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Behavioral and genomic characterization of scheduled ethanol deprivation

Jonathan Warner
Virginia Commonwealth University

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

by

Jonathan Andrew Warner

Bachelor of Science, Virginia Commonwealth University, 2007

Director: Michael F. Miles, M.D., Ph.D.

Professor, Department of Pharmacology and Toxicology

Virginia Commonwealth University

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Abstract

BEHAVIORAL AND GENOMIC CHARACTERIZATION OF SCHEDULED ETHANOL DEPRIVATION

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Alcoholism is a persistent substance abuse disorder that is associated with negative health, social, and economic outcomes. Treatment strategies for alcohol use disorders are limited, and only three drugs have been approved by the FDA for treatment. Although behavioral therapy and drug combination strategies improve abstinence outcomes, the majority of those in treatment will not achieve long-term abstinence. Therefore, better treatment strategies are needed.

While much progress has been made toward understanding the neurobiology of alcoholism, this knowledge has not been effectively translated into treatment strategies. Animal models of alcohol drinking have been crucial to this research effort, but until recently there have been few procedures that effectively model alcoholism by producing binge-like drinking, withdrawal, and relapse behavior. In the last five years the intermittent alcohol access (IAA) model, which uses repeated cycles of scheduled alcohol deprivation and reinstatement to elevate drinking, has been

established as such a procedure, with substantial evidence that escalation of drinking produced by IAA is mediated by similar mechanisms as in human alcoholics, which include transcriptional regulation that alters functioning of mesolimbocortical reward pathways. The IAA model.

The studies reported herein characterize changes in gene expression in mesolimbocortical brain regions associated with development of maladaptive binge-like alcohol drinking due to scheduled abstinence, particularly in the nucleus accumbens, which regulates motivated behavior. Furthermore the IAA model is characterized with regard to effectiveness in 2 ethanol-preferring C57BL/6 inbred mouse strains, and the influence of concurrent access to multiple alcohol concentrations is examined. Finally, the potential of naltrexone and novel mu-opioid receptor-selective antagonist NAQ to modulate alcohol drinking under continuous access and intermittent access procedures is reported.

Microarray analysis is used to analyze the transcriptome in prefrontal cortex, nucleus accumbens, and ventral midbrain of C57BL/6NCrl mice after alcohol deprivation, and to identify differentially expressed genes and gene co-expression networks in C57BL/6J mice during continuous access, as well as after six cycles of IAA. Differentially expressed genes, network hub genes, and regulation mechanisms represent high priority targets for further study in binge-like drinking behavior, with the goal of translating this knowledge to treatment strategies for alcoholism.

Chapter 1. Introduction

Alcoholism is a persistent substance abuse disorder with a high rate of relapse for patients in therapy and potentially fatal withdrawal symptoms caused by abstinence from ethanol (Powell, 1999). Ethanol is a small molecule that interacts with a diverse set of molecular targets in neurons and glia, including glutamate, GABA, and serotonin receptors. These actions at the cell surface initiate changes in cell signaling and gene expression, which are associated with altered behavior toward drugs and other rewarding and aversive stimuli. The nature of these changes varies among individuals and between brain regions, which are effected by ethanol according to their respective target abundance and composition, which in turn are dependent on environmental history and genetic background (Kalsi et al., 2009; Vengeliene et al., 2009).

Persistent exposure to ethanol alters protein composition and function, which regulates cellular processes, leading to changes in function and the transition to a dependent state, in which ethanol is required for normal functioning. From this point, absence of ethanol leads to physiological and psychological withdrawal symptoms, which include desire for ethanol and severe seizures that may lead to death (Valdez et al., 2002; Vilpoux et al., 2009). The transition to dependence is gradual, as continued ethanol exposure over time alters mRNA transcription and protein translation through diverse signaling networks, leading to changes in brain function and behavior. Neuroplasticity induced by ethanol exposure over time is not well-understood, but many of the signaling pathways and genes have been identified in recent years through

behavioral study in animal models (Kerns, 2005; Koob and Volkow, 2009). The goal of studying alcohol in animal models is to develop effective treatments for alcoholism through better understanding of the behavior and neurobiology of alcohol abuse.

The complexity of the effects of alcohol and variation among individuals have impeded the development of effective pharmacological therapeutic tools for the treatment of alcoholism. As of 2013 only 3 drugs are approved by the FDA for reduction of relapse in abstinent alcoholics: naltrexone, acamprosate, and disulfiram. These drugs do not share a direct mechanism of action, have rarely been shown to be effective for maintaining long-term abstinence, and are not effective for all alcoholics (Garbutt, 2009; Krampe et al., 2006). The variation in effectiveness of drugs used to prevent relapse shows that the physiological nature of dependence differs among individuals. Thus, detailed study is needed to identify common disease factors, which can be exploited to produce targeted therapeutic strategies that are effective in a significant subset of alcoholics (George and Koob, 2010). However, this endeavor is particularly complex in the case of ethanol because of the large number of primary targets and the diverse signaling mechanisms involved (Pignataro et al., 2009). Interaction with these targets produces myriad effects on the cell depending on abundance and function, which depend on environment and genotype (Spanagel, 2009).

Furthermore, alcohol intake shows extreme individual variation even within inbred rodent strains of identical genetic background, which indicates that differences in transcriptional regulation, which are modulated by epigenetic processes, are likely major factors that contribute to drinking behavior (Wolstenholme et al., 2011). Ethanol causes histone hyperacetylation, and in the amygdala this is accompanied by elevated expression of cAMP response element-binding protein (CREB) and Neuropeptide Y (NPY), which are known to play a role in addictive behavior and

alcohol dependence (Robison and Nestler, 2011; Sorensen et al., 2013). Withdrawal from alcohol produces anxiety-like behavior and deficits in histone acetylation, which are reversed by histone deacetylase inhibitors (Pandey et al., 2008). Transcriptional regulation in the brain caused by ethanol is complex, largely region-specific, and often correlated with ethanol-related behavior (Ahmadiantehrani et al., 2013; Kerns, 2005).

To better understand the role of transcriptional regulation in the transition to dependence, regional genomic analysis is used, which identifies changes in mRNA in brain regions associated with particular aspects of ethanol-related behavior. Genomic methods measure the abundance of tens of thousands of transcripts in a single sample from a single region of the brain, and can be applied to identify significant differentially expressed genes across experimental groups (Kerns and Miles, 2008; Miles and Williams, 2007a). Targets of interest need not be specified before the experiment, so it can be used to discover transcripts regulated by the model without known behavioral significance, rather than merely confirm changes in abundance for transcripts with known relevance to ethanol-related behavior. Thus, experimenter bias is minimized and the potential for novel discovery is maximized.

Transcripts found to be regulated by experimental manipulation can be correlated with differences in acute and chronic ethanol-related behavior, such as ethanol consumption and preference over water, sensitivity, and tolerance (Costin et al., 2013b; Iancu et al., 2013; Mulligan et al., 2011; Osterndorff-Kahanek et al., 2013; Repunte-Canonigo et al., 2010; Wolen and Miles, 2012; Wolen et al., 2012; Wolstenholme et al., 2011). Further analysis of regulated transcripts co-expression and functional networks provides biochemical context through gene ontology, as well as important hub genes and regulation mechanisms, such as transcription factors and miRNAs (Chen et al., 2009b; Ho Sui et al., 2005; Kaimal et al., 2010; Vadigepalli et

al., 2003). Identified genes and pathways represent potential pharmacological or genetic therapeutic targets for alcoholism that warrant further study (Tabakoff et al., 2009; Wang et al., 2010). Selective ligands, RNAi, and knock-out mice can be used to verify the functional relevance of particular targets (Bhutada et al., 2010; Choi et al., 2002; Cortínez et al., 2009). Indeed, perturbation of targets selected by our laboratory based on microarray analysis of diverse ethanol-related behavioral models for adeno-associated virus-mediated regional overexpression in murine prefrontal cortex (PFC) (*Clic4, Sgk1, Gsk3b*) have been generally successful in altering ethanol-related behaviors, including locomotor stimulation and sensitization produced by low ethanol doses, loss of righting reflex produced by high ethanol doses, drinking behavior, and withdrawal-induced anxiety-like behavior.

Recent years have seen the development of animal models of the transition to alcoholism that rely on voluntary ethanol self-administration combined with scheduled abstinence of varying lengths, which are known as the ethanol deprivation effect (EDE) and intermittent alcohol access (IAA) models, respectively (Khisti et al., 2006a; Simms et al., 2008). These methods produce escalation in alcohol consumption and preference at concentrations ranging from 3% to over 30%, which are similar to those typically consumed by humans, without sucrose fading or other procedures designed to facilitate drinking. Naltrexone and acamprosate attenuate escalation of drinking behavior produced by both models which indicates some shared physiological foundation for the EDE, IAA, and alcoholism (Heyser et al., 2003; Li et al., 2010a; Sabino et al., 2013). After several weeks or months of IAA the procedure produces behavior in rodents that resembles drinking in human alcoholics: binge-drinking, inflexible intake, and seizures upon withdrawal (Hopf et al., 2010; Hwa et al., 2011).

Ethanol deprivation on particular schedules significantly increases ethanol consumption, preference for ethanol over water, and operant responses for ethanol in mice (Sparta et al., 2009). Repeated deprivation in animals allowed concurrent access to multiple concentrations of ethanol causes a significant shift in preference to the highest ethanol concentration offered (30% w/v) (Melendez et al., 2006a), and near-total preference for ethanol over water (Warner, in preparation). Escalation of intake due to deprivation and IAA varies with animal strain, ethanol concentration, and access schedule, but 50% to 100% increases in ethanol consumption and significant increases in preference for ethanol over water occur immediately under most conditions (Hwa et al., 2013; Khisti et al., 2006a; Melendez, 2011; Melendez et al., 2006a; Rosenwasser et al., 2013). Extended IAA often produces 3- to 4-fold increases, and produces near-total ethanol preference over several weeks, without the use of multiple alcohol concentrations to boost intake (Hargreaves et al., 2009b; Wise, 1973). The IAA model has been associated with changes in ΔFosB abundance and enhanced excitatory synaptic strength in the ventral tegmental area, and its effect is decreased by glial cell line-derived neurotrophic factor (GDNF), which alters gene expression and promotes neuronal survival (Carnicella et al., 2009b; He, 2005; Li et al., 2010a; Stuber et al., 2008).

The need for better treatments for alcoholism can only be met by the careful use of valid animal models that represent the condition in humans. Scheduled abstinence models meet this criteria by allowing for detailed study of the molecular basis of the development of increased ethanol consumption and preference over time, using a schedule similar to human patterns of alcohol use during adolescence and early adulthood, when alcoholic behavioral patterns are usually established (Moss et al., 2010). Indeed, several candidate ligands have been tested using scheduled deprivation in recent years, showing that the IAA model is a useful initial screen to

identify compounds that alter craving during abstinence and merit further study in the context of alcohol-related behavior (Hopf et al., 2011; Li et al., 2011a; Li et al., 2012a; Moorman and Aston-Jones, 2009a; Wen et al., 2012; Yardley et al., 2012).

Interestingly, in IAA naltrexone reduces binge-like drinking and reverses accumulation of transcription factor deltaFosB, and the efficacy of naltrexone to reduce binge-like drinking is controlled by early life experience, during which epigenetic programming of gene expression is occurring (Daoura and Nylander, 2011; Li et al., 2010b; Meaney et al., 2007; Renthal et al., 2007; Siegmund et al., 2009). These results show that transcriptional regulation may be important for the behavioral changes induced by the model, both downstream of the mu opioid receptor, which is known to mediate changes in gene expression (Befort et al., 2008; Oliva and Manzanares, 2006; Yu et al., 2010), and upstream of the receptor, where epigenetic processes determine the expression of it and other genes (Hwang et al., 2010; Lin et al., 2008). Thus, transcriptional regulation induced by cycles of ethanol intake and abstinence contributes to the plasticity that leads to alcohol dependence, and a better understanding of the way genetics and gene expression influence this process will drive the study of alcohol and of other drugs of abuse and identify novel therapeutic targets for the treatment of alcoholism. Therefore, the author here reports studies performed toward this end, with the guiding hypothesis that scheduled abstinence in mice produces regional changes in gene expression in the brain, which mediate changes in alcohol drinking behavior over time. Experiments were performed with the following aims:

1. To determine the environmental and genetic factors that regulate escalation of drinking behavior produced by scheduled abstinence in mice.
2. To determine the nature of transcriptional regulation associated with scheduled abstinence in areas of the mesolimbic reward pathway.

3. To determine the functional relevance to IAA behavior of targets identified by genomic analysis of transcriptional regulation using *in vivo* pharmacological manipulation.

Chapter 2. Background and significance

Alcoholism and alcohol use disorders

Alcoholism is a persistent disorder with significant costs to the afflicted individual and to society. In the United States approximately 80,000 deaths each year are attributable to excessive alcohol use, which the Centers for Disease Control and Prevention ranks as the third-leading lifestyle-related cause of death (Mokdad et al., 2004). The average potential life lost for each individual for whom excessive drinking contributed to death was nearly 30 years, and in 2006 the economic cost of this behavior was estimated at $223.5 billion (Bouchery et al., 2011). Furthermore, alcohol use disorders (AUDs) are associated with negative employment outcomes and low marriage quality (Booth and Feng, 2002; Leonard and Rothbard, 1999). People drink despite this physical and economic damage because alcohol produces rewarding psychoactive effects, such as euphoria and anxiolysis, and the aversive effects associated with heavy use and withdrawal are not always sufficient to moderate consumption (Spanagel, 2009; Valdez and Koob, 2004).

The motivation of an organism to consume alcohol is influenced by dynamic neurobiological and environmental factors that modulate the rewarding and aversive effects of the drug. Initiation and regulation of alcohol consumption is controlled by memories of previous experiences with the drug, associations of these experiences with environmental context and cues, genetic and biological factors, and social influences (Barr et al., 2008; Behrendt et al., 2008; Goltz et al.,

2009; Pawlak et al., 2008; Siegmund et al., 2005b; Spanagel et al., 2005; Vengeliene et al., 2009). The influence and interplay of these factors varies from one individual to another, and with their respective stage of development and of alcohol addiction. Approximately 25% of Americans develop an alcohol use disorder at some point in their lives, and for some it becomes an overwhelming negative influence that results in uncontrolled drinking and disruption of normal life (DSM-IV-TR, A.P.A., 2000; Haeny et al., 2013).

Until 2013 the American Psychiatric Association listed defined criteria for two alcohol use disorders (AUD) in the Diagnostic and Statistical Manual (DSM-IV): alcohol abuse and alcohol dependence. Alcohol abuse was characterized by drinking despite social, occupational, and legal problems that are associated with impairment and stress, but not with physical dependence. Alcohol dependence reflected neurobiological adaptations to the long-term presence of alcohol, and had several defining characteristics: tolerance, withdrawal, drinking more than intended, unsuccessful attempts to quit or reduce drinking, interference with other life activities, and continued use despite knowledge of negative physical and psychological problems that are likely caused by drinking. For a diagnosis of alcohol dependence there must have existed a maladaptive pattern of drinking behavior that lead to clinically significant impairment or distress accompanied by at least 3 of the above symptoms in a 12-month period (A.P.A., 2000). Studies have shown that alcohol dependence was the more valid and reliable of these two diagnoses (Bucholz et al., 1994; Canino et al., 1999; Chatterji et al., 1997), and in 2013 the definitions for AUDs were revised for the 5th edition of the DSM (DSM-V). Instead of two distinct constructs, the DSM-V defines a single construct with moderate and severe diagnoses based on the number of criteria displayed by the patient, with the previous criteria for alcohol abuse and dependence remaining largely unchanged, but combined into a single list. A moderate AUD is a pattern of

maladaptive drinking behavior with 2-3 of the listed criteria, while a diagnosis of severe AUD requires 4 or more of these criteria (Kopak et al., 2013).

Neurobiology and treatment of alcoholism in humans

It is clear from the persistent cost of alcoholism to individuals and society that the disorder is not well understood and thus not well controlled. Alcohol use disorders are particularly difficult to manage due to the complexity of the biological and environmental factors that motivate drinking. Alcohol is widely available in the United States, where the drug may be purchased legally by persons over the age of 21 in most of the country, with the exception of some dry counties. Behavioral and pharmacological treatment strategies have not proven effective in the long-term for the majority of patients, although there is evidence to suggest that a combination of these strategies is more effective than either alone (Feeney et al., 2006; Garbutt, 2009; Krampe et al., 2006; McKay, 2006; Soyka and Rosner, 2008). Much has been learned in recent years about the neurobiology of ethanol exposure, with the goal of developing more effective treatment strategies.

Alcohol produces psychoactive effects through action at more molecular targets than is typical for an abused substance (Vengeliene et al., 2009). In the CNS, ethanol interacts with receptors for glutamate, GABA, glycine, acetylcholine, and serotonin, and inhibits ion channels for potassium and calcium. Several other neurotransmitters are known to modulate aspects of alcohol drinking behavior, including dopamine, serotonin, endogenous opioids, endocannabinoids, corticotrophin releasing factor, and neuropeptide Y. Due to this complexity of action the FDA has been able to approve only three drugs for the management of alcoholism as of 2013: disulfiram, acamprosate, and two forms of naltrexone. Although drugs approved to treat alcoholism show limited effectiveness, much can be learned about alcoholism and alcohol-

related behavior from study of the behavioral changes they produce in animal models and their mechanisms of action.

Disulfiram inhibits the action of the acetaldehyde dehydrogenase enzyme, which increases levels of acetaldehyde 5- to 10-fold (Faiman et al., 2013). Acetaldehyde is an alcohol metabolite that mediates some of the aversive effects of alcohol consumption, and genetic differences that reduce acetaldehyde dehydrogenase function are associated with reduced ethanol intake (Faiman et al., 2013). High levels of circulating acetaldehyde cause flushing of the skin, tachycardia, nausea, vomiting, headache, and other unpleasant symptoms (Bae et al., 2012). Disulfiram is relatively effective when patients are compliant, with some evidence for abstinence rates of about 50% over a 9-year study when combined with calcium carbimide, which is an ethanol-sensitizing agent (Krampe et al., 2006).Unfortunately, disulfiram does not reduce craving for ethanol and patient compliance is a major obstacle to successful therapy. A study of disulfiram treatment in cooperation with the U.S. Veterans Administration found patient compliance at 20%, which precluded any statistically significant effect of disulfiram treatment combined with therapy compared to disulfiram alone (Fuller et al., 1986).

Acamprosate and naltrexone both address more directly the psychological aspects of alcohol addiction by acting at a pharmacodynamic level to interfere with the neurobiological underpinnings of ethanol reward and craving (Soyka, 2013). When given in conjunction with cognitive behavioral therapy, each of these drugs has been shown to produce abstinence in the majority of patients over a 3-month period. There is some evidence that the combination of the two drugs is more effective than either alone, although naltrexone produced nearly the same proportion of abstinent patients alone as when combined with acamprosate (Anton et al., 2006; Feeney et al., 2006). Until 2013 it was thought that acamprosate attenuates the hyperactive

neuronal state associated with withdrawal, which is characterized by hyperactive NMDA glutamate receptors and hypoactive $GABA_A$ receptors. By acting as a partial agonist at $GABA_A$ receptors and inhibiting NMDA receptor activity, acamprosate would reduce hyperexcitability and negative aspects of withdrawal and reduces alcohol consumption (Kurokawa et al., 2013; Oka et al., 2013).

There is also some evidence *in vitro* for neuroprotective effects of acamprosate that reduce neuronal death caused by excitotoxicity induced by withdrawal and glutamate release, which could mitigate long-term deficits caused by cycles of alcohol abuse and withdrawal (al Qatari et al., 2001). However, a recent landmark *Neuropsychopharmacology* study shows that all of the actions of acamprosate (calcium-bis-N-acetylhomotaurinate) can be attributed to calcium, and that N-acetylhomotaurine is a biologically inactive molecule (Spanagel et al., 2013).

Naltrexone is a non-selective competitive antagonist at the three main subtypes of opioid receptor: mu, kappa, and delta. It is available in oral and injectable forms for prevention of relapse for heavy-drinking alcoholics and patients dependent on opioids. Naltrexone interferes with the reinforcing effects of alcohol by preventing activation of opioid receptors by endogenous opioid peptides, which is accompanied by reduced release of dopamine in the ventral tegmental area (VTA) of the midbrain (Soyka and Rosner, 2008).

The VTA is the site of origin for dopaminergic neurons that project forward to the nucleus accumbens (NAc) in the mesolimbic reward pathway, as well as efferents that project to the prefrontal cortex (PFC) in the mesocortical dopamine pathway. These two dopamine pathways are not independent, and the functioning of one may be affected by altering the other. Although differences in opinion exist as the function and extent of these regions and pathways, there is

general consensus that together with closely connected regions, they act to regulate behavior in response to drugs of abuse and other potentially rewarding stimuli. Other areas of the brain involved in these behaviors through connections with these pathways include the amygdala and the hippocampus. The mesocortical pathway is thought to be involved in attention and motivation, emotional response, decision making, and other functions associated with the frontal lobe of the cerebral cortex. The mesolimbic pathway is thought to be involved in reward, desire, and motivated behavior. There is some evidence that activity in this pathway is particularly associated with incentive salience, which is the ability of a stimulus to induce craving or wanting in an organism, as opposed to pleasure or euphoric feelings (Berridge, 2012). Study of the effects of drugs known to be useful for management of alcoholism in the context of these neural pathways and regions may point the way toward better therapeutic strategies, with respect to molecular targets and method of administration.

Animal models of excessive alcohol consumption

Better drugs are needed for the management of alcoholism, but experimentation toward this goal has been hindered by the impracticality of extensive study of neurobiological factors and response to novel compounds in humans. Thus, reliable and valid animal models of alcoholism are necessary for better understanding of the disease and the development of new treatment strategies.

The study of alcohol-related behavior in animals has been hindered by the low propensity of most rodents to self-administer the drug (Amit et al., 1970; Crabbe et al., 2012; Wise, 1973, 1974). Mice and rats do not typically drink alcohol in a manner that produces measurable signs of intoxication or significantly elevates blood alcohol content above 1.0% by volume, which is approximately the legal limit for motor vehicle operation in most states. However, animal

models have been developed for the study of alcohol abuse and dependence that reflect particular sets of their respective defining characteristics, with the aim of elucidating the biological and environmental factors that influence maladaptive excessive drinking behavior. Models of escalating alcohol self-administration are also useful for the identification and testing of potential therapeutic strategies, and for more basic study of the nature of reward processes.

Experimental strategies for producing high-level alcohol self-administration in rodents may be grouped into several categories that reflect different aspects of alcohol-related behavior (Becker, 2013): scheduled brief access to alcohol (drinking-in-the-dark, DID), ethanol deprivation following a baseline consumption period (alcohol deprivation effect, ADE/EDE), intermittent alcohol access (IAA), schedule-induced polydipsia, and induction of dependence and withdrawal via ethanol vapor or diet (chronic intermittent ethanol, CIE). Each of these models produces effects on drinking behavior that are mediated by distinct neuromolecular substrates, and depend greatly on the strain of rodent used. Much progress has been made in recent years toward illumating the behavioral and neuromolecular characterization of these models, but results have varied among laboratories with regard to the major players involved. The CIE and IAA models are thought to be particularly reliable and valid representations of the transition to alcoholism in humans, and have received much research attention in recent years.

Chronic intermittent ethanol vapor (CIE)

Induction of dependence with ethanol vapor is a well-established experimental method in rodents. A typical procedure consists of 12-14 hour sessions of ethanol vapor exposure that induce binge-like BEC levels of 100 mg% and greater (Criado and Ehlers, 2013; Griffin et al., 2009; Lopez et al., 2011; Repunte-Canonigo et al., 2013). Vapor sessions are interspersed with periods of withdrawal followed by home-cage drinking, and voluntary intake gradually increases

with repeated cycles of dependence and withdrawal. This type of CIE vapor treatment increases voluntary consumption and preference for alcohol by approximately 40-50%, but methodological concerns limit the validity of the model for representing alcoholism. High BECs are achieved through the use of the alcohol dehydrogenase inhibitor pyrazole, which is not used by human drinkers and may itself alter drinking behavior over time. Furthermore, vapor exposure is an involuntary method of ethanol intake, and involuntary drug use is known to produce neurobiological changes that are different from those produced by voluntary use (Ahmadiantehrani et al., 2013; Fernandez-Castillo et al., 2012; Tapocik et al., 2013). Nevertheless, the CIE model has been used to study the neuromolecular consequences of repeated alcohol exposure and withdrawal, which have been difficult to model in rodents.

Diverse molecular targets in several brain regions have been implicated in the production of increased voluntary ethanol intake by CIE vapor exposure. $GABA_B$ agonist Baclofen decreased operant ethanol self-administration following CIE (Walker and Koob, 2007), while the benzodiazepine lorazepam and glutamate receptor antagonist MK-801 reduced signs of seizure during the withdrawal phase of CIE, suggesting involvement of GABA and NMDA receptor subunits (Veatch and Becker, 2005). CIE altered NMDAR subunit expression (NR1, NR2A, NR2B) and excitability in the bed nucleus of the stria terminalis (BNST) (Kash et al., 2009) and hippocampus (Nelson et al., 2005; Pian et al., 2010). Hippocampal LTP was transiently blocked after 1 day of withdrawal from CIE, but recovered after 5 days (Roberto et al., 2002). These changes in hippocampal LTP have been shown to involve changes in MAPK activity and sigma-receptor-dependent mechanisms (Roberto et al., 2003; Sabeti and Gruol, 2008).

CIE has also been shown to alter expression and function of thalamic T-type voltage-gated calcium channels (Graef et al., 2010) and to interfere with synaptic plasticity and in nucleus

accumbens (NAC) (Jeanes et al., 2010). Administration of neuropeptide Y (NPY) or antagonists for corticotrophin releasing factor (CRF) receptor or mu opioid receptor attenuated increased drinking following CIE (Gilpin et al., 2008a; Gilpin et al., 2008b). Increased immobility in the forced swim test (FST) produced by CIE was correlated with CRF levels in amygdala and decreased NPY in frontal cortex (Walker et al., 2010). Genomic studies of mice following CIE have shown changes in expression of genes regulated by protein kinase A (PKA), including NPY, in medial prefrontal cortex, NAC, and amygdala, and have demonstrated alterations in transcript abundance that likely affect the insulin/PI3K, NF-KB, and JAK/STAT pathways (Repunte-Canonigo et al., 2007; Repunte-Canonigo et al., 2010). Lesion of the BNST or central nucleus of the amygdala did not prevent increased voluntary ethanol drinking due to CIE (Dhaher et al., 2008), suggesting that multiple brain regions are involved in the behavior.

The CIE model, despite its limitations, has been effectively used to study alcohol dependence processes, and molecular results have fit well with what is known of the neurobiological underpinnings of alcoholism, in addition to providing insight into heretofore unknown players. However, the model is expensive and time-consuming to use, and experimental design is complicated by the number of control groups often needed: air-exposed and home-cage drinkers, as well as vehicle controls when the effects of a drug are studied. In particular the stress of long periods in the vapor chamber must be considered, which is difficult given the inconsistent effects of stress on drinking behavior. Perhaps the most important limitation of the model is the involuntary nature of alcohol exposure, which produces distinct regional activation and transcriptional regulation compared to voluntary exposure. Thus, better animal models of the transition to alcohol dependence are needed.

Intermittent alcohol access (IAA)

In recent years, abstinence-based procedures for producing increased craving for alcohol in 2-bottle voluntary self-administration models have progressed substantially and have been adopted by several laboratories. Scheduled abstinence models have several advantages in validity and experimental utility compared to other methods for producing high or binge-like alcohol consumption in rodents. With this type of model, drinking is voluntary and occurs in the home cage, and water and food are always available. No enzyme inhibitors are required to achieve high blood ethanol concentrations, and the only experimental manipulations performed are single housing and scheduled abstinence. Thus, IAA has significant advantages in validity and feasibility compared to other procedures used to produce increased ethanol craving and dependence-like phenotypes, including vapor-chamber models that make use of enzyme inhibitors and complex experimental designs, experimenter-administered ethanol injections that are stressful to the subjects, and forced consumption through ethanol diet or lack of water availability.

