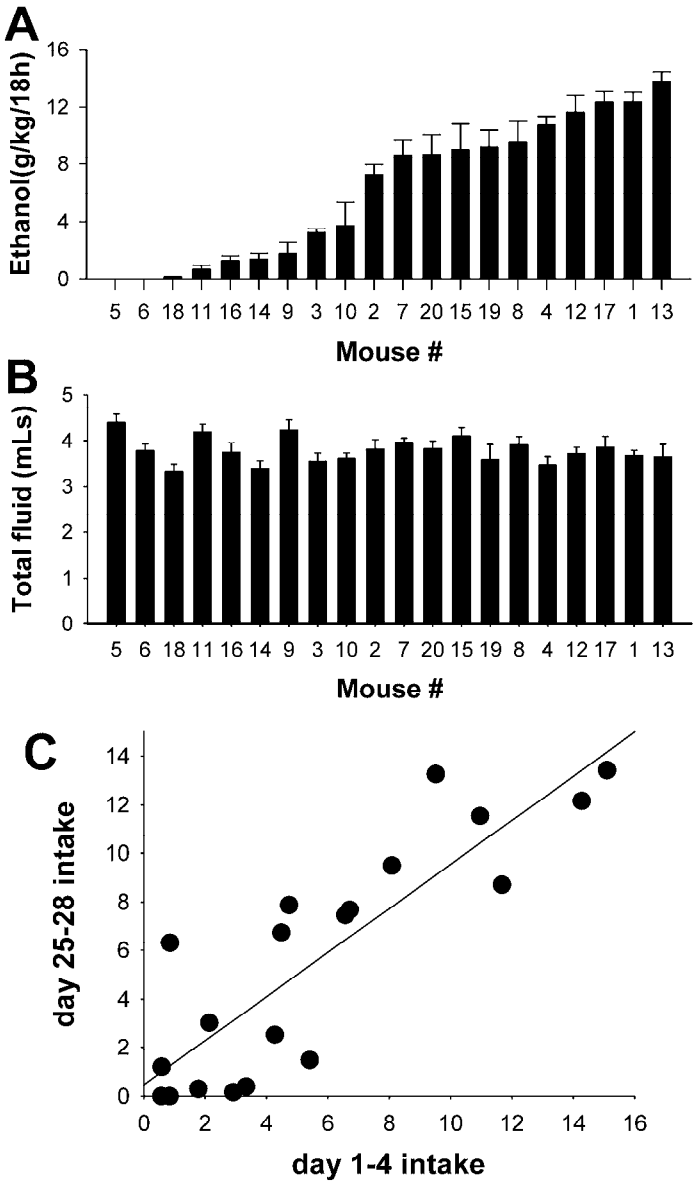

Figure 3: Ethanol Drinking in Individual C57BL/6NCrl Mice. A. Ethanol intake expressed in grams per kilogram body weight over 18 hours of ethanol access. Mice show a robust, but persistent variation in ethanol drinking. **B.** Total liquid consumed (mLs ethanol + mLs water) in 18h/day. **C.** Scattergram of ethanol drinking on days 1-4 versus days 25-28, correlation $R=0.676$, $p<0.0011$.

Table 2: Correlation of initial ethanol intake versus subsequent rounds of drinking following deprivation.

	d1-4 intake	d9-12 intake	d17-20 intake	d25-28 intake
d1-4 intake	1.000	0.703	0.772	0.676
d9-12 intake	0.703	1.000	0.850	0.779
d17-20 intake	0.772	0.850	1.000	0.993
d25-28 intake	0.676	0.779	0.993	1.000

stability is evidence that most of the variance observed is due to between-subject individual differences rather than merely to error variance.

Variation of ethanol intake and preference within the inbred C57 mice is highly reliable. Over the course of study, thirteen separate drinking studies were performed and each cohort showed similar distribution of persistent individual variation in ethanol intake over 14 days of basal drinking. Representative graphs from select studies are seen in Figure 4. Ethanol intake ranged from less than 1 g/kg to greater than 12 g/kg in each study, regardless of whether ethanol was continuously available or limited to 18h/day.

Ethanol drinking mice consume enough ethanol in a 6 hour period to raise blood ethanol concentrations (BEC) to detectable levels. The average BEC for all mice was 148.5 +/- 28.7 mg/dL. Ethanol intake was highly and significantly correlated to BEC ($R=0.721$, $p=0.005$, Figure 5). Some mice consumed enough ethanol in a 6 hour period to raise BEC over pharmacologically significant levels with BEC over 100 mg/dL in a 6 hour period. Pharmacologically significant blood ethanol levels have been previously defined as greater than 100 mg/dL (Rhodes et al. 2005).

Additionally, we determined whether the observed individual variation in ethanol drinking was due to litter effects. In two separate cohorts consisting of 10 litters (n=3-5 males/litter), ethanol intake over 14 days of baseline drinking did not differ between litters ($F(9,32)=1.258$ $p=0.2967$). Ethanol preference also did not differ between litters ($F(9,32)= 1.629$, $p=0.1489$). Representative ethanol intake in one cohort consisting of six litters can be seen in Figure 6. Figure 6A shows the average ethanol intake within each

litter, while figure 6B shows the distribution of ethanol intake between individual mice.

Within each

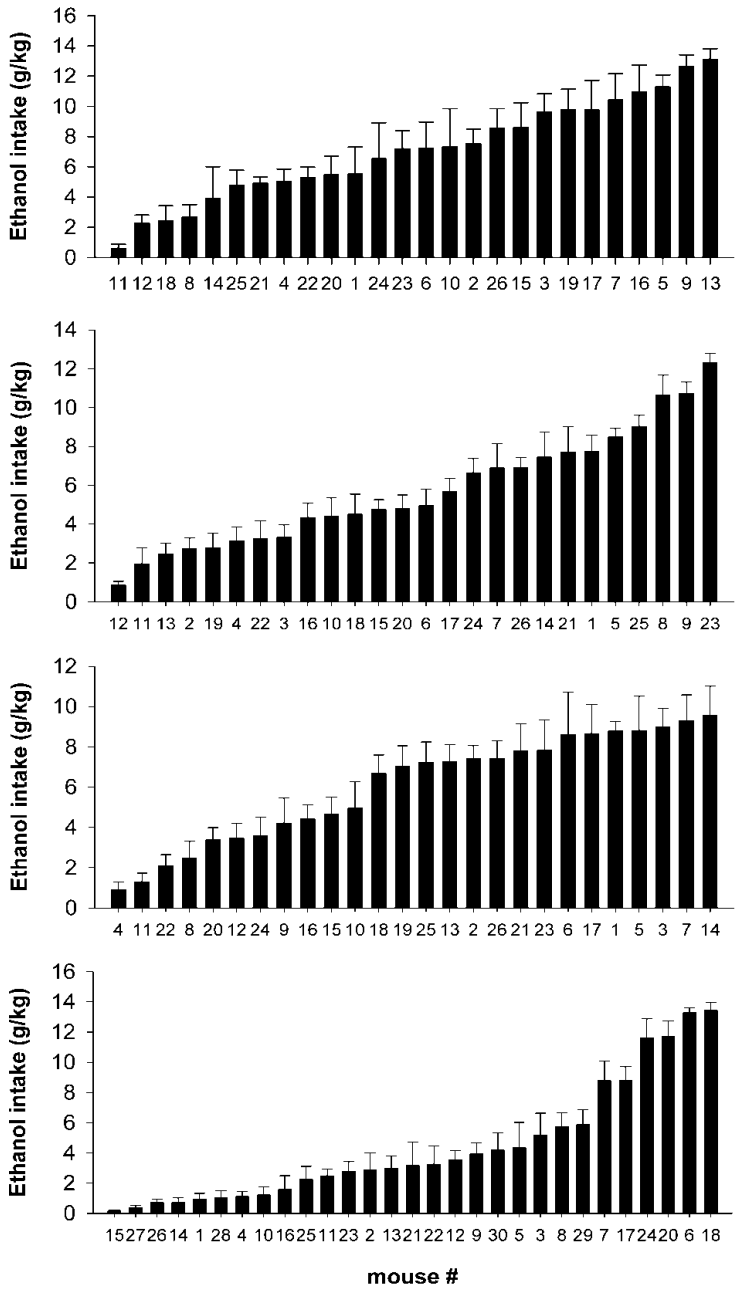

Figure 4: Repeatability of Individual Variation over Multiple Experiments. Ethanol intake in four representative experiments for baseline drinking in a 2-bottle choice paradigm. In each experiment, ethanol intake ranged from less than 2 g/kg to over 12 g/kg.

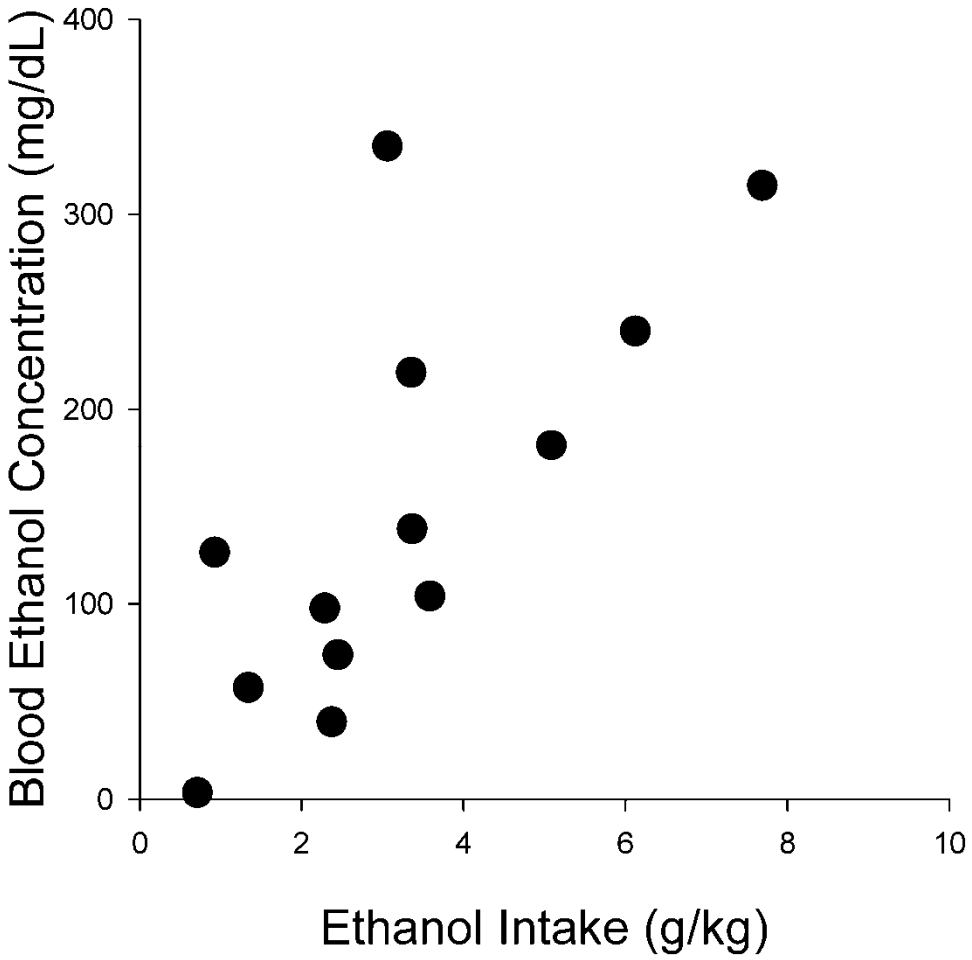

Figure 5: Blood Ethanol Concentration is Highly Correlated to Ethanol Intake. Ethanol intake over 6 hours of access is highly and significantly correlated to blood ethanol concentration (R=0.721, p=0.005) and reaches pharmacologically significant levels.

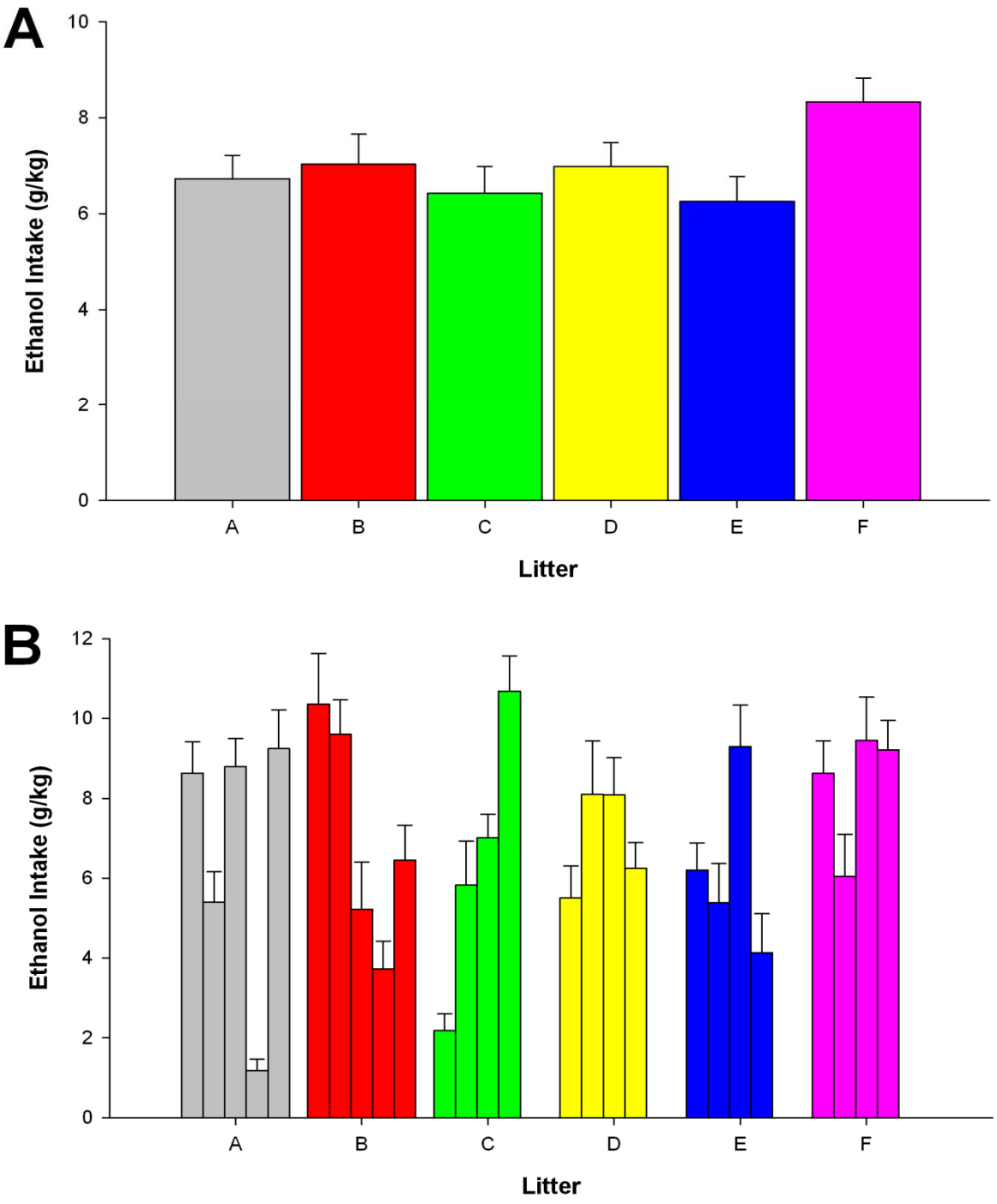

Figure 6: Litter is not a Major Factor in Individual Variation of Ethanol Drinking. A. Average ethanol intake is not significantly different between litters ($F(5,20)=0.314$, $p=0.899$). Each bar represents the average for all the males in the litter (n=4-5/litter). **B.** Average ethanol intake over 14 days of baseline drinking for each mouse in the litters represented in panel A.

litter there tends to be a range of ethanol intake suggesting that simple litter effect differences do not contribute to the inter-individual variability of intake in these mice.

In order to assess whether taste discrimination was contributing to the variation in ethanol drinking, we performed an experiment where mice (n=16) were assessed for ethanol drinking, followed by studies on preference for quinine (0.1 mM) or saccharin (0.033%). While there was some individual variation in quinine consumption, preference for saccharin (R=0.142, p=0.589) or quinine (R=0.196, p=0.468) showed no significant correlation to ethanol preference (Figure 7). These results argue against taste as a contributing factor for individual differences in ethanol preference.

Discussion

C57BL/6NCrl mice show a large degree in inter-individual variation in their ethanol drinking behaviors. In the present study, ethanol intake ranged from less than 1g/kg to over 14 g/kg of ethanol intake in an 18 hour period. Each mouse remained stable in its ethanol preference, where the first four days of drinking accurately predicted the animal's preference over the course of the experiment. Thus, among genetically identical B6 mice, some mice reliably consumed large amounts of ethanol while others almost totally avoided it. We suspect these differences were generated by subtle environmental differences such as rearing behaviors (Meaney et al. 2002; Brake et al. 2004; Weaver et al. 2004; Weaver et al. 2006), intrauterine position, social interactions and stress (Lathe 2004; Holmes et al. 2005). Individual variation has been reported for ethanol drinking behaviors as well as in stress responsivity (Krishnan et al. 2007) which may be a contributing factor

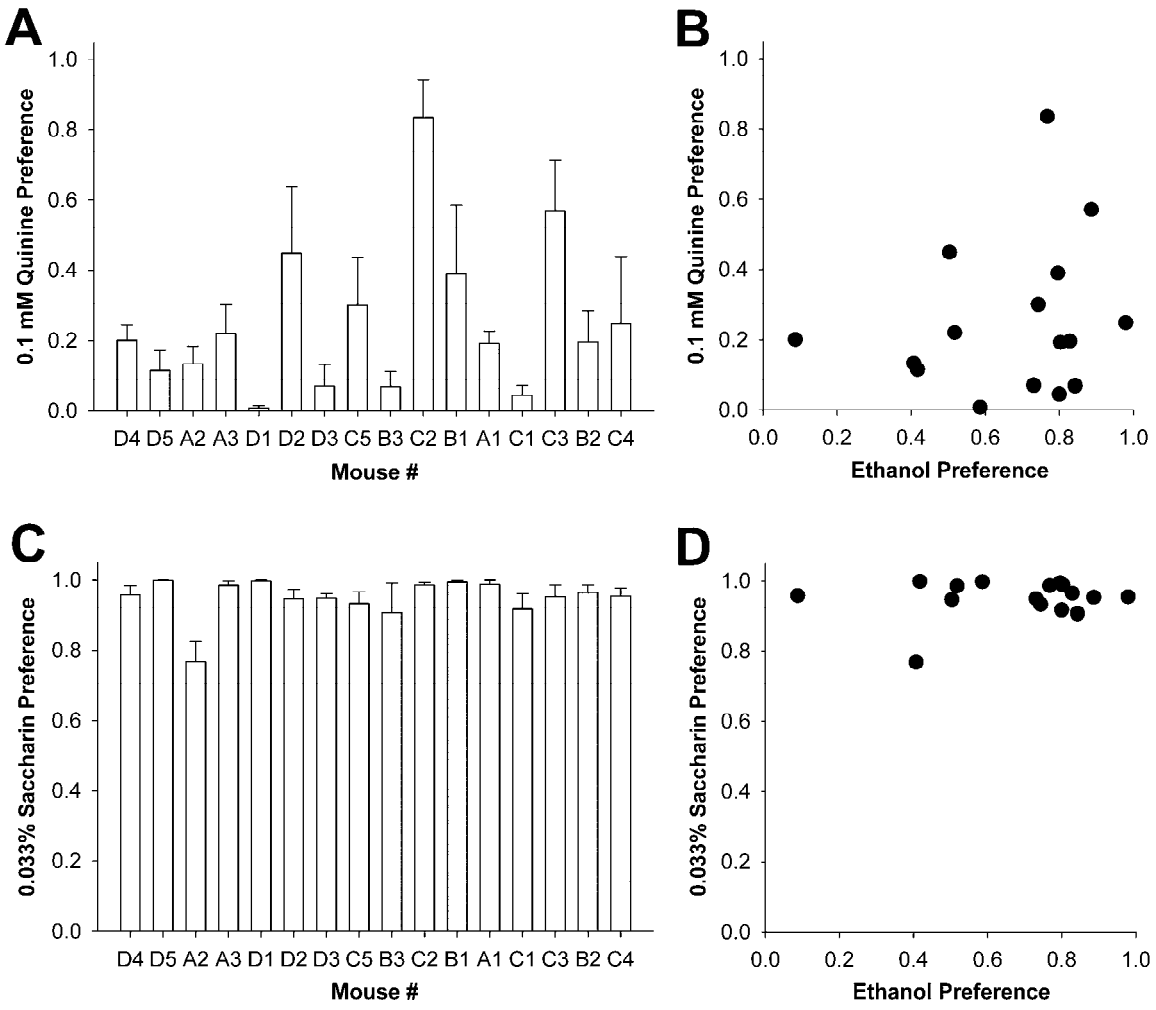

Figure 7: Ethanol Preference Does Not Correlate to Preference for a Bitter or Sweet Solution. **A.** Average preference for 0.1 mM qunine solution in individual mice over 3 day 2-bottle choice test. **B.** Ethanol preference does not correlate to quinine preference (R=0.196, p=0.467). **C.** Average preference for 0.033% saccharin in individual mice over 3 day 2-bottle choice test. **D.** Ethanol preference does not correlate to saccharin preference (R=0.142, p=0.599).

to ethanol preference (Rockman et al. 1984; Rockman et al. 1987; Volpicelli et al. 1990; Chester et al. 2004; O'Callaghan et al. 2005; Boyce-Rustay et al. 2007). Regardless of which environmental conditions may have contributed to the variation in ethanol drinking behaviors, we hypothesize that the differences could be mediated by individual variation in gene expression (investigated in Chapter 6), caused by social stress (investigated in Chapter 4) or due to underlying differences in innate anxiety-like behavior (investigated in Chapter 5).

Other investigators have also reported individual variation in ethanol intake within C57 inbred strains (Dole et al. 1988; Little et al. 1999; O'Callaghan et al. 2002; O'Callaghan et al. 2002; Rhodes et al. 2005). Early reports on ethanol preference in B6 mice by Dole et.al (Dole et al. 1988) analyzed the non-genetic factors in ethanol drinking and showed persistent differences between individual animals. Each animal's individual variation accounted for the major part of the variance in drinking behavior. A paper describing the drinking in the dark model also shows individual ethanol drinking variation within the C57BL/6J mice (Rhodes et al. 2005). Although the authors did not further investigate this variation, they have suggested that individual differences in ethanol intake could be due to subtle environmental differences that occurred during rearing or normal animal caretaking and handling. In a typically high alcohol preferring sub-strain, C57/BL10 mice, Little et al. have shown a bimodal distribution of ethanol drinking patterns. Ethanol preference was not correlated with gender or ethanol metabolism, and could not be altered by simple environmental disturbances (O'Callaghan et al. 2002). Selective breeding did not show a simple genetic link to this variation (Little et al. 1999),

where low preference mice bred to other low preference mice also produced offspring with a bimodal distribution of ethanol preference. Our findings also did not show a simple litter effect in the C57BL/6NCrl mice since ethanol intake and preference were not significantly different between litters which suggests that differences in rearing behaviors does not play a major role in the individual variation.

The current study showed a significant correlation between blood ethanol concentration and ethanol intake over a 6 hour session. In fact, BECs reached pharmacologically significant levels, averaging 148 mg/dL. This is higher than expected, but not overly surprising considering that these mice had a short ethanol deprived period, albeit during their inactive phase when little ethanol is expected to be consumed. Regardless, ethanol intake as measured by volumetric assays on the drinking bottles was indeed highly correlated to blood ethanol concentrations.

Dole *et al.* have suggested that C57 mice may drink ethanol to excess due to an inability to discriminate based on taste while others have proposed that C57 mice drink ethanol for its caloric value rather than its pharmacological effect (Dole et al. 1985; McMillen et al. 1998). However, studies by Middaugh have shown that C57 mice indeed consume ethanol for its postingestive pharmacological effects, i.e. hedonic value (Middaugh et al. 2000). It is also believed that deficiencies in taste discrimination alone cannot account for the high ethanol drinking in B6 mice. In fact, C57 mice can discriminate ethanol concentrations as low as 3% (v/v), and concentrations of 6-12% ethanol are preferred over water (Middaugh et al. 2000; Becker et al. 2004). Here, we have shown that, while there may be some individual variation in a bitter tasting solution,

quinine, it is not correlated with differences in ethanol intake and preference. Saccharin preference was not significantly different between mice, nor was it correlated to ethanol intake. Additionally, our laboratory and others have demonstrated ethanol craving in two substrains of C57 mice using the alcohol deprivation effect, a temporary increase in ethanol drinking following a period of forced abstinence (Khisti et al. 2006; Melendez et al. 2006). Together, this shows that while C57 mice from Charles River Laboratories display a persistent and robust variation in ethanol intake and preference, these differences are most likely not due to mice consuming ethanol simply for its caloric value or due to their inability to discriminate taste. The remaining chapters will further investigate the potential behavioral (Chapter 4 and 5) and molecular (Chapter 6) factors which contribute to these differences in ethanol intake and preference.

CHAPTER 4 Social Stress and its Effects on Ethanol Drinking Behavior

Introduction

Stress, anxiety state and the anxiolytic properties of ethanol play important roles in the predisposition of ethanol intake, initiation of ethanol abuse, dependence and relapse drinking. The tension-reduction hypothesis which asserts that stress and stressful events increase drug seeking and consumption, is perhaps the most popular theory explaining stress induced drug seeking and relapse behavior (Conger 1956). Individuals with a history of stress and mood disorders have a three-fold increased risk for developing an alcohol-related disorder. (Regier et al. 1990) and alcoholics report increased alcohol drinking following periods of stress to reduce feelings of anxiety (Pohorecky 1981; Newlin et al. 1990). Social stressors such as early family adversity, including abuse, emotional neglect, and harsh inconsistent punishment are also risk factors for alcohol and drug abuse (Zoccolillo et al. 1999). During periods of chronic abstinence, alcoholics can experience mood disturbances, negative affect and anxiety (Begleiter et al. 1979; Roelofs 1985) which are correlated with relapse drinking (Hershon 1977; Annis et al. 1998). These clinical findings have been supported by a number of studies in animals showing that social

isolation or maternal separation in early life increases alcohol and drug self-administration (Meaney et al. 2002; Brake et al. 2004).

Like humans, rodents are social creatures and a variety of social factors influence ethanol intake and preference in rodents. These factors include social isolation (Parker et al. 1974; Schenk et al. 1990; Wolffgramm et al. 1991), overcrowding (Hannon et al. 1976), social rank (Ellison et al. 1983; Blanchard et al. 1987) and social defeat stress (Kudryavtseva et al. 1991; Hilakivi-Clarke et al. 1992; van Erp et al. 2001; van Erp et al. 2001). Early studies in rodents have shown that subordinate rodents have higher ethanol intake as compared to their dominant cage mates (Ellison et al. 1983; Blanchard et al. 1987; Kudryavtseva et al. 1991; Wolffgramm et al. 1991; Hilakivi-Clarke et al. 1992). Interestingly, Hilakivi-Clarke and Lister have shown that dominant and subordinate status may predict ethanol preference, but these preference differences do not exist prior to establishment of social hierarchies (Hilakivi-Clarke et al. 1992). In fact, it appears that becoming a subordinate mouse increases ethanol intake and preference since alpha mice and mice in cages without fighting have similar ethanol intakes. Therefore, they suggest that the experience of social stress in the form of subordination may cause increases in ethanol intake and preference. Similarly, five consecutive daily defeats in the resident-intruder test increased ethanol preference and intake in low preferring mice as compared to control mice (Croft et al. 2005). Although decreases in ethanol intake following social defeat have also been seen (van Erp et al. 2001; van Erp et al. 2001), forced subordination through continuous fight-stress tends to increase ethanol consumption (Blanchard et al. 1987; Wolffgramm et al. 1991; Blanchard et al. 1993).

Experiments have identified a potential interaction between the HPA axis and the mesocorticolimbic system in social stress. Social defeat stress acutely increases neuronal activity in several limbic areas many of which (the dorsal and median raphe, the paraventricular nucleus, the medial amygdala, and the central grey) remain high after repeated defeats (Martinez et al. 1998). In mice, repeated defeats increase neuronal activation (via increased cFos expression) in several limbic regions such as the forebrain, cingulate cortex, and hippocampus, as well in areas of the HPA axis, the hypothalamus and amygdala (Matsuda et al. 1996). The experience of social stress (in the form of repeated social defeat as opposed to the threat of defeat or non-aggressive social contact), causes alterations in neurochemical measures of anxiety and increased cardiac and adrenocortical responsiveness (Engler et al. 2005). Repeated defeat also induces a long-term increase in neuronal activity in the mesolimbic dopamine circuitry (Miczek et al. 2004). Episodic social stress increases neurochemical correlates of stress (i.e. corticosterone and ACTH) without the habituation that occurs in chronic social stress (Tornatzky et al. 1993; Tidey et al. 1996; Engler et al. 2005).

Ethanol activates the HPA axis and mesocorticolimbic system acutely (Han et al. 1993; Carson et al. 1996; Weiss et al. 1996; Wu et al. 1997), even though ethanol is an anxiolytic. Although the mechanisms whereby stress increases ethanol consumption are not fully understood, it has been suggested that ethanol drinking behavior may be modified by stress through the action of neural pathways responding to stress or by HPA axis effectors such as CRF and corticosterone (CORT) (Grant et al. 2003; Funk et al. 2004; Zimmermann et al. 2004; Breese et al. 2005). Recent work in a primate model shows an

inverse correlation between responsiveness of the HPA axis and ethanol consumption (Porcu et al. 2006).

As outlined above, prior evidence supports an important role for social stress in modifying ethanol drinking behavior. We have previously characterized a large and persistent variation in ethanol drinking behavior across individual mice from the same inbred strain. We hypothesize that these individual differences in drinking behavior originate from an environmental factor, namely, social stress. The studies described in this chapter were designed to test the influence of social stress on ethanol drinking behaviors as a means to help explain the persistent variation of basal ethanol intake within an inbred strain. Additionally, we harnessed the power of the individual variation in genetically similar animals to further explore stress-influenced drinking behavior. We have used two models of social stress, a modified form of social disruption in a group-housed situation and repeated social defeat, to investigate the influence of an ethologically relevant stress on the initiation of ethanol drinking behavior.

Methods

Social rank assessment: Mice were observed and videotaped in their home cages for 30 minutes following each cage change. Dominance was assigned based on offensive behaviors (attack, chase, tail-rattling, and biting). Subordination was assigned based on defensive, avoidance or active escape behaviors (immobility, flee, and jump) including the characteristic defensive posture such as standing on hind legs with the ventral body surface directed towards the aggressor and forelegs raised off the ground (e.g., (Miczek et al. 1982; Hilakivi-Clarke et al. 1992). The frequency of these offensive (# bites, attack, mount, chase

and tail rattle) and defensive (bites received, flee, freeze, jump and rearing posture) measures was recorded by independent observers. The total number of defensive behaviors was subtracted from the total number of offensive behaviors to give a "social rank score." For enhanced reliability, mice are classified over three observations (Bartolomucci et al. 2001). These scores were used to rank the mice from dominant to submissive and in later studies to identify the alpha aggressive male for social defeat studies.

Social rank pilot study: Six week old male C57BL/6NCrl mice (n=16) were quarantined for 1 week with four mice per cage upon arrival to the vivarium. The mice were individually housed for one week before being reassigned to a new group of four mice per cage. Mice were weighed, had their tails painted for identification with non-toxic water-based paint before being videotaped in their home cages for 30 minutes immediately after cage changes. Cages were changed twice a week when group housed allowing for three recordings of social interaction. Tapes were scored for offensive and defensive behaviors as described in Social Rank Assessment. The four mice in each cage were ranked from dominant to submissive based on the total number of offensive and defensive behaviors. Observations were scored from videotapes by two independent observers. Because mice closely matched in weight show less aggressive attacks than mice which have greater differences in weight (Hilakivi-Clarke et al. 1992), mice were chosen to have less than 1 gram body weight difference when housed with non-siblings. In addition, no mouse showed visible hair-loss or bleeding from attacks while housed in groups. These mice were only used for screening social behaviors and did not have any ethanol drinking sessions.

Social rank in littermates: 26 male C57BL/6NCrl littermates from 6 litters were housed as littermates (4-5 mice per litter per cage) upon arrival to the vivarium. Offensive and defensive behaviors in each cage were videotaped and scored on two separate occasions for 30 minutes following cage changes. Social rank scores were calculated as described in Social Rank Assessment. Mice were singly housed for three weeks with 2 weeks of ethanol drinking sessions in a two-bottle choice paradigm. Half of the mice were housed back together with their littermates (cages A, C and D), while the other half of the mice were housed with non-littermates (cages 1, 2 and 3). Observations were recorded and scored for 30 minutes immediately after cage changes on 3 occasions during the week of group housing.