The scientific lineage of intermittent access (IA) models is complex. Multiple lines of investigation have converged in recent years, although significant design differences remain in access schedules, ethanol concentrations offered, and use of baseline access periods. In 2008 Simms and colleagues (Simms et al., 2008) resurrected the IAA model pioneered by Roy Wise decades earlier in rats, and this every-other-day, single-concentration (20% v/v), no-baseline model has been well-characterized in recent years, and extended to mice (Melendez, 2011; Wise, 1973). A further line of research originated in the study of the so-called alcohol deprivation effect (ADE), a well-characterized single-deprivation model, which was extended to a multiple deprivation or intermittent access model in mice in 2006 by Melendez and colleagues, who

allowed simultaneous access to multiple alcohol concentrations for one day per week, after an initial baseline access period (Khisti et al., 2006a; Melendez et al., 2006a).

In ADE studies deprivation occurs after a baseline continuous access period, and elevated intake is transient upon resumption of continuous access. A consensus model has begun to emerge, and studies published in the last 5 years utilizing these types of models have tended to use a single ethanol concentration between 15 and 20% v/v, offer access with water for 24 hours on 3 non-consecutive days per week, do not compare levels to a baseline access period, and make use of a continuous access (CA) group as a time-matched control. Due to experimental limitations rodents are always housed one animal per cage, which has been shown to induce stress and may contribute to the effects produced by intermittent access. It is surprising that concurrent access to multiple alcohol concentrations is rarely used, given the high levels of drinking it produces, and the obvious similarity to human drinking (Bell et al., 2004b; Holter et al., 1998; Melendez et al., 2006a; Obara et al., 2009b; Wolffgramm and Heyne, 1995).

The intermittent alcohol access (IAA) procedure increases ethanol consumption and preference for ethanol over water in a manner thought to model the early stages of the transition from controlled to uncontrolled alcohol consumption that occurs in human alcoholics (Melendez et al., 2006a; Simms et al., 2008; Wise, 1973). Alternating periods of voluntary oral ethanol self-administration and abstinence produce two-fold increases in ethanol consumption and significant increases in preference for ethanol over water in as little as 1 week, which increase over subsequent weeks of IAA to reach binge-like levels of consumption (> 20 g/kg/day) and BEC (> 80 mg/dl). The magnitude of escalation produced by IAA depends on animal strain, ethanol concentrations offered, and access schedule (Crabbe et al., 2012; Khisti et al., 2006a; Simms et al., 2008). Changes indicative of dependence-like phenotypes have been shown after months of

IAA: increased handling-induced convulsions during withdrawal (Hwa et al., 2011) and resistance to decreased alcohol consumption produced by quinine adulteration (Hopf et al., 2011).

Influence of age, genetics, and environment on IAA

The degree to which intermittent access produces binge-like drinking behavior depends on environmental and genetic factors, as well as the developmental stage of the animal utilized. Drinking during adolescence is characterized by binge consumption, which may interfere with brain development (Maldonado-Devincci et al., 2010; Silveri, 2012; Spear, 2000). Interestingly, the IAA model produces greater binge-drinking in adolescent rodents than in adult rodents, but these differences tend to disappear by adulthood. Group-housed adolescent rats drank beer on an IA schedule and achieved high drinking levels of consumption (approximately 8 g/kg/day), which gradually declined as the rats aged, and by postnatal day 55 mean consumption was approximately 4 g/kg/day, and did not differ from intake in adult rats with continuous access.

Adolescent rats under intermittent access conditions drank more beer than adolescents with continuous access, and more than adult rats under intermittent access (Hargreaves et al., 2009b). Adolescent C57BL/6J mice under IA conditions increased intake more quickly and by a greater relative amount, compared to adult mice (Melendez, 2011). Adolescent mice displayed significantly decreased water intake from session 2, whereas adult mice did not show this effect until session 5. This effect seems to be a later consequence of intermittent alcohol access that reflects a loss of interest in fluids that do not contain alcohol. More study is needed to determine the role of adolescent IAA in adult drinking behavior, to determine the extent of similarity between binge-drinking in rodents and humans, and thus the utility of the model for study of early intervention to prevent the development of AUDs. Differences in the effects of adolescent

binge-drinking on drinking in adulthood are likely caused by social and cognitive factors that may be difficult or impossible to model in rodents, such as peer pressure.

Few studies have examined the role of stress and anxiety in the IAA model, despite the established role of CRF signaling in IAA drinking behavior, and the utility of CRF antagonists to reduce binge-like consumption in dependent and non-dependent animals (Finn et al., 2007; Hwa et al., 2011). Furthermore, AUDs have shown significant comorbidity with anxiety disorders such as post-traumatic stress disorder (PTSD) in epidemiological studies, and alcohol is used by humans as an anxiolytic (Coffey et al., 2010). In rodents, foot-shock procedures induce anxiety-like behavior such as conditioned freezing and reduced exploration. Stress induced by foot-shock in Long-Evans rats produced elevated drinking behavior in the IAA model, but only when given prior to the start of drinking. Shocked rats showed elevated drinking for the initial 70-day IAA period, and for a further 20 days following a 40 day withdrawal period (Meyer et al., 2013). Thus there seems to be a direct relationship between certain stressors and binge-like drinking behavior produced by IA, but the fact that stress did not alter previously established drinking patterns produced by IA is intriguing. This lack of effect suggests that behavior produced by IAA is relatively inflexible, which is a characteristic of drinking in human alcoholics, who drink despite negative consequences and have difficulty moderating intake.

Juvenile social isolation is also known to increase anxiety-like behaviors and ethanol consumption. Social isolation in rats during adolescence increased anxiety-like behavior in the elevated plus maze, and increased drinking in the IAA model, compared to group-housed rats (Chappell et al., 2013). Furthermore, these measures were significantly correlated across individual animals in the socially isolated group and in group-housed rats. Maternal separation is an early life stressor with lasting neurobiological and behavioral consequences that include

altered opioid signaling, increased alcohol consumption, and altered dopamine D2 receptor density in the VTA (Cruz et al., 2008; Li et al., 2013). Maternal separation during the period from postnatal day 1 to 20 has been shown to decrease the escalation of 5% alcohol consumption in adult Wistar rats (Daoura et al., 2011), but the use of a low alcohol concentration, and the lack of effect in rats with normal upbringing, may limit generalization to other versions of the model. Maternal separation also influences the efficacy of naltrexone for reducing binge-drinking during adulthood (Daoura and Nylander, 2011). Naltrexone had no effect in rats reared under normal conditions, and dose-dependently decreased intake of 5% and 20% alcohol in rats with extensive maternal separation. Interestingly, in rats with shorter periods of maternal separation naltrexone reduced intake of 5% alcohol, but increased preference for 20% alcohol. These results suggest a complex link between early life stress, opioidergic neurotransmission, and adult binge-drinking behavior that should be further explored, and the results of which could inform current treatment strategies in humans.

Inbred animal strains of the type used for nearly all behavioral research display a wide range phenotypes for drinking and other alcohol-related behaviors. Alcohol consumption varies from strain to strain under the influence of genetics, and among individuals under the influence of epigenetics and environment (Crabbe et al., 2012; Crabbe et al., 2009; Khisti et al., 2006a; Metten et al., 2010; Wise, 1974; Wolstenholme et al., 2011). Much effort has been devoted to study of the influence of genetic background on drinking behavior induced by intermittent access, with the goal of correlating differences in alcohol intake to genetic, epigenetic, and phenotypic factors. In IAA behavior the animal supplier used for drinking studies has been shown to affect drinking behavior. In Wistar rats from 5 suppliers, significant differences in drinking for 5% ethanol, with fewer differences for 20% ethanol were observed; response to

intermittent access varied greatly across the strains, and no strain displayed the elevated intake typically produced by IAA in other strains (Palm et al., 2011).

Intermittent access has been shown to increase alcohol consumption and preference in several strains of mice, although the magnitude of the effect varies. Interestingly, the IAA procedure produced similar escalation in drinking behavior across mouse strains bred for divergent ethanol-related behavior (Rosenwasser et al., 2013). Mice bred for binge-drinking and sensitivity to withdrawal-related seizures showed no difference compared to their respective genetic background strains. IAA using a shortened 4-hour access period did not produce elevated ethanol intake in C57BL/6J or HDID (High Drinking-In-the-Dark) mice, and a 24-hour access period produced this effect in C57BL/6J mice only (Crabbe et al., 2012). It is surprising that the IAA model seems to represent an aspect of alcohol-related behavior controlled by distinct genetic mediators as compared to withdrawal sensitivity and binge-drinking, when it is a model that produces precisely these effects in mice. However, the preponderance of evidence shows that binge-like drinking behavior may be altered by a wide range of pharmacological and genetic manipulations. Any of the molecular targets of these manipulations, or genes with as yet unknown relationships to alcohol behavior, could be responsible for the trait produced in the selectively-bred mice. That is, selective breeding for a trait may produce a phenotype through genetic mediators with no similarity to those that produce the trait in uncontrolled settings, and it is thus important to be skeptical regarding neurobiological and behavioral conclusions drawn through its use.

Ethanol drinking behavior in rodents is extremely sensitive to environmental change, and particularly to changes in the personnel attending to the animals. A recent IAA study in the Miles laboratory (unpublished) examined drinking behavior in 7 strains chosen from the Hybrid Mouse

Diversity Panel group, which were selected to represent a wide range of CA drinking behavior. In this study a change in personnel was found to decrease combined group mean alcohol intake more than did the initial intra-peritoneal saline injections given by the new experimenter (Miles lab, unpublished data). The initial change in drinking was due only to indirect interaction with the mice; the experimenter did not handle the mice until 1 week after initial exposure, when saline injections were begun.

Although IAA immediately elevates alcohol intake, there is mounting evidence that after weeks or months the procedure causes further behavioral and molecular changes that reflect the transition to alcohol dependence. These changes suggest a loss of interest in alternative reinforcers and the development of compulsive and inflexible drinking behavior, which are characteristics of human alcoholics. Six weeks of IA to 20% alcohol led to maintained consumption despite quinine adulteration, and this effect was not shown by CA animals. Furthermore, long-term IA caused impairment in the Rota-rod task compared to CA animals (Loi et al., 2010). Three to four months, but not 1.5 months, of IA using a hybrid model with operant self-administration and home-cage intermittent procedures prevented reduction of progressive ratio breakpoints by quinine adulteration (Hopf et al., 2010). Extended IAA also reduced the effect of quinine adulteration on home-cage drinking, and motivation for sucrose adulterated by quinine was not altered. Furthermore, quinine taste preference was not altered by extended IAA, which showed that this procedure did not seem to alter taste preference for bitter solutions.

Significant differences from continuous access rats were only observed for the first 8 weeks in alcohol-preferring Indiana University P rats offered concurrent access to 15% and 30% alcohol on an intermittent schedule (Obara et al., 2009a). However, IA rats drank significantly less water than CA rats after the 5th week, and showed differences in abundance of several proteins

involved in glutamatergic signaling in the nucleus accumbens and central amygdala. Protein levels of mGluR5 in NAc core were elevated by 24h withdrawal in IA and CA rats, but after 4 weeks of withdrawal this protein remained elevated in IA rats, and had returned to normal in CA rats. In CeA mGluR5 is upregulated after 24h withdrawal in IA rats, but not at any withdrawal time point in CA rats. These results suggest that in NAc and CeA alcohol alters glutamatergic signaling via NMDA receptors similarly in IA and CA rats, but that long-term IA causes persistent regulation of metabotropic glutamate receptor signaling in a manner distinct from CA. Thus long-term IAA seems to engender a second set behavioral and neurobiological changes distinct from the immediate increase in drinking, and these changes, which include inflexible behavior and alterations in glutamatergic signaling, are consistent with the transition to alcoholism in humans.

Neurobiology of Intermittent Alcohol Access

Escalation induced by IAA is attenuated by naltrexone and acamprosate, and thus seems to represent a valid model for the study of other compounds with potential to treat AUDs (Simms et al., 2008). A large number of studies in recent years have focused on study of the neuromolecular systems altered by IAA, changes in which are associated with increased craving and binge-like drinking. Typical experimental strategies toward this end are the use of ligands and knockout mice to alter IAA behavior, and use of molecular methods to study the effects of IAA on neurotransmission, neuronal excitability, mRNA transcription, and protein abundance.

The most extensively studied molecular mediators of the effects of IAA on behavior are the transcription factor deltaFosB, glial cell line-derived neurotrophic factor (GDNF) and the corticotropin-releasing factor/hormone (CRF/CRH) system, but perturbation of other neurotransmitter systems has been shown to modulate effects of IAA. In recent years several

laboratories have explored a diverse array of ligands for the potential to alter binge-like drinking behavior, and each of these compounds represents a potential treatment vector for AUDs (Ahmadiantehrani et al., 2013; Carnicella et al., 2009a; Hopf et al., 2011; Hwa et al., 2013; Li et al., 2011a; Moorman and Aston-Jones, 2009a; Sabino et al., 2013; Sajja and Rahman, 2013; Wen et al., 2012; Yardley et al., 2012). Some of the drugs effective in reducing IAA are already approved by the FDA for use in humans, and further study is needed to determine which vectors represent the most high priority targets for further preclinical and clinical research.

Recent studies do not agree on the role of CRF in binge-like drinking behavior. CRF is produced by the paraventricular nucleus (PVN) of the hypothalamus in response to stress, and is trafficked to the pituitary gland, where it causes the release of adrenocorticotropic hormone (ACTH), which stimulates glucorticoid release from the adrenal glands. CRF acts at 2 subtypes of CRF receptor: CRHR1 and CRHR2. The observation that CRF-R1 antagonists attenuate elevated ethanol consumption produced by chronic ethanol vapor exposure led to its study in IAA. Taken together the data thus far indicate that CRHR1 antagonism attenuates binge-like drinking behavior produced by IAA, but this effect may be dependent on strain and age.

Crucially, significant differences have been observed regarding the role of the CRF system in animals on IA and CA alcohol drinking schedules, suggesting that the system mediates maladaptive drinking in a manner distinct from controlled drinking, and is thus a promising therapeutic candidate. The CRF-R1 antagonist CP-376395 reduced consumption of 20% ethanol in Long-Evans rats on an intermittent schedule, but had no effect on rats offered continuous access. CRF-mediated signaling in the hypothalamus of IA rats was decreased compared to CA rats, which suggested that CP-376395 effects on drinking behavior are mediated by extrahypothalamic mechanisms (Simms et al., 2013).

The CRHR1 antagonist antalarmin reduced alcohol consumption for 20% ethanol under intermittent and continuous access schedules, but reduced consumption of 10% ethanol under the intermittent schedule only (Cippitelli et al., 2012). In Sardinian alcohol-preferring rats, binge-like drinking produced by IAA was suppressed by naltrexone and dopamine D1 receptor antagonist SCH 39166, but not by CRF1 antagonist R121919. Thus in sP rats, the effects of IAA appear to be mediated by the opioid and dopamine neurotransmitter systems, but not by the action of corticotropin releasing factor on CRF1R (Sabino et al., 2013). This result underscores the fact that alcohol preference and high levels of consumption may be mediated by distinct genetic factors across strains, and that study of IAA in multiple animal models is important.

The effects of CRHR1 on binge-like drinking are likely mediated by the VTA, but other regions may be involved (Hwa et al., 2011). In Long-Evans rats and C57BL/6J mice intra-VTA CRHR1 antagonism selectively decreased alcohol intake elevated by IAA, and intra-DRN CP-154,526 administration reduced intake of ethanol and water. Individual animals with relatively high alcohol intake were more severely affected by CRHR1 antagonism than animals with relatively low intake. Effectiveness in the VTA, but not the dorsal raphe nucleus, implied that dopaminergic inputs to the nucleus accumbens were more involved in maintenance of IAA behavior than were serotonergic projections from DRN to amygdala and striatum. In adolescent rats, a hybrid operant self-administration IAA model reduced CRF cell counts in the central amygdala and increased adult drinking during intermittent access to alcohol, but not constant access to alcohol. Rats with a history of binge drinking in adolescence also showed decreased anxiety-like behavior in the elevated plus maze, but this difference was abolished by a history of dependence induction with ethanol vapor (Gilpin et al., 2012). Thus, the CRF system appears to be regulated by IAA and to mediate some aspects of IAA drinking behavior.

DeltaFosB (ΔFosB),a persistent form of the Fos family of transcription factors that is associated with addictive behavior (Krasnova et al., 2013; McClung and Nestler, 2003; Pitchers et al., 2013). ΔFosB accumulates in neurons over time in response to cocaine and other drugs of abuse, and eventually becomes the predominant form of Fos. Targets of ΔFosB transcription include dynorphin, the GluR2 AMPA glutamate receptor, Cdk5, NFκB, and c-Fos (Robison and Nestler, 2011). Changes in the function of such ΔFosB targets in the nucleus accumbens seem to enhance the reinforcing properties of some drugs of abuse, and contribute to dendritic remodeling and other neuroplastic processes (Robison et al., 2013). Thus ΔFosB appears to play the role of a molecular switch involved in the initiation and maintenance of motivated behavior for drugs of abuse, as well as for food and natural reward (Pitchers et al., 2013).

In an early IAA publication Li and colleagues showed that Sprague-Dawley rats exposed to alcohol 15 times over 35 days on a 3-day-per-week IAA schedule significantly elevated their consumption over 2 to 3 sessions, and increased alcohol consumption due to IAA was associated with significantly increased ΔFosB levels in the nucleus accumbens core and the dorsolateral striatum, but not in the nucleus accumbens shell or the dorsomedial striatum. Similar effects were observed in the prefrontal cortex, where ΔFosB immunoreactivity was increased in the ventral orbitofrontal cortex, but not in the medial prefrontal cortex. Acute naltrexone (2 mg/kg I.P.) treatment attenuated the effects of IAA on drinking behavior, and reduced ΔFosB levels in nucleus accumbens core, dorsolateral striatum, and orbitofrontal cortex compared to IAA rats given saline (Li et al., 2010a). The mechanism by which naltrexone interferes with ΔFosB accumulation seems to be downstream of opioid receptors, because treatment with Cytisine, a nAChR antagonist, reduced alcohol intake of C57BL/6J mice in constant access and IAA

drinking, and attenuated ΔFosB accumulation in the dorsal and ventral striatum following drinking (Sajja and Rahman, 2013)

Interestingly, further studies examined the effects of electroacupuncture on drinking behavior produced by IAA and regional ΔFosB accumulation, and found that electroacupuncture at a particular point in rats at 100Hz, but not 2Hz, decreased consumption and preference induced by IAA. This decrease was maintained for 72 hours following treatment, and intake of sucrose was unchanged. ΔFosB levels in the prefrontal cortex, striatum, and VTA were elevated after binge-like ethanol consumption due to IAA, but these effects were abolished by 6 days of electroacupuncture treatment. (Li et al., 2012b). These studies demonstrate that regional ΔFosB accumulation is an important mediator of behavioral changes produced by IAA, and that diverse methods of therapeutic intervention are effective at reducing ΔFosB levels and maladaptive drinking behavior.

Naltrexone has been shown to be effective at reducing IAA-induced drinking, but it is a nonselective opioid ligand, and the relative contribution of each opioid receptor subtype to its effects has not been determined. While the contribution of kappa and mu type opioid receptor subtypes to IAA behavior remains unclear, the delta opioid receptor seems to be involved. In Long-Evans rats, intermittent alcohol access increased δ-opioid receptor (DOR) function in the dorsal striatum compared to CA and water-drinking rats. This upregulation led to persistent DOR-mediated analgesia in adulthood, when this effect is normally seen in young animals only. Administration of the DOR antagonist naltrindole, both systemic and into the dorsal striatum, decreases ethanol consumption and DOR activity in the dorsal striatum (Nielsen et al., 2012). Thus DOR activity in the dorsal striatum seems to play a role in binge-like drinking induced by IAA.

Unlike other drugs of abuse, alcohol has caloric value, and thus drinking behavior is connected to hunger and satiety more directly than is self-administration behavior for other drugs of abuse. In the brain, satiety is partially mediated by the action of glucagon-like-peptide-1 (GLP-1), which has receptors in the VTA and NAc, and modulates food intake through the mesolimbic dopaminergic system (Dickson et al., 2012; Dossat et al., 2011). Administration of Glucagon-like-peptide and analogs reduced intake in the IAA model, and blockade of the GLP-1 receptor exacerbated the effects of IAA. Microinjection studies indicated that this effect was produced by action in the VTA, although other regions were not studied (Shirazi et al., 2013). Furthermore, GLP-1 agonist Exendin-4 reduced ethanol-induced dopamine release in the nucleus accumbens of mice, and altered several ethanol-related behaviors. Exendin-4 abolished conditioned place preference for alcohol, reduced alcohol-induced locomotor activity, and reduced consumption of 20% ethanol in the IAA model (Egecioglu et al., 2013). Mesolimbic signaling through GLP-1receptors thus represents an important mediator of the acute behavior effects of alcohol and of escalation of drinking behavior due to IAA, and a promising therapeutic candidate.

Some further evidence for a role of processes involved in food reward in IAA binge-like drinking was provided by the studies of Moorman and Aston-Jones (2009). Rats exposed to IAA showed significantly greater preference for ethanol than sucrose-faded rats, and in both groups the orexin-1 receptor antagonist SB-334867 reduced preference selectively in rats with high ethanol preference (Moorman and Aston-Jones, 2009b). Orexin is produced only by a subset of several thousand hypothalamic neurons, although their axons extend throughout the CNS, where they exert a primarily excitatory influence. This result suggests that the orexin system, which is associated with appetite and arousal, is causally related to ethanol preference.

Alcoholism is associated with inhibited inhibitory signaling in the brain, which, with elevated glutamatergic signaling, contribute to hyperexcitability and seizures associated with withdrawal (Caputo and Bernardi, 2010; Hughes, 2009). Therefore the role of inhibitory signaling has been investigated in IAA drinking behavior. Intake of ethanol, but not sucrose or water, was reduced by infusion of glycine into the VTA of rats under IA and CA schedules. Glycine also reduced responding for ethanol under operant self-administration conditions, and these effects were mediated by strychnine-sensitive glycine receptors (Li et al., 2012a). In Long-Evans rats, IAA upregulates GABAA receptor signaling in the hypothalamus, which is associated with lowered HPA axis function and blood corticosterone levels. Administration of GABAA antagonist picrotoxin into the paraventricular nucleus of IAA rats reduced alcohol intake and increased blood corticosterone levels. This effect was specific to alcohol over sucrose, and blocked by GABAA antagonist muscimol (Li et al., 2011a). Therefore it is clear that IAA alters inhibitory signaling through receptors for GABA and glycine, and the IAA model is a useful test for effectiveness of drugs for alcoholism that act on these systems.

Further evidence for changes in excitability due to IAA was provided by studies on potassium channels involved in action potential regulation. Alcohol is known to alter function of SK-type potassium channels in limbic areas, and Chlorzoxazone, an activator of these channels, selectively and dose-dependently reduced alcohol consumption in rats exposed to intermittent, but not continuous, alcohol access (Hopf et al., 2011). This effect was partially explained by changes in excitability of nucleus accumbens core medium spiny neurons. These core neurons showed increased firing, reduced control of SK-channels over firing, and more potent Chlorzoxazone-induced inhibition of firing, compared to neurons from rats with continuous alcohol access. Chlorzoxazone is an FDA-approved muscle relaxant and represents a high

priority target for further preclinical and clinical study as a treatment for AUDs. These results implicate changes in NAc excitability in the control of binge-like drinking behavior.

In a surprising result that further implicated changes in excitability and neurotransmission, the antiparasitic agent Ivermectin was shown to interfere with alcohol-related behavior in a dose-dependent manner (Yardley et al., 2012). In arthropods, Ivermectin acts at glutamate-gated chloride channels, but in mammalian cell culture the drug blocked ethanol-induced inhibition of P2RX4 (Purinergic receptor P2X, ligand-gated ion channel, 4) function. P2RX4 is a ligand-gated cation channel with high calcium permeability that is activated by ATP and involved in depolarization (Koshimizu et al., 2000; Shigetomi and Kato, 2004). Interestingly, P2X4R activation has been linked to BDNF release from microglia, and increased BDNF is known to reduce alcohol drinking and alter the rewarding properties of alcohol (Bahi and Dreyer, 2013; Ulmann et al., 2008). Systemic Ivermectin reduced alcohol consumption in a 2-bottle-choice voluntary drinking model and a 4-hour limited access model. The drug also reduced operant alcohol self-administration, and the authors showed that significant reductions in intake corresponded to peak Ivermectin levels in the brain. Ivermectin is therefore an intriguing therapeutic candidate, with a mechanism of action supported by the literature that has not been pharmacologically exploited.

Despite the established role of transcriptional regulation mediated through cyclic AMP-responsive element binding protein (CREB) in alcohol drinking and addiction to other drugs of abuse, the role of cyclic AMP (cAMP) signaling and CREB in IAA drinking behavior has not been well established (Green et al., 2006; McClung and Nestler, 2003; Misra and Pandey, 2006; Misra et al., 2001; Pandey, 2004b; Robison and Nestler, 2011; Uddin and Singh, 2007). Interference with cAMP signaling using the phosphodiesterase-4 inhibitor Rolipram reduced

operant responding for ethanol, as well as continuous access and intermittent access drinking. Interference with phosphodiesterase-4 function raises cellular cAMP levels and thus may activate CREB signaling and alter transcription of genes involved in drug-related behavior, such as BK-type calcium-activated potassium channels and BDNF (Wen et al., 2012). Further study of the role of cAMP signaling and CREB-mediated gene expression in IAA will illuminate the transcriptional mechanisms that mediate changes in behavior, and establish the similarities and differences of IAA and other preclinical models of addictive behavior.

Other than those examining ΔFosB, relatively few studies have examined the role of regulation of particular mRNA transcripts in IAA behavior. Transcript abundance of *Prkcz*, which in the brain encodes primarily for protein kinase M zeta (PKMζ), is increased by ethanol exposure, and is correlated with ethanol consumption in mice (Mulligan, 2006; Mulligan et al., 2011). Furthermore, PKMζ has been shown to be involved in maintenance of long-term memories, including memories of cocaine and morphine reward that cue reinstatement of drug-taking behavior (Li et al., 2011c; Shabashov et al., 2012), Knockout of the *Prkcz* gene in mice reduced intermittent access drinking, but not constant access drinking or drinking-in-the-dark. IAA drinking increased PKMζ protein in the ventral striatum of wild-type littermates, and ethanol up-regulated Prkcz transcript in cultured PC12 cells (Lee et al., 2013). These results are important because they highlight differences in neurobiology associated with drinking behavior in models that produce binge-like drinking. Specifically, the IAA model appears to produce binge-drinking through mechanisms that are more similar to those involved in relapse behavior for other drugs of abuse than does the drinking-in-the-dark model. The IAA model may therefore be considered as one of repeated abstinence and relapse, supporting validity for representing behavior in alcohol humans.

Several recent studies have focused on the role of glial cell line-derived neurotrophic factor (GDNF) as an ethanol-responsive gene and negative regulator of IAA drinking behavior, as well as the role of dopaminergic signaling in the NAc and VTA. GDNF is a growth factor that is required for the development and function of midbrain dopaminergic neurons (Airaksinen and Saarma, 2002). Following GDNF binding to GDNF family receptor alpha-1 (GFRA1), Ret tyrosine kinase is activated, which leads to transcriptional regulation and increased activity of dopaminergic neurons (Yang et al., 2001). The primary site of GDNF production is the nucleus accumbens, from which it is retrogradely transported to the VTA, where it alters gene expression and excitability of dopaminergic neurons of the mesolimbic reward system(Wang et al., 2010).

Immediately following an IA consumption period dopamine levels in NAc begin to fall, and differ significantly from levels in water drinkers after 1 hour of withdrawal. Decreased NAc dopamine is maintained for at least 24 hours in abstinent rats, and intra-VTA GDNF increased NAc dopamine to normal levels (Barak et al., 2011). Furthermore, intra-VTA GDNF reduced ethanol intake in the IAA model, operant responding for ethanol, and relapse (Carnicella et al., 2009b). One week of IA elevated GDNF levels in VTA, but after 7 weeks of IA this effect was no longer present. Following 7 weeks of IAA, an abstinence period of 24 hours reduced GDNF levels, which were in turn elevated by a subsequent period of alcohol exposure and binge-like drinking. Rats that drank low amounts of alcohol during this post-deprivation binge showed greater GDNF elevation than did rats that drank greater amounts of alcohol (Ahmadiantehrani et al., 2013). These findings suggest that GDNF represents a potential treatment for alcoholism, and that GDNF tone and responsiveness to alcohol contribute to individual variation in binge-like drinking.

Other studies have examined the effects of IAA on neuronal signaling, connectivity, and excitability. Intermittent access to 20% alcohol in rats enhanced postsynaptic AMPA glutamate receptor function and spontaneous tonic glutamate release, but did not alter evoked presynaptic glutamate release (Stuber et al., 2008). In Wistar rats 7 weeks of IAA altered endocannabinoid and GABAergic signaling in the dorsolateral striatum (Adermark et al., 2011). Endocannabinoid signaling was inhibited at a level downstream from the CB1 receptor, and was accompanied by reduced inhibitory control of striatal output mediated through GABAA receptors. Interestingly, similar effects were seen in age-matched control animals that had received no experimental manipulation other than being housed one rat per cage. This effect suggests that social isolation may contribute to some of the changes in signaling that occur during IAA, and demonstrates the need for more sophisticated experimental techniques that do not require single housing.

Examining Fos expression in medial prefrontal cortex (mPFC), central amygdala (CeA), hippocampus, and NAc, indicated that IAA impairs inhibitory control of the mPFC on the CeA to produce changes in drinking behavior and cognitive impairment (George et al., 2012). Abstinence during IAA activated mPFC, and the number of Fos-positive neurons was significantly correlated with impairment of working memory in the Y-maze and binge-like drinking behavior. Interestingly, 2 hours of alcohol access normalized numbers of Fos-positive neurons to control levels, suggesting that craving induced by IA is at least partially due to altered mPFC function. Differences in regional connectivity between prefrontal cortex, nucleus accumbens, and amygdala were induced by intermittent alcohol access as compared to constant access. In mPFC the authors showed that abstinence from IA increased numbers of CRF-positive neurons, and activated GABAergic interneurons. As a correlate to altered PFC functioning, IA rats displayed impaired working memory after 24h of withdrawal, but not immediately following

alcohol access. CA rats showed no deficits in working memory during immediate withdrawal, and no group differences were observed after 50 days of withdrawal. Taken together these data suggest that IAA activates CRF and GABA neurons in the mPFC during withdrawal, and that changes in PFC/CeA connectivity impair executive control over drinking behavior and working memory. Furthermore, activation of mPFC and CeA during abstinence seems to be predictive of subsequent binge-like drinking behavior, and may represent an early test for the propensity to develop AUDs.

Despite extensive data supporting IAA as a model for the transition to alcoholism, little effort has been aimed at elucidating transcriptional regulation occurring over time that is associated with the development of binge-drinking. Changes in mRNA abundance can provide insight into the mediators of neuroplasticity associated with changing behavior, and thus potential therapeutic targets for future study. Genomic study using DNA microarray technology allows for the unbiased examination of thousands of mRNA transcript species at once. Patterns of gene expression can then be associated with phenotypic data and experimental manipulation, such as a disease state or drug response. Because differences in gene expression mediate the effects of genotype on behavior, studies of this type can provide novel mechanistic information and inform hypothesis generation for future experimentation (Abiola et al., 2003; Kerns et al., 2005; Miles and Williams, 2007b; Mulligan et al., 2008a; Trabzuni et al., 2013; Wolen and Miles, 2012).

Alcoholism and the response to alcohol in the CNS have been extensively studied using genomic methods, because alterations in transcription caused by alcohol and other drugs of abuse are thought to underlie neuroplasticity that leads to addiction (Mulligan, 2006; Mulligan et al., 2008b; Mulligan et al., 2011; Nestler and Aghajanian, 1997). Experimental approaches used to study the relationship of alcohol and gene expression in animal models have tended to use two

approaches, which are sometimes combined: study of alcohol-induced changes in gene expression, and study of differences in basal gene expression across strains of mice with divergent alcohol-related behavior (Kerns and Miles, 2008; Singh et al., 2007; Xu et al., 2001). Studies have generally produced results consistent with knowledge of the neurobiological underpinnings of alcohol-related behavior, such as alterations in glutamatergic signaling, but have also been used identified novel players in those processes (Bowers et al., 2006).

Alcohol causes persistent changes in gene expression that are apparent within 4 hours of exposure (Miles et al., 1991), and differences in the transcriptional response to ethanol seem to contribute to the genetic heritability of alcoholism (Chesler et al., 2005; Schadt et al., 2003). Expression profiling in whole brain of C57BL/6J and DBA/2J mice given a high dose of ethanol identified ethanol-responsive genes gene expression, cell signaling, and response to stress (Treadwell and Singh, 2004). Interestingly, although these strains show divergent ethanol-related behavior, only a small subset of 16 transcripts were differentially regulated in the two strains by alcohol, which was attributed to the use of whole brain tissue, rather than a particular region (Crabbe and Belknap, 1993; Crabbe et al., 1998; Goldstein, 1973; Phillips et al., 1994) In recent years most studies have opted for a regional approach for the study of ethanol-induced gene expression and the contribution of basal transcript abundance to ethanol-related behaviors. Regional approaches allow for more refined hypothesis generation through association of expression and behavioral data in the context of regional roles in behavior.

Recent studies have shown that ethanol-responsive gene expression varies with brain region and cell type (Kerns, 2005; Mulligan et al., 2011; Ozburn et al., 2012; Ponomarev et al., 2012). In regions of the mesolimbic dopaminergic reward pathway, ethanol has been found to alter transcription of genes associated with distinct functional areas: BDNF signaling in the NAc,

myelination in the PFC, and retinoic acid signaling in the VTA (Kerns, 2005). Other studies have found region-specific differences in alcohol-responsive gene expression in the NAc shell and core, subregions of the amygdala, striatum, hippocampus, cerebellum, and other regions (McBride et al., 2010; Vanderlinden et al., 2013). Regional gene expression has been shown to be significantly correlated with alcohol drinking across strains and individuals (Mulligan et al., 2011; Wolstenholme et al., 2011). Interestingly, distinct changes in gene expression have been associated with drinking behavior, operant self-administration, and experimenter-administered alcohol, suggesting that the method of alcohol administration strongly influences its effects on gene expression (McBride et al., 2010; Rodd et al., 2008). Studies such as these, which examine the acute transcriptional response to alcohol, are especially useful for determining the mechanisms through which alcohol exerts its initial effects on neurons, and the immediate consequences of this action.

However, the primary purpose of preclinical study of alcohol in animal models is to better understand alcoholism in humans, which is characterized by binge-drinking, dependence and withdrawal, and in recent years some progress has been made toward understanding the contribution of transcriptome alteration to the development of dependence over time. The chronic intermittent ethanol (CIE) vapor model induces cycles of dependence of withdrawal, and leads to progressive increases in ethanol consumption similar to that seen in human alcoholics (Lopez and Becker, 2005; O'Dell et al., 2004). In the CIE model changes in drinking behavior are associated with changes in gene expression in cortex and amygdala for genes involved in synaptic plasticity and glutamatergic neurotransmission, which are processes involved in neuroplasticity leading to alcoholism (Rimondini et al., 2002). The utility of this type of study was further illustrated by the confirmation of the role of metabotropic glutamate receptor

signaling in nucleus accumbens in drinking produced by intermittent alcohol access (IAA) (Meinhardt et al., 2013; Obara et al., 2009a).

No preclinical model can serve as a fully valid representation of the human disease of alcoholism, and even models that appear to involve similar behavior can produce significantly different regional changes in gene expression. For example, the DID model and the IAA model produce binge-like drinking, but transcription in PFC induced by IAA was more closely correlated with changes induced by lipopolysaccharide treatment than with those induced by DID (Osterndorff-Kahanek et al., 2013). It is therefore important to apply genomic study of the ethanol transcriptome across preclinical models of alcohol drinking and other alcohol-related behaviors, to achieve the most complete understanding of alcoholism and progress toward its successful treatment.

Therefore, herein are reported the results of studies performed with the goal of better understanding genetic and environmental factors that contribute to binge-like drinking behavior produced by scheduled abstinence in the EDE and IAA models, and the transcriptional mechanisms operating in the nucleus accumbens that lead to the development of this behavior over time. These results should contribute to basic understanding of addictive behavior and of alcoholism, and provide novel targets for the study of therapeutic intervention in alcohol use disorders. The guiding hypothesis for these studies was that scheduled abstinence in mice produces regional changes in gene expression in the brain, which mediate changes in alcohol drinking behavior over time.

Chapter 3. Genomic analysis of the alcohol deprivation effect identifies alterations in mesocorticolimbic gene expression functioning in neuroplasticity

Introduction

Alcoholism is a strongly heritable disorder characterized by recurrent relapse despite severe social and biomedical consequences, and availability of several pharmacological and psychiatric treatment strategies (Garbutt, 2009). Even the most effective treatments rarely produce long-term abstinence (Krampe et al., 2006; McKay, 2006). Chronic ethanol exposure produces behavioral changes that are thought to be mediated in part by gene expression alterations in discrete neuronal populations (Vengeliene et al., 2009; Vilpoux et al., 2009). These changes gradually produce a state of dependence, during which ethanol withdrawal is associated with severe dysphoria that is thought to contribute to relapse and progressive consumption (Breese et al., 2011; Powell, 1999; Valdez et al., 2002). Moreover, alcoholics are prone to relapse long after halting abusive ethanol intake, due perhaps to neuroplasticity evoked by chronic ethanol exposure (Heinz et al., 2009; Heinz et al., 2005; Vollstadt-Klein et al., 2011).

Several prior studies have identified changes in gene expression in response to ethanol administration or basal gene expression correlated with ethanol consumption. Such transcripts regulated in brains of humans and animals provide insight into the nature of neuroplasticity occurring with repeated exposure (Goldowitz et al., 2006; Kerns, 2005; Mulligan, 2006; Uddin and Singh, 2007; Wolstenholme et al., 2011). However, these studies have not addressed

molecular events that might be specifically associated with escalation of ethanol consumption following a period of abstinence, or mechanisms underlying relapse drinking behavior.

The alcohol deprivation effect (ADE) is an increase in ethanol consumption and preference following abstinence in animal models of voluntary self-administration, and has been demonstrated using two-bottle-choice and operant self-administration procedures (Sparta et al., 2009; Wise, 1973). Although the ADE is not a model of alcohol dependence, it is considered to model increased craving for ethanol following abstinence in human alcoholics, because it is attenuated by naltrexone and acamprosate (Heyser et al., 2003). The ADE has been demonstrated in rodents and primates under various experimental parameters, including varied ethanol concentrations and lengths of deprivations, and has been shown to be specific for ethanol over sucrose and water (Bell et al., 2004a; Khisti et al., 2006b; McBride et al., 2002; Vengeliene et al., 2006). Repeated deprivations of 1 to 6 days on various schedules produce gradual, but significant, increases in ethanol consumption and preference over a period of several weeks in ethanol-preferring animals. The ADE may thus model events related to recidivism following abstinence and the transition from casual use to abuse, addiction and dependence (Hopf et al., 2010; Melendez et al., 2006a).

The current study performs a detailed genomic analysis of changes in the brain transcriptome of C57Bl/6NCrl mice after 4 days of deprivation from *ad libitum* ethanol access in a two-bottle choice model, a time point at which reinstatement of alcohol access produces significant increases in ethanol consumption and preference compared to baseline, and to non-deprived animals. Microarrays and bioinformatics analysis were used to assess changes in gene expression associated with ethanol deprivation in nucleus accumbens (NAC), prefrontal cortex (PFC) and ventral midbrain (VMB), regions of the mesocorticolimbic dopaminergic pathways that have

been shown to be important in reward and motivated behavior (George and Koob, 2010). Our results showed significant gene regulation by the ADE, particularly in NAC, as compared to PFC and VMB. Bioinformatics analysis revealed a striking enrichment for genes involved in neuronal plasticity and ion channel function in NAC, particularly regarding calcium signaling. Our results provide novel insight into molecular adaptations occurring with the ADE and identify novel targets for preclinical study of alcohol-related behavior and pharmacotherapy.

Materials and Methods

Animals

39 male C57BL/6NCrl mice were obtained from Charles River Laboratories (Wilmington, MA) at 60 – 80 days of age. This substrain of C57BL/6 mouse was used since we had previously demonstrated that they show a robust ADE (Khisti et al., 2006b). Mice were given *ad libitum* access to standard chow (Harlan TekLad#7912, Madison, WI) and water throughout the experiment and were housed individually in an AAALAC-approved colony room under a 12hr/12hr light/dark cycle with weekly cage and bedding (Harlan Sani-chips, #7090A, Harlan TekLad, Madison, WI) changes. All procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University Medical Center and conformed to the *NIH Guide for the Care and Use of Laboratory Animals.* Mice were acclimated to the colony for one week before experimentation. Mice were separated into 3 groups: ethanol deprivation/sacrifice (N = 12), ethanol deprivation/reinstatement (N = 18), and water only (N = 9).

Two-bottle choice drinking

Mice in the ethanol deprivation group (N = 30) were allowed access to tap water and 10% w/v ethanol in tap water for 18 hours/day, and to water only for the remaining 6 hours as described previously (Khisti et al., 2006b). Bottle positions were alternated every 2 days to control for bias. Mice were given access to ethanol for 13 consecutive days prior to a single 4-day deprivation period, and 12 mice were sacrificed on the final day of deprivation for microarray studies. The remainder of the ethanol deprived mice (N = 18) were allowed continuous access to alcohol after the 4-day deprivation period to confirm the EDE and measure the transience of increased drinking behavior. The water control group (N = 9) was treated identically but with access only to water for the length of the experiment. Water group mice were sacrificed concurrently with mice used for microarray studies. Statistical analyses of behavioral data were performed with GraphPad Prism 5 software using one-way ANOVA with repeated measures and Dunnett's Multiple Comparison Test to compare days of ethanol exposure *post hoc*. The dependent variables were ethanol intake (in g/kg/18 hours) and ethanol preference (the ratio of ethanol intake to total fluid intake).

Tissue collection and RNA isolation

Mice were sacrificed by cervical dislocation and tissue from nucleus accumbens, prefrontal cortex, and ventral midbrain was collected according to published procedures (Kerns, 2005). Tissue for each brain region was randomized to produce 5 groups of 3 mice each for both treatment groups (water vs. ethanol deprivation), which was pooled and homogenized in RNA STAT-60 reagent (Tel-Test, Friendswood, TX) using a Tekmar homogenizer. RNA isolation and quality control was performed as described previously (Kerns, 2005). RNA from each pooled sample (7 μg) was processed to cRNA for array hybridization as per standard protocols from the array manufacturer (Affymetrix).

Microarray hybridization and scanning analysis

RNA samples were hybridized to individual Affymetrix murine 430A 2.0 arrays for each of the three brain regions (10 arrays per region) and analyzed on an Affymetrix GeneChip Scanner 3000. Hybridization quality was assessed via standard RNA degradation functions, scatter plot and histogram analyses for each array. One sample (VMB EDE 2) had an excessively large scaling factor and was removed from further analysis. Inter-array correlations for remaining chips all exceeded 0.98.

Probesets were subjected to absent-present-call quality control procedures using open-source R software, and analyzed using the S-score algorithm as described previously (Kerns, 2005), but using the R implementation of the S-score algorithm (Kennedy et al., 2006a; Kennedy et al., 2006b). Following global normalization, probe level values for control animals for each probeset in each brain region were averaged, and each ethanol deprivation sample array was subjected to pair-wise S-score analysis versus this averaged control file. S-scores are normally distributed with a mean=0, have a standard of deviation of 1, and are proportional to fold change (Kerns et al., 2003). Probesets consistently called "absent" across all samples in a brain region were removed from the analysis. This absent-present filtering left 10,417 probesets in NAC, 15,321 probesets in PFC, and 13,784 probesets in VMB for significance analysis, which was performed separately for each region. S-scores across biological replicate arrays were analyzed using One-class Significance Analysis of Microarrays (SAM) for each brain region (Tusher et al., 2001) with a false discovery rate <0.05 for PFC and NAC and <0.1 for VMB considered significant. A higher FDR was used for VMB due to having fewer control samples (n=3) for statistical analysis of that region. Gene lists were finally filtered to remove probesets with S-score values between -1.5 and 1.5, to eliminate very low magnitude expression changes.

Bioinformatic analysis of microarray data

Toppgene (http://toppgene.cchmc.org/), an open-source online gene ontology analysis tool, was used for gene set enrichment analysis with a FDR correction of p-values. Results were filtered for FDR < 0.05 in NAc and FDR < 0.1 in VMB. No significant over-represented gene groups were obtained from PFC at this statistical threshold. Results were further filtered to remove terms containing fewer than 2 genes or greater than 350 genes, and terms with similar definitions and gene lists were trimmed for clarity. Toppgene was also used to rank candidate genes within and across each region using the ToppNet analysis tool. ToppNet uses a user-defined "training set" of genes (see Results), which are mapped onto a protein-protein interactions network (PPIN) along with the "test set", which are the genes to be prioritized. The test set is ranked according to topological distance and interactions with the training set (Chen et al., 2009a; Chen et al., 2009b).

Ingenuity Pathways Analysis (Ingenuity Systems Inc., Redwood City, CA, USA) was used to analyze gene sets for overrepresentation of genes in canonical pathways and specific gene networks highly regulated by ethanol deprivation. Networks were limited to 35 molecules, and scored by IPA based on the hypergeometric distribution, calculated using the right-tailed Fisher's Exact test. Results were reported as the –log of this value, such that a score of 20 indicates that there is a 1 in 10^{20} chance of producing a network containing at least the same number of genes of interest from 35 randomly chosen genes.

Gene sets regulated in each brain region were also analyzed for overrepresentation of transcription factor binding sites in promoter regions ±2000 bp from the transcription start site

using the oPossum Single Site Analysis (http://burgundy.cmmt.ubc.ca/oPOSSUM/) algorithm with a matrix match threshold of 80% (Ho Sui et al., 2005).

Filtered gene sets were further analyzed for overlap with published gene sets relevant to ethanol-related behaviors using Gene Weaver, an open-source tool for gene set analysis (http://geneweaver.org). Significance of these overlapping genes was determined based on the likelihood of an equal sized overlap in two random gene sets of the same size, chosen from a total gene population of 14,000, the number of genes represented by probesets on the Affymetrix M430A 2.0 chip.

Quantitative Real-Time Polymerase Chain Reaction (Q-rtPCR)

Q-rtPCR was used to confirm microarray results for select genes as described in Results. cDNA (Superscript First Strand Synthesis, Invitrogen, Carlsbad, CA) was generated from DNAse-treated (DNA-Free, Ambion, Austin, TX) total RNA (1 µg) isolated as above. Q-rtPCR was performed with SYBR Green I-based detection according to the manufacturer's instructions (iQ SYBR Green, Bio-Rad, Hercules CA) using the iCycler iQTM system (BioRad, Hercules, CA).

Results

Alcohol Deprivation Effect (ADE) in C57Bl/6NCrl Mice

C57Bl/6NCrl mice consumed 2.6 g/kg/18h of 10% w/v ethanol during the 13 day baseline access period, during which their ethanol preference (ethanol/total) was 0.19. The 4-day ethanol deprivation period significantly increased ethanol consumption (4.9 g/kg/18h; $F_{4,17} = 21.18$, $p < 0.0001$) and preference for ethanol over water (0.29; $F_{3,17} = 10.43$; $p < 0.0001$) upon reinstatement (see Figure 3.1), similar to previously published results (Khisti et al., 2006b). Elevated ethanol consumption and preference in 18 mice not sacrificed for microarrays gradually

extinguished over the following 6 days. On the seventh day of access following deprivation ethanol consumption and preference were not significantly different from baseline, but showed a slight trend towards decreased preference.

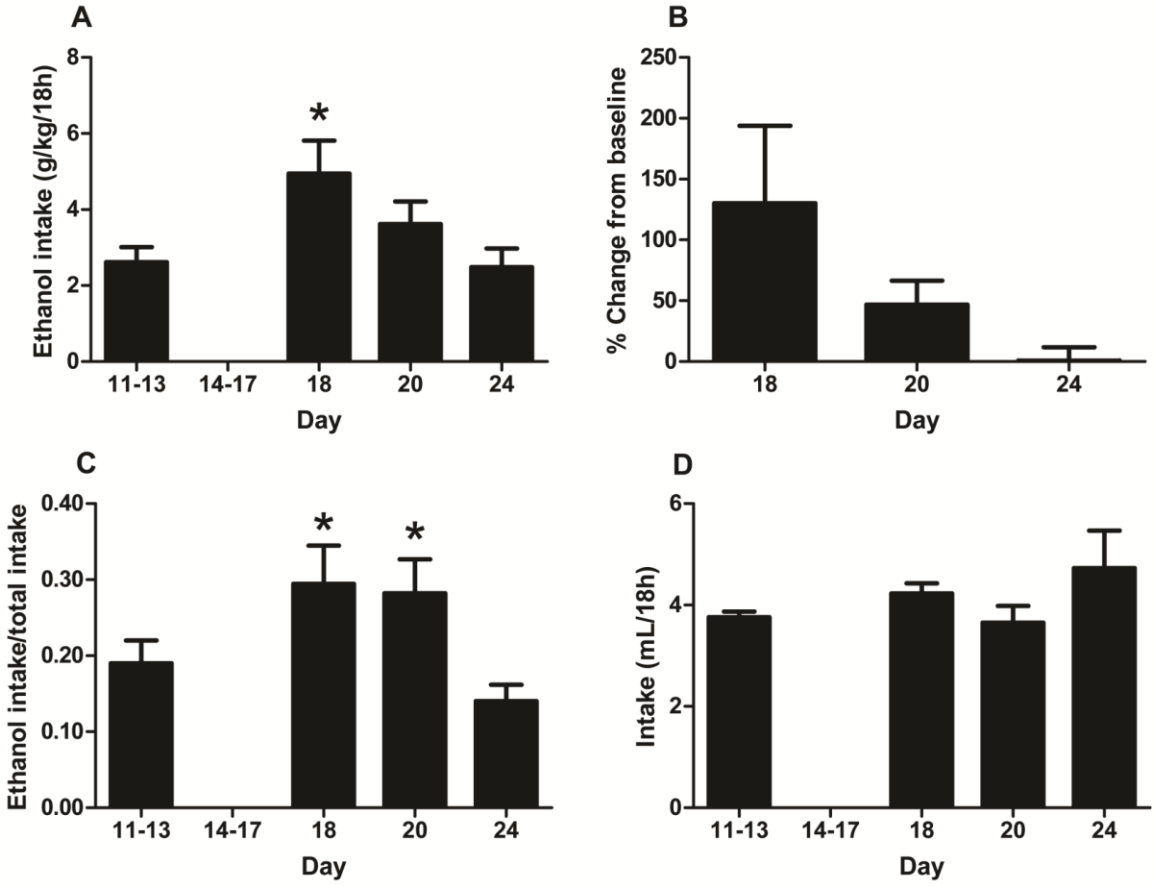


Figure 3.1. Alcohol deprivation effect in C57BL/6NCrl mice. A 4-day abstinence period following 13 days of ethanol access produced significant and transient increases in (A,B) ethanol consumption and (C) preference for ethanol as a proportion of total fluid intake compared to baseline. Total fluid intake (D) did not change significantly. Data are expressed as group average + SEM. Analysis was one-way ANOVA with repeated measures and Dunnett's post-hoc test, with a value of $p < .05$ (*) indicating statistical significance.

Microarray Analysis

To identify genes regulated by ADE vs. water, we used the S-score algorithm to directly compare arrays at the probeset level as described previously (Kerns, 2005; Wolen et al., 2012). This analysis yielded groups of probesets in each brain region significantly regulated by ethanol drinking and deprivation as compared to water drinking animals, but with a striking predominance in NAC. We observed 525 significantly different probesets in NAC, 189 in PFC, and 93 in VMB (see Figure 2, Supplementary Tables 1, 2, 3). These probesets represented 498 genes in NAC, 186 genes in PFC, and 87 genes in VMB. More transcripts were down-regulated by ethanol deprivation than up-regulated in NAC (341 vs 184) and PFC (111 vs 78), but in VMB all identified probesets were up-regulated.

Q- rtPCR Verification of Select Genes

Nine transcripts identified by microarray analysis were selected from the NAC gene set for confirmation by Q-rtPCR. Six genes were found to be significantly regulated in the same direction indicated by the microarray analysis: *Cacna1d*, *Cacna1g*, *Eif2c2*, *Gria1*, *Kif5c*, and *Smarca4* (see Figure 3.2). Three genes tested did not show significant differences on Q-rtPCR (*Cacna1h*, *Aprl1*, and *Clns1a*).

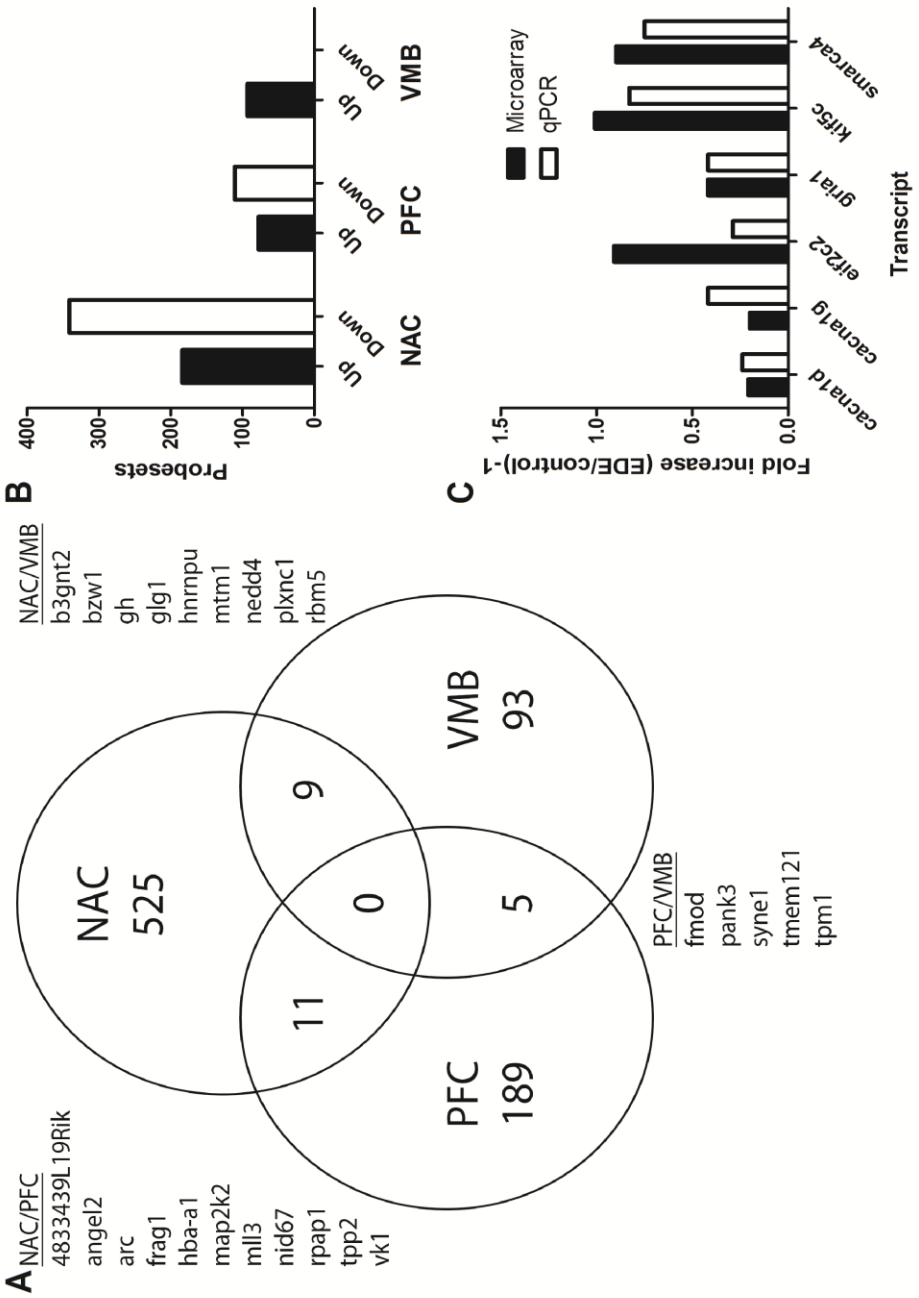


Figure 3.2. Regional changes in gene expression associated with ethanol deprivation. In (**A**) the regional magnitude of changes in transcript abundance are indicated for NAC, VMB, and PFC, with overlap between regions. The directionality of these changes is indicated in (**B**), and in (**C**) the fold-change for selected transcripts confirmed by quantitative PCR is shown next to the fold-change indicated by microarray analysis.