Repeated social defeat paradigm: The repeated social defeat paradigm (RSD) is a variation on the resident–intruder paradigm. In this case, experimental mice were used as intruders. Steps were taken to prevent physical injury in the intruder mice. The RSD protocol requires a separate cohort of animals (5 cages, n=5/cage) obtained as randomly housed from the supplier. These mice were observed serially at the time of cage changing for social dominance. Dominant, aggressive males in each cage were identified and singly housed as "residents" for at least four days without bedding changes prior to having an intruder mouse added to the cage. RSD consisted of three phases: priming, defeat and threat of defeat. Intruder mice were placed into the cage of an aggressive resident mouse. In order to reduce the variability in aggressive behavior by the resident stimulus animal, the intruder animal is exposed to the resident initially behind a protective screen for a brief period (5 min); the priming phase. Thereafter, the protective screen was removed, and the

intruder was attacked very quickly and frequently. The experimental session was terminated with the display of the defeat behavior, as illustrated in (Miczek 1991). In mice, the upright defeat posture with retracted ears, limp forelimbs, audible squeals upon approach by the aggressive stimulus animal are clear signs of defeat (Miczek et al. 1982). This defeat phase lasts until the intruder showed the defeat posture for 3 seconds or until a maximum of 5 minutes. The intruder was covered by a wire cage (8x8x5 cm) for the threat of defeat phase and remained in the resident cage for 30 minutes before returned to single housing. Social defeat was repeated daily for 5 consecutive days. Resident mice failing to show dominant behaviors were replaced. No resident was paired with the same intruder more than once.

Social Defeat in C57BL/6NCrl mice with continuous ethanol access: 50 male C57BL/6NCrl mice were housed in groups of 4/cage for 2 weeks on a reverse light cycle upon arrival to the facilities. At 7 weeks old, mice were singly housed for 7 days then started on two bottle choice drinking (10% ethanol (w/v) or tap water). Mice were given 24 hours of ethanol access beginning 4 hours into their dark cycle (1200 hours). Standard mouse chow was supplied ad libitum throughout all studies. The position of ethanol and water bottles was switched every other day to avoid side preferences (i.e. L, L, R, R). 30 mice were given 14 days for baseline ethanol drinking, while 20 mice were given two bottle of water. After baseline drinking, 20 ethanol drinkers and 10 water drinkers were given 5 consecutive days of social defeat by an aggressive male C57 mouse. The remaining 10 ethanol drinkers and 10 water drinkers remained in their home cages as controls. Ethanol drinking mice had continuous access to ethanol during the days of defeat

and for 3 weeks following social defeat except for the time (35 minutes/day x 5 days) that they were placed in the cage of an aggressor.

Social defeat in C57 mice without continuous ethanol access: 29 male C57BL/6 mice from Charles Rivers were housed in groups of 3-4/cage for 1 week upon arrival to the facilities. At seven weeks old, mice were individually housed for 7 days then started on two bottle choice ethanol drinking. A bottle of 10% (w/v) ethanol in tap water and a bottle of tap water were placed onto the home cages of each mouse at 1600 hours each day and the amount drank from each bottle was recorded at 1000 hours after which the bottles were replaced with a standard water bottle. Standard mouse chow was supplied ad libitum throughout all studies. The position of ethanol and water bottles were switched every other day to avoid side preferences. After 14 days of baseline drinking ethanol drinking sessions were halted and 15 mice were defeated in the home cage of an aggressive male C57BL/6 mouse, while 14 control mice were placed into a clean empty cage for 1 hour a day. Social defeat was repeated daily for 5 days followed by 2 days without defeat before reinstituting two-bottle choice drinking. Mice were given 2 days of rest without ethanol or defeat to study longer lasting effects of social stress on ethanol drinking and not acute effects. Two bottle choice drinking was resumed for 2 weeks following the repeated social defeat period.

Social defeat in 129SvJ mice: 22 male 129X1Sv/J mice were housed in groups of 4/cage for 2 weeks on a reverse light cycle upon arrival to the facilities. At 7 weeks old, mice were singly housed for 7 days then started on 2 bottle choice drinking (10% ethanol or tap water) exactly as the C57 mice with continuous ethanol access. Mice were given 24

hours of ethanol access beginning 4 hours into their dark cycle (1200 hours). Standard mouse chow was supplied ad libitum throughout all studies. All mice were given 13 days of baseline ethanol drinking prior to social defeat. 15 mice were socially defeated by an aggressive C57 male, while 7 mice remained in their home cage. Ethanol was continuously available to both groups of mice during the days of defeat and for 3 weeks following defeat.

Statistical Analysis: Pearson correlations were used to determine the correlation between observers for social rank assessments. Chi Square analyses were used to determine if effects were greater than would be expected by chance in social rank studies and to determine the effect of social defeat between experiments. The Mann Whitney rank sum test was used as a non-parametric equivalent of the standard t-test for social observations. Appropriate one-way and two-way Repeated Measures Analysis of Variance (RM-ANOVA) were used to evaluate changes in ethanol intake, ethanol preference and total fluid consumed in ethanol drinking experiments. Dunnett's post hoc tests were used, when appropriate for comparisons to baseline. One-way ANOVA was used to test for differences in individual animals. For each statistical test a p value <0.05 was considered to be statistically significant. To correct for the confounding effects of regression to the mean in these test-retest drinking experiments, the average baseline intake of all the subjects was subtracted from each subject's post-test score. The difference was multiplied by the factor (1-r), where r is the Pearson correlation coefficient between the baseline and post-test scores. The result was then added to the post-test score for each subject. This is the corrected post-test score and is free of the confound (Hopkins 2002; Kelly 2005).

Results

Social rank assessments

Social stress experiments depend on the ability to reliably determine aggressive alpha males while housed in a group setting. Thus initial experiments were performed to show that aggressive alpha males could be identified through scoring dominant and offensive behaviors. Social rank assessment scores for each mouse in each of 4 cages are seen in Figure 8A. The number of observations was variable between each cage of group housed mice ranging from less than 15 to more than 40 scored behaviors, indicating that the total level of offensive and defensive behaviors was variable between groups and is presumably dependent on the members in each cage. Social rank scores from two independent observers, however, were highly correlated ($R=0.991$, $p<0.0001$, see Figure 8B). Thus, even though the number of offensive and defensive behaviors is variable between cages of mice, the behaviors are easily and reliably scored suggesting that this method can be used to determine a social hierarchy of mice in a group housed environment.

In littermates, the frequency of offensive and defensive behaviors was very low (less than 10 observations in any given mouse) making it difficult to determine a dominant alpha male in each cage of littermates (Figure 9A). Consequently, the number of alpha males was significantly lower in littermates housed together since birth than when the mice were evaluated after three weeks of single housing ($\chi^2=8.571$, $p=0.0034$). Following three weeks of single housing with two weeks of ethanol drinking in a 2 bottle choice paradigm, three litters (cages A, C and D) were returned to their original group-housing while three

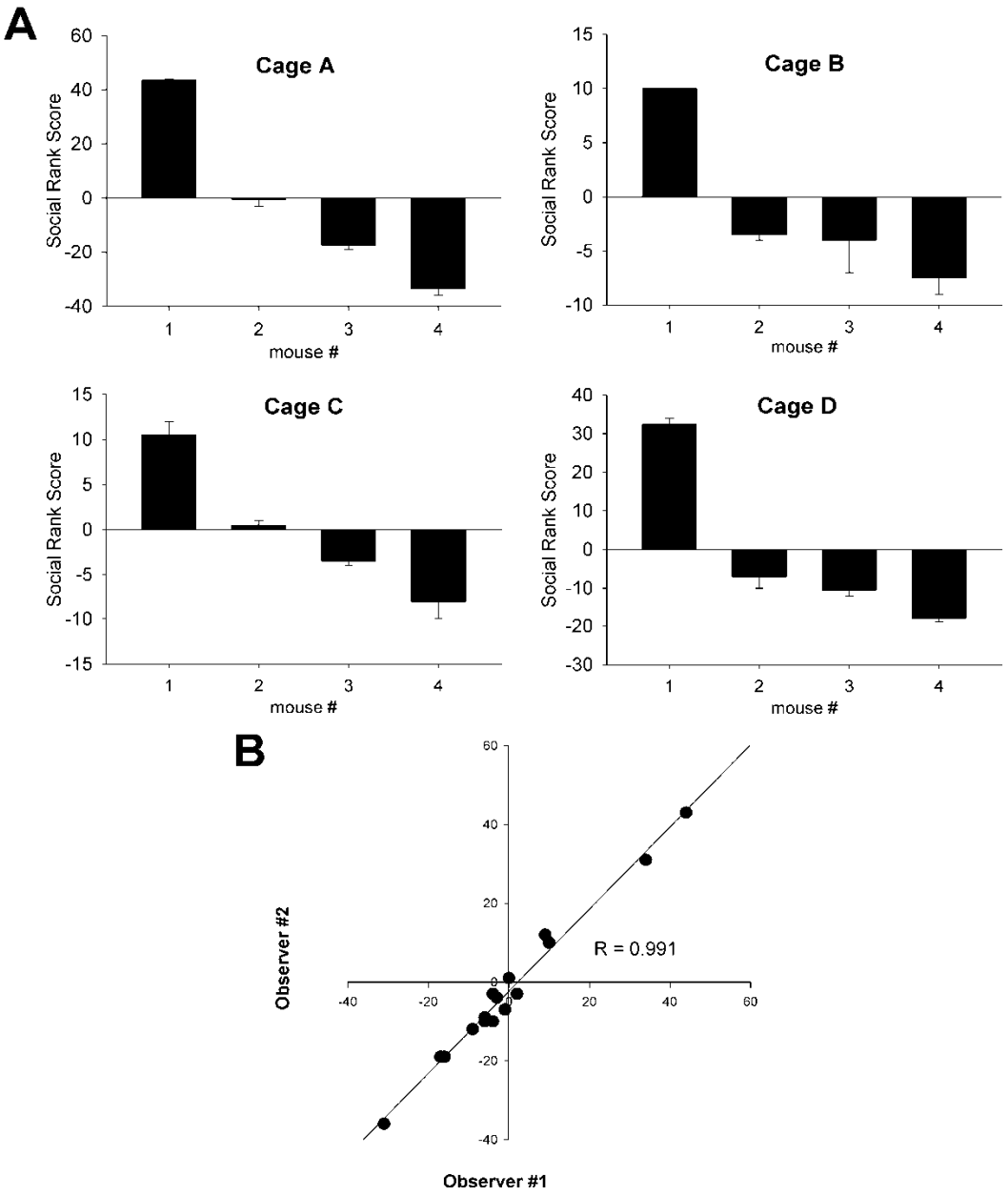

Figure 8: Social Rank Assessments in Non-Littermates. A. Rank scores represent a single observation session of social behaviors. Offensive and defensive behaviors were scored by two independent observers in four cages (4 mice/cage). In each graph, along the x axis mice are ranked from dominant to most submissive. Social rank score calculated by subtracting the total number of defensive behaviors from the total number of offensive behaviors for each observer is on the y-axis. **B.** Scattergram of social rank scores for two independent observers. Social rank score was highly correlated between observers at R= 0.991, $p<0.0001$.

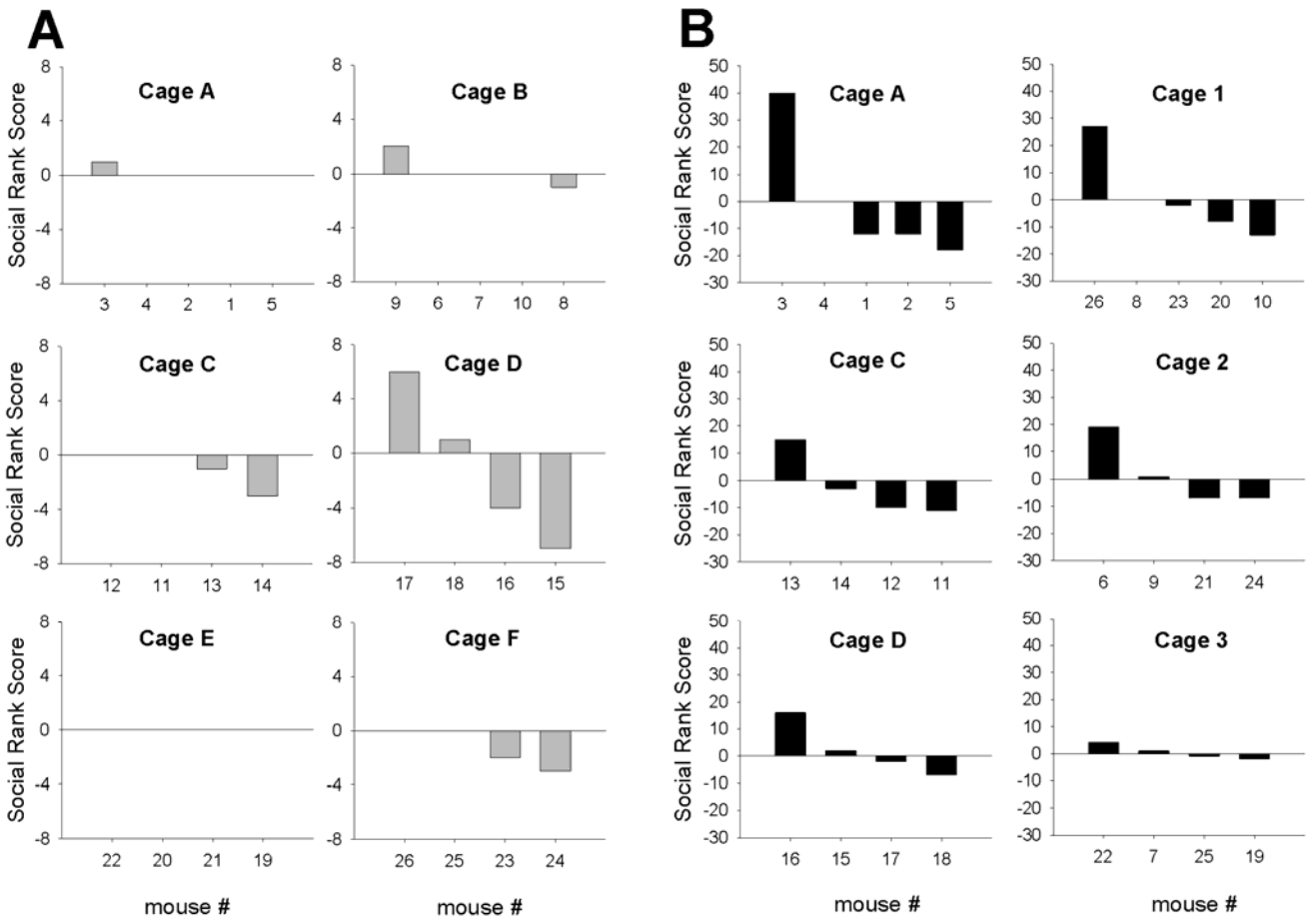

Figure 9: Social Ranking in Littermates and Group Housed Mice. Mice are ranked from dominant to subordinate on the x-axis and social rank scores on the y-axis. Positive scores represent a higher number of offensive behaviors, while negative scores indicate more defensive behaviors. Observations were made while group housed after cage changes. **A.** Social rank scores in 6 littermate cages. Male C57 mice housed as littermates at 6 weeks old display very few offensive and defensive behaviors. **B.** Social rank scores of male littermates (cages A, C, D) and non-littermates (cages 1, 2, 3). Following 3 weeks of single housing, littermates and non-littermates display more offensive and defensive interactions.

litters were placed into a new group setting (cages 1, 2 and 3) ensuring that mice from the same litter were not housed together. Interestingly, the total number of offensive and defensive displays increased in both littermate and non-littermate cages (Figure 9B). Due to the low level of social dominance and subordinate interactions in littermate mice, we had expected the degree of social interaction in littermate cages to be lower than in group housed cages. Indeed, by Mann Whitney rank sum test, the total number of offensive behaviors scored was significantly higher in mice housed as non-littermates ($U(24)=43.5$, $p=0.037$) as was the total number of defensive behaviors ($U(24)=28.0$, $p=0.004$). However, the social rank scores were not significantly different in cages of mice housed as littermates and non-littermates ($U(24)=68.5$, $p=0.426$).

Social rank and ethanol intake

Previous studies in rodents and monkeys have suggested that dominant animals consume lower amounts of ethanol. Subordinate animals, on the other hand, consume high amounts of ethanol (Ellison et al. 1983; Blanchard et al. 1987; Hilakivi-Clarke et al. 1992). In the current studies, mice were scored again for aggressive behaviors after 2 weeks of ethanol drinking. In general, the dominant mice tended to be among the highest ethanol drinkers. In the five cages where a dominant alpha male could be reilably identified, the dominant male was never the mouse with the lowest intake (Table 3). In cage A, the alpha male had the highest ethanol intake following a week of group housing. In cages 2 and C, the alpha males had the second highest intake and in cages 3 and D, the alpha males had the third lowest intake (see Table 3). There were no differences in ethanol intake in mice which had been returned to housing with their littermates as compared to mice which were

Cage #	Mouse #	Social Rank Score	baseline intake	intake after group housing
1	22	4	2.10	6.13
1	7	1	9.29	9.98
1	25	-1	7.23	9.49
1	19	-2	7.05	4.29
2	6	19	8.60	8.11
2	9	1	4.21	3.76
2	21	-7	7.81	11.10
2	24	-7	3.57	4.11
3	26	27	7.43	6.34
3	8	0	2.48	5.81
3	23	-2	7.83	9.84
3	20	-8	3.39	3.31
3	10	-13	4.96	7.22
A	3	40	8.99	9.58
A	4	0	0.92	2.17
A	1	-12	8.79	8.89
A	2	-12	7.41	5.11
A	5	-18	8.81	6.65
C	13	15	7.26	5.92
C	14	-3	9.57	12.42
C	12	-10	3.46	2.40
C	11	-11	1.30	1.35
D	16	16	4.42	6.00
D	15	2	4.64	5.98
D	17	-2	8.66	8.66
D	18	-7	6.68	6.60

Table 3: Social Rank and Ethanol Intake. Social rank score and ethanol intake at baseline and after 1 week of group housing. Mice identified as dominant alpha males are highlighted in pink. Cages 1, 2 and 3 were group housed with non-siblings. Cages A, C and D were returned to housing with littermates. An alpha male could not be determined in Cage 1.

group housed with non-siblings (T(24)=0.522, p=0.607). Therefore, the relationship between ethanol intake and social rank remains unclear in C57 mice.

Social defeat and ethanol drinking

Based on the findings that alcoholics report increased drinking during periods of social stress, we hypothesized that repeated social defeat would increase ethanol intake in mice. To test this hypothesis we have performed three experiments investigating the effect of social defeat stress on ethanol drinking behaviors. First, male C57 mice (n=30) were allowed to freely consume ethanol for a baseline period of 2 weeks and then separated into two groups based on their initial ethanol intake in a counterbalanced design. Basal ethanol intake in the two groups was not significantly different (t(27)=0.174, p=0.863). In this experiment, mice had continuous access to ethanol 24 hours per day throughout the entire study. 20 mice were socially defeated once a day for 5 consecutive days, while 10 mice remained in their home cage. Social defeat decreased ethanol intake during the period of social defeat and up to two weeks following the last defeat (Figure 10). Ethanol intake in socially defeated mice was significantly decreased from baseline during the defeat period, and on weeks 2 and 3 following social defeat (RM ANOVA (F(18,4)=4.386, p=0.003, Dunnett's post hoc test). Ethanol preference was also significantly decreased from baseline during the defeat period (RM ANOVA F(18,4)=3.708, p=0.008, Dunnett's post hoc test). Ethanol intake and preference in the home cage mice was increased over baseline only during the second week following the defeat period (RM ANOVA F(9,4)=5.548, p=0.001 and F(9,4)=11.071, p=0.001 respectively). Repeated social defeat did not alter total fluid consumed over the course of the experiment. Total fluid intake was not significantly

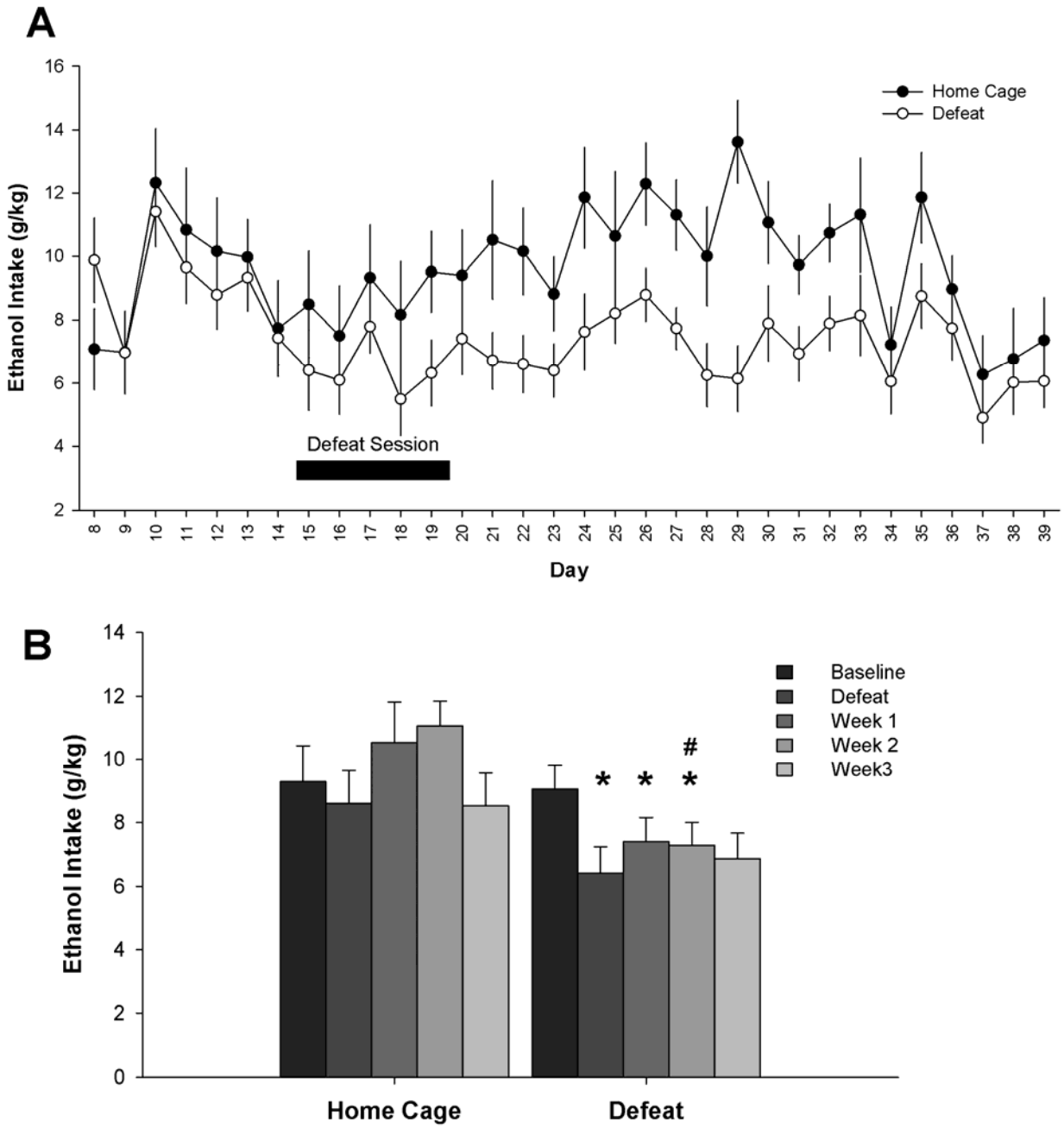

Figure 10: Repeated Social Defeat Decreases Ethanol Intake in C57 Mice. **A**. Daily ethanol intake in socially defeated (n=20) and home cage (n=10) mice **B**. Average ethanol intake at baseline, during and for 3 weeks following social defeat.* $p<0.01$ vs. baseline, # $p<0.05$ vs. home cage.

different at any time point between socially defeated and home cage mice by Two-way Repeated Measures ANOVA. There was a main effect of time ($F(4,27)=9.367$, $p<0.001$) where fluid intake was slightly lower on weeks 2 and 3. But no main effect of group ($F(1, 27)=1.427$, $p=0.243$), or significant interaction between the two factors, time and group ($F(4,4)=1.772$, $p=0.140$). Therefore, social defeat decreased ethanol intake and preference during social defeat and ethanol intake remained at depressed levels throughout the course of the study.

Since previous reports have suggested a delayed effect of social stress on ethanol drinking (Sillaber et al. 2002; Croft et al. 2005), we conducted a second experiment where mice did not have ethanol access during the days of social defeat. This design avoids any potential confound from ethanol intoxication affecting defensive maneuvering during social defeats and also temporally separates ethanol access from being associated with immediate effects of defeat stress. Again, ethanol intake and ethanol preference were significantly reduced following social defeat (Figure 11). Repeated Measures ANOVA revealed that ethanol intake on week 1 and week 2 was significantly lower than baseline ($F(14,2)=5.939$, $p=0.007$). Ethanol preference was also significantly lower in weeks 1 and 2 following social defeat ($F(14,2)=5.214$, $p=0.012$). The total amount of fluid consumed was not altered throughout the experiment ($F(14, 2)= 2.877$, $p=0.073$). Mice which were exposed to a clean empty cage during the defeat period did not significantly alter their ethanol intake ($F(13,2)=1.544$, $p=0.233$) or their ethanol preference ($F(13,2)=0.505$, $p=0.609$) throughout the course of the experiment. Thus, the long term and acute effects of

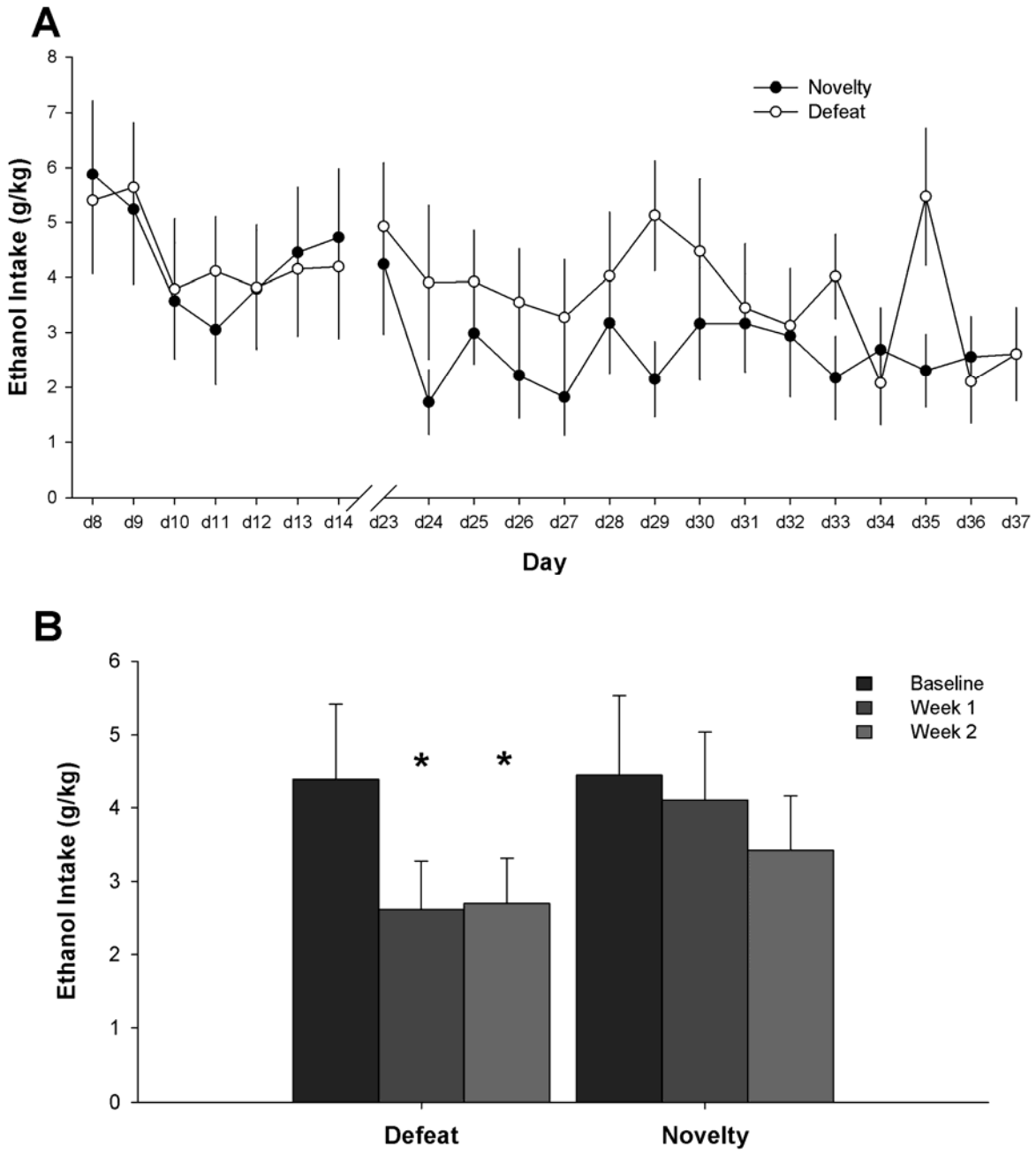

Figure 11: Repeated Social Defeat Decreases Ethanol Intake After Delayed Access to Ethanol. A. Daily ethanol intake in socially defeated mice (n=20) and mice exposed to a novel cage (n=10) during the defeat session. **B**. Average ethanol intake at baseline and 2 weeks after the defeat session. * $p<0.05$ vs. baseline intake.

social defeat stress decrease ethanol intake and preference in C57 mice which undergo five days of repeated social defeat.

Individual differences in ethanol drinking following social defeat

These experiments were conducted to study individual variation of ethanol drinking behaviors and Figure 12 summarizes ethanol intake in individual mice. When mice had continuous ethanol access, eight out of nineteen mice significantly decreased ethanol intake during at least one week after social defeat (separate One-way ANOVAs on daily intake in individual mice, $p<0.05$, Figure 12A). One mouse significantly increased ethanol intake during the first week following defeat ($p<0.05$) and four mice showed a trend towards an increase. When ethanol was not available during the week of social defeat, only four mice significantly decreased their ethanol intake for at least one week after social defeat (Figure 12B). Although control mice which remained in the home cage and mice exposed to a clean empty cage during the defeat period as a group did not significantly change their ethanol intake over the course of the experiment, a few individual mice did alter their ethanol intake. In home cage control mice, one mouse significantly increased ethanol intake over baseline on drinking days 27 to 33 ($p=0.0203$, data not shown). In mice exposed to a clean empty cage during the defeat period, one mouse increased ethanol intake the first week ($p=0.0289$) and four mice significantly decreased from baseline at week 1 or 2 after exposure to a novel cage (by separate One Way ANOVA, $p=0.0037$, $p=0.0073$, $p=0.0148$, and $p=0.0021$, respectively, data not shown). This raises the possibility that exposure to a novel cage may be a stressor.

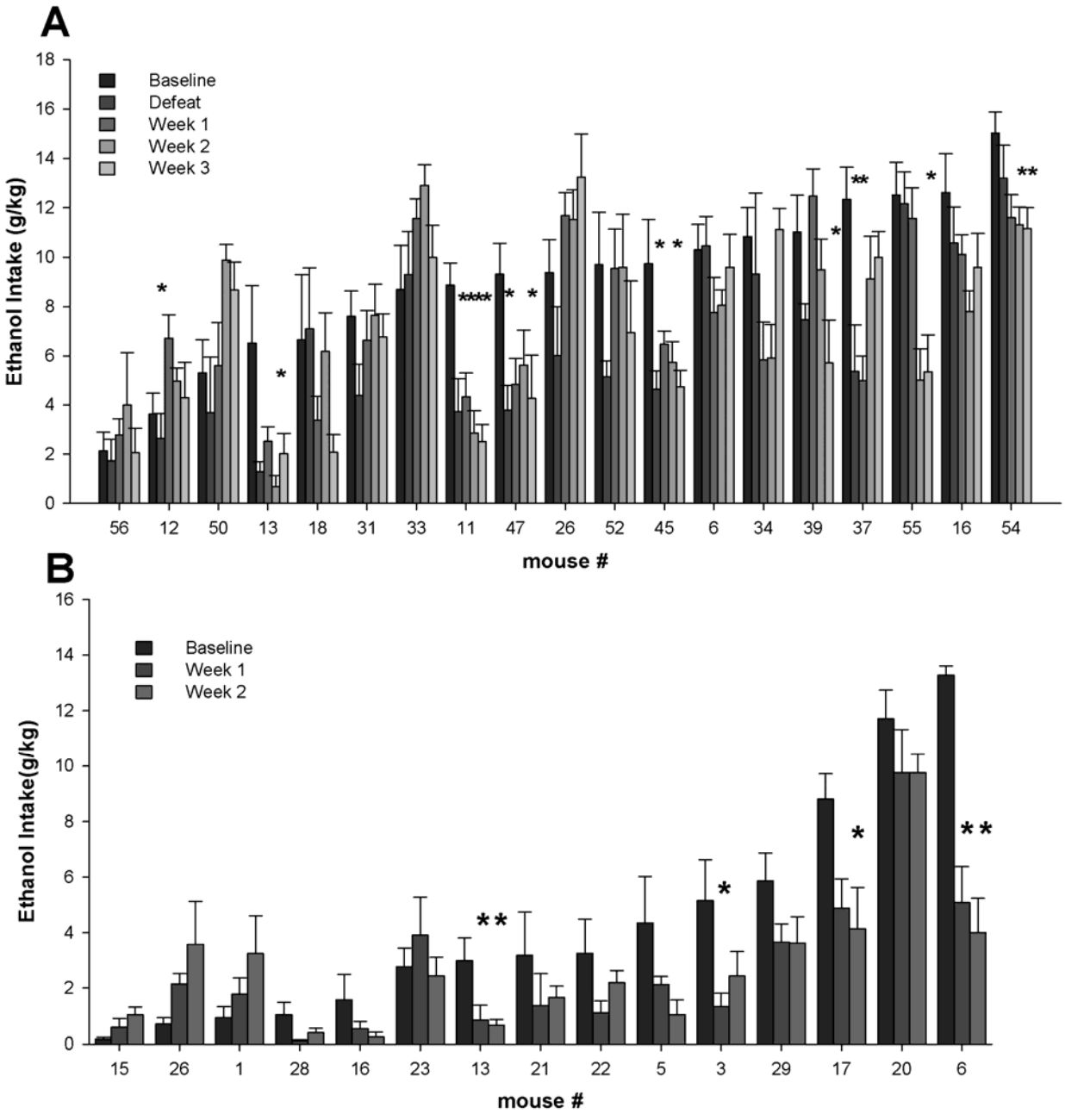

Figure 12: Ethanol Intake Following Social Defeat Stress in Individual Mice. Socially defeated mice altered ethanol intake following defeat stress when ethanol was continuously available (**A**) or when access was delayed until defeat stress was terminated (**B**). There is a trend for the lowest drinking mice to increase intake following defeat and high preferring mice to decrease intake. * $p < 0.05$ vs. baseline intake.

In order to determine whether social defeat's effects on ethanol intake were greater than would be expected by chance, a Chi Square analysis was conducted for each experiment. When mice had continuous access to ethanol and were compared to a control group which remained in the home cage, repeated social defeat decreased ethanol intake in more mice (χ^2= 7.09, p=0.0289). However, when mice did not have continuous ethanol access and were compared to mice exposed to a novel cage, social defeat did not have a greater effect on ethanol intake (χ^2= 1.167, p=0.558). Thus, social defeat suppresses ethanol drinking in more mice when ethanol is continuously available. Exposure to a novel cage acts similarly to social defeat and is a poor control group for these studies.

Ethanol intake and social defeat in 129SvJ mice

Even though social defeat decreases ethanol intake in C57 mice, there was a suggestion that mice with the lowest ethanol intake or preference may increase drinking following defeat (see Figure 12). In order to more systematically test this hypothesis, a different inbred mouse strain was tested for ethanol drinking following social defeat. 129/SvJ mice are known to consume moderate amounts of ethanol (Belknap et al. 1993; Bachmanov et al. 1996; Bachmanov et al. 1996) and have recently been shown to be more sensitive to the effects of footshock stress than C57 mice (Yang et al. 2008). 129/SvJ mice consume significantly less ethanol than C57 mice during 24 hours of ethanol drinking in a two bottle choice paradigm (Mann-Whitney Test, $p<0.001$). 129SvJ mice consume on average 2.5 g/kg ethanol in a 24 hour period, whereas C57 mice consume 6-8 g/kg ethanol. Ethanol intake in C57 and 129SvJ mice were not normally distributed, but C57 mice

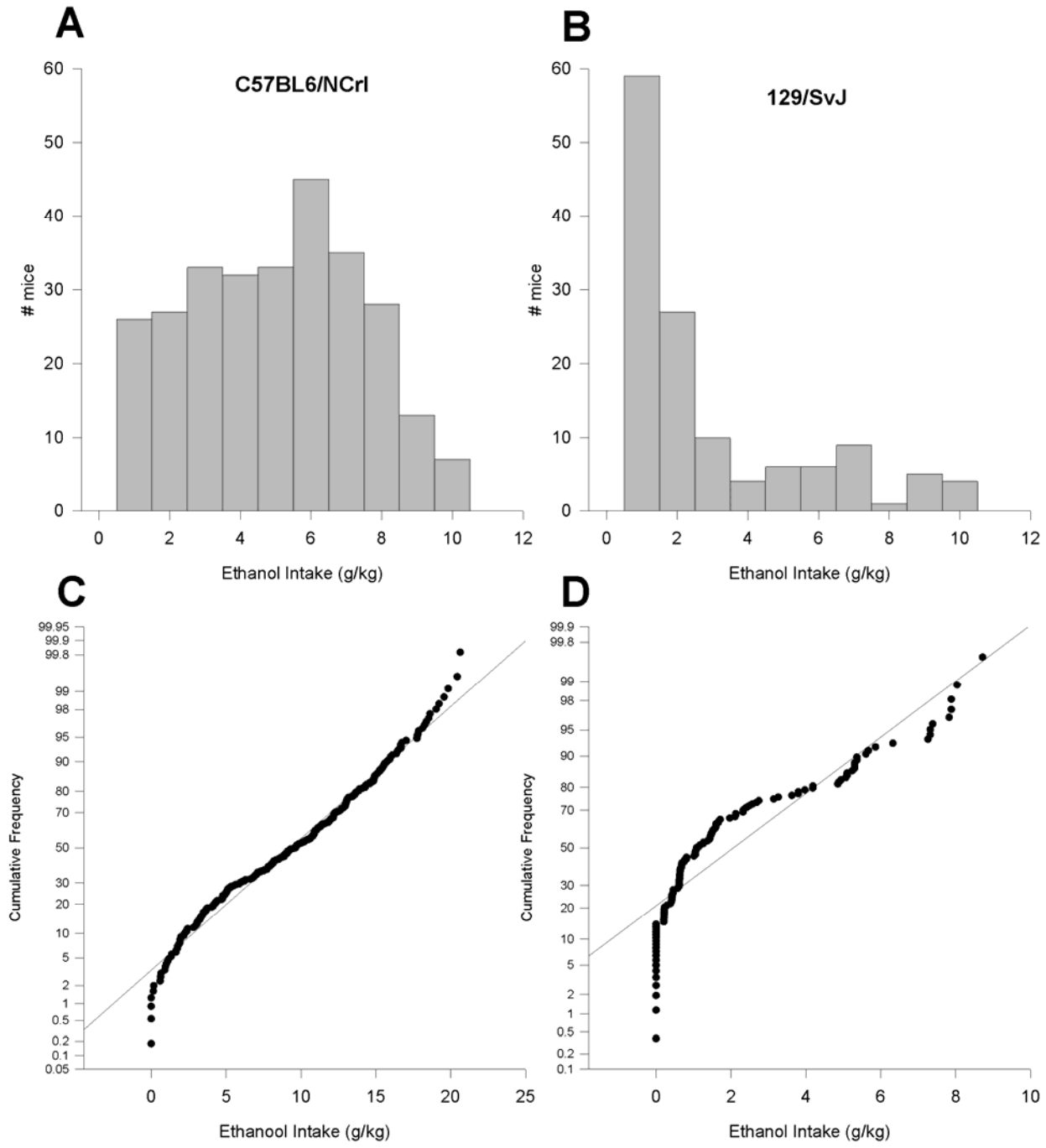

Figure 13: Histogram and Normal Probability Plots of Baseline Ethanol Intake in C57 and 129SvJ Mice. A and C. C57 mice show a more normal distribution of basal ethanol intake. **B and D.** 129SvJ mice show a right-skewed distribution of basal intake where a majority of mice consume less than 4 g/kg ethanol.

approached a more normal distribution, while 129SvJ mice have a more bimodal distribution (Figure 13).

129SvJ mice underwent the same ethanol drinking and repeated social defeat paradigm as C57 mice with continuous ethanol access. Baseline ethanol intake and ethanol preference were highly correlated in 129SvJ mice ($R=0.982$, $p<0.0001$) and total liquid consumed did not vary over the course of the experiment or between treatment groups (data not shown). Surprisingly, we found that 129SvJ mice were not aggressive enough to be reliably used as residents in the social defeat paradigm. Therefore, C57 mice were used as aggressive residents for the social defeat stress. Initially, repeated social defeat did not appear to have an effect of ethanol intake in 129SvJ mice (Figure 14). However, when the bimodal distribution of basal ethanol intake was taken into consideration, intriguing differences were found. Socially defeated mice were subdivided into 2 groups (less than 4 g/kg and greater than 4g/kg) based on basal ethanol intake. Two-way RM ANOVA revealed that ethanol intake significantly increased in mice with low initial ethanol intake. There was a main effect of group ($F(1,13)=5.027$, $p=0.043$), no main effect of time ($F(4,13)=0.183$, $p=0.943$) but a significant interaction between the group and time ($F(4,4)=2.839$, $p=0.033$). Post hoc comparisons within each group showed that ethanol intake was significantly increased over baseline during the defeat period, and at weeks 1, 2 and 3 after social defeat in mice with low (<4 g/kg) baseline intake (Student Newman Keuls post hoc test, $p<0.05$). Ethanol intake in defeated mice which consumed high amounts of ethanol (> 4g/kg) did not significantly alter their ethanol intake at any point during the course of the experiment (Figure 14B). Additionally, mice in the two subdivided

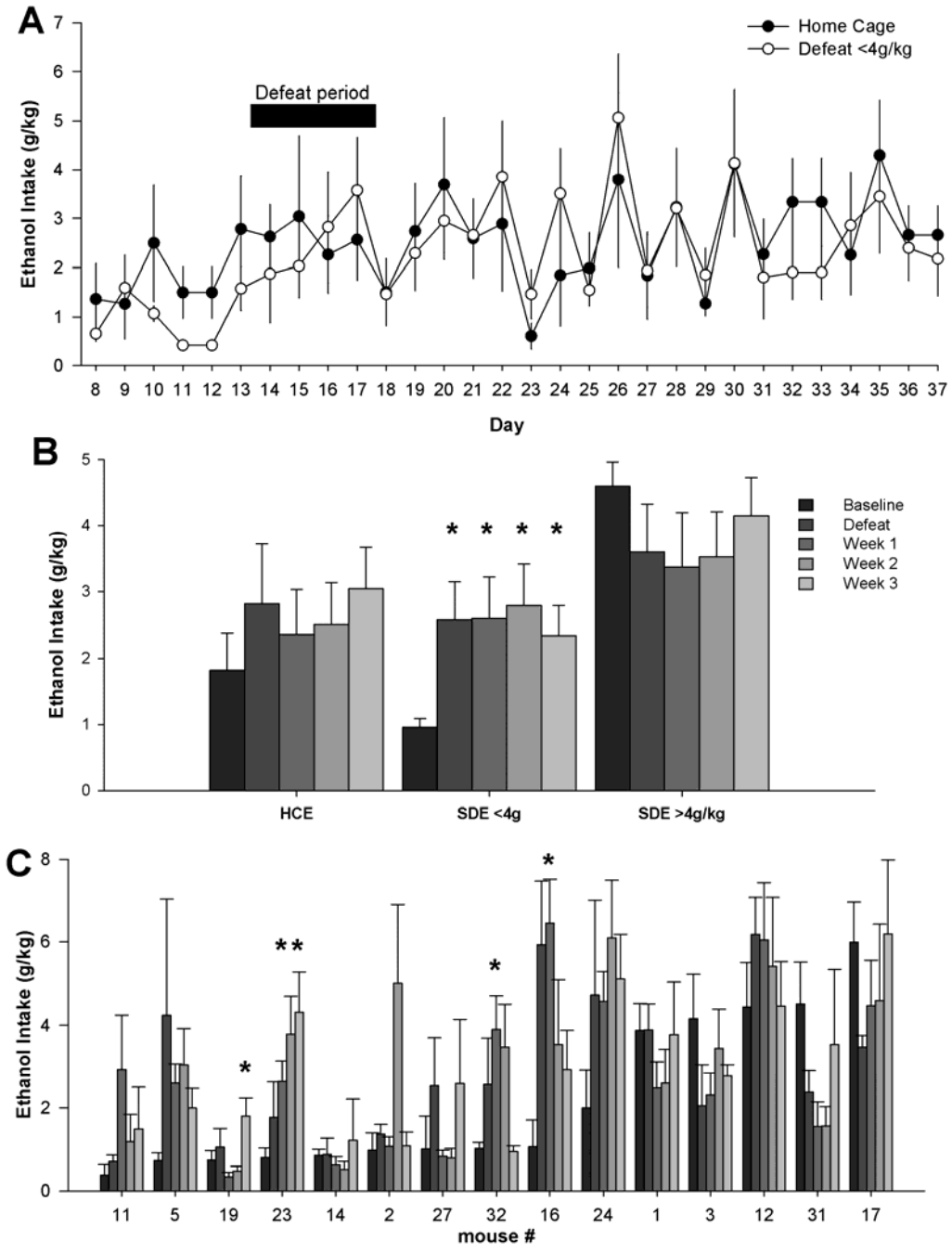

Figure 14: Social Defeat Increases Ethanol Intake in 129SvJ Mice. A. Daily ethanol intake in defeated and home cage control mice. **B.** Summary of ethanol intake over the drinking session of home cage mice and defeated mice consuming less than 4 g/kg (n=10) or greater than 4 g/kg (n=5) at baseline. **C.** Ethanol intake at the same time points in individual socially defeated mice

groups (high and low ethanol intake) were only significantly different from each other at basal intake. Ethanol intake in mice which remained in their home cage did not change over the course of the experiment ($F(6,4)=1.628$, $p=0.200$).

Looking at the effects of individual variation in 129/SvJ mice, social defeat significantly increased ethanol intake in 5 out of 11 low drinkers (separate ANOVA on individual mice, $p<0.05$). The remaining 5 low drinkers showed a trend towards an increase, but their daily variation was too great to reach significance (see Figure 14C). None of the highest drinking mice significantly altered ethanol intake ($p>0.05$ by ANOVA). Interestingly, two of the home cage mice showed increased intake at week 1 or week 3 over baseline drinking ($p=0.0126$, $p=0.0007$). The finding that ethanol intake increased in the lowest drinking C57 and 129SvJ mice raises the possibility that we may be observing a regression to the mean in these drinking experiments.

Effects of regression to the mean

In a test-retest experiment, there is the potential to observe a regression towards the mean where subjects at the drinking extremes during baseline (far from the mean on the first set of observed behaviors) will tend to be closer to the mean on the second test. One method to correct for regression to the mean is explained in Methods. Ethanol intake at each time point (during defeat and at weeks 1, 2 and 3 after defeat) was corrected to remove potential regression to the mean in 129SvJ and C57 mice. In the 129SvJ mice, home cage control mice still did not alter their ethanol intake over the course of the experiment ($F(6,4)=1.921$, $p=0.139$). Socially defeated 129SvJ mice with high ethanol intake also did not change their intake over the course of the experiment ($F(3,4)=0.042$,

p=0.996). Importantly, low drinking socially defeated 129SvJ mice showed a trend to increase ethanol intake following social defeat and just missed significance ($F(10,4)=2.535$, $p=0.057$).

Additionally, socially defeated C57 mice still significantly decreased their ethanol intake ($F(18,4)=4.211$, $p=0.004$) even once the data was corrected for potential to regression to the mean. All time points were decreased as compared to baseline (Dunnets post hoc test, $p<0.05$). Therefore, repeated social defeat in C57 mice decreases ethanol intake although the effect appears to depend on initial ethanol intake. Social defeat tends to increase ethanol intake in 129SvJ mice with low basal drinking which cannot solely be attributed to the confound of regression to the mean.

Discussion

Social stress has been hypothesized to increase ethanol and drug taking in both human and preclinical studies (Conger 1956; Pohorecky 1981; Newlin et al. 1990). However, the findings have not always been consistent as increases (Kudryavtseva et al. 1991; Hilakivi-Clarke et al. 1992; Croft et al. 2005) and decreases (van Erp et al. 2001; van Erp et al. 2001) in ethanol drinking behaviors have been found. Initial ethanol preference is a major factor in the way stress influences ethanol drinking (Rockman1987, Volpicelli 1990, Croft 2005) and the present studies have confirmed these findings. We initially designed these experiments to test the hypothesis that early social stress experience in the inbred C57 strain has affected their individual ethanol intake and preference leading to the persistent individual variation described in Chapter 3. Secondarily, these experiments take

advantage of the large variance of ethanol drinking within the C57 mice to study the effects of social defeat stress on ethanol drinking behaviors.

The earliest social stress studies utilized group-housed settings and elaborate colony models in rodents to show that subordinate animals consumed more ethanol than their dominant and non-stressed counterparts (Blanchard et al. 1987; Wolffgramm et al. 1991) (Ellison et al. 1983; Hilakivi-Clarke et al. 1992). Differential ethanol preference did not exist in animals prior to establishment of a social hierarchy (Hilakivi-Clarke et al. 1992). Social subordination stress, however, increased ethanol intake over that of alpha males, which remained similar to intake in non-fighting control cages. Our current studies did not replicate these early findings. In littermates housed as siblings from birth, a clear social hierarchy could not be determined and thus we did not detect differences in ethanol intake prior to the establishment of a social hierarchy.

A major finding in social rank scoring of littermates is that littermates do not display as many offensive and defensive behaviors as non-littermates, even after cage-changes. The number of alpha males was significantly lower in littermates when scored upon arrival than when the mice were evaluated after three weeks of single housing. While it is tempting to assume that littermates may not establish a social hierarchy or a dominant alpha male, it is more likely that a period of single housing prior to group housing may be necessary to observe these behaviors in littermates. Hilakivi-Clarke *et al* have shown that a week of single housing is crucial to reliably establish a social hierarchy. In their studies, when 8 cages of mice were immediately group housed upon arrival, none of the cages showed signs of fighting or contained an alpha mouse. But, when mice were singly housed

for 1 week then housed in groups of 5, all seven cages contained an alpha mouse (Hilakivi-Clarke et al. 1992). As others have suggested, this week of single housing may be necessary to increase territorial aggression in singly housed mice and increase the likelihood of a social hierarchy (Miczek et al. 2008). Thus, it appears that a week of single housing is critical to reliably establish a social hierarchy and an alpha male in each cage and should have been used in our case.

Ethanol intake did not correlate to social subordination or dominance upon return to a group housed setting, even though we could reliably determine alpha aggressive males in this instance. There are a few reasons which can account for these differences. It is possible that our studies did not produce a significant social stress in these mice as there were no visible signs of injury in any cage. A clear social hierarchy could only be determined in 5 out of 6 cages. Additionally, in 3 of the cages, mice were housed back with siblings that may not be as stressful as living with new cage mates and being forced to establish a new social structure. Further complicating the interpretation, the number of offensive and defensive behaviors observed in each cage was highly variable. Our studies only used a week of group housing for social stress, while previous studies showing increased ethanol intake in subordinate animals used colony housing or visible burrow systems and were long term studies lasting weeks to months (Ellison et al. 1983; Blanchard et al. 1987). A final issue further complicating the influence of social dominance and subordination on ethanol consumatory behaviors is that social hierarchies may not be as stable as originally assumed. A recent study by Avitsur *et al* measured dominance order within a cage before and after a social disruption test and found that social order could be

altered by the brief introduction of an aggressive animal. Even in controls, not all cages showed the same dominance order one week after the first assessment (Avitsur et al. 2007). Considering these factors, our studies of offensive and defensive behaviors in group housed mice have shown considerable variability between cages in the frequency of these displays and the experimental design of group housing to inflict social stress is perhaps not the best design.

Since our initial studies had possible significant structural flaws complicating the interpretation of social dominance and subordination influences on ethanol drinking, we switched to a model of repeated social defeat for our social stress paradigm. This model gives the investigator substantially more control over the variability of interaction between two individuals as well as the duration and intensity of social attack behavior (Miczek et al. 2004; Miczek et al. 2008). We conducted two social defeat studies in C57 mice altering the timing of ethanol access following defeat. Social defeat stress decreased ethanol intake and preference with both continuous and delayed ethanol access. This result is not overly surprising as other investigators have reported decreased ethanol intake (van Erp et al. 2001; van Erp et al. 2001; Funk et al. 2004) or no change in intake (Keeney et al. 1999) following social defeat stress. Interestingly, having ethanol "on board" did not appear to significantly alter the response to social defeat stress as both conditions decreased ethanol intake. We had expected that having ethanol continuously available would give more time for the mice to associate ethanol consumption with relief from the negative aspects of social defeat stress, although these studies did not explicitly create an environment where ethanol anxiolysis was paired with social defeat or learned prior to defeat experience.

Alternatively, with ethanol access in such close proximity to the defeat sessions, there is the possibility that mice may associate ethanol with the negative aspects of defeat stress and not its anxiolytic properties. Future studies would need to be designed to directly pair ethanol access following defeat with its anxiolytic properties.

Although some clinical and animal studies point towards a positive relationship between stress and ethanol drinking, the tension-reduction hypothesis for alcohol-use has not been uniformly supported. Many clinical studies are inherently biased by self-reporting where it may be difficult for subjects to distinguish between events which resulted from alcohol use and relapse and events which precipitated alcohol use (Brady et al. 1999). Additionally, studies on the direct effects of stress on alcohol use and craving use contrived laboratory situations which may have their own inherent limitations. In animal models, the type, strength and frequency of the stressor and timing of ethanol presentation affect the drinking outcome (see Table 1 in Background and Significance). In two lines selectively bred for alcohol preference, 10 days of unpredictable restraint stress moderately decreased ethanol intake in preferring P and HAD1 rats during the stress period. Five days following the stress application, ethanol intake increased in P rats but not HAD1 rats (Chester et al. 2004). Footshock-induced stress decreased drinking in Sprague-Dawley rats (Brunell et al. 2005), but increased ethanol intake in P and HAD rats (Vengeliene et al. 2003). On the other hand, swim stress increased drinking in Wistar rats, but decreased intake in the alcohol preferring P and HAD lines (Vengeliene et al. 2003). In studies where stress increases ethanol drinking, intake decreases during the stress

application, but increases in the days following termination of the stress (Yavich et al. 2000; Sillaber et al. 2002; Croft et al. 2005).

Seeing as these social stress experiments were originally undertaken to explain individual differences in ethanol drinking behaviors, we found that in general, social defeat stress decreased ethanol intake in less than half of the mice. In both C57 experiments, there was a suggestion that mice with the lowest ethanol intake or preference increased drinking following social defeat. In order to more systematically test this hypothesis, 129SvJ mice, a low-preferring strain, were tested for ethanol drinking following social defeat. Ethanol intake increased in a majority of individual 129SvJ mice after social defeat stress. In the subpopulation of mice basally consuming less than 4 g/kg, ethanol intake increased in each of these mice. Mice consuming more that 4 g/kg did not significantly alter their ethanol intake and neither did mice which remained in their home cage.

There are several possibilities which could explain why C57 mice decreased drinking while a portion of 129SvJ mice increased drinking following social stress. One possibility for increased effects of ethanol drinking in the 129SvJ mice is that the social stress was more salient in this strain. We originally planned to use conspecifics as aggressive residents in the social defeat studies, but 129SvJ mice appear to be less aggressive and display fewer offensive attacks in a group housed setting. When tested for aggressive behavior, only 2 mice out of 8 cages could be reliably assigned as aggressive. Therefore, C57 mice were used as aggressors. In line with this possibility, Yang et al have shown that corticosterone is significantly higher in 129SVEV mice as compared to C57B/6J mice after 4 days of restraint stress (Yang et al. 2008). It is not clear if these

differences in glucocorticoid response are directly responsible for the differential drinking response to stress, but is a likely possibility that requires further study. Genetic background may also play an important role in stress-induced effects on ethanol consumption. Two rat lines genetically selected for ethanol preference have divergent ethanol responses to restraint stress (Chester et al. 2004) where alcohol-preferring P rats decrease drinking during the stress period, but following termination increase consumption above baseline levels. Alcohol-preferring HAD rats also moderately decrease drinking during the stress, but never increase above baseline levels following termination. Recently, Matthews and colleagues showed that moderate footshock increased voluntary ethanol drinking and plasma corticosterone in C57 mice but did not alter ethanol consumption or corticosterone levels in DBA or A/J mice (Matthews et al. 2008). However, the increases seen in C57 mice could be an artifact due to a large and significant decrease of ethanol intake in control mice and relatively minor increase in stressed mice in those experiments. Since initial ethanol preference appears to be an underlying factor in a number of stress-induced ethanol drinking studies, there is a possibility that we may be observing a ceiling effect in mice with the highest ethanol preference where stress cannot induce the mice to consume more than their baseline. Consequently, social stress-induced ethanol drinking increases in 129SvJ mice could be due to differences in initial ethanol preference, stress responsivity, genetic background, or a combination of these.

Further complicating interpretation of our results, recent studies have reported individual variability in response to a social stress. Avitsur *et al* have demonstrated individual variability in immunological response to social defeat stress depending on the

animal's social rank (Avitsur et al. 2007). Koolhaas *et al* have characterized active and passive "coping styles" as the prevalent measure for these differences (Koolhaas et al. 1999; Koolhaas 2008) and have suggested that individual differences in coping styles are reflected in behavioral and sympathetic stress reactivity. Recently, Krishnan *et al* have elegantly characterized behavioral and physiological differences in susceptible phenotypes following social defeat stress (Koolhaas et al. 1999; Krishnan et al. 2007) and argue that molecular adaptations within the mesolimbic dopamine circuitry mediate these differences. Together, these studies point towards underlying differences in dopamine and/or sympathetic reactivity which may account for individual variations in response to social stress. Further studies will be needed to determine whether similar differences could explain our observed variation in stress-induced drinking response, or even initial preference for ethanol. Gene expression analysis in Chapter 6 begins to address the molecular factors involved in initial variation of ethanol drinking behaviors and long term effects from social defeat stress.

In summary, contrary to the tension-reduction hypothesis, social defeat stress decreases ethanol intake in C57 mice regardless of whether ethanol was continuously available or deferred until after the stress period. Social dominance and subordination also were not indicative of ethanol preference or intake. Individual differences in basal ethanol preference appear to play a role in the stress-influenced ethanol drinking behavior. In each of these studies mice with the lowest ethanol preference display stress-induced increases while the highest preferrring animals decrease or do not change their ethanol intake. Similarly, other studies have shown dramatic differences in coping and susceptibility to

social stress (Koolhaas et al. 1999; Krishnan et al. 2007). These studies have suggested that alterations in neuroendocrine responses, emotional reactivity and even the mesolimbic dopamine system are mechanisms involved in the individual responses to social stress. We have further investigated the molecular factors involved in social defeat stress and individual variation of ethanol drinking in Chapter 6.

CHAPTER 5 Anxiety-Like Behaviors in Ethanol Drinking and Social Defeat

Introduction

Stress and anxiety are commonly thought to play a major role in the development of alcohol abuse and relapse drinking. Alcoholics report anxiety-reduction as a major motivational factor for drinking (Conger 1956; Pohorecky 1981; Newlin et al. 1990). In fact, 80% of alcoholic patients report alcohol drinking to reduce feelings of anxiety, depressed mood and negative emotional states (Hershon 1977; Annis et al. 1998). Alcohol abuse and alcoholism show a high degree of comorbidity with anxiety-related disorders (Bibb et al. 1986; Cornelius et al. 2003). Social stressors such as early family adversity, including abuse, emotional neglect, and harsh inconsistent punishment are also risk factors for alcohol and drug abuse (Zoccolillo et al. 1999). These clinical findings have been supported by a number of studies in animals showing that social isolation or maternal separation in early life increases alcohol and drug self-administration (Meaney et al. 2002; Brake et al. 2004). Additionally, ethanol can act as an anxiolytic to reduce several measures of anxiety and neurochemical markers of HPA axis activation (Sinha et al. 2000; Sher et al. 2007). Likewise, ethanol is a well-documented anxiolytic in multiple rodent

models of anxiety, including the elevated plus maze (LaBuda et al. 2000; LaBuda et al. 2001; Boehm et al. 2002), the light-dark test (Costall et al. 1988; Bilkei-Gorzo et al. 1998; Boehm et al. 2002), the social interaction test (Varlinskaya et al. 2002), and the mirrored chamber test (Cao et al. 1993; Kliethermes et al. 2003).

However, a majority of clinical studies involve self-reports or co-occurrence of disease and it is not always clear whether the proclivity to abuse alcohol precedes the anxiety-related disorders. Thus, a variety of animal models have been employed to investigate the relationship between anxiety-like behaviors and ethanol consumption. Variability in ethanol consumption in rodent lines may be, at least in part, due an animal's basal anxiety levels suggesting a role for basal anxiety states in the predisposition towards ethanol consumption in a rodent model. Two lines of rats selectively bred for ethanol preference, P and Sardinian P rats, show higher anxiety in three different behavioral measures as compared to ethanol-nonpreferring (NP and sNP rats) (Stewart et al. 1993; Colombo 1997; Pandey 2003). Additionally, when sP rats are allowed to freely self-administer ethanol, they display reduced anxiety-like behavior in the elevated plus maze as compared to ethanol-naïve sP rats. A direct relationship between ethanol drinking and anxiety-like behavior is not always consistent. Other strains selectively bred for alcohol preference, HAD/LAD and AA/ANA rats, alcohol-preferring rats are more anxious, less anxious or do not differ from non-preferring rats (Tuominen et al. 1990; Tuominen et al. 1990; Spanagel et al. 1999; Badia-Elder et al. 2003). The finding that not all selectively bred rats show a link to anxiety and ethanol consumption is not necessarily surprising and

it is likely that during the selective breeding, genes responsible for alcohol preference did not segregate with genes responsible for innate anxiety.

Outbred rodent studies provide evidence which strengthens this link between emotionality and ethanol consumption. Using Wistar rats selected for individual differences in anxiety-related behavior on the elevated plus maze, Spanagel *et al.* reported a positive correlation between ethanol consumption and anxiety levels (Spanagel et al. 1995). Rats selected for differences in anxiety-like behaviors show increased place preference for the ethanol paired compartment (Blatt et al. 1999). Furthermore, bilateral lesions of the central nucleus of the amygdala, a region important in anxiety-related behavior, reduced anxiety-like behavior and voluntary ethanol intake (Moller et al. 1997). Ethanol and stress responsive pathways share common overlapping neurobiological substrates and brain regions as previously discussed in Background and Significance.

The current studies were designed to test the hypothesis that innate anxiety may contribute to the variation of ethanol drinking in the C57 mice. We have used the light-dark transition model to measure anxiety-like behaviors. The light-dark transition model is a neophobia test which takes advantage of a mouse's natural aversion to light coupled with a preference to explore a novel environment (File 1995). It allows automatic measurements of the amount of time and distance traveled in the light versus dark compartment. An increase in time or distance traveled in the light is interpreted as a low anxiety phenotype. Treatment with an anxiolytic drug increases exploration of the illuminated compartment while an anxiogenic drug decreases light chamber exploration (Crawley et al. 1980). Similarly, ethanol increased the time in the light chamber three-fold in rats with mCPP-

induced anxiety (Bilkei-Gorzo et al. 1998). Although some researchers use multiple behavioral models of anxiety to study "generalized" anxiety, our rationale for choosing only one model is due to evidence that even similar behavioral models of anxiety measure different forms of anxiety-like behavior (Belzung et al. 1994; File 1995; Ramos et al. 1997). This idea is supported by basal anxiety level QTL analysis in mice, showing that different behavioral models of anxiety correlate with different chromosomal regions (Flint 2003).

Three separate experiments were performed to test whether basal anxiety contributes to ethanol drinking behaviors, and if the response to defeat stress is reflected in anxiety-like behaviors. We asked several question with these experiments. First, can basal anxiety predict future ethanol intake and preference? Second, does ethanol drinking alter anxiety phenotypes in the light-dark model of anxiety? Third, since social defeat both increases and decreases ethanol intake (see Chapter 4), can measuring anxiety-like behavior shed any light on the response to social defeat?

Methods

Anxiety-Related Behaviors in the Light-Dark Transition Model: C57BL/6NCrl mice were tested for basal anxiety-like behaviors in the light-dark transition model of anxiety. The light-dark box was adapted from the originally described apparatus (Crawley et al. 1980). Our light-dark box contained two equally size compartments (30 cm x 15 cm x 15 cm), separated by a black plastic partition with an opening in the middle to allow for light-dark transitions (Med Associates, St. Albans, VT). The box was enclosed in a sound-attenuating box equipped with overhead lighting and fan ventilation and interfaced with

Med Associates software to allow for automatic measurement of activity using a set of 16 infrared beam sensors along the X-Y plane. Data was collected in both chambers and includes distance traveled, time spent, rears, light-dark transitions, and velocity. Following a 1-hour acclimation period to the behavioral room, animals were placed in the center of the light chamber facing the entrance to the dark chamber. Studies consisted of a 5 minute test session, once the animal entered the dark compartment. Results are expressed as percent time spent in the light, percent distance traveled in the light and number of transitions into the light compartment to avoid locomotor activity contamination. An increase in any measure is interpreted as less anxiety-like behavior. Each experimental mouse group consisted of 10 or 15 mice.