Bioinformatics Analysis

Toppgene analysis identified overrepresented gene ontology (GO) terms among sets of regulated genes for biological processes (BP), molecular functions (MF) and cellular component (CC). Significant results were obtained from NAC with a FDR ≤0.01 but we elected to relax the statistical significance to an FDR of ≤0.05 to increase our ability to identify potentially important functional gene networks (see Supplementary Table 4). Significant results were obtained for VMB at FDR<0.1 (see Supplementary Table 5). No significantly over-represented GO categories were observed in PFC at any FDR < 0.2, so analysis was focused on NAC and VMB.

Analysis with a FDR of 0.05 identified 19 MF terms and 75 BP terms as over-represented in genes regulated by ADE in NAC. These GO terms largely converged on a few related areas: myelination, ion channel activity, RNA splicing/translation, peptide and steroid hormone function, neurogenesis and synaptic function. Of particular interest, there was a striking number of calcium-signaling and potassium channel related genes and terms within the ToppGene results.

The most significant over-represented MF term was "*structural constituents of the myelin sheath*", with 4 out of 5 transcripts down-regulated by ethanol deprivation. Both genes in the related term "*ceramide glucosyltransferase activity*" (*Ugcg*, *Ugt8*), which are involved in synthesis of myelin sphingolipids, were also down-regulated (Bosio et al., 1996; Watanabe et al., 2010). Regulation of 4 genes involved in "*RNA cap binding"* suggested inhibition of mRNA translation via enhancement of RNAi (*Eif2c2*) and inhibition of translation initiation. The MF term "*purine nucleoside binding*" was identified in connection with 23 genes involved in diverse processes: cell signaling, transport, and mRNA transcription and translation. Potential enhancement of protein degradation processes was indicated by the down-regulation of several

genes in the *"small conjugating protein-specific protease activity"* term involved in ubiquitin removal.

Many of the remaining MF terms identified in NAC related to ion channel and ion channel regulator activity. Genes identified in the "*gated channel activity"* term primarily affected cation currents, but 4 genes were involved in chloride transport: gamma-aminobutyric acid (GABA) receptor subunits α1 (*Gabra1)* and γ2 (*Gabrg2),* as well as chloride transporters coupled to transport of protons (*Clcn5*) and potassium (*Slc12a5).* Several transcripts modulating potassium transport were regulated by deprivation and tended to be down-regulated. Two regulators of sodium channel activity were down-regulated (*Commd1, Nedd4*), while two regulators (*Slc9a3r1, Slc9a3r2*) of solute carrier family 9, member 3 (*Slc9a3*), a sodium/hydrogen exchanger, were up-regulated. The transcript coding for the pore-forming α subunit (*Scn8a*) of the type VIII voltage-gated sodium channel, which is involved in formation of the action potential in excitable neurons, was down-regulated (Chatelier et al., 2010).

MF results in NAC featured several significantly over-represented calcium-related terms, due to the presence of several genes coding for subunits of calcium-permeable ion channels, including 6 voltage-gated calcium channel subunits, 2 transient receptor potential (TRP) channels (*Trpc1, Trpc7*), 2 glutamate receptors (*Grin1, Gria1*), and the inositol 1,4,5-triphosphate receptor 1 (*Itpr1*). In addition, several genes in the term "*calmodulin binding*" were identified, suggesting coordinated regulation of calcium-related signaling at the transcriptional level during ethanol deprivation.

Over-represented BP terms in NAC indicated potential regulation of diverse cellular processes in neurons in this region. Several subsets of related terms with similar transcript membership were

identified. First, a set of 14 similar terms containing genes involved in ion transport and homeostasis, primarily of calcium, were identified. A related set of 13 terms was identified that contained transcripts involved in neurotransmission. These genes were involved in calcium-ion dependent exocytosis, membrane fusion, signal release, and transport and secretion of acetylcholine, glutamate, and GABA. Steroid hormone receptor signaling and cellular response to peptide hormone stimulus were represented by 7 BP terms that contained diverse transcripts involved in cell signaling and gene expression. Two deprivation-regulated transcripts were contained in 6 of 7 hormone-related terms: growth hormone 1 (*Gh1*) and v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (*Src*). Interestingly, more genes were up-regulated than down-regulated by deprivation in the terms related to peptide hormone response, which was a reversal of the trend of the gene set as a whole. Four terms loosely related to kinase activity were identified, which contain a wide variety of genes involved in cell signaling.

A set of 11 BP terms involved in transcription, RNA processing, and translation were identified that contained diverse transcripts involved in regulation of DNA binding, transcription initiation from RNA polymerase II promoter, spliceosome assembly and RNA splicing, and ribonucleoprotein complex biogenesis and assembly. Four terms contained genes that were related to protein modification and metabolism, primarily by ubiquitination. A set of 14 BP terms was produced that contained deprivation-regulated transcripts involved in regulation of neurogenesis, cell differentiation, development, and apoptosis.

Three significantly over-represented BP terms did not clearly form a biologically cohesive set with any other terms: *positive regulation of glucose metabolic process*, *negative regulation of molecular function,* and *peptidyl-amino acid modification*. Transcripts in these terms were primarily involved in cell signaling, but 3 transcripts had known or suspected histone

acetyltransferase or methyltransferase activity (*Mll3, Naa15, Prmt7*), and thus the ability to induce and maintain changes in gene expression that could influence behavior (Maze and Nestler, 2011; Pandey et al., 2008). Three identified terms pertained to higher level sensory and behavioral processes: *adult behavior*, *feeding behavior*, and *regulation of sensory perception of pain*. All identified transcripts in the latter two terms were found to be down-regulated by ethanol deprivation, and represented 9% and 17%, respectively, of the genes in these terms.

In VMB gene ontology analysis identified 8 over-represented MF terms and 18 over-represented BP terms, which converged on a few key areas: cell adhesion and cell skeleton regulation, neuron apoptosis, mRNA processing/modification, and cell signaling via phosphoinositide 3-kinase cascade, steroid hormone signaling, and vascular endothelial growth factor (VEGF) signaling (see supplementary table 5).

Ingenuity Pathways Analysis

Ingenuity Pathways Analysis (IPA) was used to identify gene networks and over-represented canonical signaling pathways among genes identified as significantly regulated by ethanol deprivation (-log(p-value) $\geq$ 1.30). In NAC 62 canonical signaling pathways were identified as significantly over-represented, 16 pathways were identified in PFC, and in VMB 2 pathways were identified (see Figure 3.3, Supplementary table 6).

The most significantly (-log(p-value) > 3) over-represented canonical signaling pathways were *cAMP-mediated signaling, G-protein coupled receptor signaling, insulin-like growth factor 1 (IGF-1) signaling, corticotropin releasing hormone (CRH) signaling, androgen signaling, and CXCR4 signaling.* The majority of identified pathways had –log(p-value) between 2 and 3, and included *calcium signaling, synaptic long term potentiation, glucocorticoid receptor signaling,*

CREB signaling in neurons, and *PI3K/AKT signaling*, among others. Less significantly over-represented pathways included *ephrin receptor signaling*, *insulin receptor signaling*, and *axonal guidance signaling*, among others.

Several genes were represented in several identified pathways: adenylate cyclase 6 (*Adcy6)*, v-akt murine thymoma viral oncogene homolog 1 (*Akt1)*, calmodulin 3 (*Calm3)*, guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2 (*Gnai2)*, guanine nucleotide binding protein (G protein), gamma 4 (*Gng4)*, inositol 1,4,5-triphosphate receptor, type 1 (*Itpr1)*, mitogen-activated protein kinase kinase 2 (*Map2k2)*, 3-phosphoinositide dependent protein kinase-1 (*Pdpk1)*, catalytic (*Prkacb)* and regulatory *(Prkar1a)* subunits of cAMP-dependent protein kinase (PKA), protein kinase C, eta (*Prkch)*, and v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) (*Src*).

All significantly over-represented canonical pathways in PFC had –log(p-value) between 1.30 and 1.96, and included *LPS-stimulated MAPK signaling, insulin receptor signaling, VDR/RXR activation, VEGF signaling, and MIF-mediated glucocorticoid regulation*. Transcripts common to several pathways included dual specificity mitogen-activated protein kinase kinase 2 (*Map2k2)*, phosphatidylinositol 3-kinase regulatory subunit alpha (*Pik3r1)*, and NF-kappa-B inhibitor beta *(Nfkbib).*

Only 2 canonical signaling pathways were significantly over-represented in VMB: *thyroid cancer signaling* and *EGF signaling*.

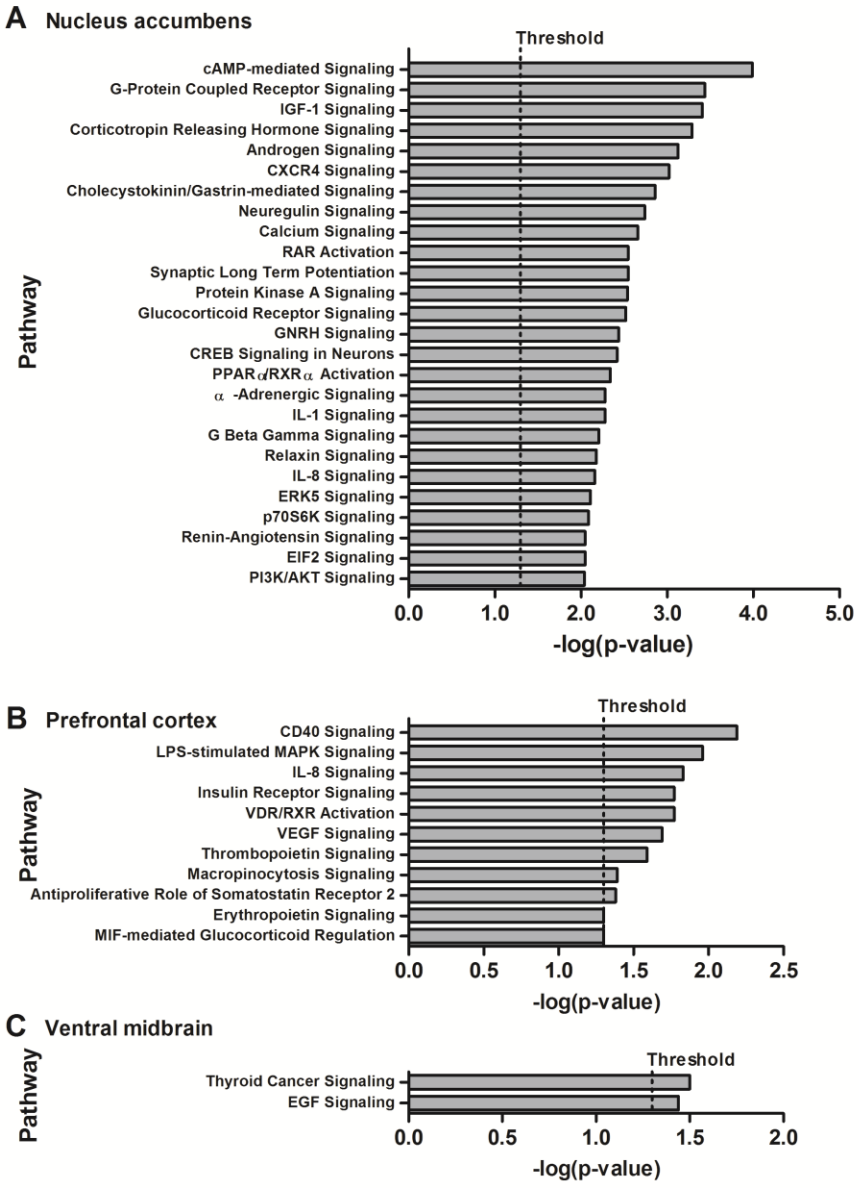


Figure 3.3. Over-represented canonical signaling pathways identified by Ingenuity Pathways Analysis. (A) nucleus accumbens, (B) prefrontal cortex, and (C) ventral midbrain. Pathways in NAC have been filtered to remove those specific to non-neuronal cell types. A –log(p-value) > 1.30 indicates statistical significance.

More than 25 gene networks were identified in NAC, while in PFC and VMB only 10 and 6 networks that contained more than 2 molecules were identified, respectively. Top functions for each network were identified based on the IPA literature database. In NAC it was found that the 2 top-scoring networks contained a gene in common, *smarca4*, and were merged to create a gene network with RNA post-transcriptional modification, gene expression, and protein trafficking as top functions (see Figure 3.4). For PFC networks 1 and 3 were merged to create a network with gene expression, cancer, and carbohydrate metabolism as top functions (see Figure 3.5).

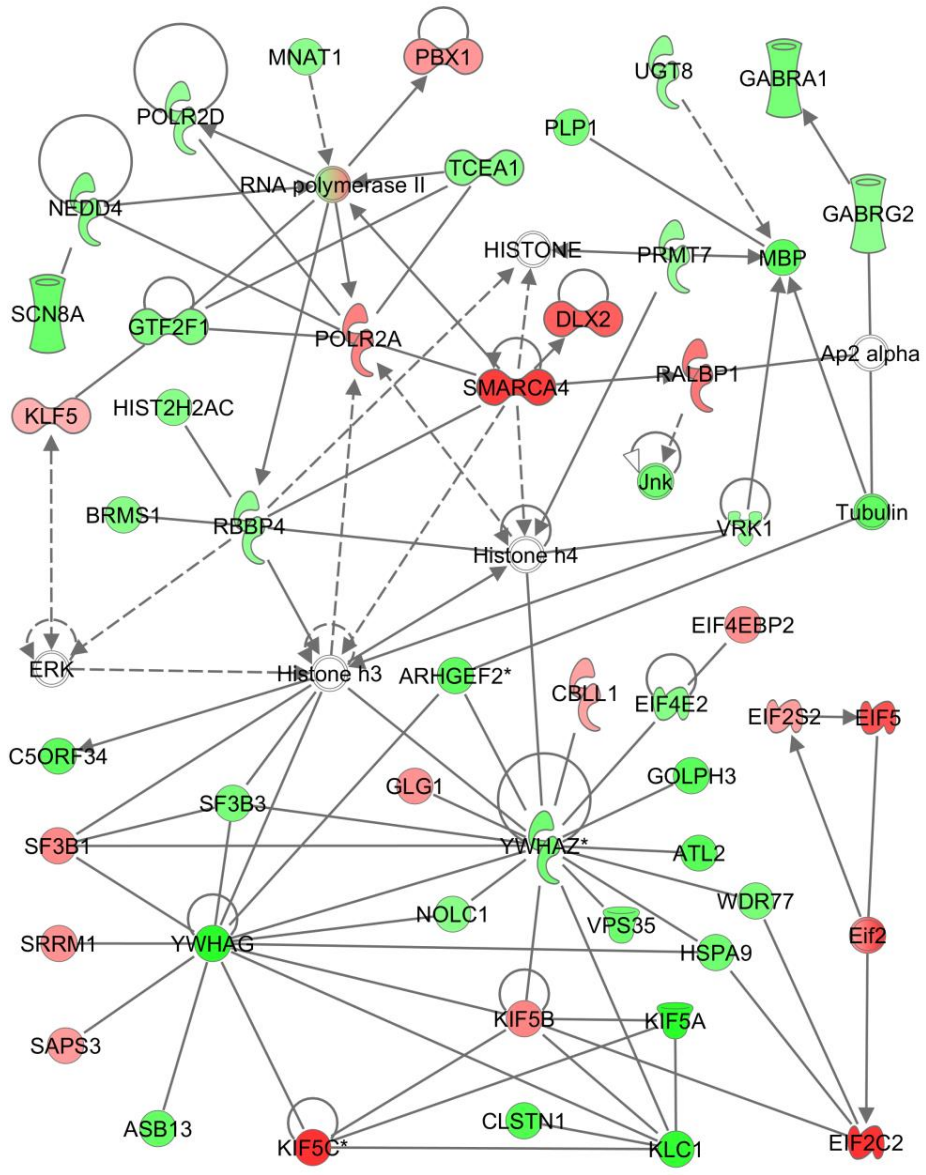


Figure 3.4. Coordinately regulated gene network identified by Ingenuity Pathways Analysis in nucleus accumbens. Top functions for this network are RNA post-transcriptional modification, gene expression, and protein trafficking. Network hubs (clockwise from top-left) are POLR2A, SMARCA4, MBP, Histone H4, YWHAZ, YWHAG, and Histone H3. Red shading indicates up-regulation, while green shading indicates down-regulation. Color saturation indicates relative significance of regulation, such that greater saturation indicates more significant regulation in the direction indicated.

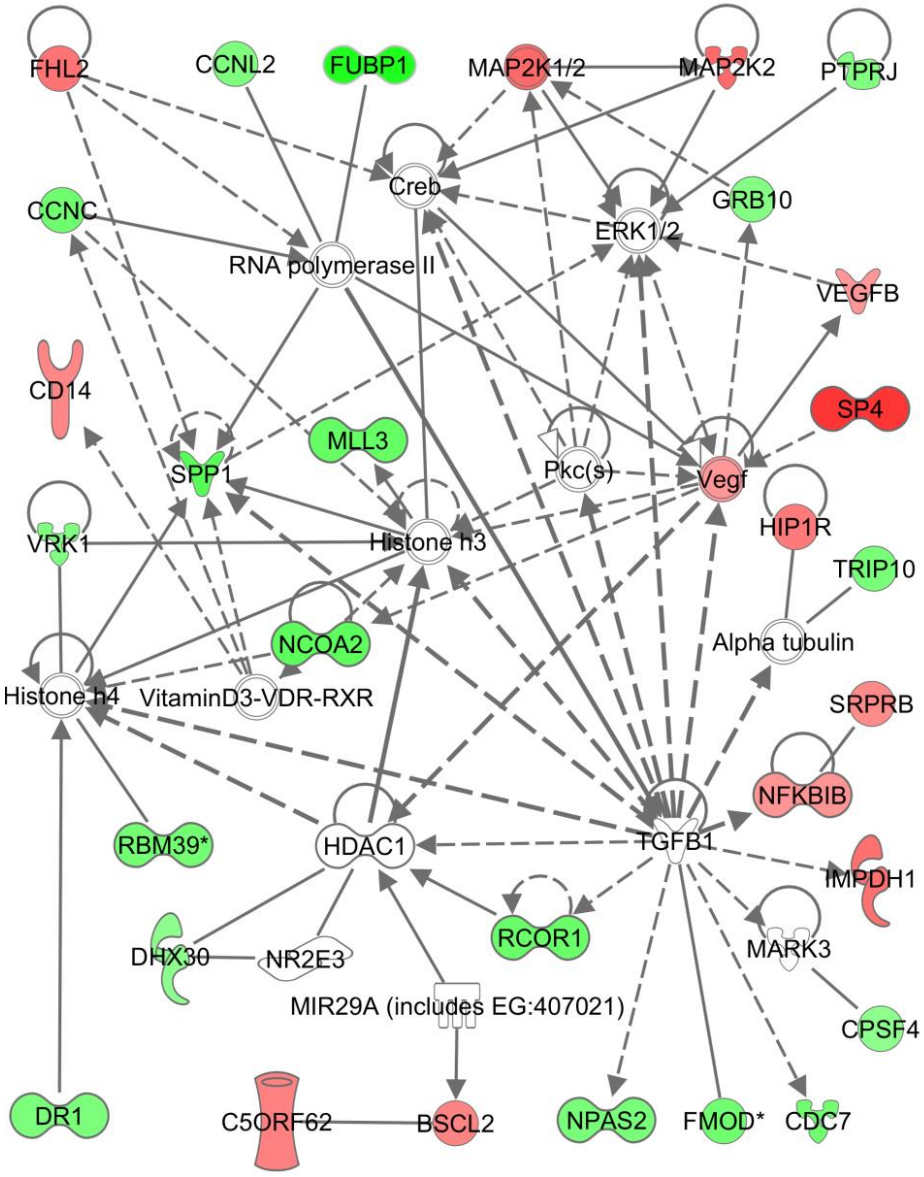


Figure 3.5. Coordinately regulated gene network identified by Ingenuity Pathways Analysis in prefrontal cortex. Top functions for this network are gene expression, cancer, and carbohydrate metabolism. Network hubs (clock-wise from top-left) are RNA polymerase II, CREB, ERK1/2, PKC, VEGF, TGFB1, HDAC1, Histone H4, Histone H3, and SPP1. from Red shading indicates up-regulation, while green shading indicates down-regulation. Color saturation indicates relative significance of regulation, such that greater saturation indicates more significant regulation in the direction indicated.

Transcription factor binding site analysis

A total of 66 significantly over-represented consensus sequences were identified in promoter regions of genes regulated in NAC, 25 sequences were identified in VMB, and 9 sequences were identified in PFC ($p < 0.05$**,** see Table 3.1). All sequences identified in VMB and PFC were also identified in NAC, and binding sites for 3 transcription factors were found to be significantly over-represented in all 3 regions: SRY (sex determining region Y)-box 5 (SOX17), E74-like factor 5 (ets domain transcription factor) (ELF5), and myeloid zinc finger 1 (MZF1).

Table 3.1. Top 25 results in NAc from transcription factor binding site analysis performed with oPossum (http://burgundy.cmmt.ubc.ca/oPOSSUM/) in promoter regions +/- 2000 base-pairs from transcription start site for genes regulated by ethanol deprivation in nucleus accumbens, prefrontal cortex, and ventral midbrain of C57BL/6J mice. A total of 66 consensus binding sequences were significantly over-represented in NAC. Three sequences were over-represented in genes from all regions: ELF5, MZF_1-4, and SOX17.

Consensus Sequence	Transcription Factor Class	NAC Z-score	NAC P-value
GABPA	ETS	5.42	6.14E-07
E2F1	E2F_TDP	2.06	8.63E-07
ELK4	ETS	6.14	2.19E-06
Arnt-Ahr	bHLH	7.14	3.98E-06
MAX	bHLH-ZIP	5.07	8.37E-06
USF1	bHLH-ZIP	4.38	1.34E-05
Ddit3-Cebpa	bZIP	10.14	1.62E-05
MZF1_5-13	ZN-FINGER, C2H2	-1.19	2.19E-05
RORA_1	NUCLEAR RECEPTC	-1.7	2.76E-05
Cebpa	bZIP	11.05	2.86E-05
FOXD1	FORKHEAD	12.45	3.23E-05
CREB1	bZIP	3.76	3.55E-05
Mycn	bHLH-ZIP	3.74	3.89E-05
Arnt	bHLH	3.79	3.95E-05
ELK1	ETS	-3.44	6.85E-05
PBX1	HOMEO	8.37	1.30E-04
SP1	ZN-FINGER, C2H2	-5.28	1.36E-04
SRY	HMG	22.93	1.51E-04
HNF4A	NUCLEAR RECEPTC	2.09	1.94E-04
Bapx1	HOMEO	7.65	2.07E-04
Sox5	HMG	19.2	2.14E-04
Myb	TRP-CLUSTER	0.75	2.17E-04
Lhx3	HOMEO	12.99	2.49E-04
HLF	bZIP	7.36	2.59E-04

The majority of over-represented transcription factor binding sites were only identified in NAC. The cAMP response element (CRE), which is bound by the transcription factor cAMP responsive element binding protein 1 (CREB1) was identified in the promoter regions of 142 genes. Many of these genes were involved in ion transport (*Cacna1g, Gabra1, Kcnb1, Trpc7*) and GPCR-mediated cell signaling (*Adcy6, Htr2c, S1pr5*). Several genes were identified with functions related to nervous system development and plasticity: *Bdnf, Dlx2, Fgf11, Foxp2, Map1b, Mef2c, Naa15, Nr2c2, Tcf12,* And *Tnfrsf21.* The CRE site showed a trent for over-representation in genes from PFC and VMB, with p-values of 0.09 and 0.06, respectively.

Consensus binding sequences for 4 transcription factors or protein complexes with known chromatin remodeling functions were significantly over-represented in NAC: FOXA2, REST, the MYC-MAX complex, and the STAGA complex. The FOXA2 and the MYC-MAX complexes tend to act as transcriptional activators (Duncan et al., 1998), while REST represses gene expression by binding to the neuron-restrictive silencer element (NRSE) and to corepressors MSIN3 AND COREST, followed by recruitment of histone deacetylases (Andres et al., 1999). In PFC the *Rcor1* transcript was reduced by 18% following ethanol deprivation, and it showed a non-significant increase of 20% in NAC. The multi-protein STAGA complex is a chromatin remodeling complex with acetyltransferase activity (Martinez et al., 2001). Sites for binding of PAX6 and ROAZ, which function in neurogenesis and development, were over-represented in NAC (Cocas et al., 2011; Hata et al., 2000). It has recently been shown that prenatal ethanol exposure in rats impairs PAX6 function and cerebral cortex development (Aronne et al., 2011). Binding sequences for 2 transcription factors with functions related to neuronal differentiation and maintenance were also identified: MEF2A and FOXD3 (Guo et al., 2002; Shalizi et al., 2006).

Consensus binding sites for 4 transcription factors involved in epigenetic regulation of gene expression by histone modification and chromatin remodeling were over-represented in NAC and VMB: Sp1 transcription factor (SP1), MYC associated factor X (MAX), YY1 transcription factor (YY1), and growth factor independent 1 transcription repressor (GFI). SP1 regulated gene expression is involved in diverse cellular processes, and has been implicated in mu opioid receptor regulation via recruitment of SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (*smarca4*, also known as BRG1), which is up-regulated nearly 2-fold by ethanol deprivation (Hwang et al., 2010). MAX is a transcriptional regulator that binds to MYC or MAD proteins to activate or repress transcription, respectively (Hurlin and Huang, 2006). YY1 is involved in targeting of histone acetyltransferase (HAT) and histone deacetylase (HDAC) proteins to gene promoter regions (Ren et al., 2009), and GFI associates with HDAC1, HDAC2, and HDAC3 to direct histone deacetylation processes (McGhee et al., 2003).

Overlap with published ethanol-related gene sets

GeneWeaver, an online gene set analysis tool, was used to study similarity in gene sets identified by SAM analysis to gene sets with relevance to alcohol drinking behavior. For NAC the set of significantly regulated genes was compared to 2 other gene sets: 854 genes influencing individual variation in consumption within the C57BL/6NCrl strain (INV) (Wolstenholme et al., 2011) and 305 genes found to be important for basal ethanol consumption by meta-analysis across several experiments and strains of mice (MMT) (Mulligan, 2006). The INV set was specific to the NAC, while the MMT set was derived from whole brain. The overlaps between the gene set regulated by ethanol deprivation in NAC and the INV ($p < 1.828e-11$) and MMT ($p < 2.262e-04$) strains were highly significant (see Figure 3.6).

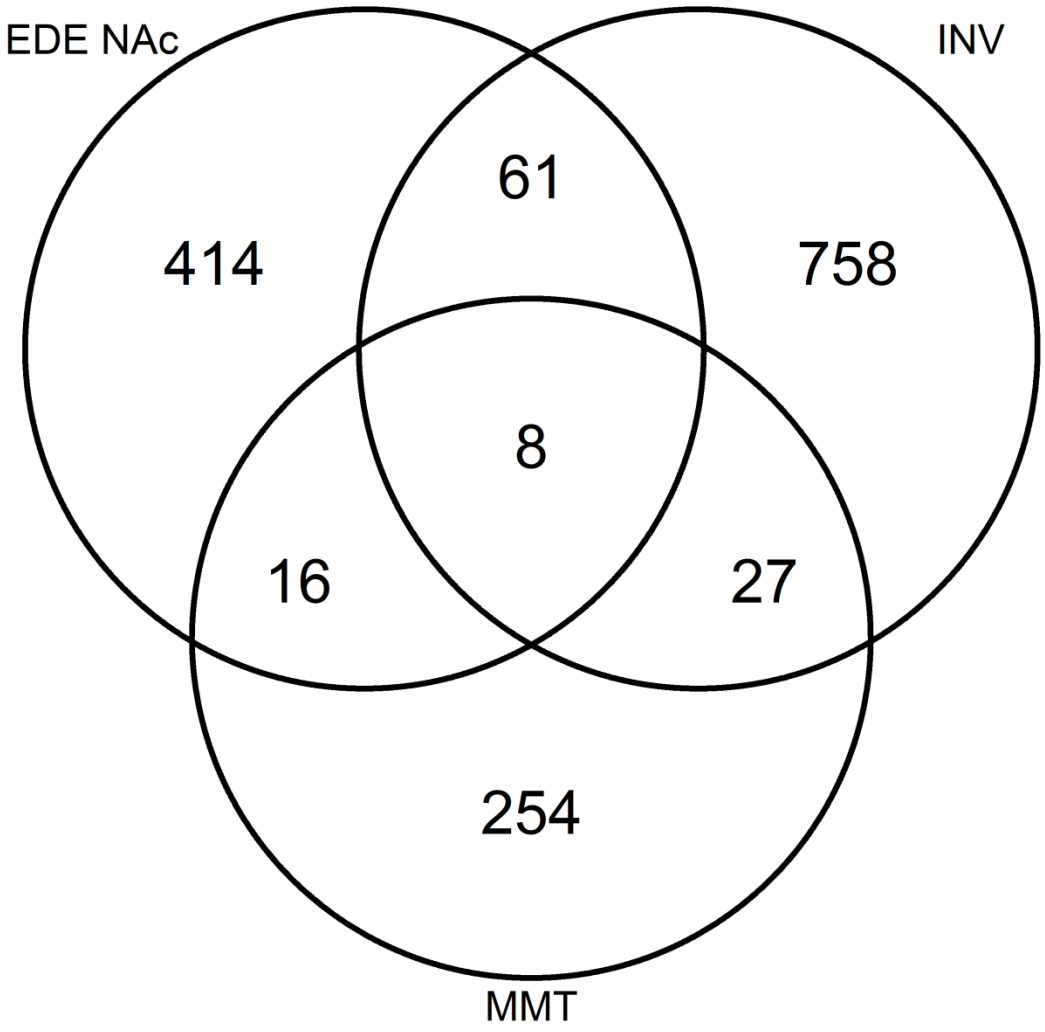


Figure 3.6. Overlap of two gene sets associated with ethanol-related behavior and the gene set identified as regulated by ethanol deprivation in nucleus accumbens.

ToppNet Candidate Gene Prioritization

The open-source *ToppNet* tool was used to rank transcripts identified in each brain region based on a protein-protein interaction network (PPIN). The test set (deprivation-regulated transcripts in a particular brain region) and a training set (1 of 4 sets of genes with relevance for ethanol consumption behavior) were mapped to the PPIN and genes in the test set were ranked based on relative location to the training set genes using global network-distance measures. The training sets used for each region were the full (*MMF*) and top (*MMT*) results from the meta-analysis performed by Mulligan et al. (2006), genes associated with individual variation in ethanol consumption in C57BL/6NCrl derived from whole brain (*INV*), and the subset of *INV* genes from the region from which the test set is derived (Wolstenholme et al., 2011). The scores derived from ranking with these 4 gene sets were averaged to obtain a regional score and ranked gene list for each brain region prioritized for likely biological and behavioral relevance for ethanol drinking behavior. Genes were then ranked by regional scores across brain regions to produce an overall ranking (see Table 3.2). The top 6 genes in NAC were the top 6 genes overall, and the majority of the top 35 ranked genes (27 of 35) were from the NAC gene set.

Table 3.2. Top 20 candidate genes for deprivation-induced elevated ethanol consumption within and across brain regions based on ranking with ToppNet (http://toppgene.cchmc.org/) using 5 gene lists related to ethanol consumption as training sets. Gene lists were scored and ranked separately using each training set, and these scores were averaged to produce a regional score, which was used to rank genes within each region. Regional scores were averaged across all brain regions to produce an overall average score, which was used to rank candidate genes across brain regions.

Rank	Top 20 Cross-region Genes	Cross-region Average Score	Top 20 NAC Genes	NAC Score	Top 20 PFC Genes	PFC Score	Top 20 VMB Genes	VMB Score
1	Ywhag	3.47E-03	Ywhag	3.47E-03	Pik3r1	9.74E-04	Fn1	6.53E-04
2	Src	1.64E-03	Src	1.64E-03	Ndrg1	5.33E-04	Cdh1	5.17E-04
3	Eif4e	1.64E-03	Eif4e	1.64E-03	Sfrs12	5.10E-04	Cbx5	4.31E-04
4	Ywhaz	1.58E-03	Ywhaz	1.58E-03	Ncoa2	3.61E-04	Nedd4	4.24E-04
5	Akt1	1.10E-03	Akt1	1.10E-03	Fhl2	3.46E-04	Khdrbs2	3.75E-04
6	Usp7	1.09E-03	Usp7	1.09E-03	Strn4	2.83E-04	Bcr	3.37E-04
7	Pik3r1	9.74E-04	Polr2a	9.36E-04	Rpap1	2.68E-04	Apc	2.94E-04
8	Polr2a	9.36E-04	Hsp90aa1	7.85E-04	Trim27	2.51E-04	Atg12	2.51E-04
9	Hsp90aa1	7.85E-04	Rhoa	7.84E-04	Ccne1	2.38E-04	Nrip1	2.47E-04
10	Rhoa	7.84E-04	Mdm2	5.87E-04	Rfc1	2.16E-04	Hnrnpu	2.05E-04
11	Fn1	6.53E-04	Jak2	5.59E-04	Nfkbib	1.97E-04	Map2k7	2.03E-04
12	Mdm2	5.87E-04	Crk	5.20E-04	Tmpo	1.90E-04	Itgb5	1.89E-04
13	Jak2	5.59E-04	Acvr1	5.13E-04	Rhog	1.77E-04	Itsn1	1.88E-04
14	Ndrg1	5.33E-04	Usp39	4.71E-04	Cyth3	1.68E-04	Braf	1.74E-04
15	Crk	5.20E-04	Smarca4	4.69E-04	Smarcc1	1.68E-04	Safb	1.64E-04
16	Cdh1	5.17E-04	Efemp2	4.45E-04	Spp1	1.68E-04	Kcna2	1.57E-04
17	Acvr1	5.13E-04	Rasa1	4.27E-04	Grb10	1.62E-04	Gm3579	1.34E-04
18	Sfrs12	5.10E-04	Fos	4.24E-04	Trip10	1.54E-04	Racgap1	1.33E-04

19	Usp39	4.71E-04	Mbp	4.19E-04	Gtf2a1	1.41E-04	Cux1	1.25E-04
20	Smarca4	4.69E-04	Gnai2	4.12E-04	Fubp1	1.40E-04	Gstm2	1.19E-04

Discussion

It has been reported by our laboratory and others that ethanol and other drugs of abuse produce regional changes in neuronal gene expression (Costin et al., 2013b; Iancu et al., 2013; Kerns, 2005; Lewohl et al., 2000; Nunez and Mayfield, 2012; Osterndorff-Kahanek et al., 2013; Robison et al., 2013; Serrano et al., 2012; Tapocik et al., 2013; Wolen et al., 2012; Wolstenholme et al., 2011). However, rodents allowed constant access to ethanol maintain steady intake over time, demonstrating that changes in gene expression produced by ethanol consumption are not sufficient to produce elevated voluntary intake. Short periods of abstinence or ethanol deprivation produce striking increases in ethanol consumption and preference without induction of dependence or withdrawal (Heyser et al., 2003; Khisti et al., 2006b; Lopez-Moreno, 2004; Serra et al., 2003a; Spanagel and Holter, 2000). Repeated deprivations produce progressive increases in binge-like drinking that lead to inflexible drinking behavior and altered glutamatergic signaling (Hopf et al., 2010; Loi et al., 2010; Obara et al., 2009a). Thus such models have become increasingly used to study brain adaptations that may be similar to those involved in the transition to alcohol use disorders in humans. Our laboratory and others have documented that exposure to ethanol and other drugs of abuse can alter gene expression within brain regions comprising the mesolimbocortical dopamine pathway. Within this pathway the most studied regions have been the ventral midbrain or ventral tegmental area, the nucleus accumbens, and the prefrontal cortex; interconnected regions involved in reward processes, motivated behavior, and decision making (Bardo, 1998; George and Koob, 2010). We have thus proposed that neuroplasticity during abstinence, mediated in part by changes in gene expression within the mesolimbocortical dopamine pathway, is a potential important contributor to elevated consumption upon reinstatement. However, genome-wide studies on gene expression after

multiple days of deprivation following voluntary continuous access drinking—the period directly preceding elevated consumption—have not been previously reported. Here we confirmed the production of an alcohol deprivation effect (ADE) in C57BL/6NCrl mice, as reported by (Khisti et al., 2006b), further demonstrating the potential of this model to produce significantly elevated ethanol consumption and preference. We further show using this model that striking changes in gene expression occur following 2 weeks of alcohol drinking and a four day abstinence period, particularly within the nucleus accumbens.

Novel gene networks and canonical signaling pathways composed of functionally-related transcripts regulated by ethanol deprivation were identified, and these tended to be region-specific, although there were some biological themes common to one or more regions: PI3K signaling, regulation of cell fate and neuronal connectivity, hormone signaling, and regulation of mRNA expression and processing. These networks and pathways together form a molecular picture of the initial program of neuroplasticity associated with elevated ethanol consumption in mice due to deprivation. Regulated genes in NAC were found to significantly overlap with gene sets associated with differences in voluntary ethanol consumption between mouse strains and among individuals of the C57BL/6NCrl strain, supporting the role of these genes in ethanol-related behavior.

Promoter regions of transcripts found to be regulated in each brain region were subjected to transcription factor consensus binding site analysis and several over-represented sequences were identified. Three sites were found to be significantly over-represented across the brain regions examined and may represent common mediators of the transcriptional effects of ethanol deprivation. Finally, genes in each region were ranked based on protein-protein interaction and similarity to ethanol-related gene sets to identify potential therapeutic targets for which

manipulation will likely produce the greatest effect on cell signaling related to ethanol consumption.

The magnitude of transcript regulation was greatest in NAC, and the 341 down-regulated genes in NAC represented approximately 42% of all significant results. Interestingly, in VMB only 93 probesets were identified, all of which were up-regulated. Thus, the transcriptional effects of alcohol deprivation are most pronounced in nucleus accumbens, and this may be a primary site of plasticity associated with changing behavior. The lack of down-regulated transcripts in VMB may be an artifact caused by exclusion of 1 control microarray. However, it seems likely that this result does provide an accurate indication of the most robust changes in gene expression in VMB associated with ethanol deprivation, because use of a far less stringent FDR (> 0.20) for SAM is required to identify downregulated genes in this region.

Several primary targets of ethanol and genes related to these targets were identified as regulated in NAC: NMDA receptors, $GABA_A$ receptors, and L-type calcium channels. These genes represent potential primary mediators of neuroplasticity associated with deprivation following ethanol consumption (Spanagel, 2009; Vengeliene et al., 2009). Ethanol inhibits glutamatergic signaling and enhances GABAergic signaling, and compensatory regulation of receptors for these ligands may represent an early mediator of neuroplasticity during ethanol access and subsequent deprivation. It has been shown that L-type calcium channels are up-regulated in cerebral cortex of mice physically dependent on ethanol (Katsura et al., 2006). Although mice in this experiment were not ethanol-dependent, inhibition of L-type calcium channels by ethanol may result in the compensatory up-regulation of these and other calcium channels seen in NAC, which during abstinence may produce abnormal neuronal function and behavior, driving the animal to seek ethanol to restore calcium homeostasis and normal functioning.

Gene ontology (GO) analysis identified over-represented molecular functions among the protein products of regulated transcripts and the biological processes to which these functions contribute. The NAC—with the most regulated transcripts—yielded the most robust GO results, while the VMB also produced several significantly over-represented terms related to biological processes and molecular functions. Interestingly, the PFC gene set—with more than twice as many significantly regulated transcripts as VMB—did not produce meaningful GO results. Thus it is seems that regulation of gene expression associated with ethanol deprivation is more likely to produce relevant changes in biological processes and molecular functions in NAC and VTA than in PFC, despite the fact that differences in overall magnitude of regulation might indicate otherwise.

Although experimental verification—including immunoblotting and electrophysiology—will be required to draw conclusions regarding the effects of ethanol deprivation on neuronal function, GO results indicated several areas that may be fruitful for future study, based on the ratio of regulated to unregulated genes in the significant terms, and the direction of regulation for each gene(Watanabe et al., 2010).

Most striking is the downregulation in NAC of transcripts coding for 4 of 5 structural constituents of the myelin sheath and 2 of 2 ceramide glucosyltransferase enzymes involved in synthesis of myelin sphingolipids, indicating potential inhibition of myelin maintenance processes and neurotransmission (Bosio et al., 1996). Alterations in neurotransmission through the NAC would be expected to affect behavior towards rewarding stimuli (Bardo, 1998; Kapasova and Szumlinski, 2008; Middaugh et al., 2003; Thielen et al., 2004). For example, axonal segments of projections from the VTA were likely represented in our NAC sample, and demyelination of these neurons would attenuate or cease dopamine release in the NAC in

response to reward, and lead to compensatory increased alcohol consumption. Similarly, altered transmission within NAC and to other connected regions, such as PFC or amygdala, could lead to maladaptive processing of internal and external stimuli, and so to increased drinking (Bardo, 1998; George and Koob, 2010; Hwang et al., 2004; Koob, 2009; Orozco-Cabal et al., 2008; Smith and Aston-Jones, 2008).

Nearly half of the GO terms over-represented in the NAC gene set were related to voltage-gated and ligand-gated channels (11/19 MF terms; 29/75 BP terms). These terms were represented by genes involved in transport of and response to: calcium, sodium, potassium, chloride, and hydrogen.

Calcium is tightly regulated in the cell, and changes in neuronal calcium levels and calcium-related signaling may have diverse consequences for biological processes related to excitability, neurotransmitter release, gene expression, and cell fate (Catterall et al., 2005). Indeed, several of these processes were identified GO terms in the NAC gene set: membrane fusion and calcium ion-dependent exocytosis; neurogenesis, neuron differentiation, and apoptosis; and alterations in glutamatergic, GABAergic, and cholinergic signaling.

Several transcripts coding for subunits of voltage-gated calcium channels (VGCCs) were found to be up-regulated in NAC, with abundance of 3 α subunit mRNAs (*Cacna1a, Cacna1d, Cacna1g)* increased by about 25%. These subunits are most responsible for determining the nature of the channel, and they correspond to the $Ca_V2.1$ (P/Q-type), $Ca_V1.3$ (L-type), and $Ca_V3.1$ (T-type) channels, respectively. Ethanol is known to alter function of VGCCs at concentrations at concentrations achieved by human drinkers (Walter and Messing, 1999). T-type VGCCs are potentiated by ethanol *in* vitro at low concentrations and inhibited at high

concentrations (Mu et al., 2003). In addition, acute ethanol inhibits $Ca_V2.2$ (N-type) channels, while chronic ethanol increases function and density of these channels, and $Ca_V2.2$ (-/-) mice show decreased voluntary ethanol consumption and reduced duration of the loss of righting reflex caused by a high dose of ethanol (McMahon et al., 2000; Newton et al., 2004; Solem et al., 1997). Administration of NP078585, an antagonist of T-type and N-type channels with concentration-dependent selectivity, reduced conditioned place preference associated with ethanol in mice and operant self-administration of ethanol in rats (Newton et al., 2008). Other subunits of these channels can modify channel function, and 3 were found to regulated by ethanol deprivation in NAC: *Cacna2d1*, *Cacnb2*, and *Cacnb4*.

Several other calcium-permeable channels were also found to be regulated by ethanol deprivation. The GRIN1 (*aka* NR1, NMDA1) subunit of the NMDA-type glutamate receptor was down-regulated (27%) and the GRIA1 (*aka* GLUR1, AMPA1) subunit of the AMPA-type glutamate receptor was up-regulated (42%). The decrease in *Grin1* transcript likely represents a decrease in NMDA receptor density, because each receptor consists of 1 of these subunits combined with 1 or more NR2 or NR3 subunits (Kumar and Mayer, 2013). In contrast, the increase in *Gria1* mRNA may represent an alteration in receptor function, because AMPA-type glutamate receptors containing only the GRIA1 subunit are highly permeable to calcium, while those also containing the GRIA2 subunit have low calcium permeability (Chen et al., 2001). Alterations in density and function of glutamate receptors are thought to be necessary for learning processes, and thus may mediate plasticity leading to changes in behavior following ethanol deprivation, during which a subject learns that abstinence is less rewarding than inebriation. On a more basic physiological level, changes in calcium homeostasis in the NAC and connected regions could lead to hyper-excitability. In the amygdala, for example, this could

trigger anxiety or fight-or-flight responses, driving the organism to seek alcohol to restore normal activity and relieve these symptoms. When considered in light of identified transcriptional regulation of genes involved in cellular calcium homeostasis (*Atp2b2, Itpr1, Trpc1, Trpc7)* and calmodulin signaling (*Iqgap2, Marcksl1, Ppp3r1*) it appears likely that changes in NAC calcium signaling occur with deprivation.

Transcripts coding for products related to other ion species were well-represented in results from NAC. Two voltage-gated potassium channels (*Kcnab1, Kcnb1*) were regulated, as were 2 voltage-gated potassium channel-interacting proteins (*Kcnip3, Kcnip4*). These channel-interacting proteins were both down-regulated, and function as calcium sensors to alter both potassium channel function and gene expression in response to changes in intracellular calcium, thus providing a connecting mechanism from direct effects of ethanol on intracellular calcium to modulation of excitability and transcription. The product of *Kcnip3* is also known as calsenilin or DREAM, and functions as a calcium-dependent transcriptional repressor that may be involved in nociception (Carrion et al., 1999). The mRNA transcript *Scn8a*, which codes for a pore-forming voltage-gated sodium channel involved in the action potential, was reduced by 33%. Chloride channel expression also appeared to be regulated by ethanol deprivation, as transcript for the proton-coupled chloride transporter CLCN5 was increased, while those for 2 GABA receptor subunits, GABRA1 and GABRG2 were decreased. Extensive changes in transcript abundance for ion channels indicates that ethanol deprivation is causing elevations in neuronal excitability and neurotransmitter release in NAc, which are consistent with known effects of long-term ethanol exposure and withdrawal (Kapasova and Szumlinski, 2008; Melendez et al., 2005; Moghaddam and Bolinao, 1994; Obara et al., 2009a; Piepponen et al., 2002; Szumlinski et al., 2008; Szumlinski et al., 2007).

Although little overlap was found in GO terms for molecular functions between regions, the biological processes affected by alterations in these functions revealed similarities in terms related to neuroplasticity: RNA splicing, RNA modification, mRNA processing, neurotransmitter release, and regulation of neurogenesis and apoptosis. The prevalence of these terms indicated adaptation of protein composition, inter-neuron communication, and neuron population to the condition of ethanol deprivation across regions.

In NAC terms related to signaling by peptide hormones and steroid hormones, in particular androgen receptor signaling, represent genes that may play a role in regulation of gene expression during ethanol deprivation. Ethanol drinking activates the HPA-axis, and this activation has been shown to be necessary for some changes in gene expression induced by alcohol in PFC (Costin et al., 2013b). This activation causes changes in levels of circulating hormones, which cross the blood-brain-barrier and regulate transcription in the brain. Repeated deprivation in rats during adolescence has been shown to reduce anxiety-like behavior in adulthood, which may be caused by reduced HPA-axis function, as seen in alcohol dependence (Gilpin et al., 2012; Richardson et al., 2008). Interestingly, IAA during adulthood did not alter anxiety-like behavior, but induced working memory deficits (George et al., 2012).

Several terms related to mRNA processing and splicing were identified in NAC, which may be a result of drastic changes in transcript abundance in this region. It should be noted that changes in splicing could be responsible observed differences in abundance; microarray probeset hybridization efficiency may be altered by splice-variants, causing apparent changes in abundance of a particular transcript, when in fact only the balance of isoforms has been altered.

Deprivation also appears to alter translation and degradation of proteins. Transcripts coding for eukaryotic translation initiation complex proteins were regulated in NAC: *Eif2s2, Eif4a1, Eif4e, Eif4e2, Eif4ebp2,* And *Eif5*. It has previously been shown that ethanol inhibits protein translation in skeletal and cardiac muscle tissue, contributing to myopathy in humans (Lang et al., 2001). In CNS ethanol exposure decreases protein synthesis in developing neuronal tissue, leading to apoptosis (Chen et al., 2006). A group of terms related to small protein conjugation and removal, primarily of ubiquitin, were significantly regulated, with several transcripts coding for proteins involved in de-ubiquitination found to be decreased in abundance. This regulation may indicate enhanced protein degradation via hyper-ubiquitination of target proteins. and indicates that the ADE may be mediated in part by alterations in protein translation and degradation in the NAC.

In NAC and VMB the canonical pathways identified tended to be similar to the biological processes identified by GO analysis. For NAC these pathways included several functioning in neuroplasticity, gene expression, and addictive behavior: corticotropin releasing hormone signaling, glucocorticoid receptor signaling, CREB signaling in neurons, calcium signaling, synaptic long term potentiation, and axonal guidance signaling.

Transcription factor binding site analysis allowed further examination of the mechanisms of gene regulation during deprivation. Consensus binding sequences for 3 transcription factors were significantly ($p < 0.05$) over-represented in promoter regions (+/- 2000 bp from transcription start site) of genes regulated by deprivation in all brain regions studied: ELF5, MZF1, and SOX17. None of these factors were regulated at the transcriptional level by ethanol deprivation, but represent potential targets for therapeutic interference to prevent maladaptive regulation of entire groups of genes.

Epigenetic mechanisms of gene regulation via histone modification have been posited to play a role in the acute and chronic effects of ethanol, and on susceptibility to stress and drug addiction (Bardaggorce et al., 2007; Liu et al., 2009; Meaney et al., 2007; Pandey et al., 2008; Renthal et al., 2007; Wolstenholme et al., 2011). Several transcription factor binding sites in NAC and VMB genes were associated with proteins or protein complexes with chromatin remodeling functions, indicating that epigenetic mechanisms of gene regulation may mediate some transcriptional regulation associated with ethanol deprivation. For example, transcription factor SP1 regulates gene expression via the ATP-dependent SWI/SNF chromatin remodeling complex (Murphy et al., 1999; Trotter, 2007). After ethanol deprivation the *Smarca4* transcript, which codes for the protein that acts as the ATPase and helicase for this and several other chromatin remodeling complexes, showed a two-fold increase in NAC compared to control mice, and may represent an important mediator of deprivation-induced changes in gene expression. This degree of regulation placed it in the top 2% of regulated transcripts, as measured by magnitude of change in transcript abundance compared to controls. Of particular interest would be alcohol deprivation experiments in regional conditional knockout mice for *Smarca4*, and to inactivate the gene at particular phases of the experiment, such as immediately after alcohol cessation, or prior to reinstatement. These mice have not yet been developed, but the production of the appropriate constructs is an ongoing research effort by the International Knockout Mouse Consortium, so the generation of such mice should be possible in the near future.

The CRE consensus sequence was over-represented in promoter regions of genes from NAC, and chromatin remodeling is implicated in transcription mediated by CREB through the action of two closely related coactivator proteins with histone acetyltransferase activity: E1A binding protein p300 (P300) and CREB-binding protein (CBP). CREB-mediated transcriptional activation has

been implicated in addiction-related studies for alcohol (Hu et al., 2008; Zou and Crews, 2006) and other drugs of abuse (Barrot et al., 2002; Green et al., 2006; McClung and Nestler, 2003). CREB-mediated transcription acts as a gating mechanism for emotional stimuli in the NAC, serving to blunt behavioral responses to emotional stimuli (Barrot et al., 2002). Ethanol has been shown to decrease CREB activation and DNA binding in the nucleus accumbens (Misra and Pandey, 2006; Misra et al., 2001; Zou and Crews, 2006). Furthermore, ethanol drinking behavior is increased by partial deletion of CREB or inhibition of cAMP-dependent protein kinase A (PKA) (Misra and Pandey, 2006; Pandey, 2004b). Over-representation of CREB binding sites in deprivation-regulated genes is consistent with a previous report analyzing ethanol responsive gene expression (Uddin and Singh, 2007). This evidence, when considered in the context of previously reported involvement of CREB in the nucleus accumbens in addiction, suggests that gene expression mediated by CREB may be integral to the ADE.

The mechanism by which modulation of CREB-mediated transcriptional regulation contributes to the ADE is likely to be complex, due to the large number of transcripts regulated by CREB, and the diverse molecular effects of ethanol, which include potentiation of GABAergic signaling. In NAC, animals deprived of ethanol showed decreased expression of GABAA receptor subunits α1 (GABRA1) and γ2 (GABRG2). In VMB, these animals showed increased expression of the α6 (GABRA6) receptor subunits. GABAA receptor surface expression has been reported to be controlled by CREB and the dominant-negative inhibitor inducible cAMP early repressor (ICER) (Hu et al., 2008), which has also been implicated in addiction-related behavior in NAC (Green et al., 2006). These proteins bind to the promoter of the GABRA1 gene and decrease expression of its transcript and thus limit production of α1-containing receptors. In addition, it has been shown that the γ2 subunit is phosphorylated by protein kinase C in response to ethanol exposure,

serving to decrease sensitivity to ethanol (Qi et al., 2007). Altered GABAA receptor expression in NAC of deprived animals could increase the activity of the mesolimbocortical dopaminergic pathways and thus alter ethanol consumption behavior, and this mechanism could be dissected through regional quantitative PCR to examine expression of CREB and related genes, in combination with regional administration of GABAA antagonists, such as picrotoxin (Carpenter et al., 2013; Li et al., 2011a).

Another mechanism responsible for gene regulation associated with ethanol deprivation may be RNA-mediated gene silencing (RNAi), a process which is altered in brains of human alcoholics and produces teratogenic effects (Lewohl et al., 2011; Nunez and Mayfield, 2012; Wang et al., 2008a). The *Eif2c2* transcript coding for the eukaryotic translation initiation factor 2C, 2, also known as argonaute 2, is necessary for RNAi and was significantly up-regulated by ethanol deprivation (Cenik and Zamore, 2011). Upregulation of *Eif2c2* may be indicative of potentiation of RNAi processes in NAC neurons, and account for the prevalence of downregulated transcripts in this region. Future studies should use microarrays that measure miRNA abundance in addition to mRNA, which will allow for the discovery of regulatory networks and involved biological processes, and thus potential therapeutic vectors (Chavali et al., 2013; Lau et al., 2013; Zadran et al., 2013).

The primary utility of genomic analysis of the type reported in this study is to identify genes not previously examined in the context of ethanol- or drug-related behaviors, which may provide insight into biological processes leading to addiction, and novel targets for therapeutic intervention for drug abusers. However, due to the large number of genes identified, it is necessary to apply a method of prioritization for further study. Toward this end genes in each region were ranked based on the nature of their known protein-protein interactions—specifically

the similarity of these interactions to those found in other gene sets related to ethanol consumption, as well as compared to other microarray-derived gene sets relevant to ethanol consumption. Genes thus identified may play central roles in plasticity due to ethanol deprivation that leads to elevated ethanol consumption, possibly as hubs in gene networks regulated during abstinence.

When interpreting these results caution must be taken with regard to the attribution of cause and effect to regulation of specific genes. Because a single time point was examined, and this at the end of the deprivation period, the sequence of changes in gene expression during the previous 4 days of ethanol deprivation remains unknown, as do the actual cell types containing the expression changes identified in our studies. Future studies will address these concerns by examining the time course of expression changes following cessation of ethanol access, and studying candidate genes at the cellular level to identify neural/glial networks contributing to the ADE. Perhaps the biggest caveat of the study is that only one strain was examined, and it is unclear the degree to which results are generalizable to other strains. Future experimentation should verify functional mechanisms and gene expression in other inbred strains. High priority targets for verification of this type are strains known to differ in ethanol-related behavior, and strains with diverse and well-characterized genotypes and phenotypes, for example the DBA/2J, C57BL/6J, BXD panel, and the Hybrid Mouse Diversity Panel (Costin et al., 2013b; Ghazalpour et al., 2012; Li et al., 2010c; Vanderlinden et al., 2013).