Corticosterone Radioimmune Assay (RIA): Trunk blood was collected from individual mice (n=50) one hour after the last defeat episode from a separate cohort treated exactly the same as mice in experiment #3, except they were not tested in the light dark box after their last defeat. All animals were sacrificed between 1330 and 1530 hours during the dark cycle. This time point was chosen to be during a period of low circulating corticosterone levels (Filipski et al. 2004). Plasma was isolated by centrifugation at 2500 x g for 15 minutes and stored at -80^{o}C until RIA assay. Corticosterone RIAs were performed using a commercially available double antibody radioimmune assay containing I^{125} labeled corticosterone (MP Biomedicals, Orangeburg, NY). Assays were performed exactly according to manufacturer's instructions. The lower limit of detectability in this assay was 7.7ng/mL. The intra-assay coefficient of variation for the kit was 7.2%.

Experiment #1 Reliability test in the light-dark transition model: Naïve male C57BL/6J mice (n=7) were tested twice in the light-dark box to determine if mice can be repeatedly tested for anxiety-like behavior without showing habituation to the test apparatus. Following a one hour habituation to the behavioral room, mice were tested for basal anxiety-like behavior on day 1. Mice were returned to the home cage and tested again for anxiety-like behavior on day 14. In this experiment, mice did not have access to ethanol drinking.

Experiment #2 Anxiety-like behavior following ethanol drinking: 24 male C57BL/6NCrl mice at 6-7 weeks were habituated to the vivarium for 1 week housed in groups of 4/cage. Mice were singly housed for 1 week then tested in the Light-Dark transition model for basal anxiety-like behaviors. All mice were allowed to consume ethanol (10% w/v) or water in a two bottle choice drinking paradigm for 18h/day (see Methods in Chapter 4) for 14 days, and were tested again for anxiety-like behaviors in the light-dark model on day 15.

Experiment #3 Anxiety-like behavior following social defeat: 50 male C57BL/6NCrl were housed in groups of 4/cage for 2 weeks on a reverse light cycle upon arrival to the facilities (lights on at 1800 hours). At 7 weeks old, mice were singly housed for 7 days. Each mouse was tested for basal anxiety-like behavior in the light-dark box then started on two bottle choice drinking (10% ethanol (w/v) or tap water). Mice were given 24 hours of ethanol access at 4 hours into their dark cycle (1200 hours). A reverse light-cycle with 24 hour access to ethanol was used so defeat session would occur during the active cycle (in the dark) and mice would have continuous access to ethanol. Standard

mouse chow was supplied ad libitum throughout all studies. The position of ethanol and water bottles were switched every other day to avoid side preferences (i.e. L, L, R, R). 30 mice were given 14 days of baseline ethanol drinking, while 20 mice were given two bottles of water. After baseline drinking, 15 ethanol drinkers (SDE) and 15 water drinkers (SDW) were given 5 consecutive days of social defeat by an aggressive male C57 mouse. Defeats consisted of three phases: 1. acclimation -- 5 minutes of interaction with the experimental mouse under a protective cage, 2. defeat – up to 5 minutes of physical interaction stopped when the experimental animal displays the characteristic defeat posture, 3. threat -- 30 minutes of threat under protective cage. Defeated animals were then returned to their home cage. The remaining 10 ethanol drinkers (HCE) and 10 water drinkers (HCW) remained in their home cages as controls. Ethanol drinking mice had continuous access to ethanol during the entire experiment. All mice were tested again for anxiety-like behavior in the light-dark box 24 hours after the last defeat session.

Results

Test-retest reliability in the Light-Dark box

In our initial test, mice (n=7) did not display detectable habituation to the light-dark box test apparatus. The percent distance traveled in the light and percent time in the light were not significantly different on day 1 versus day 14 (paired t-test, p=0.822 and p=0.720, respectively). Total distance travelled in the activity boxes was also not significantly different between day 1 and day 14 (paired t-test p=0.615, Figure 15). Thus, mice do not appear to show habituation to the novelty aspects of repeated exposure to the apparatus

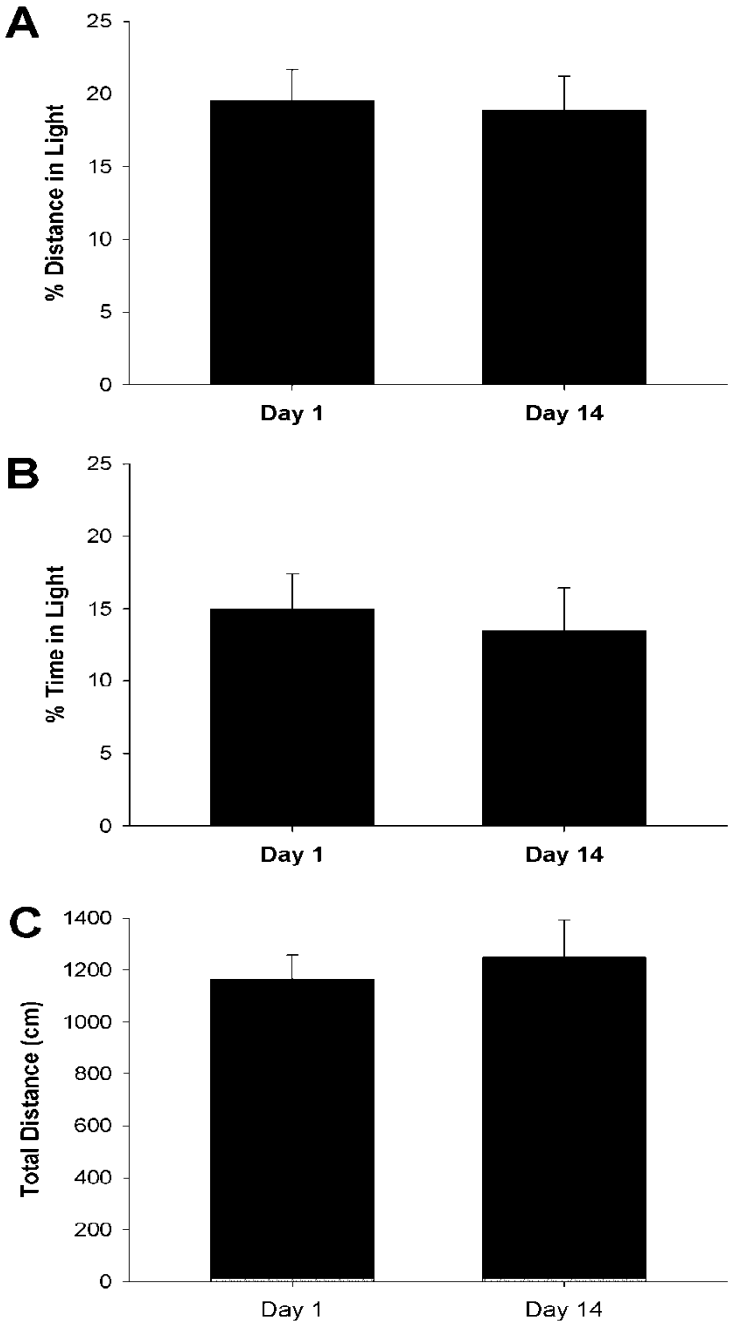

Figure 15: Test-Retest in the Light-Dark Box. A. Percent distance traveled in the light is not significantly different on the first day of testing and 14 days later ($t(6)=0.235$, $p=0.822$). **B.** Percent time in the light is not significantly different between day 1 and day 14 ($t(6)=0.375$, $p=0.720$). **C.** Total distance traveled in the activity box is not significantly altered on day 14 from first exposure to the light-dark box ($t(6)= 0.531$, $p=0.615$).

since anxiety-like behavior and locomotor behavior did not significantly change from day 1 to day 14.

Anxiety-like behavior following ethanol drinking

Basal anxiety-like behavior was significantly correlated to anxiety-like behavior after 14 days of ethanol drinking for percent distance in light (R=0.445, p=0.0292) and percent time in light (R=0.599, p=0.0045, Table 4). The number of entries into the light was not significantly correlated (R=0.318, p=0.1298), but did show a trend in the same direction. This initially suggests a high test-retest reliability in the light-dark box.

Anxiety-like behavior was significantly decreased on day 15 as compared to basal anxiety-like behavior (by paired t-tests, see Figure 16 and Table 4) since each of the measures, percent distance in the light, percent time in light, and entries into the light, was increased following 14 days of ethanol drinking. Initially, this could suggest that ethanol drinking decreased anxiety-like behaviors. However, this is not likely since anxiety-like behavior was not significantly correlated to ethanol intake at any time point over the 14 days of drinking in experiment #2. Additionally, the total distance traveled was increased after the second exposure to the apparatus. It raises the possibility that the decreased anxiety-like behavior on day 14 could be from repeated exposure to the activity box and the number of mice used in the initial test-retest experiment (experiment #1) was not large enough to show a significant effect.

In experiment #3, however, ethanol intake at each time point throughout the drinking session in home cage control mice showed a trend to be correlated negatively to the number of entries into the light on the first anxiety test (Table 5). While previous

Table 4: Basal Anxiety Phenotypes Versus Anxiety Phenotypes After Ethanol Drinking.

	Pearson Correlation		Paired T-test
Anxiety Phenotypes	R value	p value	p value
% Distance in light	0.445	0.0292	0.0001
% Time in light	0.599	0.0045	0.0014
# Entries in light	0.318	0.1298	0.0003
Total Distance	0.260	0.2194	0.0457

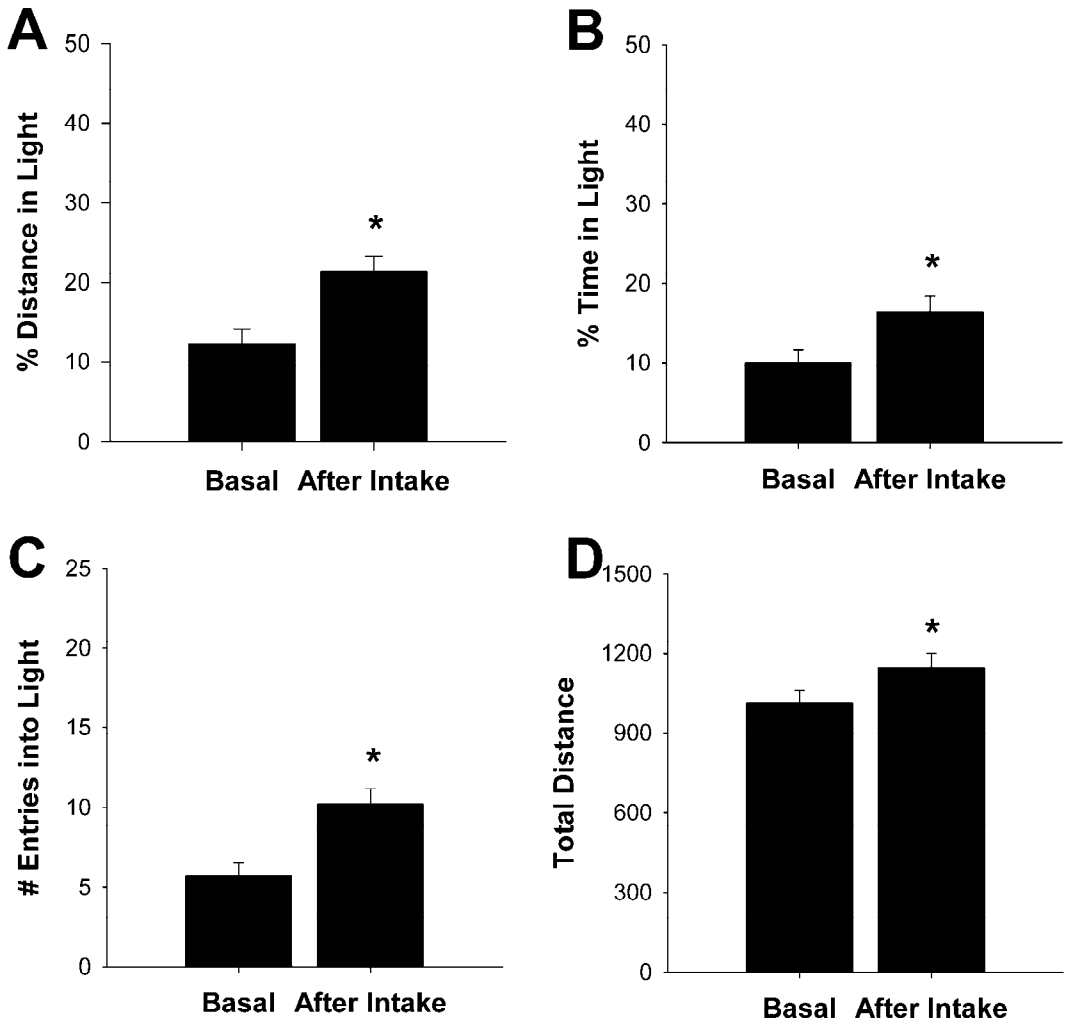

Figure 16: Repeatability in the Light-Dark Box Following Ethanol Drinking A. Percent distance traveled in the light is increased upon re-exposure to the apparatus following 14 days of ethanol drinking ($t(23)=-4.58$, $p=0.0001$). **B.** Percent time in the light is increased on day 15 from day 1 upon re-exposure ($t(23)=-3.63$, $p=0.0014$). **C.** Number of entries is increased on day 15 from day 1 ($t(23)=-4.28$, $p=0.0003$). **D.** Total distance traveled in the activity box is increased on day 15 from first exposure to the light-dark box ($t(23)= -2.11$, $p=0.04$). *$p<0.05$

Table 5: Correlation of Basal Anxiety-like Behavior with Ethanol Intake

Ethanol Intake	R value	p value
Baseline	-0.477	0.072
Defeat period	-0.421	0.118
Week 1	-0.483	0.068
Week 2	-0.482	0.069
Week 3	-0.462	0.083

experiments did not show a direct relationship between ethanol intake and basal anxiety-like behavior, mice which remained in their home cage and consumed ethanol for 40 days suggest the potential for a relationship between ethanol intake and basal anxiety-like behavior.

Effects of social defeat and ethanol drinking on plasma corticosterone levels

Repeated social defeat induced an increase in corticosterone (CORT) levels in defeated mice, (35.78 +/- 8.5 ng/ml in SDW, and 38.12 +/- 4.3 ng/ml in SDE mice) while ethanol drinking did not significantly affect corticosterone levels (25.27 +/- 6.0 ng/ml in HCW and 19.22 +/- 2.5 ng/mL in HCE mice; Figure 17). Two-way ANOVA revealed a main effect of social defeat ($F(1, 45)=7.952$, $p<0.007$) on CORT levels. There was no main effect of ethanol drinking ($F(1,45)=0.200$, $p=0.657$) or interaction between social defeat and ethanol drinking ($F(1,45)=0.525$, $p=0.473$). Ethanol intake was not significantly correlated to CORT levels for baseline intake or following social defeat in either the defeated mice or the home cage controls (data not shown). Thus, five days of social defeat activates the HPA axis as seen by increased corticosterone levels in defeated mice. Voluntary ethanol drinking does not appear to have a significant effect on corticosterone levels.

Social defeat and anxiety-like behavior

Mice were socially defeated for 5 days then tested for anxiety-like behavior 24 hours after their last defeat. Unfortunately, contrasted to studies in Chapter 4, social defeat did not significantly alter ethanol intake in this experiment. In fact, only one mouse out of fifteen significantly decreased ethanol intake following defeat ($F(4,28)=5.532$, $p=0.0021$).

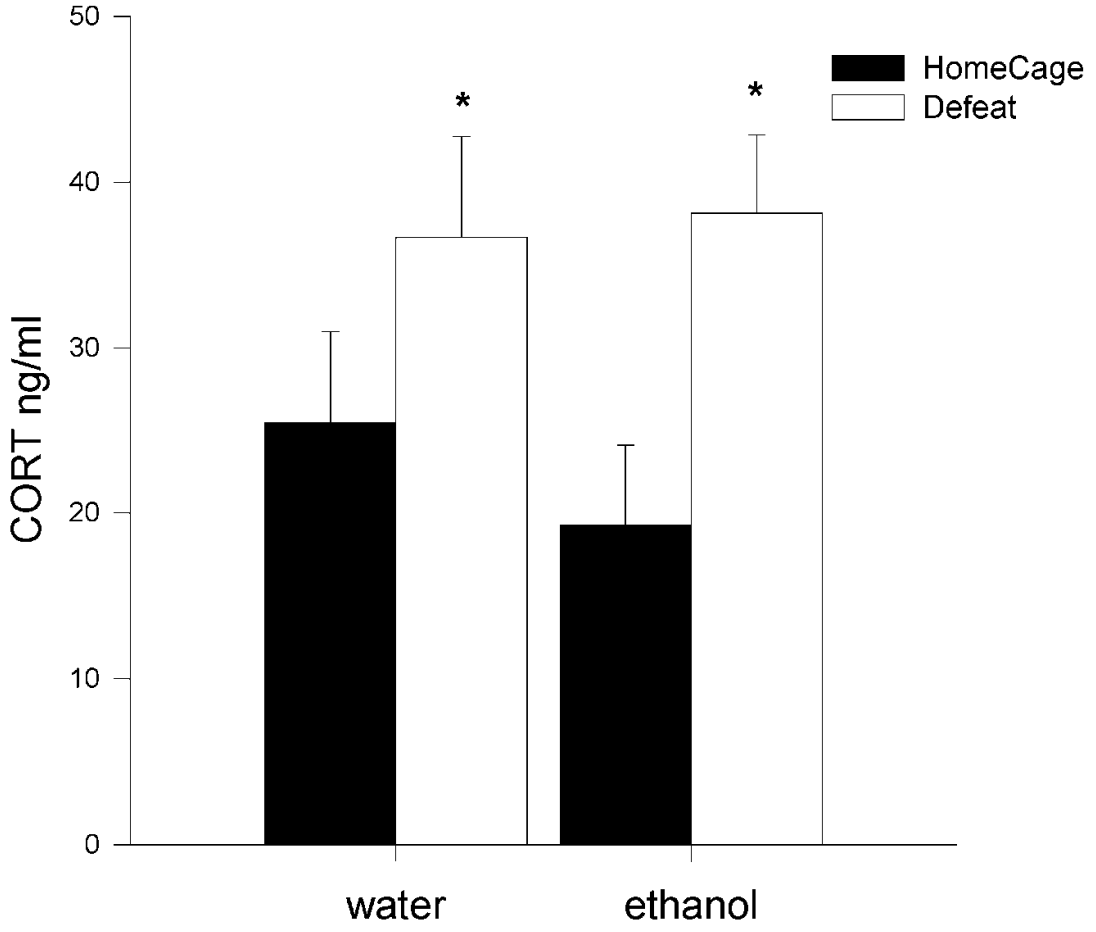

Figure 17: Social Defeat But Not Ethanol Drinking Increases Corticosterone. Plasma CORT levels are increased 1 hour following the last defeat session. Two-way ANOVA revealed significant main effect of social defeat ($p=0.007$), but no main effect of ethanol drinking ($p=0.657$) or interaction ($p=0.473$).

Anxiety-like behavior was also not significantly altered in defeated mice or home cage control mice 24 hours after the last defeat session (Figure 18). However, ethanol intake during the defeat period showed a trend to be negatively correlated to the percent distance travelled in the light (R= -0.499, p=0.058) and percent time in the light (R= -0.458, p=0.086; Figure 19) following social defeat. To further test this trend, the lowest quartile of ethanol drinkers (n=4) were compared to the highest quartile of ethanol drinkers (n=4) by t-test. Indeed, the lowest drinking mice spent more time (p=0.05) and traveled farther (p=0.042) in the light than the highest drinking mice (Figure 19). Mice with the lowest ethanol intake displayed a low anxiety phenotype, while mice which consumed the most ethanol displayed more anxiety-like behaviors. These findings suggest a relationship between ethanol intake and response to social stress which can be reflected in anxiety-like behavior.

Discussion

The current experiments have not shown a simple, direct relationship between ethanol intake and innate anxiety-like behaviors in the light-dark transition model of anxiety. However, these results point towards a relationship between ethanol intake and anxiety related behavior following a period of social stress. For the most part, we did not consistently find a strong correlation between innate anxiety phenotypes and initial voluntary ethanol consumption. Intriguingly, one experiment suggested a trend towards a relationship between long-term ethanol intake and basal anxiety-like behavior. When mice were allowed to voluntarily consume ethanol for forty days, there was a tendency for

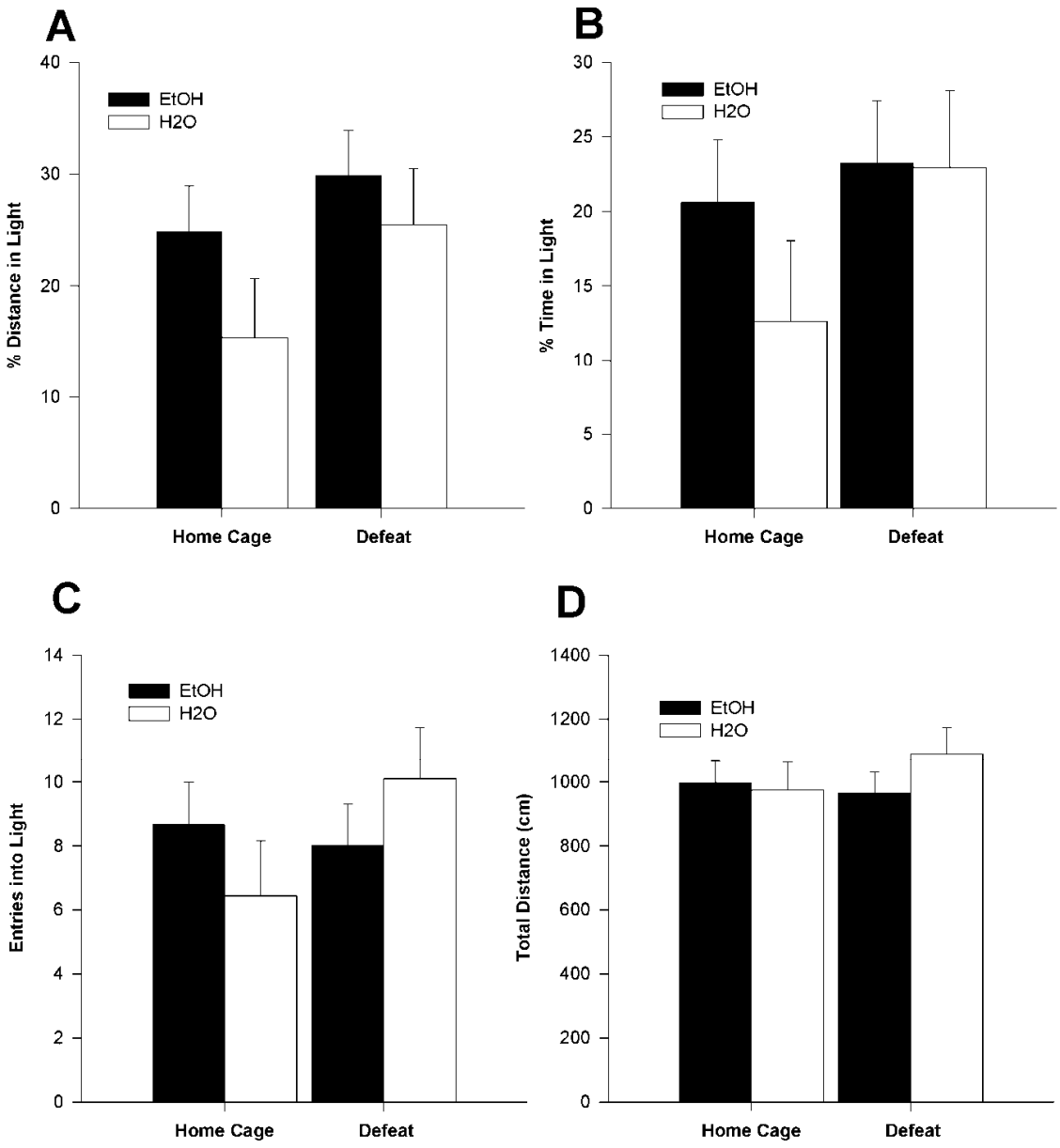

Figure 18: Social Defeat and Ethanol Intake Did Not Significantly Alter Anxiety-like Behaviors. A. Percent distance traveled in the light was not different in mice which were socially defeated (p=0.111) or consumed ethanol (p=0.144). **B.** Percent time in light was not different in mice which were socially defeated (p=0.182) or consumed ethanol (p=0.391). **C.** The number of entries into the light was not different between socially defeated (p=0.968) or ethanol drinking mice (p=0.324). **D.** Locomotor activity was not different between socially defeated (p=0.597) or ethanol drinking mice (p=0.507).

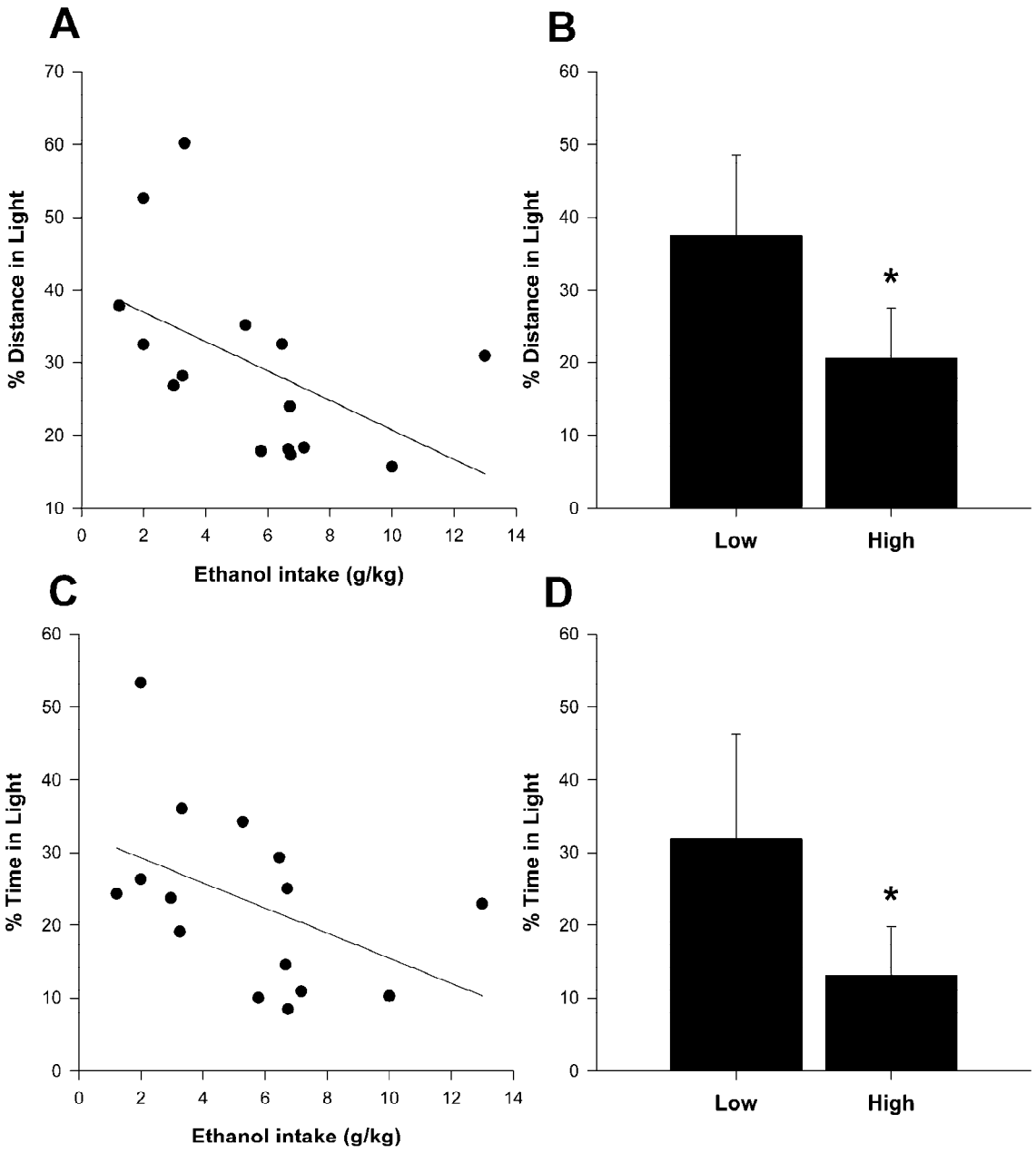

Figure 19: Ethanol Intake in Socially Defeated Mice is Correlated to Anxiety-like Behavior. A. Percent distance in the light after the last defeat is negatively correlated to ethanol intake during the defeat period (R=0.499, p=0.058). **B.** Mice which consumed the least amount of ethanol (n=4) traveled more in the light than mice with the highest ethanol intake (T-test, p=0.042). **C.** Percent time in the light after the last defeat is negatively correlated to ethanol intake during the defeat period (R=0.458, p=0.086). **D.** Mice which consumed the least amount of ethanol spent more time in the light than the heaviest drinking mice (T-test, p=0.05). * p<0.05

ethanol intake to be negatively correlated to the number of entries into the light (see Table 5). These data point towards a positive relationship between innate anxiety-like behavior and ethanol intake where mice which consume the lowest amount of ethanol display a low anxiety phenotype. Early studies in selectively bred rats suggest that innate anxiety may predispose an animal's ethanol drinking behavior (Stewart et al. 1993; Colombo et al. 1995) although these findings are not consistent between other selectively bred strains (Spanagel et al. 1999; Badia-Elder et al. 2003). Additionally, in a recent study investigating individual variation of ethanol drinking in adolescent Sprague Dawley rats, basal scores in the elevated plus maze and open-field test were not correlated to forced ethanol intake, voluntary ethanol intake or ethanol intake following deprivation (Schramm-Sapyta et al. 2008). The present studies have added to the previous findings, where we have a suggestion that basal anxiety-like behavior may predict future ethanol intake. But our findings do not show a simple and consistent relationship and further studies will be needed to elucidate this role. Perhaps, as previously suggested in other studies, the response to a stress may be more indicative of ethanol preference in these mice.

The second aim of these studies was to determine if voluntary ethanol consumption could reduce anxiety related behaviors. Alcohol preferring sP rats are more anxious in the plus maze than nonpreferring sNP rats. But after 2 weeks of voluntary ethanol drinking, sP rats displayed a lower anxiety phenotype than ethanol naïve sP rats (Colombo et al. 1995) suggesting that sP rats may consume ethanol to relieve their higher anxiety. Our experiments were designed as a test-retest paradigm to examine the relationship between basal anxiety and anxiety following ethanol consumption. When Blumstein and Crawley

(Blumstein et al. 1983) tested the reliability of the light-dark transition model of anxiety, they reported that mice can routinely be tested repeatedly up to 3 times. Even though, our initial experiments showed that mice do not habituate to the light-dark box after repeated exposures separated by 14 days, in subsequent experiments, all measures were significantly increased upon re-exposure to the apparatus. The light-dark box is based on the conflict between a mouse's natural aversion to bright, open spaces and its preference to explore a novel environment. Therefore novelty of the arena is critical for the proper assessment of anxiety-like behavior in this test (Lister 1990). Basal anxiety-like behavior in each mouse was highly correlated to their anxiety-like behavior following 2 weeks of voluntary drinking. However, in the retest sessions, all anxiety measures were significantly increased. Since ethanol intake was not correlated to any anxiety-like behavior, the most likely interpretation is that re-exposure to the box reduces novelty and thus confounds further interpretation of the relationship between anxiety-like behavior following ethanol intake. Future experiments will need to use separate groups of mice to measure anxiety-like behavior prior to and following ethanol drinking.

A common criticism of social stress studies with ethanol drinking is that it has not always been clear if the stress paradigm produces a physiological response relevant at the time of ethanol self-administration. Stress activates the HPA (hypothalamic-pituitary-adrenal) axis causing increases in corticotrophin releasing factor, adrenocorticotropic hormone and, in rodents, corticosterone. Ethanol activates this pathway acutely (Han et al. 1993; Carson et al. 1996; Weiss et al. 1996; Wu et al. 1997), even though ethanol is an anxiolytic. Here, we measured plasma corticosterone levels in four groups of mice:

ethanol drinking socially defeated mice, water drinking socially defeated mice, ethanol drinking mice which remained in the home cage and water drinking home cage control mice. Plasma was collected 1 hour after the last defeat session. Social defeat induced close to a two-fold increase in corticosterone levels over control mice, showing that repeated social defeat activates the HPA axis and produces a physiological response. Other models of social stress have shown that a single defeat session activates the HPA axis, increasing corticosterone levels (Avitsur et al. 2001; Keeney et al. 2001) without habituation to the effect, where CORT levels remain increased even after 24 defeat sessions (Keeney et al. 2001). Ethanol was freely available to mice immediately after defeat sessions for 24 hours a day allowing mice to immediately consume ethanol and potentially self-medicate or mitigate their increased stress response. Thus, our social stress paradigm produces a measurable physiological response in mice at a time when ethanol is available for consumption, yet we still did not see an increase in ethanol consumption with repeated social defeat episodes.

Voluntary ethanol drinking did not alter corticosterone levels in our experiments and ethanol intake was not correlated to corticosterone levels. This is not overly surprising since previous studies have similarly shown no effect of ethanol consumption on corticosterone (Ogilvie et al. 1997; Finn et al. 2004). In outbred rats, corticosterone levels were not significantly correlated to ethanol intake (Schramm-Sapyta et al. 2008). Bolus injections of ethanol, however, significantly increase corticosterone and activate the HPA axis (Han et al. 1993; Carson et al. 1996; Weiss et al. 1996; Wu et al. 1997; Finn et al.

2004). Presumably, in these experiments, mice do not consume enough ethanol to raise blood ethanol levels high enough to activate the HPA axis.

Surprisingly, in a between group analysis, there was not a difference in anxiety phenotypes in home cage versus defeated mice. Repeated social defeat did not induce anxiety-like behavior in either ethanol or water drinking mice. This is in contrast to previous social stress paradigms where mice were defeated and lived continuously opposite a dominant animal (Keeney et al. 1999) or were subjected to social disruption (Kinsey et al. 2007). Three strains, NMR1, C57 and CD-1 mice, showed increased anxiety-like behavior in the light-dark box, or elevated plus maze following social stress and the increased anxiety-like behavior persisted for at least 1 week following the last stress session in C57 mice (Kinsey et al. 2007). There are several differences between the social stress protocols which could account for these conflicting findings. Our protocol is a moderate stress with no physical signs of injury. Mice are defeated once a day for 5 days with a maximum 5 minute interaction period followed by 30 minute threat of defeat. In the social disruption protocol, a cage of mice is defeated 6 times a week in 2 hour sessions giving sufficient time for quantifiable injury to the subordinate mice. Kenney and Hogg's paradigm reduces the physical interaction time to less than 5 minutes, but the defeat procedure is coupled with the stress of continuously living opposite a dominant animal. Our protocol has never caused visible tissue injury or hair loss from the defeat sessions. Thus our repeated social defeat protocol could be considered a mild to moderate "psychological" stress. Additionally, in Kinsey et al., C57BL/6 mice appear to "prefer" the light compartment, spending 58% of their time in the light under basal conditions. Socially

stressed mice show increased anxiety-like behavior, reducing the amount of time in the light to 45%. Still, these mice are spending a significant amount of time in the light compartment which raises questions about the validity of their light-dark test. Both C57 and CD-1 strains, however, display significant increases in anxiety-like behavior in the open field test, showing that social disruption increases anxiety-like behavior. Social stress in this specific experiment is perhaps only moderately stressful as it did not alter ethanol drinking or anxiety-like behaviors.

Interestingly, the present studies do show a relationship between individual ethanol intake during the defeat period and anxiety-like behaviors in the light-dark box following the last defeat in socially stressed animals. While we did not find a consistent relationship between ethanol intake and basal anxiety phenotype, following a period of social stress, mice with a high anxiety phenotype consumed the highest amounts of ethanol. These findings are consistent with previous studies in dependent animals where following protracted abstinence, dependent animals did not show significant induction of anxiety-like behavior (Valdez et al. 2003). Once a brief stressor was employed, a heightened behavioral stress response could be detected in post-dependent rats. Thus, while no clear relationship between individual variation of ethanol drinking and basal anxiety could be determined in individual mice, the response to social stress may be altered in individual mice and may be the source for their individual variation of ethanol drinking. Future studies employing other stress modalities such as restraint stress or foot shock could strengthen this argument. Additionally a careful observation of coping mechanisms displayed during the defeat

sessions could provide further opportunities to investigate response to stress on an individual level.

CHAPTER 6 Molecular Factors Contributing to Individual Variation of Ethanol Drinking and Response to Social Stress

Introduction

Extensive studies in humans have suggested that genetic factors account for about 40-60% of the risk for alcoholism (Cloninger 1987; Gordis et al. 1990; Enoch et al. 2001; Radel et al. 2001). Work in humans and animal models over the last 20 years has documented genetic intervals (Phillips et al. 1994; Crabbe 2002; Lovinger et al. 2005) or individual genes (Shirley et al. 2004; Fehr et al. 2005) contributing to variation in behavioral responses to ethanol. Despite such progress on identifying genetic influences in alcoholism, little work at the molecular level has been done to identify mechanisms that mediate environmental influences on ethanol drinking behaviors or alcohol abuse. It is well documented that environmental influences such as stress or exposure to conditional stimuli can modify ethanol drinking or cause recidivism in abstinent alcoholics. Understanding the molecular mechanisms underlying such environmental influences on ethanol behaviors would augment the genetic progress mentioned above.

Using a model of persistent individual variation in ethanol drinking behavior within inbred mice, where genetic factors are strictly controlled, offers considerable power for

studying molecular mechanisms of environmental modulation of ethanol drinking behavior. Here, we have performed whole genome expression profiling in individual mice to finely dissect molecular factors underlying individual variation in ethanol drinking behavior. We hypothesized that an as yet unidentified non-genetic factor has caused long-lasting brain signaling alterations that influence ethanol preference and intake in these inbred mice. By characterizing gene networks differentially expressed between ethanol-preferring and avoiding mice, we can identify signaling cascades which may have been altered in these mice and influenced their drinking patterns. We expect that these studies may ultimately lead to novel targets for pharmacotherapy in alcoholism.

In a second set of experiments, we profiled gene expression in individual mice following repeated social defeat and ethanol drinking. The central hypothesis of these studies is that social stress, a non-genetic factor, causes long lasting signaling alterations in the brain which influence ethanol drinking behaviors. In mice, repeated social defeat increases neuronal activation in the HPA axis and several limbic regions. Such activation has pointed to a potential interaction between the HPA axis and the mesocorticolimbic system in social stress. These systems are also involved in ethanol drinking behaviors. Social defeat stress may increase drinking particularly in low preference mice (Rockman et al. 1986; Croft et al. 2005). However, decreased drinking or no change following defeat has also been reported (see Table 1 in Background and Significance). Regardless, the molecular mechanisms that underlie this phenomenon are poorly understood. Moreover, social defeat may have differing effects on ethanol drinking based upon an animal's baseline predilection as shown in Chapter 4. Social defeat decreased ethanol intake in a

majority of mice, but showed a tendency to increase drinking in low preferring mice (see Figure 12). This final set of experiments is an initial attempt to identify those gene networks associated with stress-influenced ethanol drinking.

Methods

Animals: Male C57BL/6NCrl mice at age 42 to 49 days of age were purchased from Charles River Laboratories (Wilmington, MA). All mice were habituated to the housing environment by group housing (5 mice/cage) for 1 week followed by individual housing for 1 week prior to beginning drinking experiments. Tissue used for experiment 1 was obtained from mice in Chapter 3 with rounds of ethanol access in a two bottle choice paradigm. Tissue used for experiment 2 was obtained from select mice used in Chapter 4 for the repeated social defeat studies with continuous ethanol access.

Two bottle choice drinking: Voluntary two-bottle choice drinking was done essentially as described previously (Khisti et al.) and in Chapter 3. Briefly, mice (n=20) had access to two bottles in their home cage containing 10% ethanol or tap water at the beginning of the dark cycle. In experiment 1, bottles were available for 18 hours/day and mice were allowed free access to water for the remaining 6h/d from standard water bottles. Mice were given four consecutive days of drinking sessions followed by four days of abstinence. This cycle was repeated four times to give a total of 16 days of drinking.

For experiment 2, mice (n=29) were habituated to a reverse light for 2 weeks then given 24 hours of ethanol access beginning 4 hours into the dark cycle (1200 hours). 19 mice were given 14 days for baseline ethanol drinking, while 10 mice were given two bottle of water. After baseline drinking, 14 ethanol drinkers and 5 water drinkers were

given five consecutive days of social defeat by an aggressive male C57 mouse. The remaining 5 ethanol drinkers and 5 water drinkers remained in their home cages as controls. Ethanol drinking mice had continuous access to ethanol during the days of defeat and for 3 weeks following social defeat except for the time (35 minutes/day x 5 days) that they were placed in the cage of an aggressor.

In both experiments, mice were sacrificed by cervical dislocation 6 days after their last drinking session and brain tissue harvested for microarray analysis as described below.

HDAC Inhibitor Studies: A separate group of mice was used to test the effects of Trichostatin A (TSA) an inhibitor of class I (HDAC isoforms 1, 2, 3, 8 and 11) and class II HDACs (isoforms 4, 5, 6, 7, 9 and 10) (Villar-Garea et al. 2004; Dokmanovic et al. 2005), on ethanol drinking. 18 male C57BL/6NCrl mice from Charles River Laboratories were acclimated to a reverse-light cycle (lights on 2000 h, lights off 0800h) for two weeks and singly housed for 1 week prior to initiation of drinking studies. Voluntary ethanol drinking was initiated similarly to the above experiment except these mice had 24 hours of access each day. Tubes were measured daily and replaced with fresh tubes at 1200h, four hours into the dark cycle. Mice had 7 days of ethanol access to determine each animal's baseline ethanol intake and preference. Mice were then divided into two groups, TSA or vehicle, based on their baseline intake in a counterbalanced design. Mice in the TSA group (n=9) were injected with TSA i.p. at 2 g/kg (dissolved in DMSO and diluted 1:5 in normal saline) for 5 consecutive days. Control mice (n=9) were injected with an equivalent volume of vehicle once daily (DMSO diluted 1:5 in saline). All mice had continuous access to ethanol during the five treatment days and up to 26 days after treatment.

Tissue Harvest: Brain tissue for microarray analysis was collected from individual mice 6 days after the last drinking session. Three brain regions were harvested for subsequent cRNA synthesis and microarray analysis: prefrontal cortex (PFC), nucleus accumbens (NAc) and ventral tegmental area (VTA). Microdissected brain regions were harvested as previously described (Kerns et al. 2005). Briefly, animals were sacrificed by rapid cervical dislocation. Mouse brains were extracted and chilled in ice-cold phosphate buffer for 1 min. Dissections were complete within 5 minutes from the time of death. Brain regions were placed into individual tubes, snap-frozen in liquid nitrogen and stored at -80^{o}C until total RNA isolation.

RNA isolations and cRNA synthesis: Total RNA was extracted from PFC, NAc and VTA from individual mice. Tissue was homogenized in STAT 60 reagent (Tel-Test, Friendswood, TX) using a glass dounce homogenizer on ice and isolated according to the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm and RNA quality was assessed by Experion automated electrophoresis (BioRad, Hercules, CA) and 28S:18S ratios. Total RNA (2 *u*g) was reversed transcribed into double-stranded cDNA using the One-cycle Targeting and Control Reagent kit from Affymetrix (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was synthesized from cDNA, purified and fragmented according to manufacturer's instructions.

Microarray hybridization and scanning: Labeled cRNA from individual animals (n=19 for experiment 1 and n=29 for experiment 2) was hybridized to a single microarray for each brain region studied. 57 total microarrays were used in experiment 1 for gene expression profiling of the PFC, NAc AND VTA. 29 total microarrays were used in

experiment 2 since only the NAc was profiled. Samples were analyzed on oligonucleotide arrays (Mouse Genome 430A 2.0 array) that contain >22,000 well-characterized genes and expressed sequence tags. Array hybridization and scanning were performed exactly according to manufacturer's protocols. Arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and scanned using the Gene Chip Scanner 3000 (Affymetrix).

Microarray data analysis: Microarray data were initially processed using the Gene Chip Operating Software v4.1 (GCOS, Affymetrix). Arrays were normalized to a mean total hybridization intensity (target average intensity) of 190. Array quality was assessed by accepting only arrays with a scaling factor of < 3 and a 3'-5'-actin ratio of <2 and by examining linearity and inter-chip correlations of intensity values. Arrays determined to be acceptable were further analyzed using the Robust Multichip Average (RMA) low level analysis algorithm to summarize probe set expression data (Irizarry et al. 2003). Probesets with RMA expression values < 4.5 consistently across all microarrays were filtered to reduce variance from low expressing genes.

In experiment 1, to identify genes with expression values correlated with ethanol drinking behavior across individual mice, RMA values for each brain region were separately template matched (Pavlidis et al. 2001) to a drinking scale using the template matching tool in T-Mev (TIGR Multiple expression viewer (Saeed AI 2003)). The drinking scale was calculated from the average ethanol intake (g/kg/18h) of each mouse over the last 8 days of ethanol access. The p values from the template matching analysis were then used in estimating the false discovery rate using the q value method (Storey et al. 2003) in

the R programming environment (Team 2009). Probe sets were considered significant using a false discovery rate of 1%. Significant probe sets were then used in subsequent bioinformatics analyses to identify genes with the most robust changes. All analyses were performed in each brain region (PFC, NAc and VTA) separately. Significantly correlated genes were further analyzed by hierarchical clustering within T-MeV program using average linkage.

In experiment 2, gene expression was profiled in four groups of mice: social defeat ethanol drinking (SDE, n=19), home cage ethanol drinking (HCE, n=5), social defeat water drinking (SDW, n=5) and home cage water drinking (HCW, n=5). In order to determine genes differentially regulated by ethanol drinking and social defeat, a two-way analysis of variance (2 way ANOVA) was run using 1000 permutations at $p<0.01$. Two factor (ethanol x defeat) analysis will identify genes with expression profiles significantly altered by either ethanol drinking, social defeat, or the interaction between the two. In subsequent analyses, we focused on genes significantly altered by stress-influenced ethanol drinking, i.e. the interaction gene list in order to determine the potential neuroadaptations following stress and ethanol drinking. Significant probesets were further analyzed by hierarchical clustering within T-MeV program using average linkage and used in subsequent bioinformatics analyses to identify genes with the most robust changes.

In both experiments, the Expression Analysis Systematic Explorer (EASE version 1.21) (Hosack et al. 2003) nonbiased annotation analysis tool was used to identify biological themes among gene expression profiles and to group genes into functional classifications developed by several public databases. The following annotation groupings

were analyzed for over-representation in gene lists: chromosome, SwissProt key word, PIR (The Protein Information Resource) key word, GenMAPP (Gene Map Annotator and Pathway Profiler) pathway, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, Pfam (Protein families database of alignments and HMMs) domain, SMART (Simple Modular Architecture Research Tool) domain, Gene Ontology Consortium biological process, molecular function, and cellular component. EASE results were filtered to remove categories with more than 250 members and EASE scores of >0.05. Redundant categories with the same gene members were removed to yield a single representative category.

Additional bioinformatics analysis of gene lists were performed with Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) and Bibliosphere (http://www.genomatix.de). These tools utilize biomedical literature associations to annotate genes with biological functions and cellular components. Ingenuity Pathway Analysis also generates networks of interrelated genes based on their curated knowledge base.

Principle Component Analysis in Experiment 1: As an alternative to correlating gene expression to the average of the last 8 days of ethanol drinking data, we used a different method for reducing the dimension of the entire set of ethanol drinking data prior to correlating ethanol drinking with the gene expression profiles. That is, there were 16 ethanol drinking observations for each of the 20 mice in the study, raising concerns that the average of the last 8 days of ethanol drinking may not be a comprehensive summary of drinking behavior to relate to gene expression. To address this concern, we conducted a

principle components analysis to reduce the number of covariates. Specifically, the aim was to explain the variance-covariance structure of the 16 days of ethanol intake using only a few independent linear combinations (principle components). While representing the data by only a few linear combinations of the original 16 variables will not enable one to reproduce the total variability in the data, the goal was to find the few principle components that would account for a sufficient proportion of the variability and so would contain almost as much information as the original full data set. It was found that the first 2 principle components accounted for 0.77 of the total variance. For each brain region, probe set-specific linear models predicting expression as a function of the two independent principle components were fit. An overall F-test was used for calculating P-values for each probe set level linear model. Genes significant at $p < 0.05$ level were selected for further bioinformatics analysis and comparison with results from using average ethanol intake over the last eight days. These PC analyses were run by Dr. Kellie Archer and Maria Cappruccini through a collaboration with the Biostatistics Department at Virginia Commonwealth University.

Association with alcohol-preferring and non-preferring mouse models: Genes significantly correlated to ethanol drinking patterns in experiment 1, using a false discovery rate of 1%, were analyzed for overlap with previously published gene sets having expression significantly different between alcohol preferring or non-preferring mouse models based on the criteria $|d| \geq 0.5$ and $q < 0.05$ (Mulligan et al. 2006). Genes intersecting between these data sets and the studies performed here were further analyzed using bioinformatics tools as previously described.

Western blot analysis: A separate cohort of C57BL/6NCrl mice were allowed to voluntarily consume ethanol in a two-bottle choice paradigm exactly as previously described (n=21). Six days following the last ethanol drinking session, mice were harvested for brain tissue as described. Select brain regions were homogenized in NP40 buffer (150mM NaCl, 150mM Tri-HCl, pH 8.0, 1% Ipegal (Sigma, St. Louis, MO), and protease inhibitors (Roche, Indianapolis, IN) and protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). Western blotting was performed as described (Kerns et al. 2005). Blots for RAB3A were probed with rabbit anti-RAB3A (Millipore, Bedford, MA) diluted 1:250 and visualized with anti-rabbit HRP (GE Healthcare, Buckinghamshire, UK) and ECL reagent (Amhersham Biosciences, Piscataway, NJ) according to manufacturer's instructions. To ensure equal protein loading, Western blots were re-probed with mouse anti-beta-actin (AbCam, Cambridge, MA) and anti-rabbit HRP (Calbiochem, LA Jolla, CA). Images were digitized and protein expression was determined as area under the curve normalized to beta-actin using ImageJ (NIH, Bethesda, MD).

Results

Experiment 1: Differential gene expression in ethanol preferring and avoiding mice

We hypothesized that persistent individual variation in ethanol drinking behaviors within an inbred strain might be caused by differential basal gene expression patterns generated by unknown environmental influences. Further, such differential gene expression patterns could be used as a surrogate measure to identify molecular pathways contributing to individual variation in ethanol drinking. We profiled 3 brain regions in individual mice: nucleus accumbens (NAc), prefrontal cortex (PFC) and ventral tegmental

area (VTA). These brain regions were chosen because they are major components of the mesocorticolimbic dopamine reward pathway activated by ethanol and other drugs of abuse (Koob 1992). Pair wise comparisons of microarrays showed gene intensities of individual arrays were highly correlated with the lowest Pearson correlation value being 0.97. Each array passed a number of standard quality control checks in our laboratory showing that dissected brain regions from individual animals could be reliably analyzed by microarrays without requiring sequential rounds of probe amplification.

To identify molecular factors related to ethanol drinking behaviors, gene expression patterns were correlated to a drinking template created from the last 8 days of ethanol access following a third round of ethanol deprivation (see Methods). This design was chosen because the mice did not show an ethanol deprivation effect after this time point (Figure 20 and (Khisti et al. 2006)). As we have reported previously, mice showed a diminishing deprivation effect after the first and second abstinence periods that disappeared with the third abstinence. Utilizing multiple rounds of ethanol deprivation enabled assessment of the stable individual ethanol intake while providing a window where tissue could be harvested with animals off ethanol. Correlations of ethanol intake and gene expression were performed separately for each brain region using a false discovery rate 1%. The number of genes significantly correlated to ethanol drinking was similar in NAc and PFC with fewer transcripts regulated in the VTA (Figure 21). Not surprisingly, there was little overlap in the identity of significant genes across brain regions. Therefore, gene expression data from each brain region was further analyzed separately.

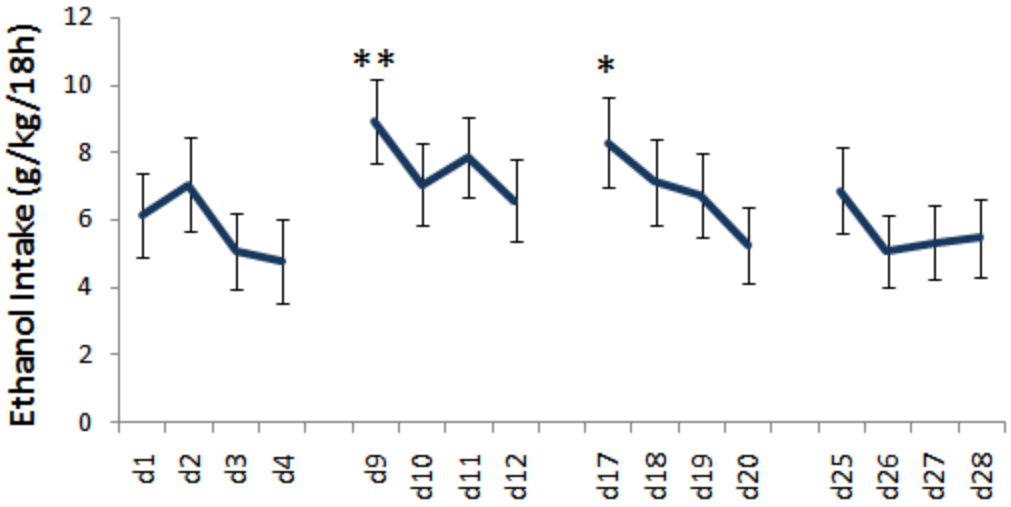

Figure 20: Average Ethanol Intake Over 16 Days of Access. Ethanol intake was significantly increased following repeated ethanol deprivations (**$p<0.001$ day 4 vs. day 9, *$p<0.01$ day 4 vs. day 17, Bonferroni Multiple Comparison test). Ethanol consumption did not differ from baseline after the third deprivation ($p>0.05$, day 4 vs. day 25).

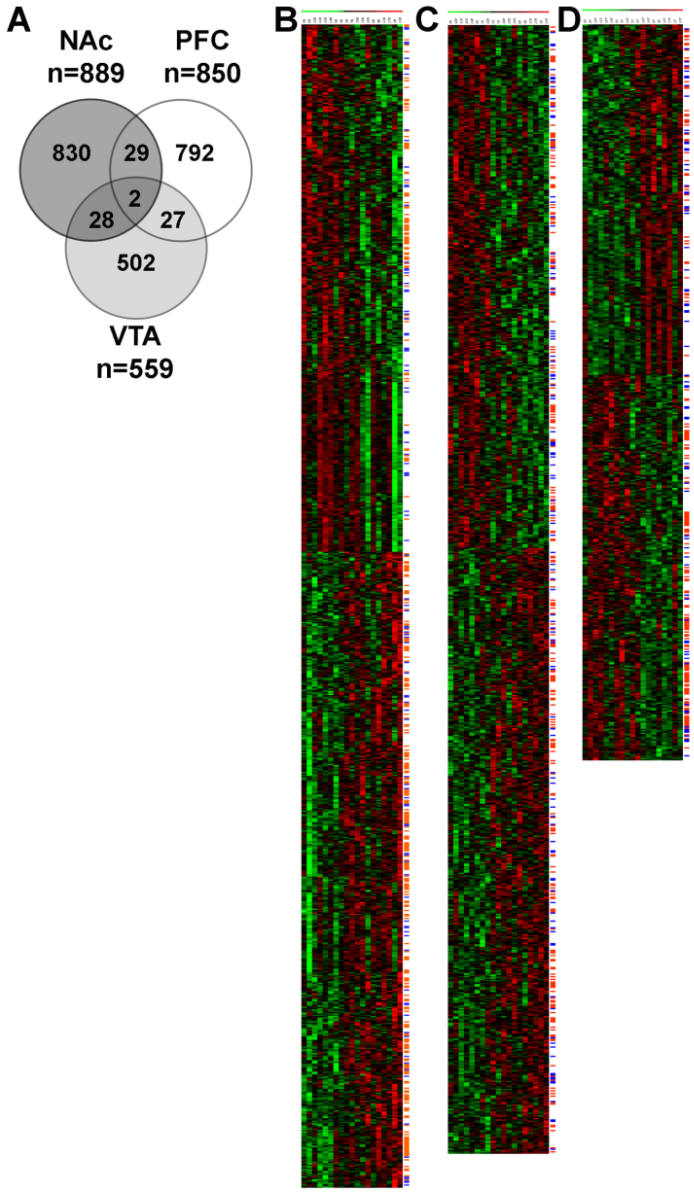

Figure 21: Genes Differentially Regulated in Ethanol Drinking Mice. A. Venn diagram overlapping and non-overlapping genes in each brain region significantly correlated to drinking at FDR<0.01. Region-specific expression patterns are represented as shaded circles (nucleus accumbens (NAc), dark; prefrontal cortex (PFC), open; ventral tegmental area (VTA), light). **B-D.** Hierarchical clustering of transcripts significantly correlated to ethanol drinking in the NAc (**B**), PFC (**C**) and VTA (**D**). Genes that overlap with the meta-analysis are labeled in blue. Genes that overlap with the principle component analysis are labeled in orange. Red color indicates higher relative expression and green indicates lower expression. Columns are arranged according to drinking behavior averaged over the last 8 days of intake, with low drinking mice on the left, progressing to higher drinking mice on the right.

To identify gene expression correlated with drinking behavior, we also performed a principle component analysis on the daily drinking activity data to reduce the number of covariates, rather than averaging the drinking data over an interval. The first two principle components (PC) accounted for 77% of the total variance. For each brain region, probe set-specific linear models predicting expression as a function of the two independent principle components were fit. The number of transcripts which fit the linear model at a level $p < 0.05$ was 547 in NAc, 670 in PFC and 725 in VTA. When these data were intersected with the results from analysis of averaged drinking intake, a highly significant degree of overlap was found between the two results. Of the number of transcript correlating with averaged drinking behavior, overlap with the PC analysis was found for 291 (33%, $p<1.29\ e^{-272}$) genes in NAc, 223 in PFC (26%, $p<6.23\ e^{-154}$) and 154 in VTA (27%, $p<2.54\ e^{-101}$).

Bioinformatics Analysis of Regional Microarray Data

Gene lists from microarray analyses were analyzed for over-representation of biological functions or gene network relationships using several different tools as described in Methods. As mentioned below, there was a striking similarity between gene lists resulting from analysis of either average drinking data or the PC data, from both PFC and NAc. Since the correlations to average drinking values generated larger gene lists, we focused our analysis on these data and the genes showing overlap with the PC analysis.

Nucleus Accumbens: The 889 transcripts from NAc correlating with average drinking values were analyzed by EASE (Hosack et al. 2003) for overrepresentation of functional categories compared with all genes on the Mouse 430Av2 chip (Table 6). Major significant groups include genes associated with synaptic vesicles, protein transport,

protein ubiquitination, chromatin modifications and histone deacetylase complex as well as categories related to small GTPase signal transduction, cytoskeletal organization and kinase activity. A majority of the categories were also identified by analysis with Bibliosphere and are bolded in Table 6. The top canonical pathways identified by Ingenuity Pathway Analysis also mirrored the Gene Ontology results. Phosphoinositol 3 kinase/Akt signaling, ephrin receptor signaling, PDGF signaling, protein ubiquitination, and inositol metabolism were among the significant canonical pathways.

Biological functions of our gene lists were further investigated using the curated knowledge base in Ingenuity Pathway Analysis. This tool generates networks of genes with known interactions or biological function. One of the top networks generated through this process is shown in Figure 22. This network included several genes related to chromatin modification and regulation of transcription through possible epigenetic mechanisms. Seven of the genes in this network were also identified in the Gene Ontology Biological Process category for establishment and/or maintenance of chromatin architecture and are identified with arrows. Additionally, 5 probesets were identified in the Gene Ontology Cellular Component for the histone deacetylase complex: *Hdac11, Rbbp4, Rbbp7, Sap18 and Suds3*. All genes in this network were significantly correlated to average ethanol drinking (Table 7).

The relative expression of select genes in this network in the top 25% of high ethanol drinkers and the bottom 25% of low ethanol drinkers are summarized in Figure 22. *Myst3, Hdac11* and *Ehmt2* were significantly different in high versus low drinkers by t-test at $p<0.05$. *Myst3*, Myst histone acetyl transferase 3, is a member of a mouse histone acetyl

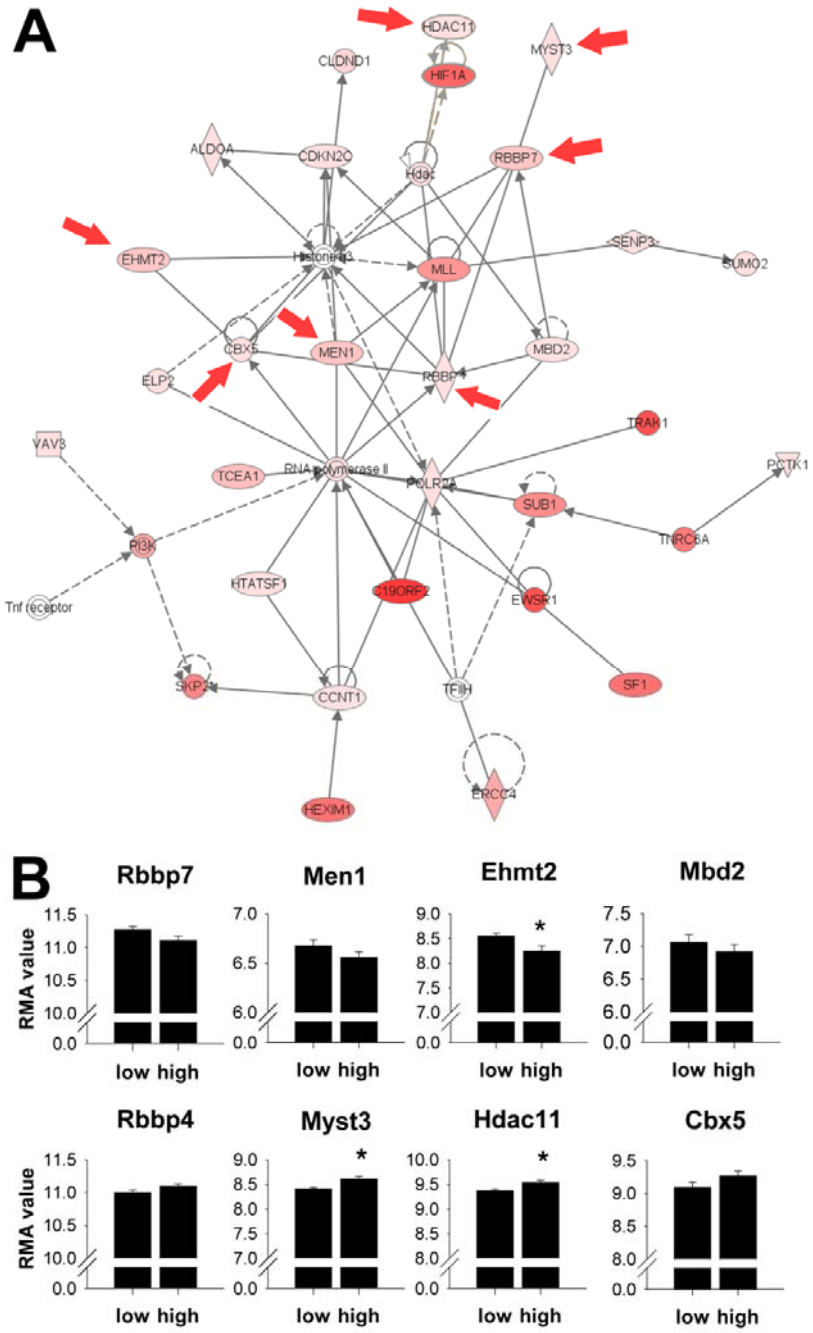

Figure 22: Chromatin Modification Genes Differentially Regulated in the Nucleus Accumbens of Ethanol Drinking Mice. A. Network of genes involved in chromatin modification generated by Ingenuity Pathways Analysis. Red arrows indicate genes identified in Gene Ontology Biological Process for maintenance of chromatin architecture. Genes significantly correlated to ethanol drinking are colored pink to red based on significance. **B**. RMA expression of transcripts in networks involved in chromatin architecture. * $p<0.05$ by t-test.

transferase (HAT) complex which increases DNA transcription (Kitabayashi et al. 2001) by acetylating histone tails. Acetylation of histone tails opens up the chromatin structure to allow transcription factors and associated proteins access to the DNA and increase gene transcription. Many genes were in HDAC complexes (*Rbbp4, Rbbp7*), had intrinsic HDAC activity (*Hdac11*) or were involved in methylating DNA (*Men1, Mbd2, Mll1, Ehmt2*). These genes are believed to be involved in transcriptional silencing by removing acetyl groups from chromatin or methylating DNA.

Further network analysis identified genes involved in synaptic vesicle formation and recycling (see Table 8). Genes involved in dynamin-dependent vesicle recycling (*Ap2a1, Ap2a2, Ap2m1, Dnm1, Dnm1l, Vamp3*, and *Vamp4*) and synaptic vesicle biogenesis (*Sh3gl2, Sh3glb1*) were generally positively correlated to ethanol intake. This suggests that synaptic vesicle recycling may be increased in mice prone to drinking greater amounts of ethanol. Conversely, low drinking mice had higher *Bdnf* expression. *Bdnf* may play a role to increase synaptogenesis in these studies as it has been implicated in plasticity from multiple drugs of abuse (Hyman et al. 1991; Poo 2001; Akbarian et al. 2002; Angelucci et al. 2007; Russo et al. 2008; Thomas et al. 2008). Moreover, BDNF has been demonstrated to increase expression of genes correlated with synaptic vesicle release.

The gene lists generated from PC analysis were similar in biological function. Overrepresented Gene Ontology categories included the synaptic vesicle, chromatin modification, histone methylation, Na^+K^+ ATPase activity and protein kinase activity (Table 9). Many of the genes highlighted in the chromatin modification and synaptic

vesicle formation and recycling networks described above were present in the principle component analysis (highlighted in bold Tables 7 and 8).