Persistent perturbation of the brain by ethanol induces neuroplasticity to maintain homeostasis and organism function (Francesconi et al., 2009; George and Koob, 2010; George et al., 2012). Although in the ADE model these changes do not reach the level of clinical dependence—in which withdrawal symptoms occur with abstinence—upon cessation of ethanol use this plasticity

seems to become maladaptive and causes the organism to seek the drug. In light of recent studies—utilizing repeated deprivation cycles and intermittent ethanol access in rodents—these behavioral and genomic results supports the idea that limited ethanol access schedules represent a valid animal model for the development of alcoholism that does not rely on forced dependence (Hopf et al., 2010; Melendez, 2011; Melendez et al., 2006a). In particular the transition after an extended period of 3 days-per-week access to inflexible intake behavior, in which quinine adulteration does not reduce voluntary ethanol consumption, may mark a second behavioral indicator in the development of alcohol addiction—after the initial increase in consumption and preference—that would not otherwise be apparent, and study of the molecular correlates of this behavioral state change will further illuminate the transition to uncontrollable alcohol consumption.

Alcoholism is a condition partially defined by relapse (DSM-IV-TR, APA, 2000; Haeny et al., 2013; Kopak et al., 2013). Research into changes in gene expression resulting from acute ethanol exposure provides a starting point for study of the cellular response to ethanol exposure, but knowledge of gene expression changes after cessation of ethanol intake can provide important insights into vulnerability to alcohol abuse and treatment of abusive behavior. Thus, alteration in gene expression during abstinence following periods of use is vital to understanding of the maintenance of alcoholism. The molecular and behavioral effects of acute ethanol exposure following a period of abstention, which are predictive of relapse behavior, depend on the state of gene and protein expression in the subject at the time of reinstatement. In reporting several molecular pathways containing ethanol responsive proteins regulated at the mRNA level after 4 days of withdrawal from voluntary ethanol exposure, this study provides evidence that the level of expression of particular genes relevant to the behavioral response to ethanol re-exposure is

altered by cessation itself. This report identified genes, pathways, and networks regulated days after cessation of ethanol intake which likely contribute to the magnitude of the ADE, and thus potentially to relapse in alcoholics, providing direction for future studies on the molecular determinants of relapse to alcoholism.

Chapter 4. Intermittent alcohol access elevates ethanol intake in C57BL/6J and C57BL/6NCrl mice

Introduction

The transition from social alcohol drinking to alcohol use disorders (AUDs) is often characterized by binge drinking, which is defined by the National Institute on Alcohol Abuse and Alcoholism as a pattern of consumption that leads to blood alcohol levels (BALs) of greater than 80 mg/dl . For a typical adult BALs representative of binge-like drinking are achieved by consumption in a 2 hour period of 5 drinks for males, and 4 drinks for females (Fillmore and Jude, 2011). Binge drinking is not rare; approximately 23% of the U.S. population engages in binge drinking at least once per month. Frequent binge drinkers, who consume 5 or more drinks on 5 or more occasions per month, make up 7% of the U.S. population, and drink 45% of the alcohol consumed by American adults. Remarkably, in drinkers 18 to 20 years of age, binge drinking accounts for 96% of alcohol consumed by the age group (NSDUH, 2003). Although binge drinking does not always lead to alcohol dependence, it is associated with diverse negative health effects, as well as negative social and economic outcomes (Standridge et al., 2004). Better understanding of this behavior will contribute to understanding of the genetic and environmental factors that contribute to binge drinking, and help identify patterns of binge drinking that may lead to dependence. Effective preclinical models that produce high alcohol consumption in a short period of time are crucial to this understanding, and in general may be classified by whether or not they induce alcohol dependence (Crabbe et al., 2011).

In rodents, binge-like drinking that leads to dependence is hypothesized to be motivated by negative reinforcement, or the desire to mitigate the negative symptoms associated with alcohol withdrawal. Preclinical models of binge-like drinking that do not lead to alcohol dependence are hypothesized to be motivated by the positive reinforcing and rewarding effects of alcohol (Barak et al., 2011; Becker, 2013; Lopez et al., 2012). Models of binge-like alcohol exposure that lead to dependence manipulate animals to achieve extremely high BALs, which may exceed 200 mg/dl (Criado and Ehlers, 2013). Following this exposure animals are allowed to drink under voluntary conditions, and display elevated consumption with repeated exposures. Examples of this type of model are those using ethanol vapor exposure, alcohol-containing liquid diets, and alcohol offered as the only source of liquid (Cheaha et al., 2013; Lopez and Becker, 2005; Roberts et al., 2000a; Wise, 1973). In contrast, drinking-in-the-dark (DID), saccharin-sweetened solutions ("supersac"), schedule-induced polydipsia, and intermittent alcohol access (IAA) produce binge-like drinking without associated alcohol dependence (Broadwater et al., 2013; Crabbe et al., 2009; Falk and Samson, 1975; Melendez, 2011; Simms et al., 2013). Non-dependent models have several advantages in validity and feasibility compared to models that induce binge-like drinking through dependence, and the IAA model in particular has received much research attention in recent years.

The IAA model was developed in the early 1970s by Roy Wise and colleagues at Sir George Williams University (now Concordia University) in Montreal, while studying intra-cranial self-stimulation in rats. In adult Wistar rats, scheduled abstinence punctuated by 24-hour access periods produces consumption of nearly 5 g/kg/day, compared to approximately 3 g/kg/day in continuous access (CA) subjects (Amit et al., 1970; Wise, 1973). The model has recently been readopted by several laboratories because it requires minimal experimental manipulation and

produces high levels of alcohol intake and blood ethanol concentration (BEC) in rodents (Hwa et al., 2011; Melendez, 2011; Simms et al., 2013; Simms et al., 2008). In the typical IAA procedure, alcohol is offered along with water for one or three nonconsecutive 24-hour consumption periods per week. IAA gradually increases alcohol consumption and preference for alcohol over water, although significant differences compared to CA subjects are often observed by the second drinking session. IAA drinking behavior is influenced by environment and genotype, and not all strains of rodent display increases in alcohol intake (Crabbe et al., 2012; Palm et al., 2011; Rosenwasser et al., 2013). Concurrent access to multiple alcohol concentrations and the temporary use of sweetened alcohol solutions significantly increase the efficacy of IAA, as do early life stressors such as maternal separation and social isolation (Chappell et al., 2013; Daoura et al., 2011; Hwa et al., 2011; Melendez et al., 2006a).

There is significant molecular and behavioral evidence that IAA is a useful model for the transition to alcoholism. Binge-like drinking produced by IAA is dose-dependently attenuated by naltrexone and acamprosate, which are approved by the FDA for use in patients with AUDs (Sabino et al., 2013; Simms et al., 2008). Most studies have implied a role for the corticotropin releasing hormone (CRH) system in IAA behavior, which may exert influence through hypothalamic and extra-hypothalamic mechanisms (Gilpin et al., 2008b; Hwa et al., 2011; Sabino et al., 2013; Simms et al., 2008). After several weeks or months of IAA a distinct set of behavioral changes are observed: significantly decreased water intake, resistance to adulteration of alcohol with the bitter substance quinine, impaired performance in the Rota-rod test, and reduced preference for sweet solutions compared to alcoholic solutions (Hopf et al., 2010; Loi et al., 2010; Obara et al., 2009a). Furthermore, withdrawal from extended IAA elevated levels of several proteins involved in glutamatergic signaling in the nucleus accumbens and amygdala of

alcohol-preferring P-rats (Obara et al., 2009a). These brain regions are known to be involved in motivated behavior and stress responses, and withdrawal from alcoholism in humans is associated with upregulation of glutamatergic signaling that contributes to craving and relapse (Bauer et al., 2013; Hermann et al., 2012; Nardone et al., 2012). It is therefore useful to study the transcriptional response to intermittent access in conjunction with drinking behavior, to provide insight into plasticity that alters neurotransmission and alcohol intake over time, because the model produces behavior and neuromolecular states similar to those seen in alcoholics.

While the IAA procedure seems to show validity as a model for the transition to alcoholism, the genetic and environmental influences on its effects are not well understood, and results have varied significantly among laboratories, even within an inbred strain (Crabbe et al., 2012; Hwa et al., 2013; Rosenwasser et al., 2013; Sabino et al., 2013). Therefore, studies are reported herein that were conducted to better understand the effects of genetic background and choice of alcohol concentration on binge-like alcohol drinking behavior, and to examine associated transcriptional regulation in the nucleus accumbens produced by binge-like intake. It is hypothesized that IAA will be more effective in the alcohol-preferring C57BL/6J mouse strain compared to the closely-related C57BL/6NCrl strain of mouse, which typically drinks less alcohol, and has not been published on in the context of the IAA model. Furthermore it is hypothesized that offering multiple concurrent alcohol concentrations to C57BL/6J mice will increase alcohol intake relative to use of a single concentration, but that both procedures will significantly elevate consumption and preference over time.

Three studies are reported that were conducted toward this end. Experiment 1 reports the effect of 1-day-per-week IAA on C57BL/6NCrl mice from Charles River Laboratories. Experiment 2 reports the effect of 1-day-per-week IAA on C57BL/6J mice with access to either 15% alcohol

or 7.5%, 15%, and 30% alcohol concurrently. Finally, quantitative real-time PCR was used to examine differences between groups in nucleus accumbens gene expression of transcripts known to be regulated by a single 4-day ethanol deprivation: *Smarca4, Cacna1g, Cacna1d, Kif5c,* and *Gria1,* to examine commonalities in transcriptional regulation across deprivation models (Warner, unpublished data, thesis chapter 2).

Materials and Methods

Animals

For experiment 1, 50 male C57BL/6NCrl mice were obtained from Charles River Laboratories (Wilmington, Massachusetts) at 8 to 9 weeks of age. For experiment 2, 56 male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Upon arrival mice were individually housed in standard clear plastic mouse cages on wood-chip based bedding (Harlan Sani-chips, #7090A, Harlan, Teklad, Madison, WI). Mice were offered food and water at all times during all experiments. All experimental procedures adhered to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Two-bottle choice drinking

Animals were offered water and alcohol solutions in drinking tubes fashioned from 10 mL disposable polystyrene pipets, into which were inserted 1.5" steel ball-bearing sipper tubes (Ancare Corporation, Bellmore, NY). After filling, the tubes were sealed with standard size 0 rubber stoppers, and offered to animals by inserting the sipper tube through the metal bars of the cage tops. Solutions were changed daily, and the side on which alcohol and water solutions were

offered was alternated every other day to avoid the development of position preference. Fluid measurements were taken by the distance from meniscus to a mark made at the meniscus the previous day. Solutions were measured to the nearest 0.1 mL and changed at 5:00pm every day, and lights were off from 6:00pm to 6:00am. During IAA, alcohol was offered on Fridays, and animal weight was measured every other Wednesday to avoid stress from handling. For all experiments alcohol consumption was calculated as g/kg/day, and alcohol preference was calculated as total alcohol consumed in mL divided by total fluid consumed in mL.

For experiment 1, mice were separated into three groups: intermittent access (IAA, N = 25), continuous access (CA, N = 12), and water only (WON, N = 12). All alcohol solutions offered were 15% alcohol by volume. The IAA group was allowed a 30-day baseline period of continuous access to alcohol as a point of comparison, followed by 8 weekly cycles of 6 days of abstinence and 1 day of drinking (86 days total). The CA group was allowed continuous access to alcohol for 86 days, and the H group was allowed access to water only for the entire study period. After the 8^{th} cycle of IAA mice were abstinent for 6 days, and then sacrificed for brain collection. Nucleus accumbens tissue was obtained and frozen immediately at -80 degrees C.

For experiment 2, mice were separated into five groups: intermittent access with multiple alcohol concentrations (IAMC, N = 12), intermittent access with 15% alcohol only (IASC, N = 12), continuous access with multiple alcohol concentrations (CAMC, N = 12), continuous access with 15% alcohol only (CASC, N = 12), and water only (WOJ, N = 12). When multiple alcohol concentrations were offered, they were 7.5%, 15%, and 30% ethanol by volume. The IAA group was allowed a 17-day baseline period of continuous access to alcohol as a point of comparison, followed by 7 weekly cycles of 6 days of abstinence and 1 day of drinking (86 days total). The CA group was allowed continuous access to alcohol for 56 days after a delayed start, to give an

equal number of days of access as the IA group. The H group was allowed access to water only for the entire study period. After the 7th cycle of IAA mice were abstinent for 6 days, and then sacrificed for brain collection. Nucleus accumbens tissue was obtained and frozen immediately at -80 degrees C.

For experiment 1 (IAA in C57BL/6NCrl mice) 2-way ANOVA and Fisher's LSD test were used to determine statistical significance for changes in drinking behavior over time and between groups. Repeated measures could not be used for experiment 1 due to missing data caused by leaking drinking tubes. For experiment 2 (IAA in C57BL/6J mice) 2-way ANOVA with repeated measures and Fisher's LSD test were used to determine statistical significance for changes in drinking behavior over time and between groups.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from NAc from individual mice in STAT 60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. RNA concentration and quality was assessed by Experion automated electrophoresis and UV spectrophotometer (BioRad, Hercules, CA). All samples met quality control standards and no samples were excluded from analysis. cDNA was generated from 1 μg total RNA by reverse transcription with the iScript CDNA kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (Q-rtRCR) was performed using the iCycler iQ system (Bio-Rad) according to the manufacturer's instructions for SYBR Green-based detection.

Quantification of gene expression levels was determined based on the threshold cycle for each well using the provided software, and all results were normalized to multiple stable reference genes using Genorm (Vandesompele et al., 2002). One-way ANOVA was used to determine

statistically significant differences across groups in transcript abundance, followed by Tukey post-tests for pair-wise group differences. Significance was taken as $p < .05$ for all comparisons.

Results

Experiment 1: 2-bottle choice intermittent access drinking in C57BL/6NCrl mice

IAA caused an immediate and significant increase in ethanol consumption in C57BL/6NCrl mice, which remained significant over 8 test sessions (see Figure 4.1). Mice in the CA group did not alter consumption over the course of the experiment. Two-way ANOVA identified a significant effect of group [$F(1, 314) = 19.12$, $p < .0001$], but not of time [$F(8, 314) = 1.316$, $p = 0.2345$], and there was no interaction [$F(8, 314) = 0.6695$, $p = 0.7184$]. In IAA mice Fisher's LSD test identified significant differences in consumption compared to baseline at $p < .05$ or lower ($p < .01$ for test days 4 and 6, $p < .001$ for test days 3 and 7) for all test days. In CA mice Fisher's LSD test identified no significant differences compared to baseline on any test day. For between groups comparison Fisher's LSD identified significant differences between IAA and CA groups on test days 3, 7, and 8 ($p < .05$).

IAA also caused an immediate and significant increase in ethanol preference in C57BL/6NCrl mice, which remained significant for all but the final test session (see Figure 4.2). Mice in the CA group did not alter preference over the course of the experiment. Two-way ANOVA identified a significant effect of group [$F(1, 314) = 9.140$, $p = .0027$], but not of time [$F(8, 314) = .9818$, $p = 0.4501$], and there was no interaction [$F(8, 314) = 0.6251$, $p = 0.7567$]. In IAA mice Fisher's LSD test identified significant differences in preference compared to baseline at $p < .05$ or lower ($p < .01$ for test days 3 and 7) for all test days. In CA mice Fisher's LSD test identified no significant differences compared to baseline on any test day. For between groups comparison Fisher's LSD identified no significant differences between IAA and CA groups at $p < .05$.

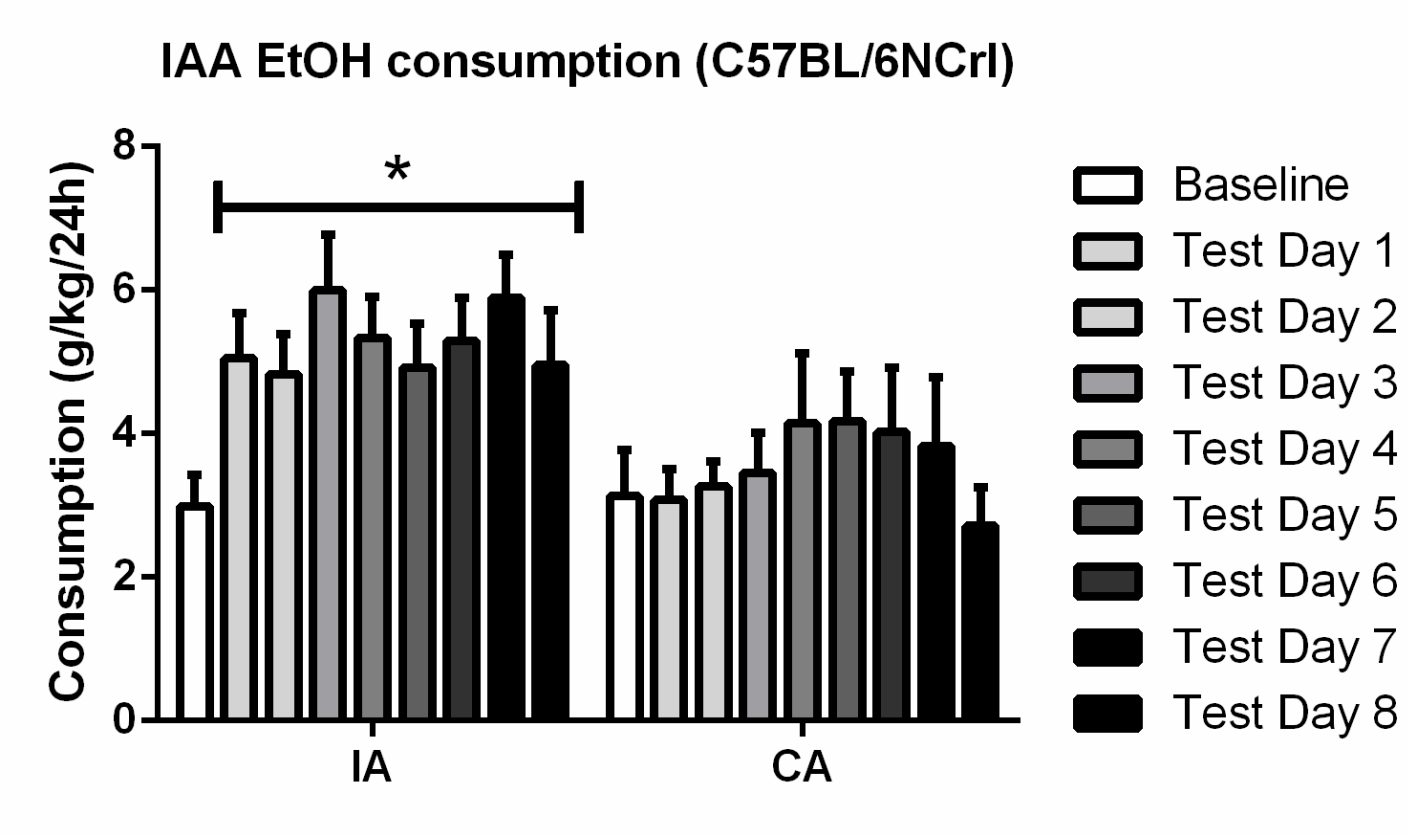


Figure 4.1. Ethanol consumption over time in mice offered intermittent access (IAA) or continuous access (CA) to 15% ethanol and water in a 2-bottle choice model. One-way ANOVA with repeated measures within each group. * : $p < .05$ in Fisher's LSD test compared to baseline.

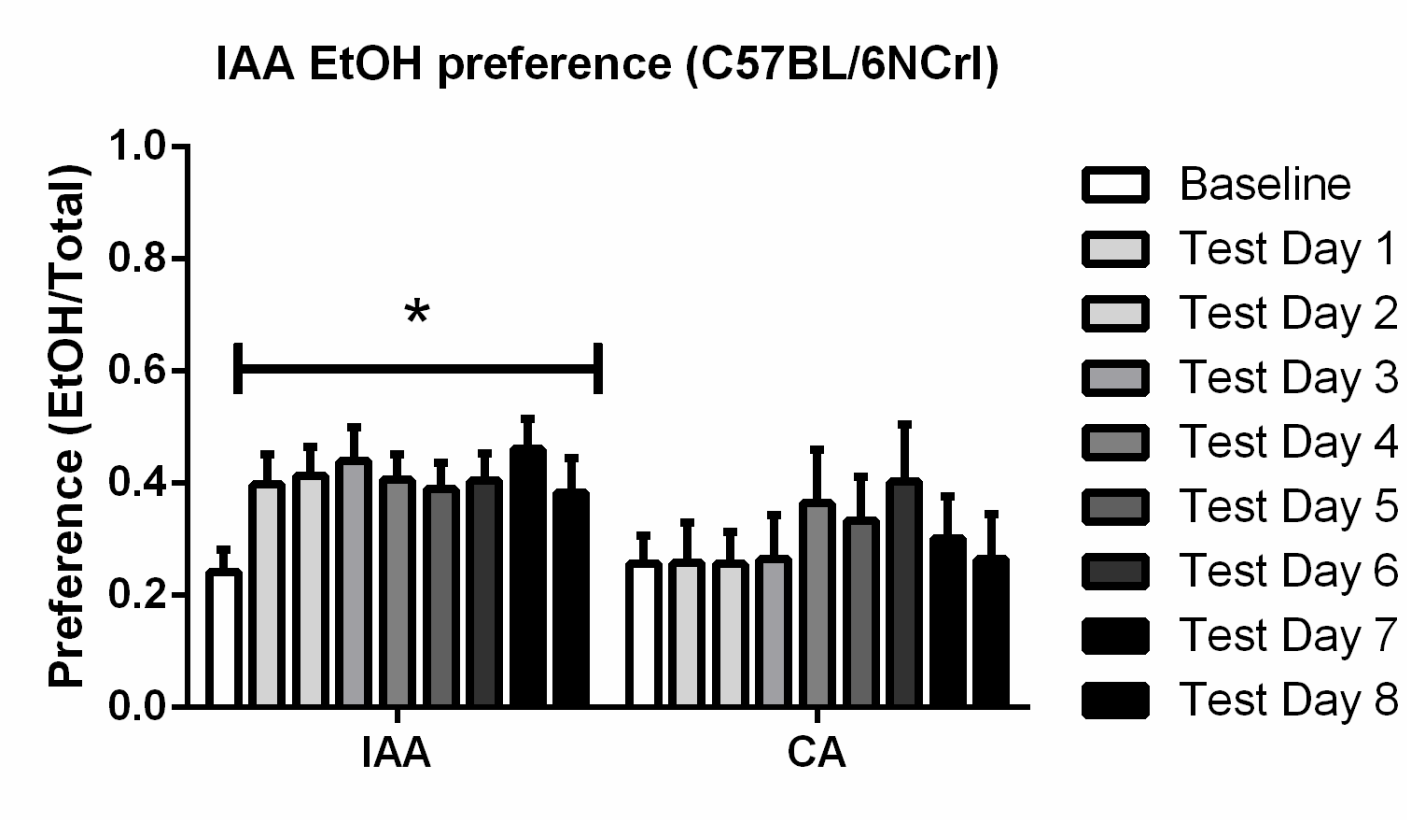


Figure 4.2. Ethanol preference over time in mice offered intermittent access (IAA) or continuous access (CA) to 15% ethanol and water in a 2-bottle choice model. One-way ANOVA with repeated measures within each group. * : $p < .05$ in Fisher's LSD test compared to baseline.

Experiment 2: 2-bottle choice and 4-bottle choice intermittent access drinking in C57BL/6J mice

Continuous alcohol access: single concentration vs. multiple concentrations

Two-way ANOVA with repeated measures revealed significant differences in ethanol consumption between C57BL/6J mice allowed continuous access to a single ethanol 15% concentration (CASC) and mice offered access to 7.%, 15%, and 30% ethanol (CAMC) (see Figure 4.3). Comparisons were drawn for the 7 days running from day 11 to day 17 of the 25 day continuous alcohol access period. There was a significant effect of group [$F(1, 22) = 62.58$, $p < 0.0001$], a trend toward a significant effect of time [$F(6, 132) = 1.978$, $p = 0.0732$], and no interaction [$F(6, 132) = 0.7509$, $p = 0.6098$]. Consumption was significantly different between the CASC and CAMC mice on all drinking days (Fisher's LSD test; $p < .0001$).

Two-way ANOVA with repeated measures also revealed significant differences in ethanol preference between C57BL/6J mice allowed continuous access to a single ethanol 15% concentration (CASC) and mice offered access to 7.%, 15%, and 30% ethanol (CAMC) (see Figure 4.4). For CAMC mice ethanol preference was calculated as total intake of fluid containing alcohol in mL divided by total fluid intake in mL, and for CASC mice ethanol preference was calculated as intake of 15% alcohol in mL divided by total fluid intake in mL. Comparisons were drawn for the 7 days running from day 11 to day 17 of the 25 day continuous alcohol access period. There was a significant effect of group [$F(1, 22) = 8.009$, $p = 0.0097$], no significant effect of time [$F(6, 132) = .6545$, $p = 0.6864$], and no interaction [$F(6, 132) = 0.7872$, $p = 0.5814$]. Preference was significantly different between the CASC and CAMC mice on days 2 through 6 of the 7 day drinking period (Fisher's LSD test; $p < .05$).

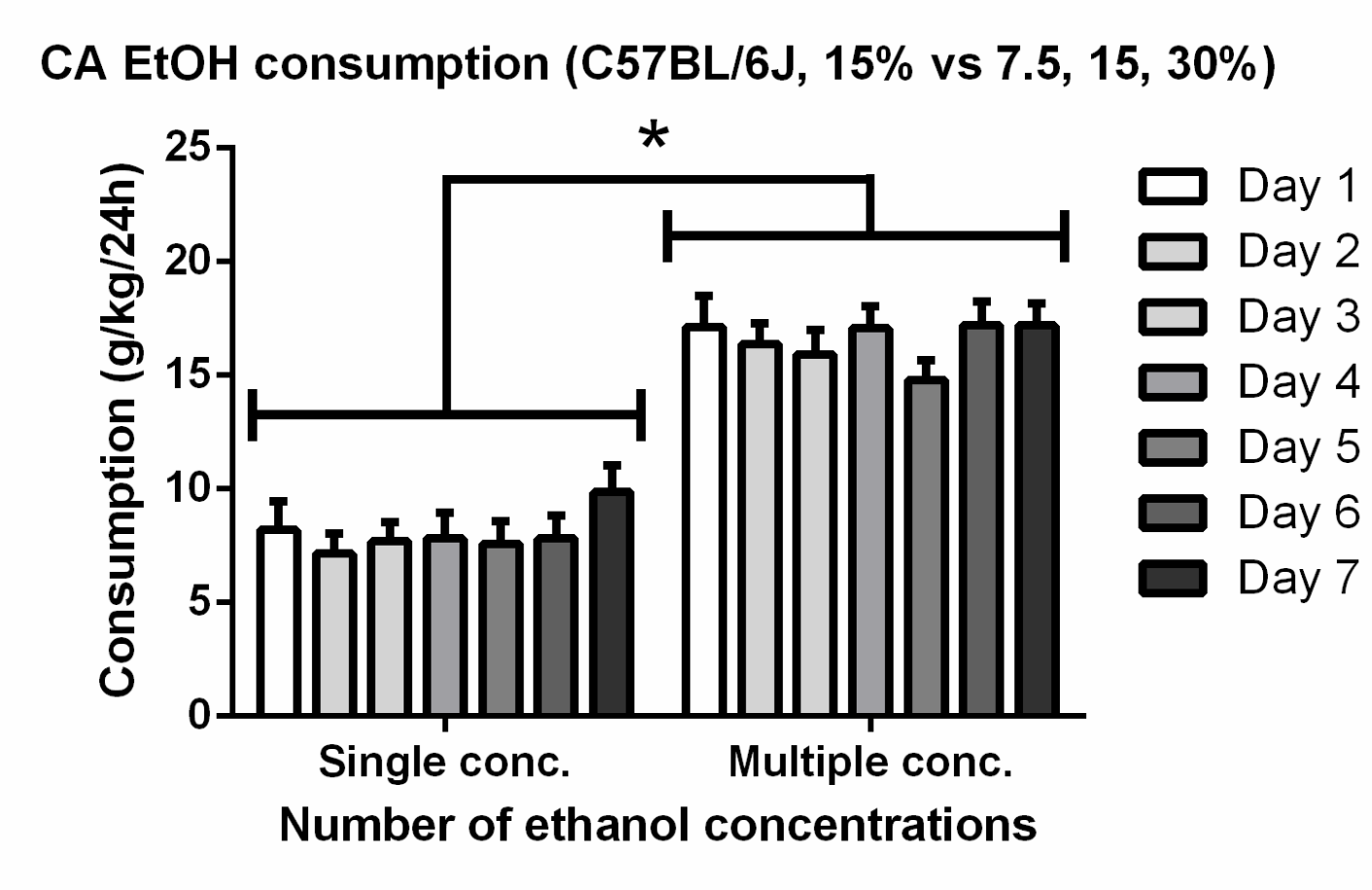


Figure 4.3. Total ethanol consumption in mice offered continuous access to water, and to either a single concentration (15%) or multiple concentrations (7.5, 15, and 30%) of ethanol. * : $p < .05$; two-way ANOVA with repeated measures, Fisher's LSD test for daily mean between groups.