Prefrontal Cortex: Primary analyses of gene transcripts differentially regulated by ethanol drinking in the prefrontal cortex yielded 850 transcripts by RMA summarization at a false discovery rate of 1% (Figure 21). The gene list was entered into EASE and Bibliosphere analysis to identify over-represented functional categories as compared to all the transcripts on the Mouse430Av2 chips. The following categories were statistically over-represented at $p<0.05$ in both analyses (see Table 6): mitochondrial inner membrane, oxidoreductase activity, cell projection and regulation of cell shape. The top canonical pathways identified by Ingenuity Pathway Analysis mirrored some results from EASE and Bibliosphere (mitochondrial dysfunction and ubiquinone biosynthesis) as well as identifying involvement of other signaling pathways: IL2, PTEN, JAK/STAT and glucocorticoid receptor signaling.

Ingenuity network analysis identified potential involvement of glutamate receptor signaling (Figure 23) in the variation of ethanol drinking behaviors. This network contained several ionotropic glutamate receptor subunits, NMDA receptor subunits 2B and 3B (*Grin2b, Grin3b*) and the kainite receptor (*Grik1*), as well as genes that bind (*Htt*) or are regulated by glutamate receptors (*Dlg4* aka *Psd95*). The NR2b subunit of the NMDA receptor was positively correlated to ethanol drinking with the lowest drinking mice having lower expression, while the NR3b and Kainate receptor (*Grik1*) were correlated negatively to ethanol drinking. Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine

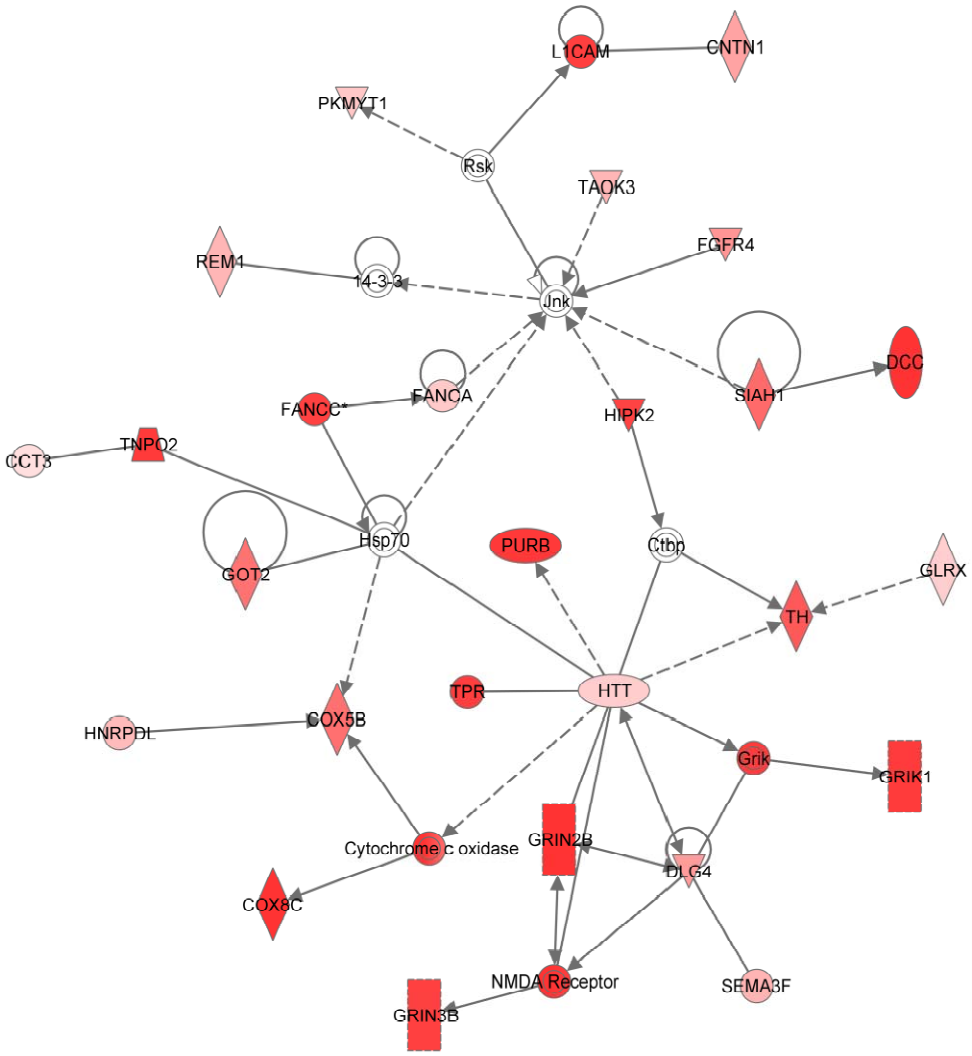

Figure 23: Network of Genes Involved in Glutamate Signaling in the Prefrontal Cortex of Ethanol Drinking Mice. Network of genes involved in glutamate signaling generated through the use of Ingenuity Pathways Analysis. Genes significantly correlated to ethanol drinking are colored pink to red based on significance.

synthesis involved in the conversion of tyrosine to dopamine, was also positively correlated to ethanol drinking.

Functional overrepresentation analysis of the PFC gene list derived from principal component derivation of the behavioral data revealed biological categories related to the mitochondria such as electron transport, respiratory chain and mitochondrial dysfunction (Table 9) that overlapped with the averaged drinking data analysis. Two glutamate receptor subunits, *Grin2b* and *Grik1*, were also present in the PC analysis. Stress activated protein kinase signaling and retinoic acid signaling were over-represented in the principle component analysis, but not in the average drinking correlation analysis.

Ventral Tegmental Area: In the VTA, 559 transcripts were significantly correlated to ethanol drinking intake at a false discovery rate of 1%. Gene Ontology analysis revealed only a few significant categories (Table 6) which were surprisingly cohesive (locomotory behavior, cell adhesion, cell projection and basolateral plasma membrane) suggesting cell migration and chemotaxis may be affected in the VTA. Corresponding analysis using Bibliosphere and the canonical pathways in Ingenuity identified many of the same categories (Table 6). Gene networks identified by Ingenuity Pathway Analysis did not reveal additional conserved biological functions for the VTA.

Despite having a low number of genes overlapping with data from the average drinking analysis, the VTA gene list correlating to principle component analysis of the behavioral data had similar functional categories were identified by Gene Ontology analysis (glucosaminoglycan degradation, locomotory behavior, and toll-like receptor signal, see Table 9).

Characterization of select genes

We used Western blot analysis to further confirm the microarray results of select genes. RAB3A was chosen for its role in synaptic vesicle trafficking. In a separate cohort of mice, RAB3A expression was determined in high (n=5) and low (n=5) drinking mice (Figure 24). RAB3A expression was significantly lower in mice consuming less than 2 g/kg ethanol than in mice consuming more than 7 g/kg ethanol ($p<0.05$, T-test). Western blot analysis showed a 1.7 fold increase of RAB3A expression in high drinking mice and was similar to mRNA expression from the microarray results.

Associations with genetic ethanol drinking phenotype

Extensive prior microarray studies have been done comparing basal brain gene expression across mouse strains with differing ethanol drinking phenotypes. A large meta-analysis of this data identified over 3000 genes correlated with ethanol drinking behavior across genetic models (Mulligan et al. 2006). We predicted that a subset of genes having correlation with individual drinking behavior within a single inbred strain would overlap with the genetically derived gene sets associated with drinking behavior. Out of 889 significantly regulated transcripts in the NAc, 202 transcripts ($p<10^{-34}$, Chi-square analysis) were also identified in the meta-analysis (see Figure 21B). Functional categories of these genes remained similar to our original analysis. PI3K/Akt signaling, protein ubiquitination and genes involved in synaptic vesicles were still highly represented. One of these genes, syntaxin binding protein 1 (*Stxbp1*) was identified as a putative candidate for an ethanol drinking locus on Chromosome 2 (Fehr et al. 2005). In the PFC, 168 genes out of 850 ($p<10^{-18}$, Chi-square) were also identified by the meta-analysis (see Figure 21C).

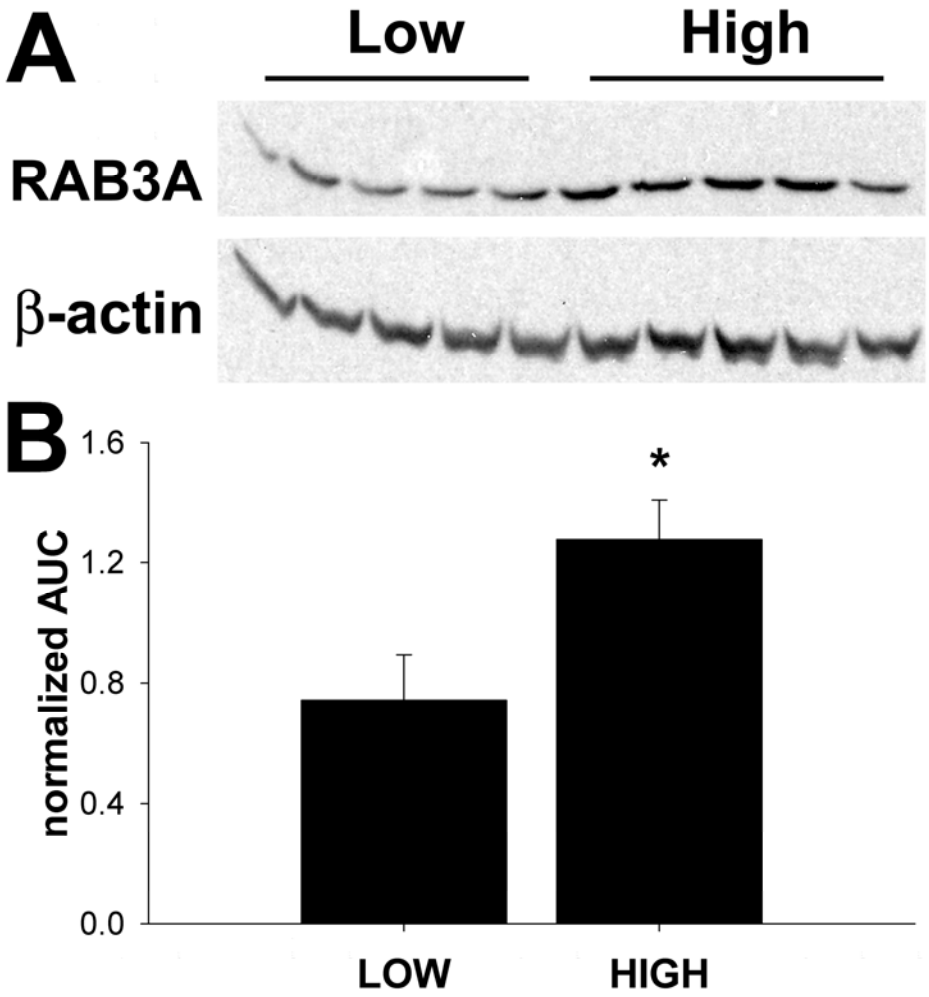

Figure 24: RAB3A Expression in High and Low Drinking Mice. **A**. Western blot of nucleus accumbens total protein probed with RAB3A and beta-actin. **B**. Quantitation of western blot analysis, area under the curve (AUC) RAB3A expression normalized to total beta-actin. *p<0.05, by t-test.

Mitochondrial dysfunction and PTEN signaling remained top biological functions. However, genes involved in glutamate receptor signaling were not represented on this list since this category was not enriched in the meta-analysis. Genes involved in retinoic acid signaling were over-represented in our principle component analysis and were also in the meta-analysis dataset. Retinoic acid signaling plays a role in the differentiation and function of dopaminergic pathways (Samad et al. 1997). In the VTA, 108 genes out of 435 ($p<10^{-4}$, Chi-square) were in common with the meta-analysis results (Figure 21D). Glucosaminoglycan degradation and cell movement were again identified as top biological functions. The highly significant overlap between our gene list and those of the meta-analysis across mouse lines genetically selected for differences in ethanol intake as well as the degree of overlap between functional gene categories in these two disparate studies both serve to further validate our findings.

Histone Deacetylase Inhibition and Ethanol Drinking

The bioinformatics analysis indicated above of genes in NAc correlating with ethanol intake showed an over-representation for genes involved in chromatin remodeling, particularly histone acetylation. Such epigenetic modifications have been shown to play a role in other drugs of abuse (Li et al. 2004; Brami-Cherrier et al. 2005). To further investigate the role of chromatin modifications in ethanol drinking Trichostatin A (TSA), a class I and II HDAC inhibitor, was examined to determine effects on ethanol drinking. We hypothesized that if chromatin acetylation events were indeed involved in the drinking phenotype, administration of an HDAC inhibitor would alter ethanol intake/preference and potentially reduce individual variation in drinking behavior. Following baseline ethanol

drinking for seven days, TSA (2mg/kg, i.p.) was administered for five consecutive days. Ethanol was freely available 24 hours/day during and for 4 weeks following TSA administration. HDAC inhibition significantly increased ethanol drinking over baseline intake by three weeks following administration. Drinking behavior remained elevated until the study was terminated (Figure 25). Repeated Measures ANOVA on the TSA treated mice over time showed a significant effect of ethanol intake over the course of the study ($F(5, 40) = 7.345$, $p<0.0001$). Tukey-Kramer post-hoc tests revealed that ethanol intake was significantly increased at week 3 and week 4 versus baseline ethanol intake and during TSA administration ($p<0.0001$) for the TSA treated mice. A separate repeated measures ANOVA on vehicle treated mice did not show a significant effect on ethanol intake ($F(5, 40) = 1.728$, $p=0.1505$). There was no effect of TSA treatment on total fluid consumed over the course of the experiment. Western blot analysis for histone H3 and histone H4 hyperacetylation confirmed elevated H3 acetylation levels at 1 day and 38 days after completion of TSA treatment.

We hypothesized that Trichostatin A treatment may reduce the variability of ethanol intake in individual mice, bringing each mouse's intake to similar levels. Indeed, TSA significantly increased ethanol intake in 6 out of nine mice over their baseline consumption values (Figure 25C). However, the amount of variance did not appreciably change over the course of the study. The change from baseline intake at week 4 in individual animals was different between TSA and vehicle treated mice. Most of the TSA animals increased their ethanol intake, consequently the correlation between baseline intake and change from baseline at week 4 was essentially flat ($R=0.027$, $p=0.6749$).

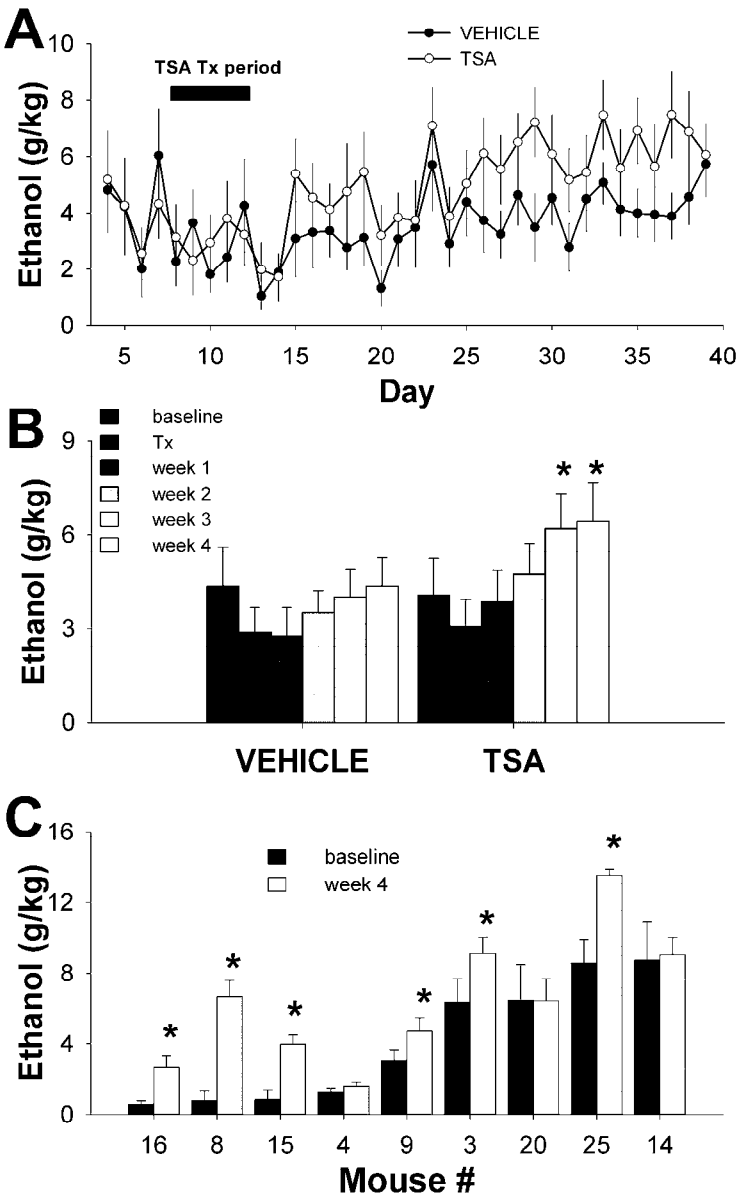

Figure 25: Histone Deacetylase Activity Inhibition Decreases Ethanol Intake. A. Daily ethanol intake of C57BL/6NCrl mice treated with 2mg/kg Trichostatin A (TSA) for 5 days (n=9) and control mice treated with vehicle (n=9). **B**. Average ethanol intake of the same mice. TSA mice significantly increased their ethanol intake in week 3 and week 4 as compared to baseline, TSA treatment week and week 1 by RM ANOVA and Tukey Kramer posthoc test * $p<0.05$. **C**. Ethanol intake of individual mice at baseline and four weeks after last TSA injection. Six mice increased ethanol intake following TSA treatment by RM ANOVA and Tukey Kramer posthoc test * $p<0.05$.

Meanwhile in the vehicle treated mice, the correlation between baseline intake and change from baseline remained significantly correlated ($R=0.738$, $p=0.0231$), showing that vehicle treatment did not significantly alter ethanol intake in individual mice.

Experiment 2: Differential gene expression following stress-influenced ethanol drinking

Despite a number of reports looking at the effects of social stress on ethanol drinking, direct study of the molecular networks involved in this behavior has not been investigated. We used genome-wide expression profiling to identify the molecular pathways activated by social stress which contribute to drinking behaviors. This initial profiling of C57 mice following social defeat and/or ethanol drinking has identified several gene networks in the NAc with differential expression patterns that may be relevant to their stress-influenced drinking. The NAc was chosen as a starting point because it is a major component of the mesocorticolimbic reward pathway activated by ethanol and receives dopaminergic input from the VTA. Episodic social stress also triggers intracellular signaling cascades in the VTA-NAc-PFC-amygdala circuit which modifies dopamine release into the NAc (Stevenson et al. 2003) Thus the NAc may be an important region for integrating salient information to influence decisions to consume ethanol following social stress. Additionally, our studies investigating the molecular factors involved in ethanol drinking preference have shown the NAc was particularly responsive and provides a good starting point for these studies.

In order to determine the potential molecular factors involved in the ethanol-response to repeated social defeat, we profiled gene expression from the NAc of ethanol drinking mice using tissue harvested six days after the last ethanol drinking session.

Accordingly, gene expression profiles are more likely indicative of neuroadaptations from chronic ethanol drinking and not merely due to having ethanol "on board" at the time of tissue collection. A two-way ANOVA (ethanol vs. social defeat, $p<0.01$) revealed 114 transcripts significantly altered by ethanol drinking, 157 transcripts altered by social defeat, and 392 transcripts significantly altered by the interaction between social defeat and ethanol drinking (Figure 26). Surprisingly, there was very little overlap between these gene lists. Since we were particularly interested the molecular neuroadaptations resulting from stress-influenced drinking behavior, our analysis focused on genes significant for the interaction of ethanol and social defeat.

Bioinformatics analysis of gene profiles responding to stress-influenced ethanol drinking

Gene lists from microarray analyses were analyzed for over-representation of biological functions or gene network relationships using several different tools as described in Methods. Gene Ontology analysis of the 392 transcripts regulated by social stress and ethanol drinking (Hosack et al. 2003) revealed several interesting functions which were significantly over-represented in our list (Table 10). Without question, energy metabolism was the most highly represented function in this gene set. Biological functions such as oxidative phosphorylation, oxidative stress, energy metabolism and electron transport or NADH dehydrogenase activity were over-represented as well as genes located in several mitochondrial compartments. Oxidative phosphorylation and mitochondrial dysfunction were also the top two canonical pathways identified through Ingenuity Pathway Analysis (figure 27). Additionally ribosomal genes and RNA polymerase activity are over-represented suggesting altered protein translation may also be occurring.

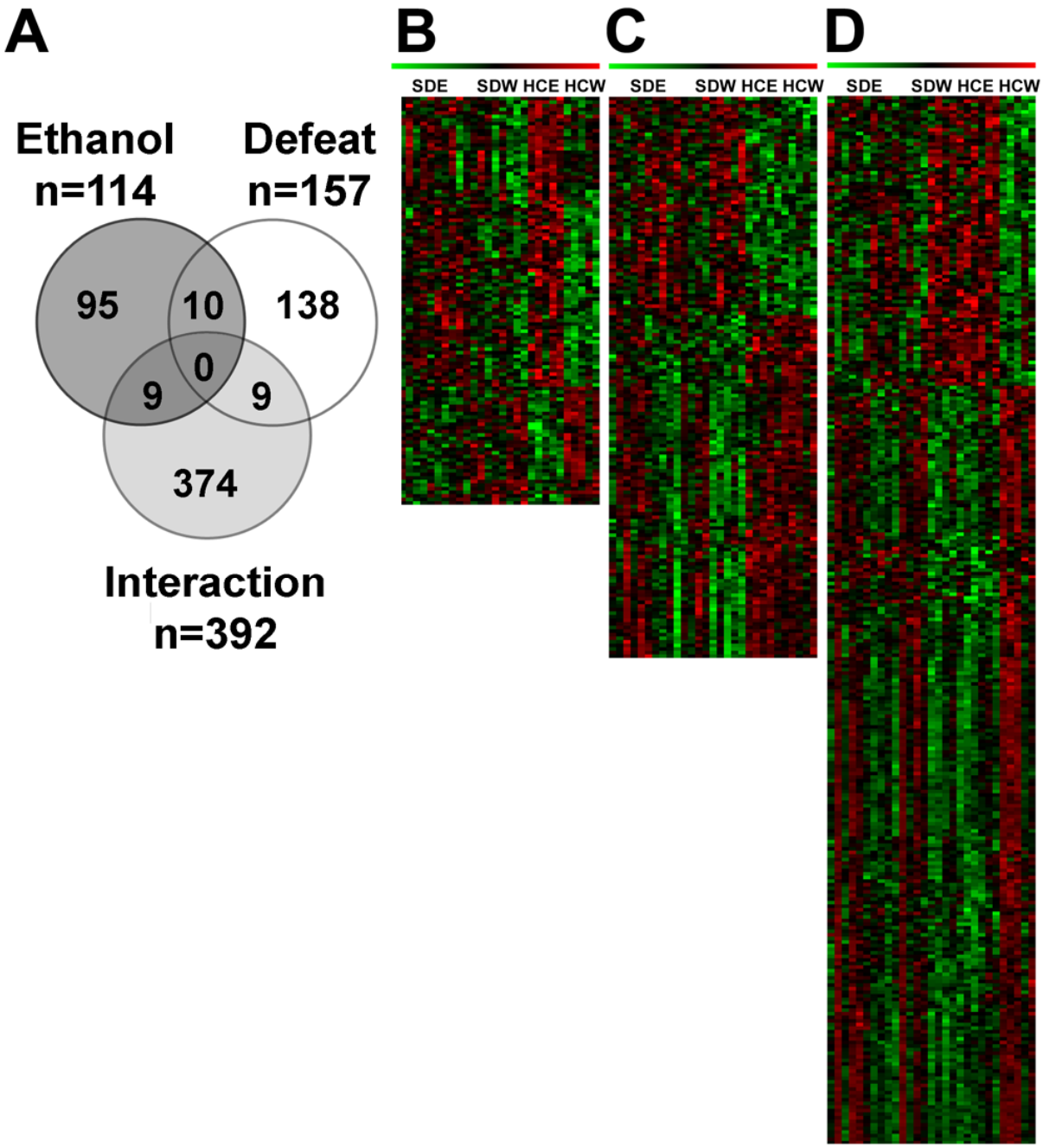

Figure 26: Genes Differentially Regulated by Ethanol Drinking and Social Defeat in the NAc. A. Venn diagram of genes from each treatment by two-way ANOVA at $p<0.01$.Treatments are represented as shaded circles (Ethanol drinking, dark; social defeat, open; interaction, light). **B-D.** Hierarchical clustering of transcripts significantly regulate by ethanol drinking (**B**),social defeat (**C**) and the interaction (**D**). Red = increase, green= decrease. SDE = social defeat + ethanol, SDW = social defeat + water, HCE = home cage + ethanol, HCW = home cage + water.

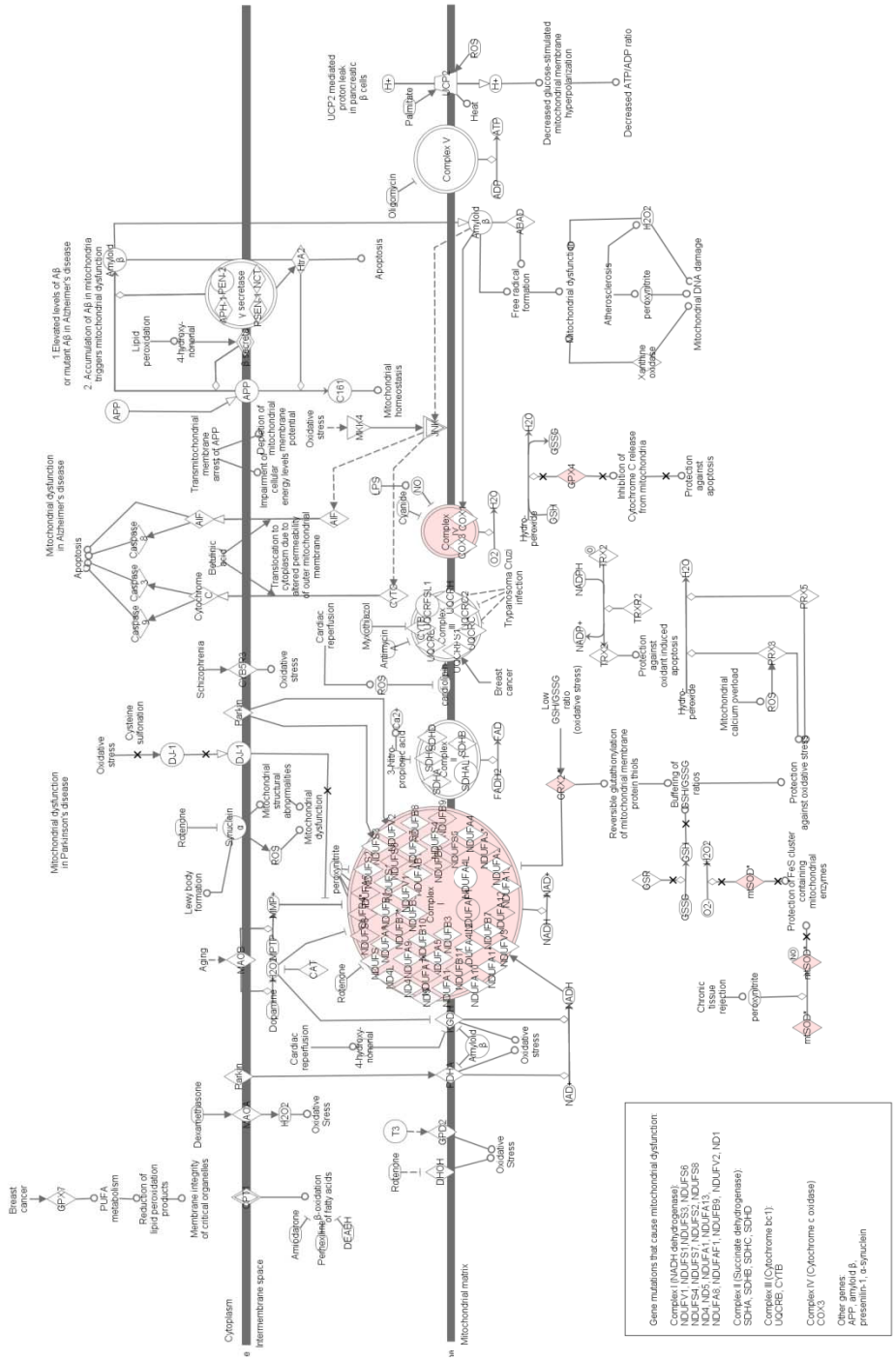

Figure 27: Canonical Pathway of Genes Involved in Mitochondrial Dysfunction. NADH dehydrogenase (Nduf) and Cytochrome c oxidase (Cox) in the electron transport chain are significantly altered by stress-influenced ethanol drinking. Genes entered into the analysis are colored pink to red based on their significance.

Interestingly, as with the our analysis of the molecular factors involved in individual variation of ethanol drinking in experiment 1, acetylation events were over-represented although the transcripts in this category were not involved in chromatin acetylation, nor did the genes in the two lists overlap.

Biological functions of our gene set was further investigated using the curated knowledge base in Ingenuity Pathway Analysis which generates gene networks based on known interactions or biological function. The top network identified 14 transcripts of the NDUF complex and 4 transcripts of the cytochrome c complex (Figure 28). These genes comprise complex I, NADH:ubiquinone oxidoreductase, and complex IV, cytochrome oxidase, of the 5 enzymes in the oxidative phosphorylation pathway in the mitochondrial electron transport chain responsible for ATP synthesis (see Figure 27). *Hsd11β1*, also present in this network, is responsible for the activation of glucocorticoids and not surprisingly has been implicated in social stress (Seckl et al. 2001; Yau et al. 2001).

Another top network also contained a few genes involved in the oxidative stress response (*DNAJc, DNAJb* and *Hspb8,* Figure 29). But more importantly, it showed genes involved in dopamine signaling including the dopamine 4 receptor (*Drd4*), growth factor receptor bound 2(*Grb2*), wingless-type MMTV integration site family, member 11 (*Wnt11*) and neuregulin 3(*Nrg3*). WNT is known to play a role in the differentiation and migration of dopaminergic cells during development (Andersson et al. 2008) and is currently being used to increase dopaminergic stem cell populations as a potential replacement therapy in Parkinsons disease (Castelo-Branco et al. 2006; Parish et al. 2008). The role of WNT on dopamine signaling pathways in adult brain tissue is less clear

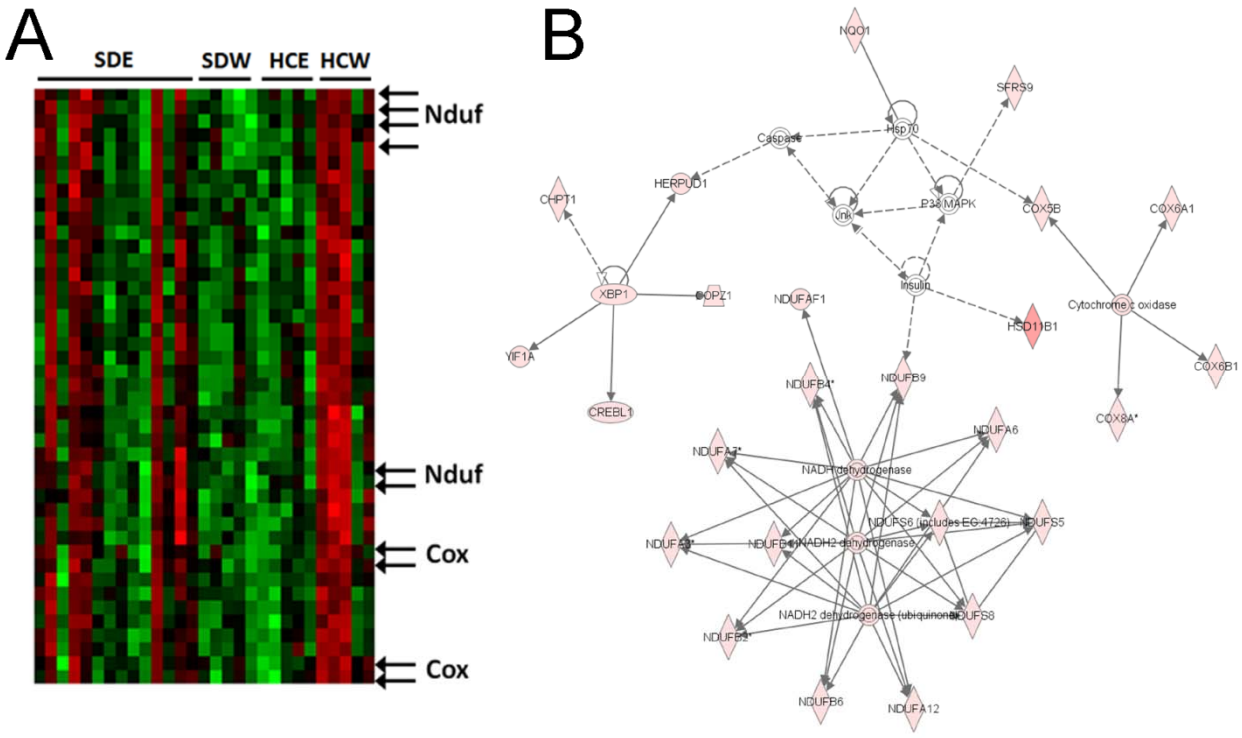

Figure 28: Clustergram and Network of Genes Involved in Mitochondrial Oxidative Phosphorylation. A. Clustergram of select genes involved in energy metabolism are down regulated in socially defeated (SDW) and home cage ethanol drinking mice (HCE), but are back to control levels (HCW) in socially defeated ethanol drinking mice(SDE). **B.** Network describing mitochondrial dysfunction generated by Ingenuity Pathways Analysis. Genes entered into the analysis are colored pink to red based on their significance. SDE = Social Defeat + Ethanol, SDW = Social Defeat + Water, HCE = Home Cage Control + Ethanol, HCW = Home Cage Control + Water. Nduf = NADH dehydrogenase and Cox = Cytochrome c oxidase. Red = increased expression, Green = decreased expression, Black = no change.

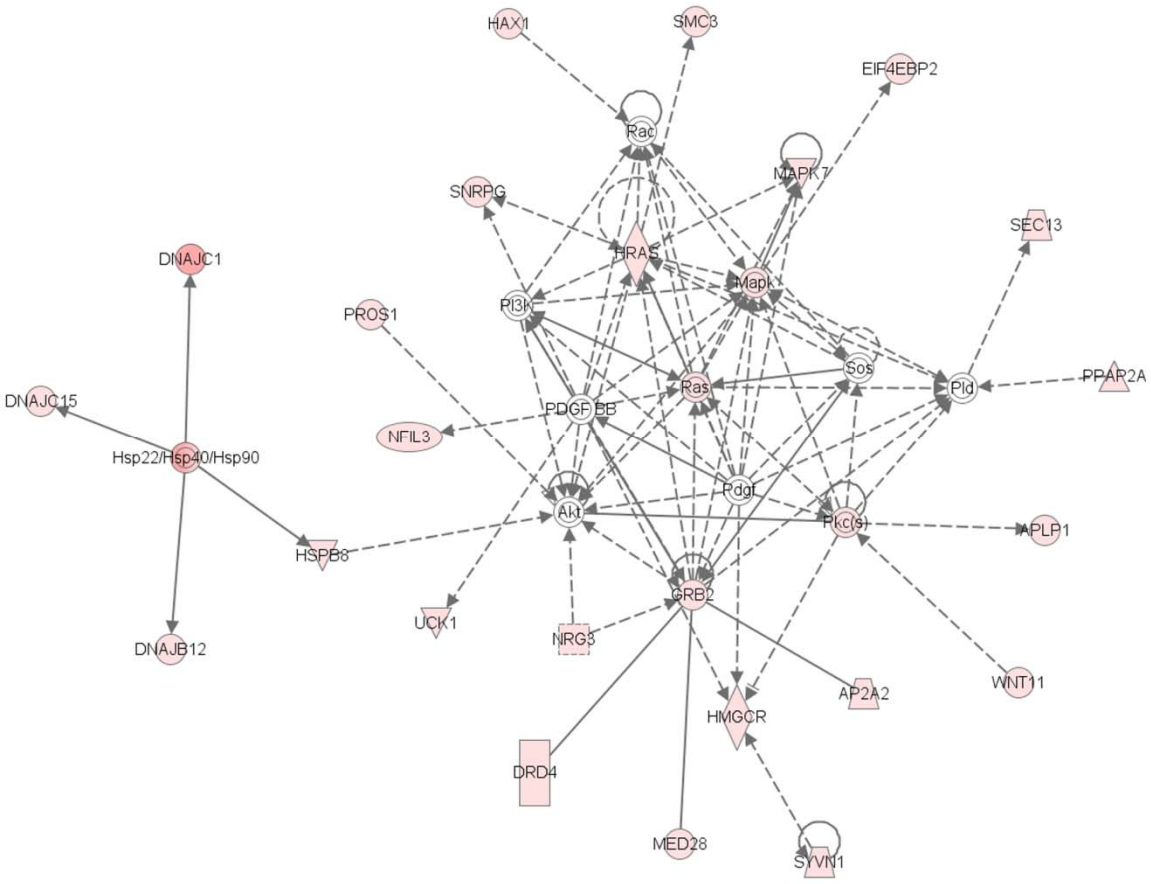

Figure 29: Dopamine and Wnt Signaling Network. This network has identified relationships between several genes involved in dopamine signaling that were altered by social stress and ethanol drinking in the NAc. This network was generated through the use of Ingenuity Pathways Analysis. Genes which were entered into the analysis are colored pink to red based on their significance.

although WNT signaling appears to play a role in the treatment of mood disorders (Gould et al. 2007). GRB2 is an SH2-SH3 adapter protein that has been shown to interact with the DRD4 receptor (Oldenhof et al. 1998). HMG CoA reductase, (3-hydroxy-3-methylglutaryl-Coenzyme A reductase, *Hmgcr*) also present in this network, has increased expression in socially defeated mice as compared to controls. This gene codes for the rate limiting enzyme in cholesterol synthesis and thus may play an important role in the steps in steroid and glucocorticoid synthesis.

Interestingly, the top biological function identified by Ingenuity was psychological disorder. Within this function, bipolar affective disorder showed 7 molecules previously identified to be involved in the disorder including (*Tp5h, Coq7, Drd4, Gpx4, Ndufs8, Pcmt1* and *Xbp* at p=0.00126). Psychological disorder of mice was also significantly over-represented (p=0.0258) where 4 molecules (*Drd4, Hsd11β1, Crhr2* and *Upc3*) were present in the list. Expression profiles of *Drd4, Hsd11β1* and *Crhr2* can be seen in figure 4. Separate two-way ANOVAs on these genes revealed significance at the level of the interactions. For *Drd4*, there was a main effect of ethanol drinking ($F(1,25)=5.53$, $p=0.027$), but no main effect of social defeat (p=0.167). There was a significant interaction between the two (p=0.022) where SDW and HCE mice had increased *Drd4* expression as compared to HCW mice. Ethanol drinking significantly decreased *Hsd11β1* expression as compared to home cage mice. There was a main effect of ethanol drinking by two-way ANOVA ($F(1,25)=4.375$, $p=0.047$), but no main effect of social defeat (p=0.315). Ethanol drinking and social defeat revealed significant interactions ($p<0.001$) where SDW and HCE mice had decreased *Hsd11β1* expression as compared to HCW mice. SDE mice

increased *Hsd11β1* expression back towards control levels and *Hsd11β1* was significantly increased over SDW mice. Finally, corticotrophin releasing hormone 2 receptor (*Crhr2*) only showed significance for the interaction between social defeat and ethanol drinking ($F(1,25)=12.744$, $p=0.001$). There was no main effect of ethanol drinking ($p=0.697$) or social defeat ($p=0.985$) alone (Figure 30).

Discussion

Experiment 1: Molecular factors involved in ethanol preference

Our studies here showed that C57BL/6NCrl mice express a striking degree of stable, inter-individual variation in ethanol drinking behavior with greater than 10-fold difference within a drinking session. We suspect these differences were generated by subtle environmental differences such as rearing behaviors (Meaney et al. 2002; Brake et al. 2004), intrauterine position, social interactions or stress (Lathe 2004; Holmes et al. 2005). As discussed in Chapter 3, individual variation has been reported for ethanol drinking behaviors (Dole et al. 1988; Little et al. 1999; Rhodes et al. 2005) as well as in stress responsivity (Krishnan et al. 2007) that may be a contributing factor to ethanol preference (Rockman et al. 1984; 1987; Volpicelli et al. 1990). Little et al. previously reported that within-strain preference variation was not correlated with gender or ethanol metabolism, and could not be altered by simple environmental disturbances (O'Callaghan et al. 2002). Regardless of which environmental conditions may have contributed to variation in ethanol drinking behaviors, we hypothesized that the differences could be mediated by individual variation in basal gene expression. The studies here employed a unique experimental design that allowed long-term measures of ethanol drinking behavior, ensured that such

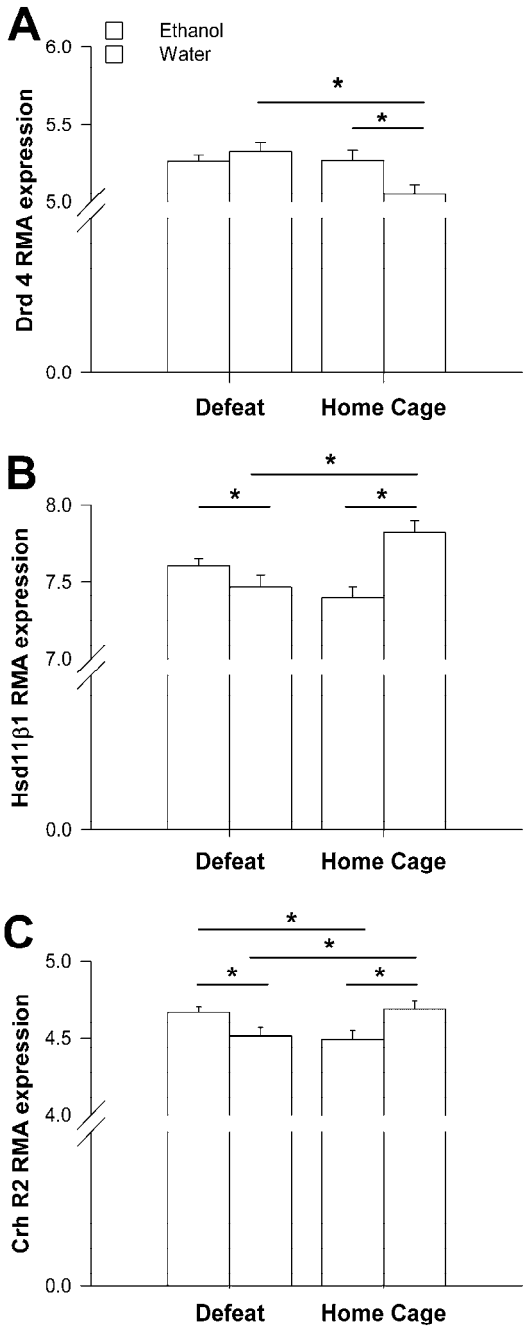

Figure 30: Gene Expression Profiles from the NAc Selected for the Interaction Between Ethanol Drinking and Social Defeat. A. Dopamine receptor 4 (Drd4), **B**. Hsd11 β1, the enzyme that activates corticosterone and **C**. corticotrophin releasing hormone receptor 2(Crhr2) are significantly regulated between treatment groups by Two-way ANOVA. * $p < 0.05$ by SNK post hoc test

behavior was stable upon reinstatement, and permitted assaying gene expression differences in individual animals off ethanol. This allowed identification of expression patterns presumably "predictive" of drinking behavior rather than simply resulting from such. However, even with the current design, we cannot totally eliminate the possibility that some of our gene expression results reflect, rather than cause, individual variation in drinking behavior.

Epigenetic mechanisms in ethanol drinking

The current studies have shown a potential role for epigenetic regulation of ethanol preference in C57 mice. Several genes with chromatin remodeling Gene Ontology function or classified in the HDAC complex had differential expression between high and low ethanol drinking animals. A majority of these transcripts were involved in histone deacetylase activity. *Rbbp4* and *Rbbp7*, retinoblastoma binding proteins, together with HDAC 1 and HDAC2, form the HDAC core which is part of both the NuRD and Sin3a complexes involved in transcriptional repression (Zoltewicz et al. 2004). We previously showed that acute ethanol treatment increases mRNA levels of *Rbbp4* (Kerns et al. 2005). *Hdac11*, histone deacetylase complex 11, functions to repress RNA expression by removing acetyl groups from the core histones allowing DNA packaging into dense chromatin structures (Gregoretti et al. 2004). Other genes in the network (*Mbd2, Mll1, Men1, Ehmt2,* and *Dnmt1*) are involved in DNA methylation events and work concurrently to repress transcription (Milne et al. 2005; Smallwood et al. 2007). *Mbd2* binds methylated DNA and may have additional de-methylase activity (Bhattacharya et al. 1999). It has been shown to direct the NuRD complex to methylated DNA. *Mll1* and *Men1* are components of

a histone methyl-transferase complex which methylates Lys4 on histone H3. MLL1 recruitment is regulated by MEN1 (Milne et al. 2005) and may additionally acetylate histone H4 and H2a allowing for transcriptional activation (Slany 2005). EHMT2 (euchromatic histone-lysine N-methyltransferase 2, the human homolog G9a) is considered to be the major euchromatic histone methyltransferase responsible for the dimethylation of lysine 9 on Histone H3 (H3K9) at transcriptionally silent regions (Tachibana et al. 2002). Dimethylation of H3K9 by EHMT2 creates a binding platform for CBX5 (HP1, hetereochromatin protein 1) (Lachner et al. 2001). HP1 associates with a variety of other chromatin remodeling enzymes including Suv39H1, HDACs and transcriptional repressors and stimulates the activity of DNMT1 which subsequently increases the levels of DNA methylation in the surrounding area. DNMT1 can also act to stabilize the binding of HP1 to chromatin. Furthermore, DNMT1 can increase the activity of EHMT2 and EHMT2 can increase DNMT1 recruitment forming a potential positive feedback loop between DNMT1, HP1 and EHMT2 to coordinate gene silencing (Smallwood et al. 2007). Thus, our genomic findings suggest an extensive and complex modulation of gene networks involved in chromatin modification.

Inhibiting HDAC activity with Trichostatin A injections increased ethanol intake above baseline levels, supporting a role for chromatin modifications in the modulation of ethanol preference. This data is the first to show modulation of drinking behavior by altering chromatin acetylation. However, evidence of ethanol-induced chromatin remodeling has been reported in rodent models and humans. For example, DNA methylation was increased by 10% in peripheral blood of alcoholic males (Bonsch et al.

2004). Epigenetic ethanol regulation of genes in hepatocytes has been well demonstrated (Shukla et al. 2008), while ethanol-mediated epigenetic regulation in brain tissue has received less attention. In mouse brain, ethanol downregulates *Smarca2*, a member of the ATP dependent Swi/Snf chromatin remodeling complex, acutely and for 2 hours following injection (Rulten et al. 2006). In cultured cortical neurons, ethanol increases NR2B transcription possibly through epigenetic modifications such as the methylation of CpG islands (Marutha Ravindran et al. 2004). Finally, Pandy and co-workers have recently shown that acute ethanol increases histone H3 and H4 acetylation and decreases HDAC activity in amygdala, while ethanol withdrawal produces the opposite response with decreased histone acetylation (Pandey et al. 2008).

Drugs of abuse, neuronal activity and even social stress have recently been shown to regulate genes by epigenetic mechanisms. Acute cocaine and antipsychotic drugs transiently increase H4 acetylation and H3 phosphosacetylation at cFos promoters (Li et al. 2004; Brami-Cherrier et al. 2005). Chronic cocaine, however, decreases H4 acetylation and H3 phosphoacetylation in favor of H3 acetylation. Chronic electroconvulsive stimulation increases *Bdnf* expression in the hippocampus presumably by H3 acetylation at its promoters (Tsankova et al. 2004). Pilocarpine-induced seizures also induce chromatin remodeling events such as acetylation of histone H4 at *Bdnf* promoter 2 which corresponded to increased *Bdnf* transcription (Huang et al. 2002). Models of depression such as social defeat induce histone H3K27 demethylation at certain *Bdnf* promoters, leading to decreased *Bdnf* transcription (Tsankova et al. 2006). Together, these studies demonstrate how environmental factors such as social stress can modify chromatin and

further supports a role for chromatin remodeling in the formation of stable neuronal adaptations that may underlie individual differences in drinking behavior.

Synaptic vesicle formation and BDNF Signaling

Our bioinformatics analysis of gene networks correlating with ethanol drinking also identified networks of genes involved in synaptic vesicle biogenesis and recycling. For example, synaptotagmin, functions as the calcium sensor for vesicular trafficking and exocytosis (Stevens et al. 2003), is involved in Ca^{2+} dependent neurotransmitter release and binds alpha and mu subunits of the AP-2 complex involved in clathrin-mediated endocytosis (Jarousse et al. 2001). Additionally, STXBP1 anchors synaptic vesicles to the plasma membrane was positively correlated to ethanol drinking in our studies. *Stxbp1* was previously identified as a candidate gene for a mouse Chr2 ethanol preference locus (Fehr et al. 2005). This is strong supportive evidence for our findings showing an important link between synaptic vesicle release gene networks and individual variation in ethanol intake.

RAB3A is a small GTPase associated with trafficking of synaptic vesicles and neurotransmitter release (Geppert et al. 1994). Our array studies showed a positive correlation between ethanol intake and *Rab3A* mRNA expression. We have confirmed this finding to show that RAB3A protein is 1.7 fold higher in heavy drinking mice as compared to low drinkers. RAB3A was recently shown to play a role in the sensitivity to the acute ataxic and sedative effects of ethanol in *C. elegans* and mice (Kapfhamer et al. 2008). Kapfhamer et al. also showed increased ethanol consumption in heterozygous *Rab3A* +/- mice, but not in *Rab3A* -/- mice. These investigators suggested that compensatory mechanisms in other RAB3 proteins (Schluter 2004) may have been triggered in *Rab3A*-/-

mice to obviate the effects on ethanol consumption in the heterozygote. The inverse relationship between *Rab3A* expression and drinking behavior in *Rab3A*+/- mice conflicts with our current findings. However, the potential compensatory mechanisms seen in Kapfhamer's studies may complicate the interpretation of the relationship between RAB3A expression and ethanol consumatory behavior. Regardless, that work and our current data clearly suggest an important relationship between RAB3A and ethanol drinking behavior.

We also identified an inverse correlation between *Bdnf* mRNA levels and individual ethanol consumption. Intriguingly, BDNF regulates multiple synaptic vesicle-related proteins, including several mentioned above or listed in Table 8, such as synaptotagmin, synaptophysin (Yamada et al. 2002), AP2 complexes (Beattie et al. 2000), STXBP1 and RAB3A (Thakker-Varia et al. 2001). BDNF may increase synaptogenesis and has been implicated in neuroplasticity from multiple drugs of abuse (Thomas et al. 2008; Russo et al. 2009). In clinical studies, peripheral BDNF is lower in dependent alcoholics and patients with a positive family history of dependence as compared to normal controls and dependent patients with a negative family history (Joe et al. 2007). McGough et al. (2004) also showed that *Bdnf* under-expression in *Bdnf*+/- mice caused increased ethanol consumption. These results are consistent with *Bdnf* mRNA expression observed in the current study, where *Bdnf* is lowest in mice with the highest ethanol intake. We do not believe, therefore, that *Bdnf* expression levels seen in our studies were secondary to ethanol exposure itself. In support of this, we and other investigators have shown that acute ethanol injection (2g/kg i.p.) in C57 or D2 mice increases *Bdnf* expression and that after 4

weeks of 2-bottle choice ethanol drinking, *Bdnf* is increased in the dorsal striatum versus non-ethanol controls (McGough et al. 2004; Kerns et al. 2005). Thus, we suggest that lower *Bdnf* expression in the low drinking mice was possibly a causal factor in individual drinking behavior variance, rather than secondary to drinking behavior itself.

Glutamate Signaling

Expression profiling in the prefrontal cortex has identified alterations in glutamatergic signaling between high and low drinking mice. The kainate receptor (*Grik1*) and NR2B and NR3B subunits of the NMDA receptor were correlated to ethanol drinking in the present study. In dependent alcoholics, consumption leads to enhanced glutamatergic activity and animals with an enhanced glutamate response to an injection of ethanol show a greater tendency to voluntarily consume ethanol (Selim et al. 1996; Szumlinski et al. 2003). Correspondingly, the NR2B subunit of the NMDA receptor was positively correlated to ethanol drinking in the present studies. Antagonists to ionotropic glutamate receptors decrease drinking, cue-elicited responding and the ethanol deprivation effect, a model of craving (Holter et al. 1996; Spanagel et al. 1996; Gabriel et al. 2005; Nguyen et al. 2007). Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis involved in the conversion of tyrosine to dopamine, was also positively correlated to ethanol drinking. This could reflect a "state" of decreased demand for dopamine synthesis in the low drinking mice, or an underlying causal "trait" leading to decreased reward from ethanol.

Intriguingly, we found significant overlap between our gene lists and previously published meta-analysis of basal brain gene expression across mouse strains with differing

ethanol preference. Not only was there a higher than expected overlap in the number of genes, several functional categories potentially involved in drinking phenotypes were also over-represented in both studies including PI3K/Akt and PTEN signaling, protein ubiquitination and mitochondrial dysfunction. These functional categories together suggest a role for cell survival pathways, altered energy metabolism or potential neuronal toxicity due to ethanol consumption. However, animals from the meta-analysis never consumed ethanol. Therefore it is possible that animals with a proclivity to drink ethanol may have altered signaling in these pathways prior to drinking.

Experiment 2: Gene expression in socially stressed ethanol drinking mice

The tension-reduction hypothesis for stress-increased ethanol drinking has not been uniformly supported, where increases and decreases in ethanol intake following social stress have been documented. Genetic predisposition and stress paradigm certainly are contributing factors for this disparity. Further complicating these studies are the striking amounts of individual variability in both ethanol drinking behavior and in social stress responsivity. Even though a tremendous amount of research has been done on stress-influenced drinking behavior, the molecular mechanisms underlying these responses remain unclear. In this final set of experiments, we have identified transcripts significantly altered by stress-influenced ethanol drinking. We have primarily focused our analysis on genes significant for the interaction of ethanol drinking and social defeat stress to identify the potential neuroadaptations which may have occurred. These studies were not ideally designed to specifically investigate the molecular pathways affected by social stress or ethanol drinking alone. The tissue collected for this experiment was temporally removed

from the social defeat episodes since mice were allowed to self-administer ethanol for 3 weeks following the last defeat episode. Future experiments will identify gene networks responsive to social defeat stress. Gene expression analysis in experiment 1 has thoroughly investigated profiles altered by ethanol drinking behaviors.

Involvement of genes in energy metabolism

One of the major biological functions from expression profiling in stress-influenced drinking behavior is mitochondrial dysfunction. The major players driving this category are 19 transcripts from NADH dehydrogenase and cytochrome c oxidase complexes. Most of these transcripts show a similar pattern of regulation where genes involved in energy metabolism are down-regulated (~20%) by ethanol drinking in the home cage or social defeat. When mice are allowed to drink ethanol following social defeat, gene expression returns to home cage control levels. This suggests that following social defeat and ethanol drinking, there may be decreased energy production in the NAc which can be "recovered" by being able to self medicate with ethanol following defeat. An alternative hypothesis is that ethanol drinking and social defeat are impinging on similar pathways and causing disinhibition of pathways regulating mitochondrial function.

Little research has linked mitochondrial dysfunction and ethanol consumption in the brain, let alone specific involvement of the accumbens. A few reports have documented reduced ATP production and mitochondrial dysfunction in the liver after chronic ethanol consumption (Young et al. 2006; Ivester et al. 2007). It has been hypothesized that oxidative stress in the liver plays an intimate role in the initiation and progression of alcoholic liver disease. (Bailey et al. 2002). Intriguingly, the amount of oxidative stress in

livers of chronically drinking monkeys was correlated to the amount of ethanol consumed over 18 months of drinking (Ivester et al. 2007). Mitochondrial oxidative phosphorylation has been proposed to be regulated by signal transduction mechanisms (Boneh 2006) mainly through cAMP, calcium and reactive oxygen species. Indeed, ethanol stimulated the production of reactive oxygen species at complex I and II (Bailey et al. 1999) causing oxidative damage to the mitochondria. Few studies have investigated the role of mitochondrial dysfunction in social stress, however it may be involved in several depression disorders such as bipolar disorder, major depression and schizophrenia (Rezin et al. 2009).

Secondarily, several genes involved in the production of steroids appear to be represented in this list. In fact, Ingenuity Pathway Analysis identified estrogen receptor signaling as a significant canonical pathway. Such genes include *HmgCoA*, *Hsd11β1* the rate limiting enzyme in cortisol synthesis, as well as other enzymes (*Hsd17β3* and *Hsd17β6)* involved in androgen and testosterone synthesis (Huang et al. 2000). *Hsd11β1* is widely expressed in the adult brain and through its reductase activity converts inactive 11 dehydroxycorticosterone into biologically active corticosterone (Jamieson et al. 1997). In the present studies, *Hsd11β1* was reduced in socially defeated animals as well as mice that consumed ethanol, congruent with studies in tree shrews showing that chronic psychosocial stress decreases *Hsd11β1* in the hippocampus (Yau et al. 2001). Additionally this enzyme had recently been suggested to play a role in local synthesis of bioactive glucocorticoids following withdrawal in dependent animals (Little et al. 2008). Mice that

were socially defeated and allowed to regulate their ethanol consumption showed a return towards control levels for this transcript suggesting that ethanol and social stress may interact and "normalize" gene expression and regulation of glucocorticoid activity.

Involvement of genes associated with psychological disorders

Additional genes shown to play a role in psychological disorders such as bipolar affective disorder were also significantly over-represented in this gene list (*Drd4, Crhr2* and *Hsd11βl*). Polymorphisms in the *Drd4* gene are correlated to higher novelty seeking behavior (Lahti et al. 2005; Laucht et al. 2007) and alcoholism (George et al. 1993), although these findings are not always replicated (Parsian et al. 1997; Sander et al. 1997). Possessing the DRD4 7R allele confers 1.5 fold increased risk for developing ADHD (Swanson et al. 1998), while the DRD4 2R allele has been associated with depression and bipolar disorder (Lopez Leon et al. 2005). Some of these association findings have been replicated in pre-clinical studies where *Drd4* gene deletion decreases activity in the open field and null mice were supersensitive to the effects of ethanol cocaine and methamphetamine (Rubinstein et al. 1997). Antagonists of DRD4 in the PFC decrease anxiety phenotypes in the elevated plus maze and defensive burying task (Shah et al. 2004). Together, this suggests that DRD4 modulates the normal coordinated and drug stimulated motor behaviors, mediation of fear-related behavior as well as the activity of nigrostriatal dopamine neurons. In the present studies, DRD4 expression was increased in ethanol drinking (HCE) and socially defeated (SDW) mice and showed a trend towards a reduction in ethanol drinking socially defeated (SDE) mice. It was surprising that DRD1 or DRD2 receptors were not significantly altered by stress-influenced ethanol consumption,

since these receptors are more highly expressed in the striatum and have been implicated in the rewarding properties of alcohol and other drugs of abuse. However, this involvement of DRD4 may be selectively regulated due to the interacting effects of social stress on the reward system pointing perhaps due to involvement of stress-influenced dopaminergic signaling through DRD4.

Crhr2, corticotrophin releasing hormone receptor 2, was also regulated by stress-influenced ethanol drinking in the current study. CRHR2 has been implicated in stress/anxiety phenotypes, but may play a modulatory role in ethanol self-administration. *Crhr2* knockout mice have more anxious behavior that is not due to changes in HPA axis activity, implying an extra-hypothalamic action of the CRHR2 system which may mediate a central anxiolytic response (Kishimoto et al. 2000). CRHR2 has been hypothesized to supply regulatory features to the HPA stress response, potentially maintaining the HPA axis drive since *Crhr2* -/- mice show early termination of the ACTH release (Coste et al. 2000). Involvement of CRHR2 in ethanol-related behaviors is less clear since mice deficient in *Crhr2* did not differ from wildtypes in several behaviors including loss-of-righting, conditioned taste aversion, ethanol-induced hypothermia, or ethanol metabolism (Funk et al. 2007). Ethanol intake and preference was also not significantly altered in a 24 h test and consumption was only modestly reduced in a limited access test (Funk et al. 2007). Urocortin 3, a *Crhr2* agonist, had bidirectional effects on ethanol consumption in dependent and non-dependent animals where agonism decreased intake in dependent rats but increased intake in non-dependent rats (Funk and Koob 2007). In the present studies *Crhr2* was decreased by chronic ethanol consumption and to a lesser extent by social

defeat. In socially defeated mice with a history of ethanol drinking, *Crhr2* expression returned to baseline levels. Thus, Crhr2 is responsive to both social stress and ethanol drinking, but further direct studies are needed to clarify its role in stress-influenced drinking behaviors.

In conclusion, the current experiments have described persistent inter-individual variation of ethanol drinking behaviors in C57 mice (see Chapter 3) and, more importantly, they describe changes in gene expression that may underlie these individual differences. This study utilizes variation within an inbred strain to minimize genetic influences, isolating changes in gene expression due specifically to environmental factors. These experiments have identified several gene networks previously implicated in responses to ethanol in the NAc and PFC: glutamate signaling, BDNF and genes involved in synaptic vesicle function. Perhaps most importantly, our expression studies and behavioral analysis following histone deacetylase inhibition implicate epigenetic factors involving chromatin acetylation and/or methylation as contributing to environmental modulation of ethanol intake. Defining specific gene networks targeted by these epigenetic modifications is an important goal of ongoing studies. These experiments have also pointed to a few potential mechanistic pathways involved in stress-influenced drinking such as altered regulation of energy metabolism through the mitochondrial electron transport chain. Although preliminary, we have strengthened earlier hypotheses that social stress and ethanol exposure converge on similar pathways and regulate dopamine and extra-hypothalamic CRH systems. Much work still needs to be done to tease out which alterations are occurring solely from social defeat or ethanol exposure. The novel findings presented here

could contribute to understanding mechanisms involved in individual risk for alcohol abuse and alcoholism in humans. Future work will focus on characterizing the genesis and implications of gene network alterations and epigenetic modifications associated with variation in ethanol drinking as well as the response to social defeat stress.

Table 6: Over-represented gene categories significantly correlated to ethanol drinking in C57BL/6NCrl mice. Categories in bold were also significant in the Bibliosphere analysis.