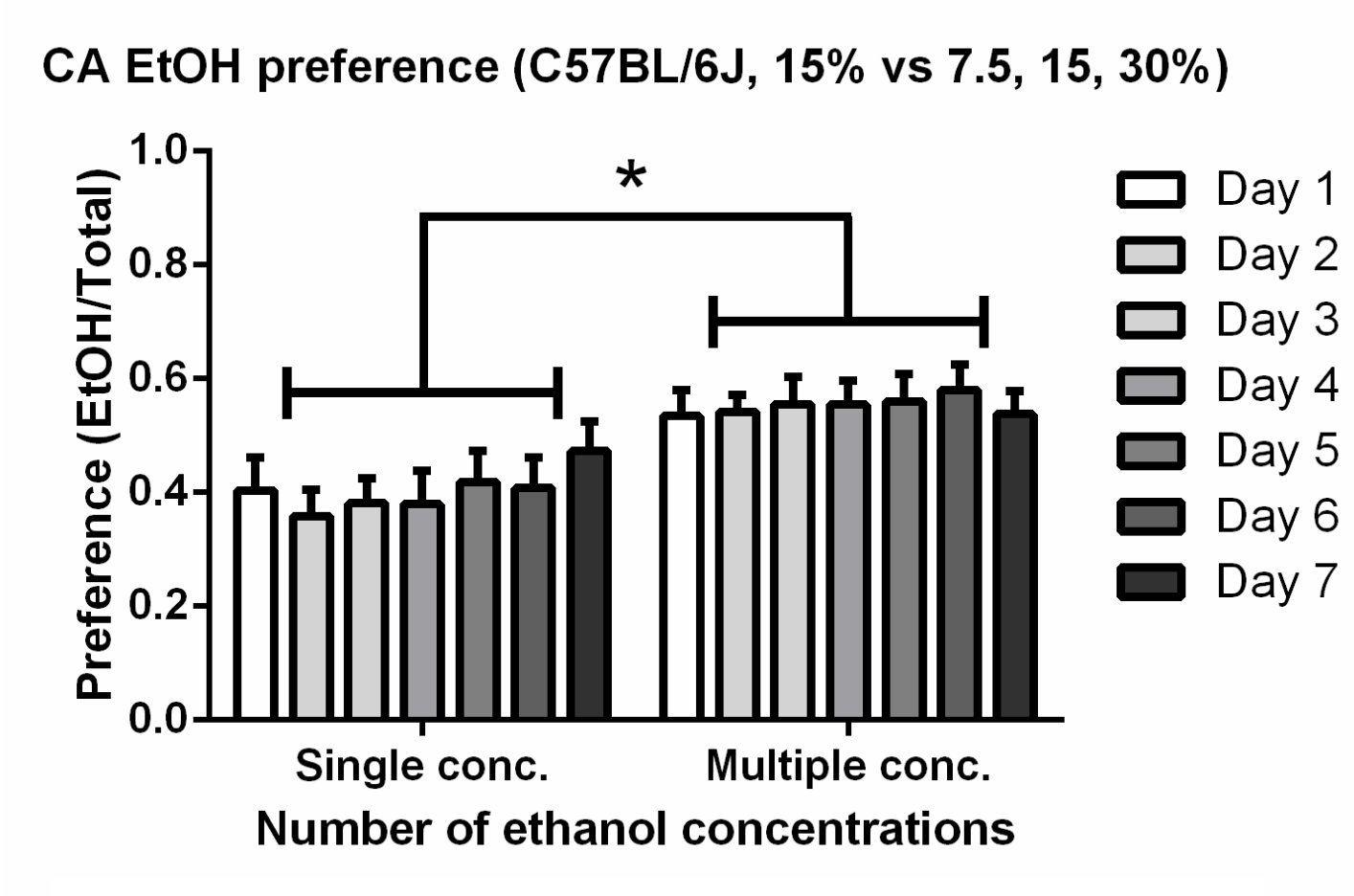


Figure 4.4. Total ethanol preference in mice offered continuous access to water, and to either a single concentration (15%) or multiple concentrations (7.5, 15, and 30%) of ethanol. * : $p < .05$; two-way ANOVA with repeated measures, Fisher's LSD test for daily mean between groups.

Intermittent alcohol access: single concentration vs. multiple concentrations

Total alcohol consumption and preference

In C57BL/6J mice IAA produced significantly increased total ethanol consumption when a single concentration (15%) or multiple concentrations (7.5%, 15%, and 30%) were offered (see Figure 4.5). Comparison of single-concentration (IASC) and multiple concentration (IAMC) mice using two-way ANOVA with repeated measures revealed significant effects of group [$F(1, 22) = 67.74$, $p < 0.0001$], and time [$F(6, 132) = 8.923$, $p < 0.0001$], but no significant interaction [$F(6, 132) = 0.9468$, $p = 0.4640$]. For IASC mice Fisher's LSD test showed significant differences in total consumption compared to baseline from test day 2 through test day 7 ($p < .05$). For IAMC mice Fisher's LSD test showed significant differences in total consumption compared to baseline from test day 1 through test day 7 ($p < .01$). Fisher's LSD test revealed significant differences in total consumption between IAMC and IASC mice for baseline and at all test days ($p < .0001$).

In C57BL/6J mice IAA also produced significantly increased total ethanol preference when a single concentration (15%) or multiple concentrations (7.5%, 15%, and 30%) were offered (see Figure 4.6). Comparison of single-concentration (IASC) and multiple concentration (IAMC) mice using two-way ANOVA with repeated measures revealed significant effects of group [$F(1, 22) = 6.942$, $p= 0.0151$], and time [$F(6, 132) = 6.423$, $p < 0.0001$], but no significant interaction [$F(6, 132) = 0.4946$, $p = 0.8115$]. For IASC and IAMC mice Fisher's LSD test showed significant differences in total consumption compared to baseline on all test days except test day 3 ($p < .05$). Fisher's LSD test revealed significant differences in total consumption between IAMC and IASC mice for baseline and on test days 1, 2, 4, 6, and 7. ($p < .05$).

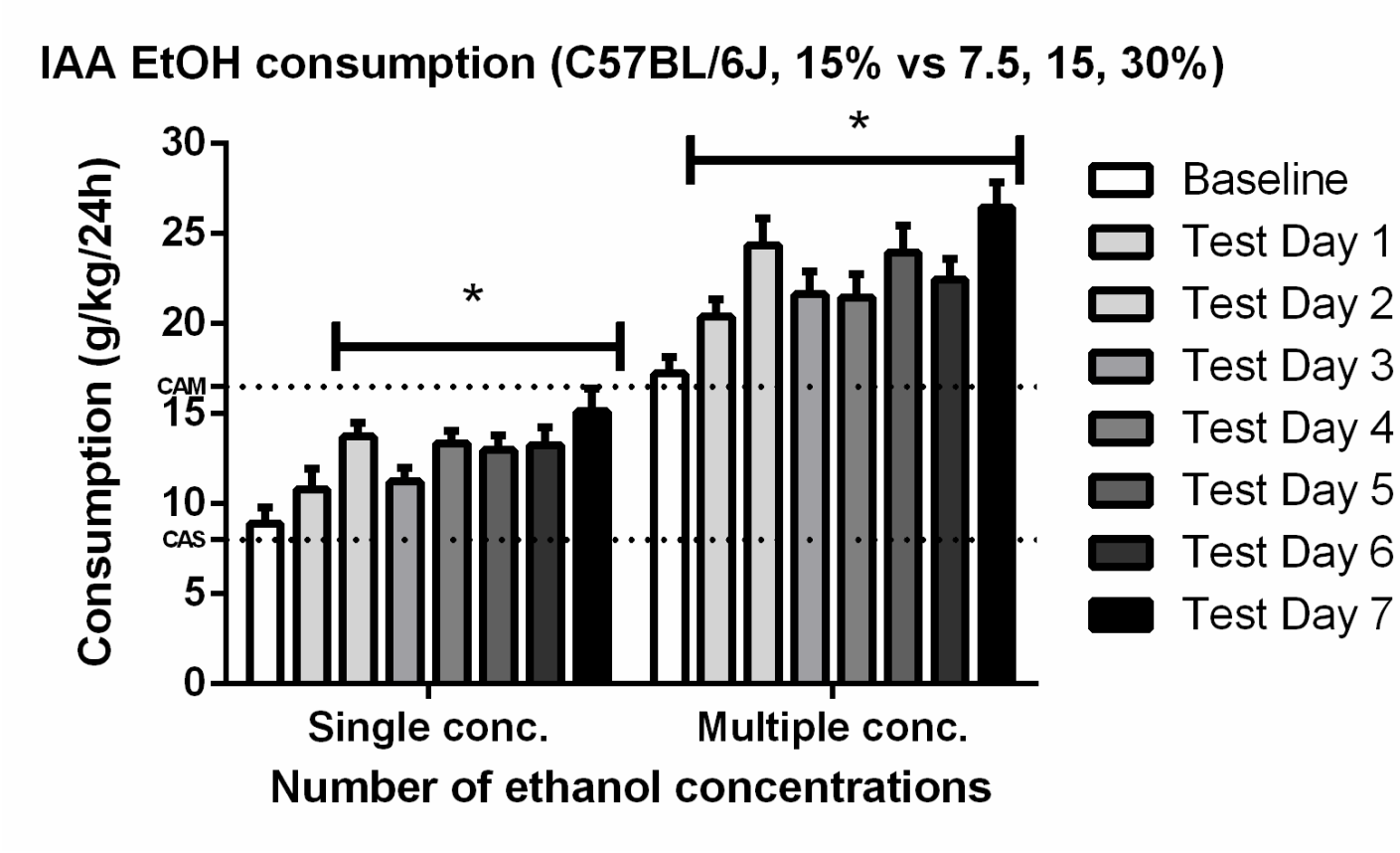


Figure 4.5. Total ethanol consumption in mice offered intermittent access to water, and to either a single concentration (15%) or multiple concentrations (7.5, 15, and 30%) of ethanol. CAS and CAM indicate 7-day average consumption for mice offered continuous access to a single concentration, or multiple concentrations, of ethanol, respectively. * : $p < .05$; two-way ANOVA with repeated measures, Fisher's LSD test for daily means compared to group baseline.

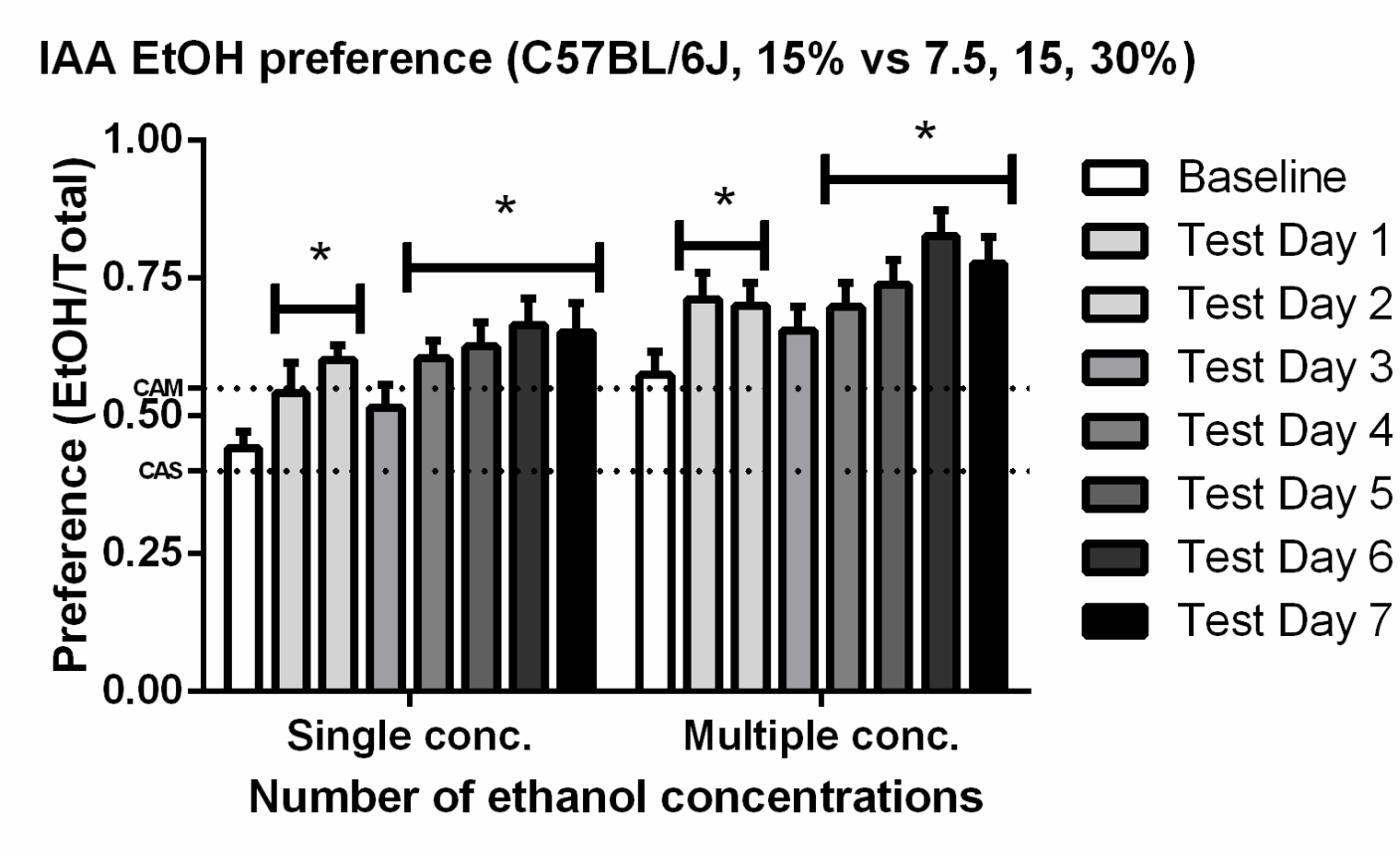


Figure 4.6. Total ethanol preference in mice offered intermittent access to water, and to either a single concentration (15%) or multiple concentrations (7.5, 15, and 30%) of ethanol. CAS and CAM indicate 7-day average preference for mice offered continuous access to a single concentration, or multiple concentrations, of ethanol, respectively. * : $p < .05$; two-way ANOVA with repeated measures, Fisher's LSD test for daily means compared to group baseline.

15% alcohol consumption and preference

IAA produced significantly increased 15% ethanol consumption when a single concentration (15%) or multiple concentrations (7.5%, 15%, and 30%) were offered (see Figure 4.7). Comparison of single-concentration (IASC) and multiple concentration (IAMC) mice using two-way ANOVA revealed a trend toward a significant effect of group [$F(1, 175) = 3.356$, $p = 0.0687$], a significant effect of time [$F(7, 175) = 4.515$, $p = 0.0001$], and no significant interaction [$F(7, 175) = 0.5680$, $p = 0.7813$]. Repeated measures could not be used due to leakage of all 15% ethanol from the drinking tube for IASC mouse #9 on test day 5. For IASC mice Fisher's LSD test showed significant differences in 15% consumption compared to baseline on test day 2 and from test day 4 through test day 7 ($p < .05$). For IAMC mice Fisher's LSD test showed significant differences in 15% consumption compared to baseline on test day 2 and from test day 5 through test day 7 ($p < .05$). Fisher's LSD test revealed significant differences in 15% consumption between IAMC (9.26 g/kg) and IASC (13.37 g/kg) mice for test day 4 only ($p = .0410$).

Differences were more apparent between IAMC and IASC mice for 15% ethanol preference, but in IAMC mice this measurement is less meaningful, because it does not represent the totality of ethanol consumed compared to water. In C57BL/6J mice IAA produced significantly increased 15% ethanol preference when a single concentration (15%) or multiple concentrations (7.5%, 15%, and 30%) were offered (see Figure 4.8). Comparison of single-concentration (IASC) and multiple concentration (IAMC) mice using two-way ANOVA revealed a significant effect of group [$F(1, 175) = 29.21$, $p < 0.0001$], a significant effect of time [$F(7, 175) = 4.331$, $p = 0.0002$], and no significant interaction [$F(7, 175) = 0.2917$, $p = 0.9565$]. Repeated measures could not be used due to leakage of all 15% ethanol from the drinking tube for IASC mouse #9

on test day 5. For IASC mice Fisher's LSD test showed significant differences in 15% preference compared to baseline on test day 2 and from test day 4 through test day 7 ($p < .05$). For IAMC mice Fisher's LSD test showed significant differences in 15% preference compared to baseline on test day 1 and from test day 5 through test day 7 ($p < .05$). Fisher's LSD test revealed significant differences in 15% preference between IAMC and IASC mice for baseline and test days 2 through 4 ($p < .05$).

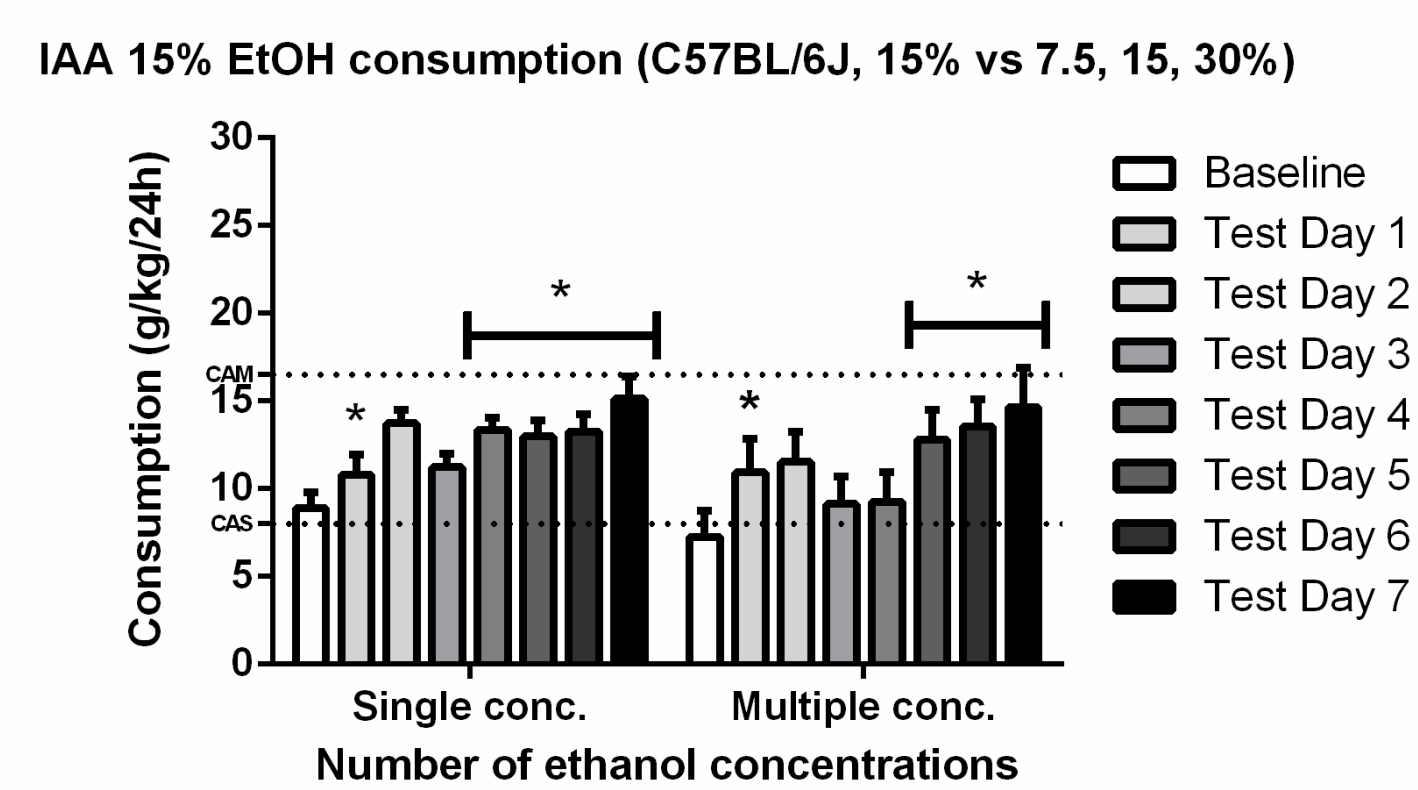


Figure 4.7. 15% ethanol consumption in mice offered intermittent access to water, and to either a single concentration (15%) or multiple concentrations (7.5, 15, and 30%) of ethanol. CAS and CAM indicate 7-day average 15% consumption for mice offered continuous access to a single concentration, or multiple concentrations, of ethanol, respectively. * : $p < .05$; two-way ANOVA with repeated measures, Fisher's LSD test for daily means compared to group baseline.

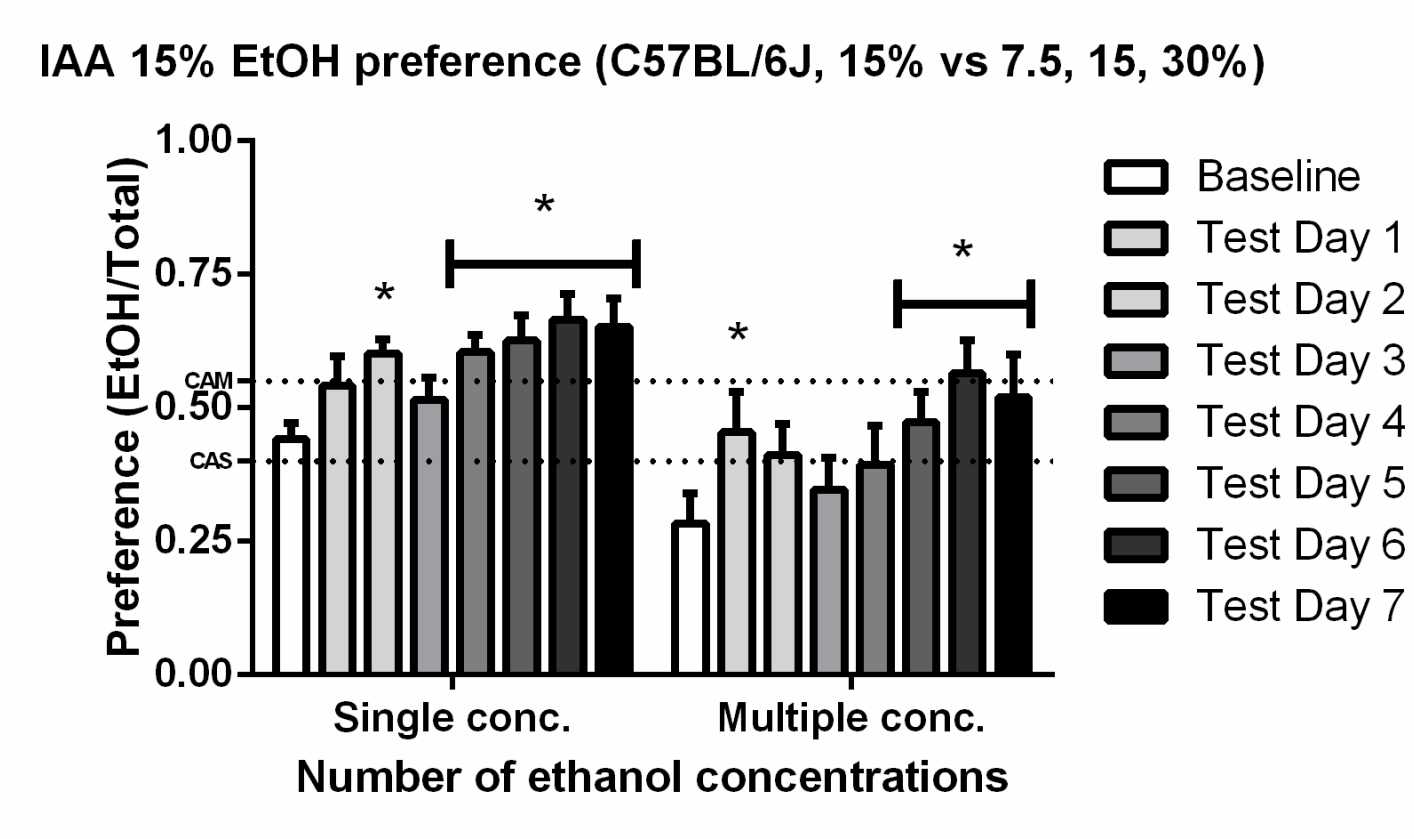


Figure 4.8. 15% ethanol preference in mice offered intermittent access to water, and to either a single concentration (15%) or multiple concentrations (7.5, 15, and 30%) of ethanol. CAS and CAM indicate 7-day average 15% preference for mice offered continuous access to a single concentration, or multiple concentrations, of ethanol, respectively. * : $p < .05$; two-way ANOVA with repeated measures, Fisher's LSD test for daily means compared to group baseline.

Intermittent alcohol access vs. continuous alcohol access

Escalation in alcohol intake for the IA group was confirmed using one-way ANOVA with repeated measures. For IA mice each test day was compared to the baseline period, which was defined as the final 7 days of continuous access in these mice. For CA mice drinking data was analyzed for stability from day 11 through 17 of the 25-day drinking period, to confirm a lack of elevation in intake due to CA. Drinking data for CA mice was collected beginning on day 42 of the study, and was collected through day 66, which in the IA mice corresponded to the period beginning 3 days prior to IA test day 4 and ending on IA test day 7. Drinking data for all IASC and IAMC were thus compared to the time-matched corresponding data for CASC and CAMC mice using unpaired two-tailed Student's t-test with a significance threshold of $p < .05$.

No differences were observed between IASC baseline consumption and preference and CASC baseline consumption and preference (unpaired two-tailed t-test, $p < .05$). Similarly, no differences were observed between IAMC and CAMC mice for baseline consumption or preference of total ethanol, or for any single ethanol concentration (unpaired two-tailed t-test, $p < .05$) (see Figure 4.9).