Annotation Groups	Gene Category	List % (n)	Chip % (n)	p value
	Nucleus Accumbens (n=965)			
SwissProt keyword	TPR Repeat	2.4 (8)	0.5 (22)	6.30E-04
GO Molecular Function	**Sulfurtransferase Activity**	0.6 (4)	0.04 (4)	1.30E-03
GO Cellular Component	**Clathrin-Coated Vesicle**	2.2 (15)	0.9 (84)	2.30E-03
SwissProt keyword	Inner Membrane	2.7 (9)	0.7 (34)	2.30E-03
GO Biological Process	**Protein Targeting**	3.4 (23)	1.7 (165)	2.80E-03
PFAM domain	FERM Domain	1.5 (6)	0.3 (17)	4.10E-03
GO Molecular Function	**Adenylate Kinase Activity**	0.6 (4)	0.1 (6)	5.90E-03
GO Cellular Component	Synaptic Vesicle	1.5 (10)	0.5 (48)	6.20E-03
GO Biological Process	**Small GTPase Mediated Signal Transduction**	3.9 (26)	2.2 (209)	6.30E-03
GO Cellular Component	Microtubule Cytoskeleton	3.6 (25)	2.1 (198)	7.30E-03
GO Cellular Component	Mitochondrial Envelope	4.2 (29)	2.5 (242)	7.50E-03
GenMAPP pathway	G13 Signaling Pathway	10.5 (8)	3.3 (33)	8.40E-03
KEGG_Pathway	Keratan Sulfate Biosynthesis	2.1 (5)	0.4 (13)	1.10E-02
GO Biological Process	**Ceramide Metabolism**	0.9 (6)	0.2 (20)	1.10E-02
SwissProt keyword	**RNA-Binding**	4.2 (14)	1.9 (90)	1.20E-02
PFAM domain	14-3-3 Protein	1 (4)	0.1 (8)	1.30E-02
SwissProt keyword	**Protein Transport**	4.5 (15)	2.2 (102)	1.40E-02
SMART domain	RNA Recognition Motif	6.9 (16)	3.6 (127)	1.70E-02
SwissProt keyword	Kinase	3.3 (11)	1.4 (65)	1.70E-02
PIR keyword	Extracellular Matrix	6 (7)	1.9 (30)	1.70E-02
GO Cellular Component	Basement Membrane	1.2 (8)	0.4 (39)	1.90E-02
PIR keyword	Nucleotide Binding	10.3 (12)	4.8 (78)	2.00E-02
GO Molecular Function	**Sodium:Potassium-Exchanging ATPase Activity**	0.6 (4)	0.1 (9)	2.10E-02
KEGG_Pathway	Galactose Metabolism	3 (7)	1 (32)	2.40E-02
GO Biological	**Rho Protein Signal Transduction**	0.9 (6)	0.3 (24)	2.40E-02

Process				
GO Biological Process	**Establishment and/or Maintenance of Chromatin Architecture**	2.8 (19)	1.6 (155)	2.40E-02
PFAM domain	Fringe-Like	0.7 (3)	0.1 (4)	2.50E-02
PFAM domain	Uncharacterized Protein Family UPF0005	0.7 (3)	0.1 (4)	2.50E-02
GO Molecular Function	**Protein Domain Specific Binding**	1.1 (8)	0.4 (42)	2.50E-02
PIR keyword	Collagen Binding	2.6 (3)	0.2 (4)	2.80E-02
GO Biological Process	**Axon Cargo Transport**	0.6 (4)	0.1 (10)	2.90E-02
GO Cellular Component	Histone Deacetylase Complex	0.7 (5)	0.2 (17)	2.90E-02
GO Biological Process	**Cell Division**	2.8 (19)	1.7 (158)	2.90E-02
SwissProt keyword	**Glycolysis**	1.8 (6)	0.5 (25)	3.00E-02
GO Cellular Component	Ribosome	2.8 (19)	1.6 (157)	3.10E-02
GO Molecular Function	Translation Factor Activity, Nucleic Acid Binding	1.8 (13)	0.9 (95)	3.30E-02
GO Biological Process	Dendrite Morphogenesis	0.7 (5)	0.2 (18)	3.40E-02
GO Molecular Function	Primary Active Transporter Activity	2.5 (18)	1.5 (151)	3.50E-02
GO Biological Process	Regulation of Kinase Activity	1.8 (12)	0.9 (85)	3.60E-02
GO Biological Process	Cellular Macromolecule Catabolism	3.6 (24)	2.3 (220)	3.70E-02
GO Cellular Component	**Microtubule**	2.3 (16)	1.3 (127)	3.70E-02
GO Biological Process	Nitric Oxide Metabolism	0.6 (4)	0.1 (11)	3.80E-02
GO Molecular Function	**Ubiquitin-Protein Ligase Activity**	2.6 (19)	1.6 (164)	3.90E-02
PIR keyword	**Ligase**	3.4 (4)	0.7 (11)	3.90E-02
GO Molecular Function	Phospholipid Binding	1.5 (11)	0.7 (76)	3.90E-02
PFAM domain	Phosphatidylinositol Transfer Protein	0.7 (3)	0.1 (5)	3.90E-02
KEGG pathway	Translation	16 (12)	8.5 (79)	4.00E-02
PIR keyword	Tandem Repeat	4.3 (5)	1.2 (19)	4.20E-02
GO Biological Process	Hydrogen Ion Homeostasis	0.4 (3)	0.1 (5)	4.30E-02
GO Cellular Component	Eukaryotic Translation Elongation Factor 1 Complex	0.4 (3)	0.1 (5)	4.40E-02
GO Molecular Function	**Unfolded Protein Binding**	1.9 (14)	1.1 (111)	4.70E-02
GO Molecular Function	**Tubulin Binding**	1.1 (8)	0.5 (48)	4.80E-02

Prefrontal Cortex (n=926)

GO Cellular Component	**Mitochondrial Inner Membrane**	4.5 (28)	2.2 (209)	4.40E-04
SMART domain	Thrombospondin Type 1 Repeats	2.7 (6)	0.5 (19)	5.10E-03
SwissProt keyword	Neurogenesis	2.7 (8)	0.8 (35)	6.10E-03
GO Biological Process	DNA Replication	2.4 (15)	1.1 (104)	7.30E-03
GO Biological Process	Glycerophospholipid Metabolism	1.1 (7)	0.3 (28)	8.20E-03
SMART domain	Src Homology 3 Domains	6.8 (15)	3.1 (111)	8.30E-03
GO Molecular Function	**Oxidoreductase Activity, Acting on NADH or NADPH**	1.5 (10)	0.5 (54)	8.70E-03
GO Molecular Function	Structural Constituent of Ribosome	2.9 (20)	1.6 (164)	1.30E-02
GO Molecular Function	Magnesium Ion Binding	3.6 (25)	2.2 (225)	1.50E-02
SwissProt keyword	Ligase	2.7 (8)	0.9 (42)	1.70E-02
GO Biological Process	**Cell Projection Organization and Biogenesis**	1.1 (7)	0.3 (33)	1.80E-02
PFAM domain	GDNF Receptor Family	0.8 (3)	0.1 (4)	2.20E-02
GO Biological Process	Antigen Presentation	1.3 (8)	0.5 (44)	2.20E-02
PFAM domain	Mitochondrial Carrier Protein	1.8 (7)	0.6 (36)	2.30E-02
GO Molecular Function	Ligase Activity, Forming Phosphoric Ester Bonds	0.4 (3)	0.04 (4)	2.40E-02
GO Molecular Function	**Metal Ion Transporter Activity**	1.5 (10)	0.6 (65)	2.80E-02
GO Molecular Function	Protein Transporter Activity	2 (14)	1.1 (109)	2.90E-02
SwissProt keyword	Serine/Threonine-Protein Kinase	5.4 (16)	3 (137)	3.00E-02
KEGG_Pathway	Axon Guidance	6.4 (14)	3.4 (113)	3.20E-02
SwissProt keyword	Zymogen	4.7 (14)	2.5 (115)	3.30E-02
GO Cellular Component	Mitochondrial Ribosome	1.1 (7)	0.4 (39)	3.80E-02
GO Biological Process	mRNA Metabolism	3.1 (19)	1.9 (177)	4.00E-02
GO Molecular Function	Phosphoinositide Binding	0.9 (6)	0.3 (29)	4.10E-02
GO Cellular Component	**Lysosome**	2.1 (13)	1.1 (106)	4.10E-02
GO Molecular Function	Primary Active Transporter Activity	2.5 (17)	1.5 (151)	4.40E-02
GO Biological Process	**Female Gamete Generation**	0.8 (5)	0.2 (21)	4.40E-02
GO Biological Process	**Regulation Of Cell Shape**	1.1 (7)	0.4 (41)	4.80E-02
SMART domain	Domain in RING Finger and WD Repeat	1.4 (3)	0.2 (6)	4.90E-02

PFAM domain	Endonuclease/Exonuclease/Phosphatase Family	0.8 (3)	0.1 (6)	5.00E-02
PFAM domain	ATP P2X Receptor	0.8 (3)	0.1 (6)	5.00E-02
PFAM domain	Nucleotide-Sugar Transporter	0.8 (3)	0.1 (6)	5.00E-02
	Ventral Tegmental Area (n=716)			
SMART domain	Band 4.1 Homologues	10.3 (3)	0.5 (3)	6.90E-03
GO Biological Process	**Locomotory Behavior**	3 (15)	1.4 (131)	9.30E-03
PIR keyword	**Cell Adhesion**	6.1 (5)	1.2 (20)	1.50E-02
KEGG pathway	Porphyrin And Chlorophyll Metabolism	9.3 (4)	1.4 (14)	1.70E-02
KEGG_Pathway	Pentose and Glucuronate Interconversions	2.3 (4)	0.4 (12)	2.10E-02
KEGG_Pathway	Toll-Like Receptor Signaling Pathway	5.8 (10)	2.5 (82)	2.40E-02
GO Cellular Component	**Cell Projection**	3.4 (17)	1.8 (178)	2.50E-02
KEGG pathway	Glycosaminoglycandegradation	7 (3)	0.7 (7)	3.00E-02
PIR keyword	Myristylation	6.1 (5)	1.5 (25)	3.30E-02
SwissProt keyword	**Lysosome**	2.9 (7)	1 (48)	3.60E-02
PIR superfamily	ADP-Ribosylation Factor	2.9 (3)	0.3 (7)	4.50E-02
GO Molecular Function	Lyase Activity	2.4 (13)	1.3 (133)	4.60E-02
GO Biological Process	Regulation of Anti-Apoptosis	0.6 (3)	0.1 (7)	4.90E-02
GO Cellular Component	**Basolateral Plasma Membrane**	1.4 (7)	0.5 (51)	5.00E-02

Table 7: Genes involved in chromatin remodeling identified in nucleus accumbens of ethanol drinking mice. Gene names in bold were also identified by the principle component analysis.

Gene Symbol	Gene Name	Affy ID	R value	p value	Function	Transcription
Mbd2	**methyl-CpG binding domain protein 2**	1425803_a_at	-0.515	1.00E-03	binds methylated DNA	silences
Men1	**multiple endocrine neoplasia 1**	1416348_at	-0.480	2.84E-03	methylated Lys4 Histone H3	silences
Ehmt2	**euchromatic histone lysine N-methyltransferase 2**	1426888_at	-0.482	2.69E-03	methylates Lys9 Histone H3	silences
Rbbp7	retinoblastoma binding protein 7	1415775_at	-0.481	2.75E-03	subunit of core HDAC complex	silences
Rbbp4	**retinoblastoma binding protein 4**	1434892_x_at	0.505	1.39E-03	member of NuRD and Sin3A complex	silences
Myst3	**MYST histone acetyltransferase (monocytic leukemia) 3**	1436315_at	0.650	7.92E-06	acetylated histones	activates
Hdac11	**histone deacetylase 11**	1451229_at	0.522	8.34E-04	deacetylates histones	silences
Cbx5	chromobox homolog 5 (Drosophila HP1a)	1454636_at	0.512	1.10E-03	binds acetylated histone 3	silences

Table 8: Genes from nucleus accumbens of ethanol drinking mice involved in synaptic vesicle formation and recycling. Genes in bold were also identified by principle component analysis.

Gene Name	Gene Symbol	Affy ID	R value	q value
adaptor protein complex AP-2, alpha 1 subunit	*Ap2a1*	1460724_at	0.484	2.55E-03
adaptor protein complex AP-2, alpha 2 subunit	*Ap2a2*	1452490_a_at	-0.485	2.45E-03
adaptor protein complex AP-2, mu1	***Ap2m1***	1450894_a_at	0.634	1.35E-05
brain derived neurotrophic factor	*Bdnf*	1422168_a_at	-0.470	3.62E-03
dynamin 1	***Dnm1***	1460365_a_at	0.541	4.38E-04
dynamin 1-like	*Dnm1l*	1428087_at	-0.447	6.21E-03
protein kinase, AMP-activated, beta 1 non-catalytic subunit	*Prkab1*	1452457_a_at	-0.455	5.27E-03
Rab acceptor 1 (prenylated)	*Rabac1*	1427773_a_at	-0.525	7.57E-04
RAB3A, member RAS oncogene family	***Rab3a***	1422589_at	0.588	8.14E-05
secretory carrier membrane protein 1	*Scamp1*	1426775_s_at	0.450	5.80E-03
SH3-domain GRB2-like 2	*Sh3gl2*	1418792_at	-0.451	5.74E-03
SH3-domain GRB2-like B1 (endophilin)	***Sh3glb1***	1418011_a_at	0.612	3.23E-05
solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter), member 1	*Slc1a1*	1448299_at	0.461	4.48E-03
solute carrier family 2 (facilitated glucose transporter), member 1	*Slc2a1*	1426600_at	0.458	4.82E-03
synaptophysin	***Syp***	1448280_at	0.573	1.48E-04
synaptotagmin II	*Syt2*	1420418_at	-0.515	9.91E-04
syntaxin 6	*Stx6*	1431646_a_at	-0.507	1.29E-03
vesicle-associated membrane protein 3	***Vamp3***	1437708_x_at	0.552	3.06E-04
vesicle-associated membrane protein 4	*Vamp4*	1422896_at	0.459	4.76E-03

Table 9: Over-represented gene categories from Principle Component Analysis in ethanol drinking mice. Categories highlighted in bold are in common with the original analysis.

Annotation Groups	Gene Category	List % (n)	Chip % (n)	p value
	Nucleus Accumbens (n=548)			
GO Biological Process	Growth	4.1 (15)	1.6 (148)	1.9E-03
GO Biological Process	Biopolymer Methylation	1.9 (7)	0.5 (43)	6.0E-03
GO Biological Process	Cellular Morphogenesis	6.2 (23)	3.4 (321)	7.1E-03
GO Biological Process	Protein Amino Acid Methylation	1.4 (5)	0.2 (22)	9.5E-03
GO Biological Process	Hydrogen Ion Homeostasis	0.8 (3)	0.1 (5)	1.4E-02
GO Cellular Component	Late Endosome	1.3 (5)	0.3 (25)	1.5E-02
GO Biological Process	Cell Growth	2.4 (9)	1.0 (90)	2.4E-02
GO Biological Process	Ubiquitin Cycle	5.7 (21)	3.4 (319)	2.4E-02
PIR pcmotif	Thyroglobulin Type-1 Repeat Signature	4.2 (3)	0.4 (7)	2.7E-02
GO Biological Process	Monovalent Inorganic Cation Homeostasis	0.8 (3)	0.1 (7)	2.8E-02
GO Biological Process	Mapkkk Cascade	2.4 (9)	1.0 (93)	2.8E-02
GO Cellular Component	Endosome	2.1 (8)	0.8 (78)	3.3E-02
GO Biological Process	Regulation of Protein Kinase Activity	2.2 (8)	0.8 (80)	3.6E-02
GO Molecular Function	Hydrolase Activity, acting on acid anhydrides, catalyzing transmembrane movement	2.6 (10)	1.1 (118)	3.6E-02
GO Biological Process	Regulation of Enzyme Activity	3.3 (12)	1.6 (153)	3.6E-02
GO Biological Process	**Chromatin Modification**	2.4 (9)	1.0 (99)	3.9E-02
GO Cellular Component	**Synaptic Vesicle**	1.6 (6)	0.5 (48)	3.9E-02
GO Biological Process	Membrane Lipid Metabolism	2.2 (8)	0.9 (83)	4.2E-02
GO Molecular Function	**Sodium:Potassium-Exchanging ATPase Activity**	0.8 (3)	0.1 (9)	4.4E-02
GO Biological Process	**Histone Methylation**	0.8 (3)	0.1 (9)	4.5E-02
GO Biological Process	**Protein Kinase Cascade**	3.5 (13)	1.9 (180)	4.8E-02
	Prefrontal Cortex (n=670)			
GO Biological Process	Ras Protein Signal Transduction	1.5 (7)	0.3 (25)	1.0E-03
GO Biological Process	**mRNA Transport**	1.3 (6)	0.2 (22)	3.5E-03
GO Biological Process	Coenzyme Catabolism	1.1 (5)	0.3 (24)	2.7E-02
GO Biological Process	**Electron Transport**	5.2 (24)	3.3 (311)	2.9E-02

GO Biological Process	Extracellular Matrix Organization and Biogenesis	1.3 (6)	0.4 (39)	3.9E-02
SwissProt keyword	**Respiratory Chain**	1.4 (3)	0.2 (7)	4.0E-02
GO Molecular Function	Heme Binding	2.2 (11)	1.1 (110)	4.1E-02
GO Biological Process	Regulation of Ras Protein Signal Transduction	0.7 (3)	0.1 (7)	4.2E-02
GO Molecular Function	Potassium Ion Binding	1.8 (9)	0.8 (81)	4.3E-02
GO Biological Process	Cofactor Catabolism	1.1 (5)	0.3 (28)	4.5E-02
GO Biological Process	Inorganic Anion Transport	2.4 (11)	1.2 (112)	4.5E-02
SwissProt keyword	Receptor	8.7 (19)	5.4 (251)	4.8E-02
	Ventral Tegmental Area (n=726)			
GO Molecular Function	Carbohydrate Binding	4.5 (23)	2.0 (208)	7.2E-04
GO Biological Process	Defense Response to Bacteria	1.8 (9)	0.4 (41)	1.1E-03
KEGG_Pathway	Cytokine-Cytokine Receptor Interaction	12.2 (22)	6.6 (217)	5.9E-03
GO Biological Process	Antimicrobial Humoral Response	0.8 (4)	0.1 (8)	6.4E-03
GO Biological Process	**Locomotory Behavior**	3.0 (15)	1.4 (131)	8.1E-03
KEGG_Pathway	**Toll-Like Receptor Signaling Pathway**	6.1 (11)	2.5 (82)	1.2E-02
GO Biological Process	Chemotaxis	2.2 (11)	0.9 (88)	1.6E-02
KEGG_Pathway	ECM-Receptor Interaction	5.6 (10)	2.2 (74)	1.7E-02
SwissProt keyword	Heparin-Binding	2.5 (6)	0.6 (30)	1.8E-02
KEGG_Pathway	**Glycosaminoglycan Degradation**	2.2 (4)	0.3 (11)	1.9E-02
SwissProt keyword	Glycosidase	2.5 (6)	0.7 (31)	2.1E-02
GO Molecular Function	Interleukin-1 Receptor Binding	0.8 (4)	0.1 (13)	2.5E-02
GO Biological Process	Cell Homeostasis	2.4 (12)	1.2 (110)	2.8E-02
SwissProt keyword	T-Cell	2.5 (6)	0.8 (35)	3.4E-02
GO Molecular Function	Monosaccharide Binding	0.8 (4)	0.1 (15)	3.6E-02
PIR pcmotif	Calcium-Binding EGF-Like Domain Pattern Signature	3.0 (3)	0.3 (6)	3.7E-02
GO Biological Process	Response To Wounding	4.1 (20)	2.5 (238)	4.2E-02
GO Molecular Function	GTPase Regulator Activity	3.3 (17)	1.9 (200)	4.3E-02
PIR keyword	Transferase	4.5 (4)	0.9 (15)	4.4E-02
GO Cellular Component	Lysosome	2.2 (11)	1.1 (106)	4.5E-02

GO Molecular Function	Pattern Binding	1.7 (9)	0.8 (80)	4.6E-02
GO Biological Process	Inflammatory Response	2.6 (13)	1.4 (134)	4.6E-02
GO Molecular Function	Cytokine Binding	1.6 (8)	0.7 (67)	4.9E-02

Table 10: Over-represented gene categories significant for the interaction of ethanol drinking and social defeat in the NAc of C57BL/6NCrl mice.

Annotrtion Groups	Gene Category	List % (n)	Chip % (n)	p value
KEGG pathway	OXIDATIVE PHOSPHORYLATION	23.1 (24)	3.2 (106)	2.7E-14
GO Molecular Function	PRIMARY ACTIVE TRANSPORTER ACTIVITY	9.5 (26)	1.4 (151)	1.6E-13
GO Molecular Function	NADH DEHYDROGENASE ACTIVITY	4.7 (13)	0.3 (35)	4.8E-11
SwissProt keyword	MITOCHONDRION	24.5 (26)	5.1 (240)	5.5E-11
GO Molecular Function	OXIDOREDUCTASE ACTIVITY, ACTING ON NADH OR NADPH	5.5 (15)	0.5 (54)	7.9E-11
GO Molecular Function	STRUCTURAL CONSTITUENT OF RIBOSOME	8.4 (23)	1.6 (164)	3.5E-10
GO Molecular Function	ELECTRON CARRIER ACTIVITY	5.5 (15)	0.6 (60)	3.7E-10
KEGG pathway	OXIDATIVE PHOSPHORYLATION	36.5 (19)	8.9 (91)	6.3E-08
GO Cellular Component	RIBONUCLEOPROTEIN COMPLEX	10.4 (28)	3.3 (317)	2.2E-07
GO Cellular Component	MITOCHONDRIAL ENVELOPE	8.9 (24)	2.5 (242)	2.7E-07
GO Cellular Component	MITOCHONDRIAL INNER MEMBRANE	8.1 (22)	2.2 (209)	3.6E-07
GO Molecular Function	ELECTRON TRANSPORTER ACTIVITY	6.2 (17)	1.4 (147)	1.7E-06
GenMAPP pathway	ELECTRON TRANSPORT CHAIN	30.3 (10)	4.4 (44)	3.3E-06
KEGG pathway	ENERGY METABOLISM	36.5 (19)	12.9 (132)	2.2E-05
SwissProt keyword	RIBOSOMAL PROTEIN	8.5 (9)	1.3 (62)	6.7E-05
SwissProt keyword	UBIQUINONE	4.7 (5)	0.3 (12)	1.1E-04
SwissProt keyword	OXIDOREDUCTASE	15.1 (16)	5 (232)	2.0E-04
GO Biological Process	NUCLEOSIDE TRIPHOSPHATE BIOSYNTHESIS	3.2 (8)	0.5 (51)	3.2E-04
GO Biological Process	RESPONSE TO OXIDATIVE STRESS	2.8 (7)	0.4 (37)	3.5E-04
SwissProt keyword	TRANSIT PEPTIDE	11.3 (12)	3.1 (143)	3.5E-04
GO Biological Process	OXYGEN AND REACTIVE OXYGEN SPECIES METABOLISM	3.2 (8)	0.6 (52)	3.7E-04
GO Biological Process	ATP BIOSYNTHESIS	2.8 (7)	0.4 (39)	4.6E-04
SwissProt keyword	NAD	7.5 (8)	1.4 (63)	5.0E-04
GO Cellular Component	MITOCHONDRIAL RIBOSOME	2.6 (7)	0.4 (39)	6.8E-04
SwissProt keyword	ACETYLATION	8.5 (9)	1.9 (88)	7.8E-04
GO Cellular Component	PROTON-TRANSPORTING ATP SYNTHASE COMPLEX	1.9 (5)	0.2 (17)	1.1E-03
GO Biological Process	PURINE RIBONUCLEOTIDE METABOLISM	3.2 (8)	0.7 (64)	1.3E-03
GO Biological Process	NUCLEOTIDE BIOSYNTHESIS	4 (10)	1.1 (103)	1.4E-03
GO Biological Process	COENZYME BIOSYNTHESIS	3.2 (8)	0.8 (80)	4.7E-03
GO Cellular Component	MITOCHONDRIAL LUMEN	2.6 (7)	0.6 (57)	5.0E-03
GO Cellular Component	MITOCHONDRIAL MATRIX	2.6 (7)	0.6 (57)	5.0E-03
GO Biological Process	RESPONSE TO REACTIVE OXYGEN SPECIES	1.6 (4)	0.1 (14)	5.1E-03
GO Cellular Component	MITOCHONDRIAL ELECTRON TRANSPORT CHAIN	1.9 (5)	0.3 (27)	6.3E-03
GO Cellular Component	CYTOSOLIC RIBOSOME	2.2 (6)	0.5 (44)	7.3E-03
GO Molecular Function	P-P-BOND-HYDROLYSIS-DRIVEN TRANSPORTER ACTIVITY	2.9 (8)	0.9 (89)	9.4E-03
GO Molecular Function	CYTOCHROME-C OXIDASE ACTIVITY	1.5 (4)	0.2 (17)	9.6E-03
GO Biological Process	ATP SYNTHESIS COUPLED PROTON TRANSPORT	2 (5)	0.4 (34)	1.1E-02
KEGG pathway	PYRIMIDINE METABOLISM	7.7 (8)	2.6 (85)	1.6E-02
KEGG pathway	PURINE METABOLISM	9.6 (10)	3.9 (129)	1.8E-02
GO Biological Process	RESPONSE TO CHEMICAL STIMULUS	5.3 (13)	2.5 (232)	1.8E-02
SwissProt keyword	HYDROGEN ION TRANSPORT	3.8 (4)	0.5 (25)	1.8E-02
KEGG pathway	MMU00130:UBIQUINONE BIOSYNTHESIS	2.9 (3)	0.2 (7)	1.8E-02
GO Molecular Function	HEAT SHOCK PROTEIN BINDING	1.8 (5)	0.4 (42)	2.5E-02
GO Cellular Component	CONDENSED NUCLEAR CHROMOSOME	1.5 (4)	0.2 (23)	2.5E-02
GO Molecular Function	UNFOLDED PROTEIN BINDING	2.9 (8)	1.1 (111)	2.8E-02
GO Biological Process	ATP SYNTHESIS COUPLED ELECTRON TRANSPORT	1.2 (3)	0.1 (11)	3.2E-02
GO Biological Process	COFACTOR METABOLISM	4 (10)	1.8 (170)	3.3E-02
KEGG pathway	TRANSLATION	17.3 (9)	7.7 (79)	3.7E-02
GO Biological Process	RIBOSOME BIOGENESIS AND ASSEMBLY	2.8 (7)	1 (97)	4.1E-02
GO Molecular Function	DNA-DIRECTED RNA POLYMERASE ACTIVITY	1.5 (4)	0.3 (30)	4.5E-02
SwissProt keyword	UNFOLDED PROTEIN RESPONSE	1.9 (2)	0 (2)	4.5E-02
SwissProt keyword	CHAPERONE	4.7 (5)	1.3 (61)	4.9E-02

CHAPTER 7 Final Conclusions and Future Directions

There are a number of reasons why an individual consumes ethanol and perhaps more compounding reasons why an individual has an increased risk for alcohol abuse and alcoholism. Initial rewarding effects of ethanol, insensitivity to aversive (i.e. withdrawal) symptoms, and impulsivity or increased novelty-seeking are hypothesized to play a role in the proclivity to drink excessively. Even in preclinical models, selected lines have differing behavioral and neurobiological mechanisms underlying ethanol preference drinking. Some alcohol preferring lines, P and sP rats, show increased anxiety-like behaviors and decreased CRF immunoreactivity (Stewart et al. 1993; Colombo 1997; Pandey 2003; Hwang et al. 2004), while other selected lines, HAD and AA rats, show the opposite (Tuominen et al. 1990; Tuominen et al. 1990; Spanagel et al. 1999; Badia-Elder et al. 2003). Using this model of persistent individual variation in ethanol drinking behavior within inbred mice, where genetic factors are strictly controlled, offers considerable power for studying the molecular mechanisms of environmental modulation of ethanol drinking behavior.

Here we have characterized a model of individual variation of ethanol drinking behavior and have shown that this variation is robust and persistent. Intra-individual variation in ethanol preference was not due to simple litter effects or differences in taste

susceptibility. The underlying premise of this work was that these differences could be mediated by individual variation in gene expression, caused by early social stress experience or due to underlying differences in basal anxiety-like behaviors. The current studies have lead to direct, testable hypotheses of signaling pathways which may regulate or be influenced by ethanol drinking preference and the interactions of social stress on ethanol drinking behavior.

One of the most likely possibilities underlying the variation in ethanol intake and preference within the inbred C57 strain are the potential differences in epigenetic regulation of gene transcription. Epigenetic variation has been referred to as the third component of natural variation, with genetics and environment being the first two components (Gartner 1990). Even in strictly controlled environments, up to 80% of random variability in quantitative traits, such as body weight, are unrelated to genetic and/or environmental influences (Gartner 1990). Epigenetic variation is increasingly used to explain how genetically identical inbred mice can display phenotypic differences. Our findings from the molecular factors which differ between ethanol preferring and avoiding mice in Chapter 6 have pointed to potential differences in genes involved in epigenetic mechanisms and regulation at the level of transcription (i.e. histone acetylation and DNA methylation). Furthermore, we have shown that inhibition of HDAC activity alters ethanol drinking preference suggesting that alterations at the level of chromatin structure can also modulate ethanol preference. While our studies in littermates did not show simple litter effects (Chapter 3), there remains the possibility that ethanol intake could be affected by social hierarchies set up early in life as well as subtle differences in rearing or variations in

pre- or post-natal development. Direct breeding and cross-fostering studies will need to be performed to determine these pre-and post-natal influences.

Many future experiments can be constructed to tease out the ways in which modulation at the level of gene transcription affects ethanol drinking behavior. A first step would be to identify the TSA-altered gene networks by genome-wide expression profiling or using gene tiling techniques to assay the entire genome and not just the transcriptome. Direct investigation of gene networks altered by the HDAC inhibitor, TSA, would aid in identifying the genes and pathways affected by increased histone acetylation that modulate ethanol drinking behavior. While we have shown that modulation of histone acetylation levels increased ethanol drinking preference, we do not know the mechanisms by which this occurs. Our studies also point to several other gene networks that are altered in these mice such as glutamate signaling, BDNF and synaptic vesicle changes. Prior studies on environmental modulation of anxiety-like behaviors have implicated epigenetic modulation at promoter regions of BDNF (Tsankova et al. 2006) or glucocorticoid receptors (Brake et al. 2004). These networks provide a good starting point for future studies extending our own experiments. Additionally, we do not know the origin of these epigenetic differences in our studies which could be due to differences in genomic imprinting or epistatic interactions. We cannot rule out the possibility that differential ethanol exposure could have caused differences in a "master regulator" such as microRNA alterations in differentially exposed high and low drinking mice. Thus, investigation of differential expression of microRNAs between high and low preferring mice would be an important first step towards identifying a potential "ethanol master regulator."

In the present studies, anxiety-like behavior was not clearly predictive of initial ethanol intake or preference in C57BL/6NCrl mice using the light-dark transition model. In one experiment, basal anxiety-like behavior in the light-dark box was correlated to initial ethanol intake for two measures, percent time in the light and percent distance in the light. In a separate cohort, there was a trend for a separate measure, the number of entries into the light, to be correlated to ethanol intake in control home caged mice. But these findings were not replicated in all experiments. We have also shown a relationship between stress-influenced ethanol drinking and anxiety phenotypes. Following social stress, mice with the lowest intake tended to display the least anxiety-like behavior. Together, these data point towards a positive, albeit inconclusive, relationship between anxiety-like behaviors and ethanol preference where mice with a low anxiety phenotype consume the lowest amount of ethanol. One possibility for these inconsistent findings could be that the light-dark model may not accurately measure differences in anxiety-like behavior in these mice. Other anxiety models may more accurately reflect differences in ethanol preference drinking such as the elevated plus maze, open field activity, or the social interaction test. The social interaction test has recently been used to show that social defeat stress decreased social approach and interaction, interpreted as increased anxiety-like behavior (Berton et al. 2006). This model may more accurately measure the "social components" involved in the anxiety and stress-induced modulation of ethanol drinking behaviors. Thus, one future experiment could test anxiety-like behavior prior to ethanol drinking and following social defeat using the social interaction test. One would expect that social defeat would decrease social interaction, but the relationship between social interaction and

ethanol preference drinking has not been investigated and would certainly be more complicated.

Studies investigating stress effects on ethanol drinking behavior have not ruled out the possibility that individual variation in ethanol intake may be due to differences in stress responsivity. While we did not find a consistent relationship between ethanol intake and basal anxiety phenotype, following a period of social stress, mice with a high anxiety phenotype consumed the highest amounts of ethanol. These findings are consistent with previous studies in dependent animals where following protracted abstinence, dependent animals did not show significant induction of anxiety-like behavior (Valdez et al. 2003). Once a brief stressor was employed, a heightened behavioral stress response could be detected in post-dependent rats. Thus, while no clear relationship between individual variation of ethanol drinking and basal anxiety could be determined in individual mice, the response to social stress may be altered in individual mice and may be the source for their individual variation of ethanol drinking. Future studies employing other stress modalities such as restraint stress or foot shock could strengthen this argument. Additionally a careful observation of coping mechanisms displayed during the defeat sessions could provide further opportunities to investigate response to stress on an individual level. Direct investigation of coping mechanisms would be a rich set of future experiments since individual variability in stress responsivity has also been shown to be correlated with differential immune responses (Avitsur et al. 2007), dopamine and/or sympathetic reactivity (Koolhaas et al. 1999; Krishnan et al. 2007). Based on the interconnected circuitry between stress- and ethanol-responsive brain regions, individual differences in

response to social stress may help explain the individual ethanol preference drinking. We already have preliminary evidence (see Chapter 6) suggesting such alterations have occurred following social defeat stress and ethanol drinking. Our initial profiling of these transcripts altered in stress-influenced ethanol drinking has pointed to several signaling pathways involved in dopamine signaling, the extra-hypothalamic stress response and alterations in steroid and glucocorticoid synthesis. Future experiments will first need to confirm these changes in expression with rtPCR, Western blotting or in situ hybridizations. Following confirmation, direct measurements of glutamate, dopamine or GABA release into the PFC or NAC by micro dialysis in high versus low drinking mice or following response to a stress challenge may further elucidate the mechanisms underlying individual ethanol preference or response to stress.

Finally, these studies did not uniformly support the tension-reduction hypothesis for alcohol abuse. Social stress, through a group-housed setting or by repeated social defeat, decreased ethanol intake in a majority of mice, although the proclivity to drink following social stress may be increased in the lowest preferring mice (see Chapter 5). This finding was supported in the 129SvJ mice, keeping in mind that different genetic backgrounds and glucocorticoid response to stress are also contributing to these results. We had expected that having ethanol continuously available would give the mice an opportunity to associate ethanol drinking with relief from the negative aspects of social stress. An alternative design would directly pair ethanol anxiolysis and social defeat stress using cue-induced reinstatement. In fact, recent work has shown that two factors are needed in order increase the likelihood of stress-induced ethanol drinking. First, ethanol

must be established as a reinforcer (e.g. the positive reinforcing properties of ethanol are established prior to the stress-induction) (Meisch 1983) and second, the animals must associate ethanol drinking with alleviation of negative symptoms (Meisch 1994). The present studies were not specifically designed to do either. Therefore, in order to reliably see increases in ethanol drinking due to a social stress, future experiments would need to establish the reinforcing and anxiolytic properties of ethanol. Such a design could include limited access training for stable voluntary ethanol drinking and long-term voluntary ethanol access with repeated social defeat in intermittent sessions allowing for a learned association between ethanol's anxiolytic properties and social stress.

Overall, this study has provided a unique contribution to the elucidation of molecular determinants underlying ethanol preference drinking and drinking responses to social stress. Specifically, these results contribute to a better understanding of the neurobiology of initial ethanol drinking behavior, and its relationship to social stress and anxiety-related behavior. Since candidate genes and their respective signaling pathways may lead to novel insight into individual differences in the susceptibility to ethanol abuse and dependence, our results may eventually lead to new therapeutic interventions for alcoholism.

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