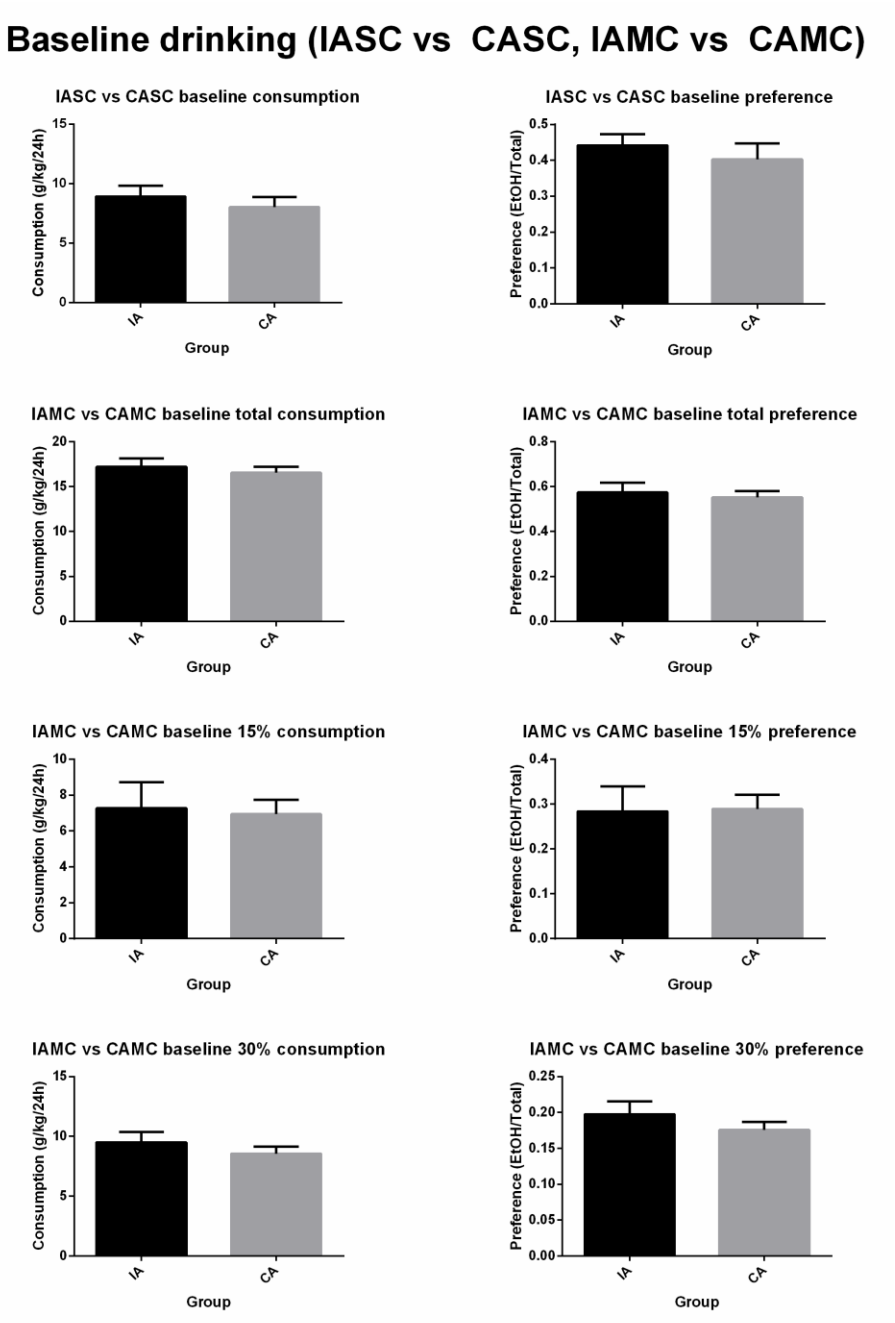


Figure 4.9. Baseline drinking in IA groups compared to corresponding CA groups for single concentration and multiple concentration drinking. For IA mice the baseline period was defined as the final 7 days of continuous access in these mice, and for CA mice drinking data was analyzed for stability from day 11 through 17 of the 25-day drinking period. No significant differences were found, which showed that IA mice did not show significantly different drinking behavior compared to CA mice prior to IA.

IASC drinking vs. CASC drinking

For IASC mice one-way ANOVA with repeated measures showed a significant effect of treatment on ethanol consumption [$F(7, 77) = 8.850$, $p < 0.0001$]. Consumption was elevated compared to baseline on test day 2 through test day 7 (Fisher's LSD, $p < .05$)(see Figure 4.10A). For ethanol preference in IASC mice there was a significant effect of treatment [$F(7, 77) = 6.527$, $p < 0.0001$], and preference was elevated on all test days except for test day 3 (Fisher's LSD, $p < .05$) (see Figure 4.10B). For CASC mice one-way ANOVA with repeated measures showed no significant differences over time in consumption [$F(6, 66) = 1.695$, $p = .1361$] or preference [$F(6, 66) = 1.359$, $p = .2445$](see Figures 4.10C, 4.10D). Comparison of time-matched daily intake between IASC and CASC mice showed significant differences on all IA test days for consumption and preference (Student's t-test, $p < .05$).

IAMC drinking vs. CAMC drinking

For IAMC mice one-way ANOVA with repeated measures showed a significant effect of treatment on total ethanol consumption [$F(7, 77) = 9.956$, $p < 0.0001$] and preference [$F(7, 77) = 6.538$, $p < 0.0001$]. Consumption was significantly elevated compared to baseline on all test days, and preference was significantly elevated on all test days except for test day 3 (Fisher's LSD test, $p < .05$)(see Figures 4.11A, 4.11B). No significant changes in total ethanol consumption [$F(6, 66) = 1.157$, $p = 0.3403$] or preference [$F(6, 66) = 0.2036$, $p = 0.9745$] over time were observed in CA mice (see Figures 4.11C, 4.11D). Comparison of time-matched daily intake between IAMC and CAMC mice showed significant differences on all IA test days for total consumption and total preference (Student's t-test, $p < .05$).

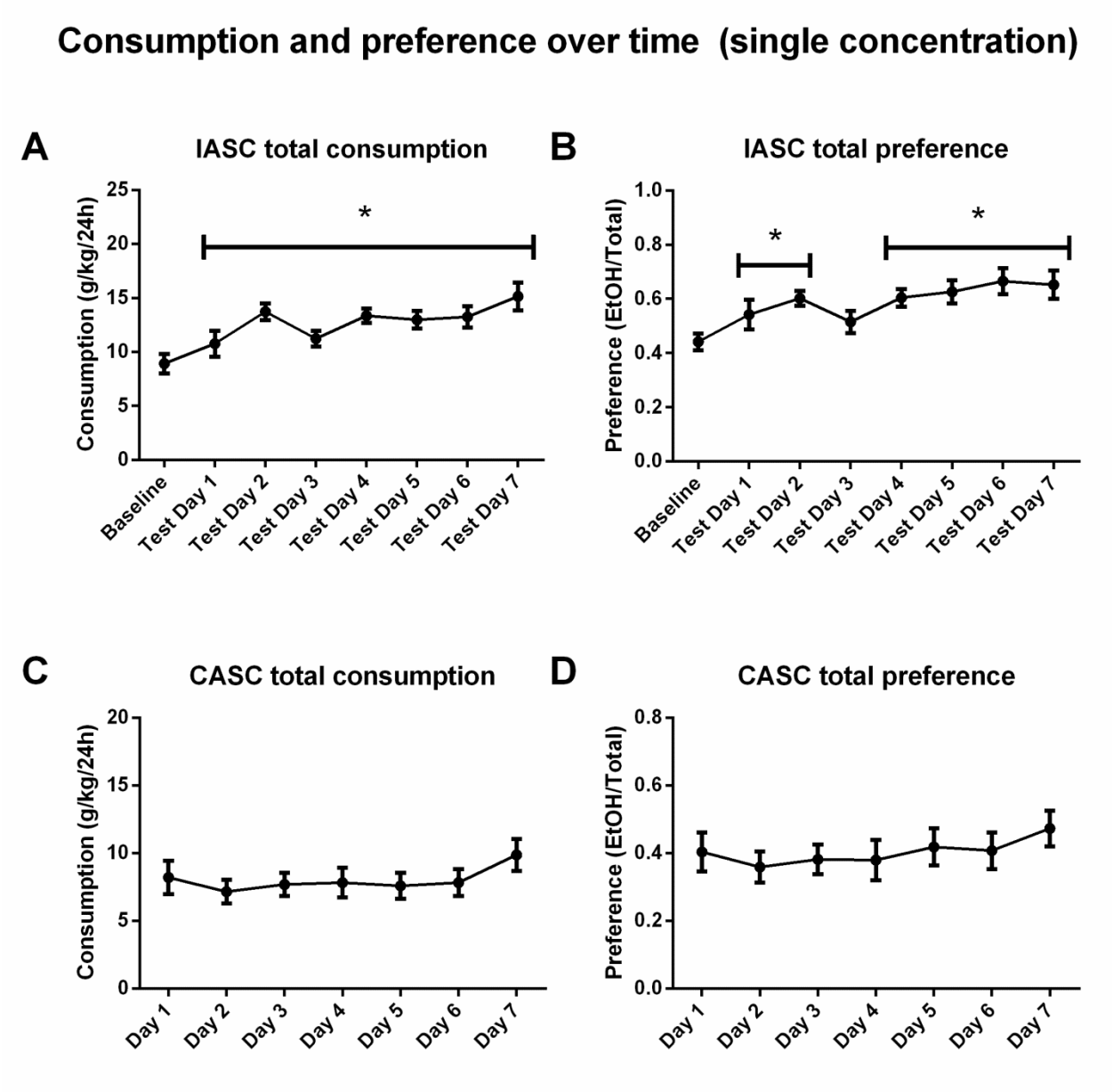


Figure 4.10. Alcohol consumption (A, C) and preference (B, D) over time for mice offered a single concentration (15%) of ethanol under IA (IASC) and CA (CASC) conditions. Data were analyzed using one-way ANOVA with repeated measures and Fisher's LSD test to compare daily drinking within groups. * : $p < .05$; Fisher's LSD test.

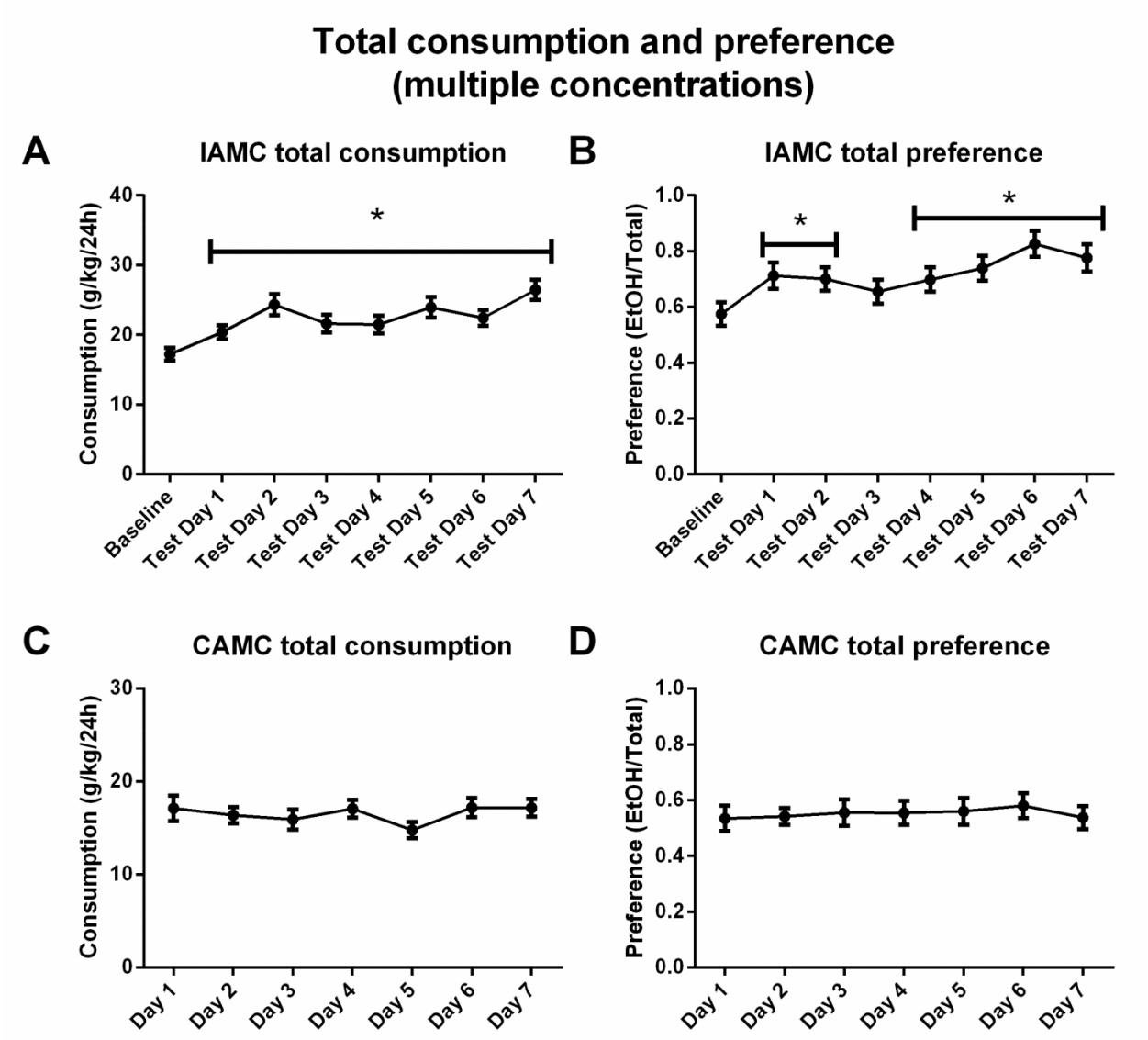


Figure 4.11. Total alcohol consumption (A, C) and preference (B, D) over time for mice offered multiple concentrations (7.5, 15, and 30%) of ethanol under IA (IAMC) and CA (CAMC) conditions. Data were analyzed using one-way ANOVA with repeated measures and Fisher's LSD test to compare daily drinking within groups. * : $p < .05$; Fisher's LSD test.

ANOVA with repeated measures in IAMC showed that consumption [$F(7, 77) = 4.499$, $p = 0.0003$] and preference [$F(7, 77) = 3.841$, $p = 0.0012$] for 15% ethanol was significantly elevated over time. Consumption of 15% ethanol was significantly elevated on test days 1, 2, 5, 6, and 7 (Fisher's LSD, $p < .05$) (see Figures 4.12A, 4.12B). Preference for 15% ethanol was significantly elevated on test day 1 and test days 5 through 7 (Fisher's LSD, $p < .05$). For CA mice ANOVA without repeated measures was used due to missing data for subject 8 on day 4 of the analyzed period. No significant changes in 15% ethanol consumption [$F(6, 76) = .2440$, $p = 0.9603$] or preference [$F(6, 76) = 0.3206$, $p = 0.9243$] over time were observed in CA mice (see Figures 12C, 12D). Comparison of time-matched daily intake between IAMC and CAMC mice showed significant differences on all IA test days for 15% consumption and 15% preference (Student's t-test, $p < .05$).

For 30% ethanol one-way ANOVA with repeated measures in IAMC showed that consumption [$F(7, 77) = 1.338$, $p = 0.2440$] and preference [$F(7, 77) = 0.6428$, $p = 0.7192$] were not altered over time by IAA (see Figures 4.13A, 4.13B). No significant changes in 30% ethanol consumption [$F(6, 66) = .9547$, $p = 0.4627$] or preference [$F(6, 66) = 0.4724$, $p = 0.8264$] over time were observed in CA mice (see Figures 13C, 13D). Comparison of time-matched daily intake between IAMC and CAMC mice showed significant differences for 30% consumption and 30% preference on IA test day 4 only (Student's t-test, $p < .05$).

For 7.5% ethanol one-way ANOVA with repeated measures in IAMC showed no changes over time in consumption [$F(7, 77) = 1.529$, $p = 0.1702$] or preference [$F(7, 77) = 1.764$, $p = 0.1068$] (see Figures 14A, 14B). Similarly, no changes were observed in CAMC mice for 7.5% ethanol consumption [$F(6, 66) = 1.760$, $p = 0.1210$] or preference [$F(6, 66) = 1.347$, $p = 0.2491$] (see Figures 4.14C, 4.14D). Comparison of time-matched daily intake between IAMC and CAMC

mice showed significant differences for 7.5% consumption on IA test day 1, and for 7.5% preference on IA test days 1 and 4 (Student's t-test, $p < .05$).

Quantitative real-time PCR

Of the five mRNA transcripts examined using qPCR, only two were found to be significantly regulated across groups (see Figure 15). *Smarca4* transcript was found to be significantly increased in CA mice compared to IA and H2O mice [$F(2, 21) = 9.039$, $p = 0.0015$; Tukey post-test: $p < .05$]. *Cacna1g* transcript was found to be significantly decreased in CA mice compared to IA and H2O mice [$F(2, 21) = 6.302$, $p = 0.0072$; Tukey post-test: $p < .05$]. Abundance of *Cacna1d* [$F(2, 21) = 2.174$, $p = 0.1386$], *Kif5c* [$F(2, 21) = 2.518$, $p = 0.1046$], and *Gria1* [$F(2, 21) = 1.737$, $p = 0.2004$] did not differ between groups.

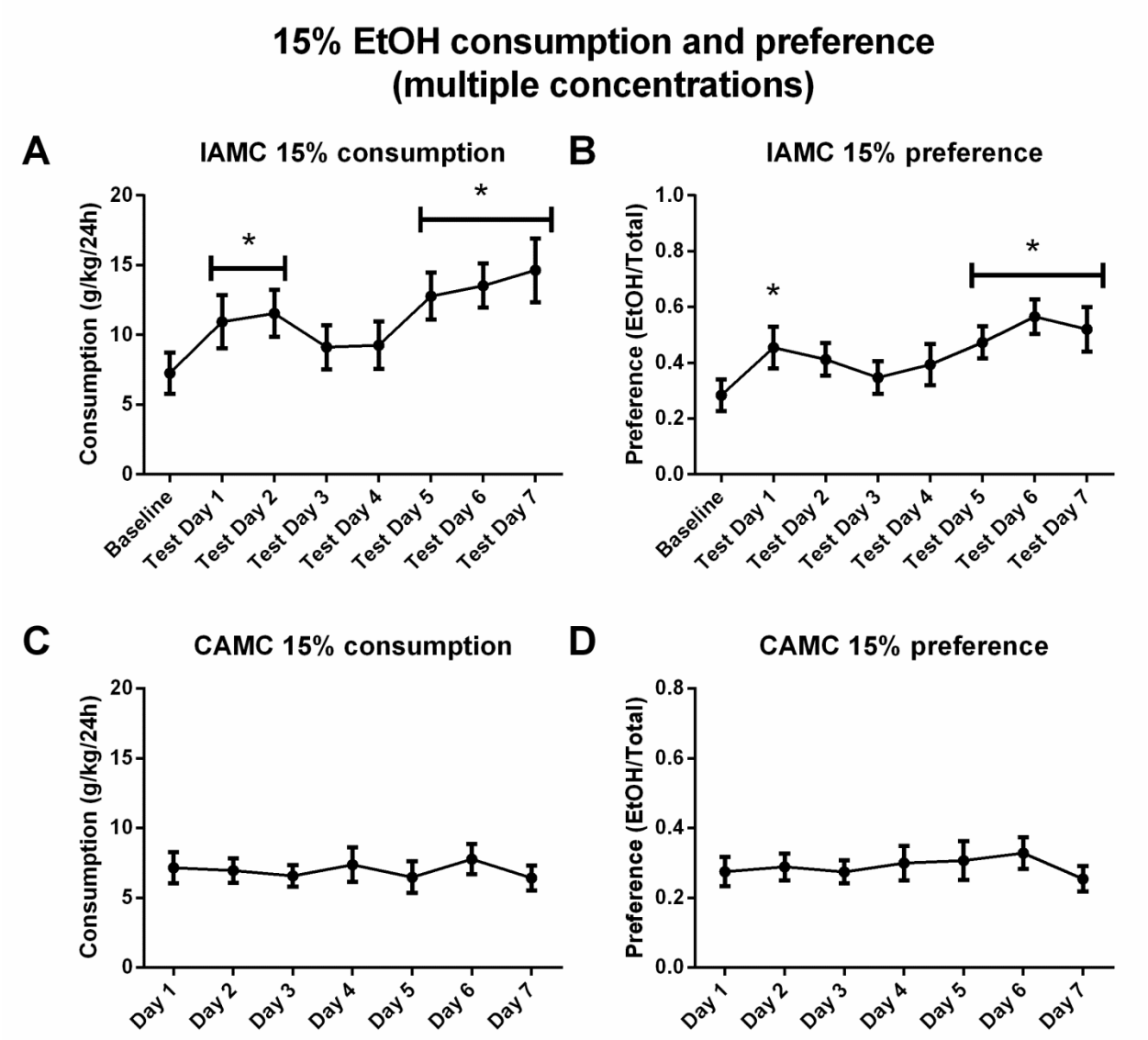


Figure 4.12. 15% alcohol consumption (A, C) and preference (B, D) over time for mice offered multiple concentrations (7.5, 15, and 30%) of ethanol under IA (IAMC) and CA (CAMC) conditions. Data were analyzed using one-way ANOVA with repeated measures and Fisher's LSD test to compare daily drinking within groups. * : $p < .05$; Fisher's LSD test.

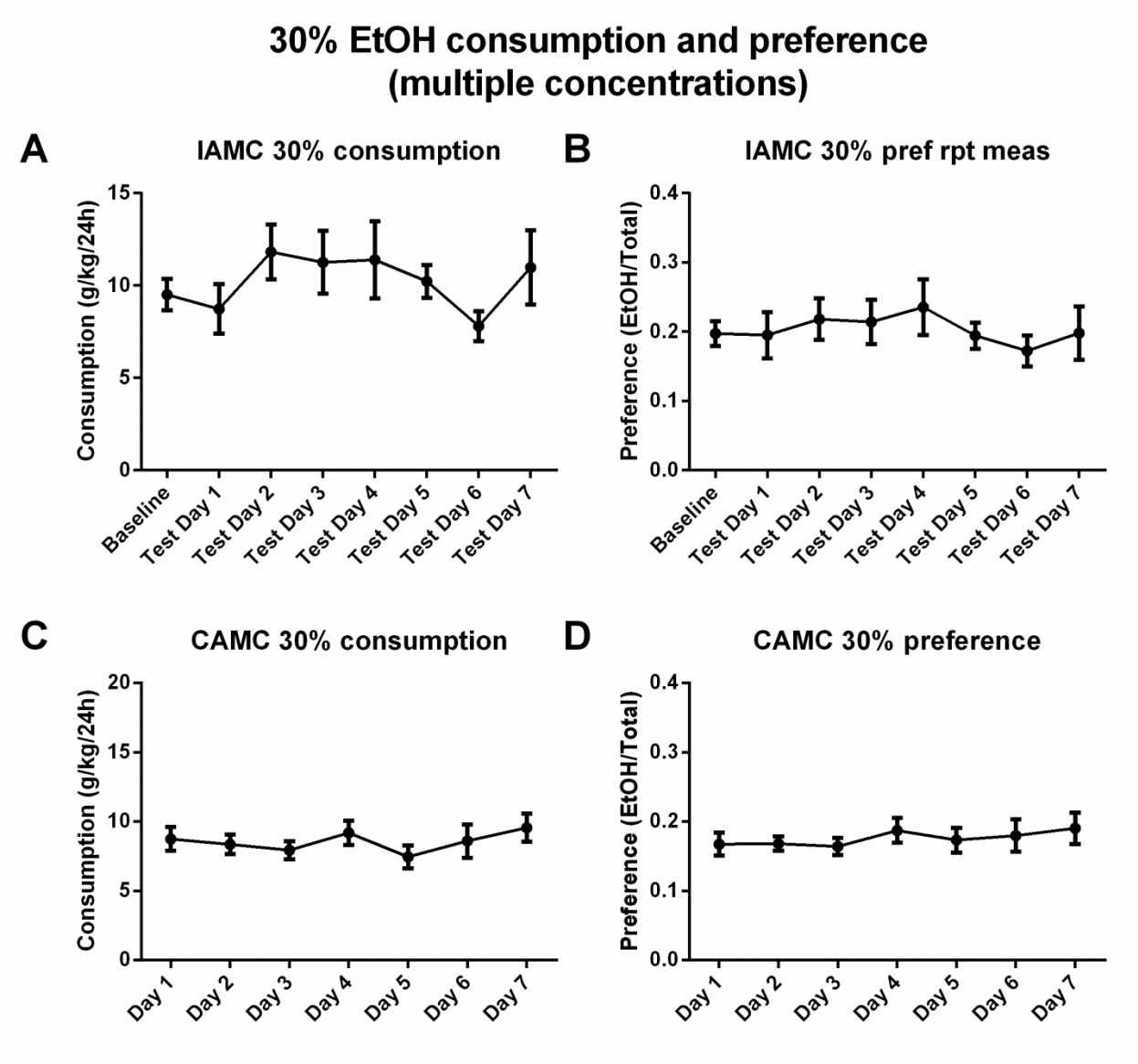


Figure 4.13. 30% alcohol consumption (A, C) and preference (B, D) over time for mice offered multiple concentrations (7.5, 15, and 30%) of ethanol under IA (IAMC) and CA (CAMC) conditions. Data were analyzed using one-way ANOVA with repeated measures and Fisher's LSD test to compare daily drinking within groups. * : $p < .05$; Fisher's LSD test.

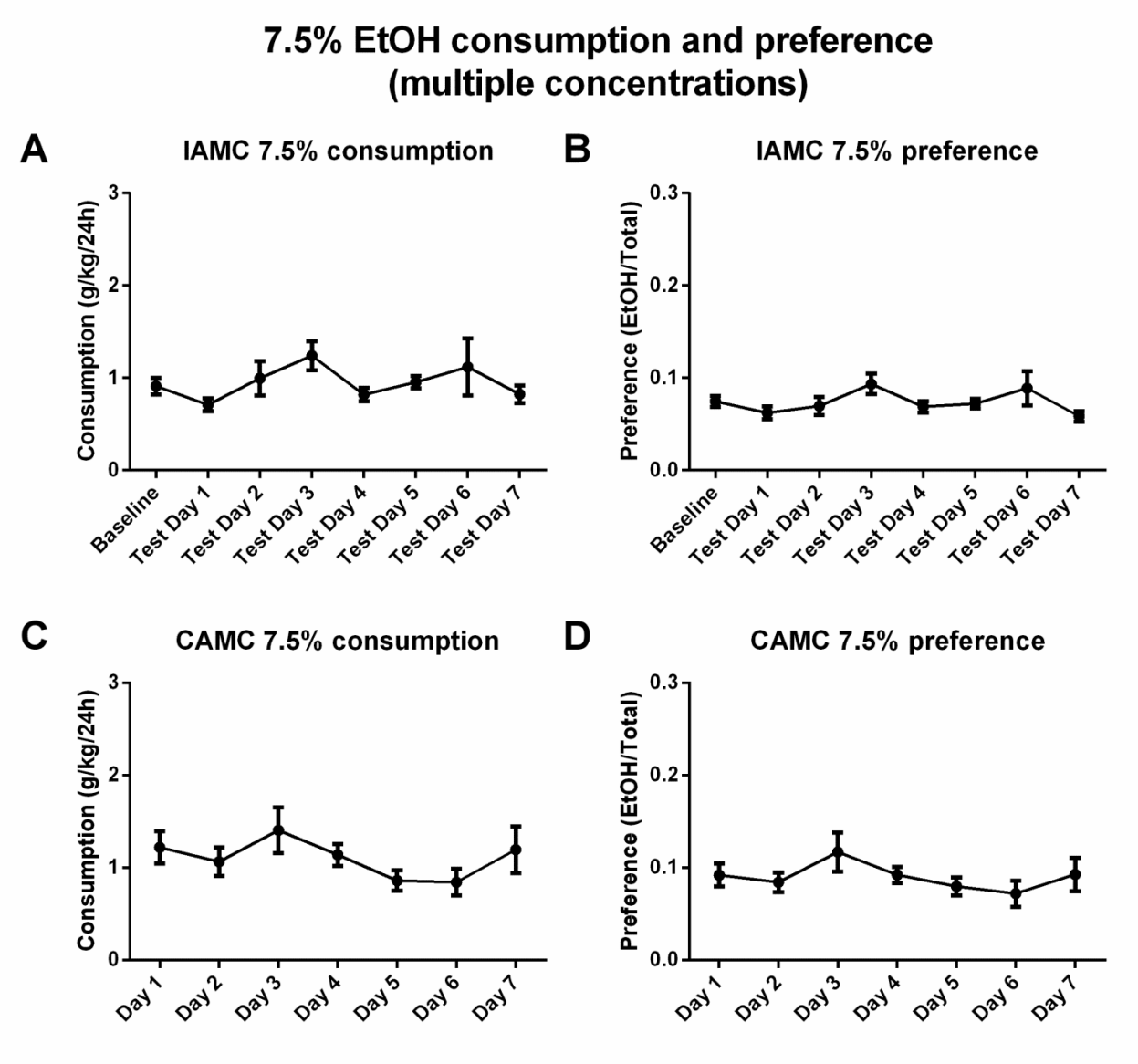


Figure 4.14. 7.5% alcohol consumption (A, C) and preference (B, D) over time for mice offered multiple concentrations (7.5, 15, and 30%) of ethanol under IA (IAMC) and CA (CAMC) conditions. Data were analyzed using one-way ANOVA with repeated measures and Fisher's LSD test to compare daily drinking within groups. * : $p < .05$; Fisher's LSD test.

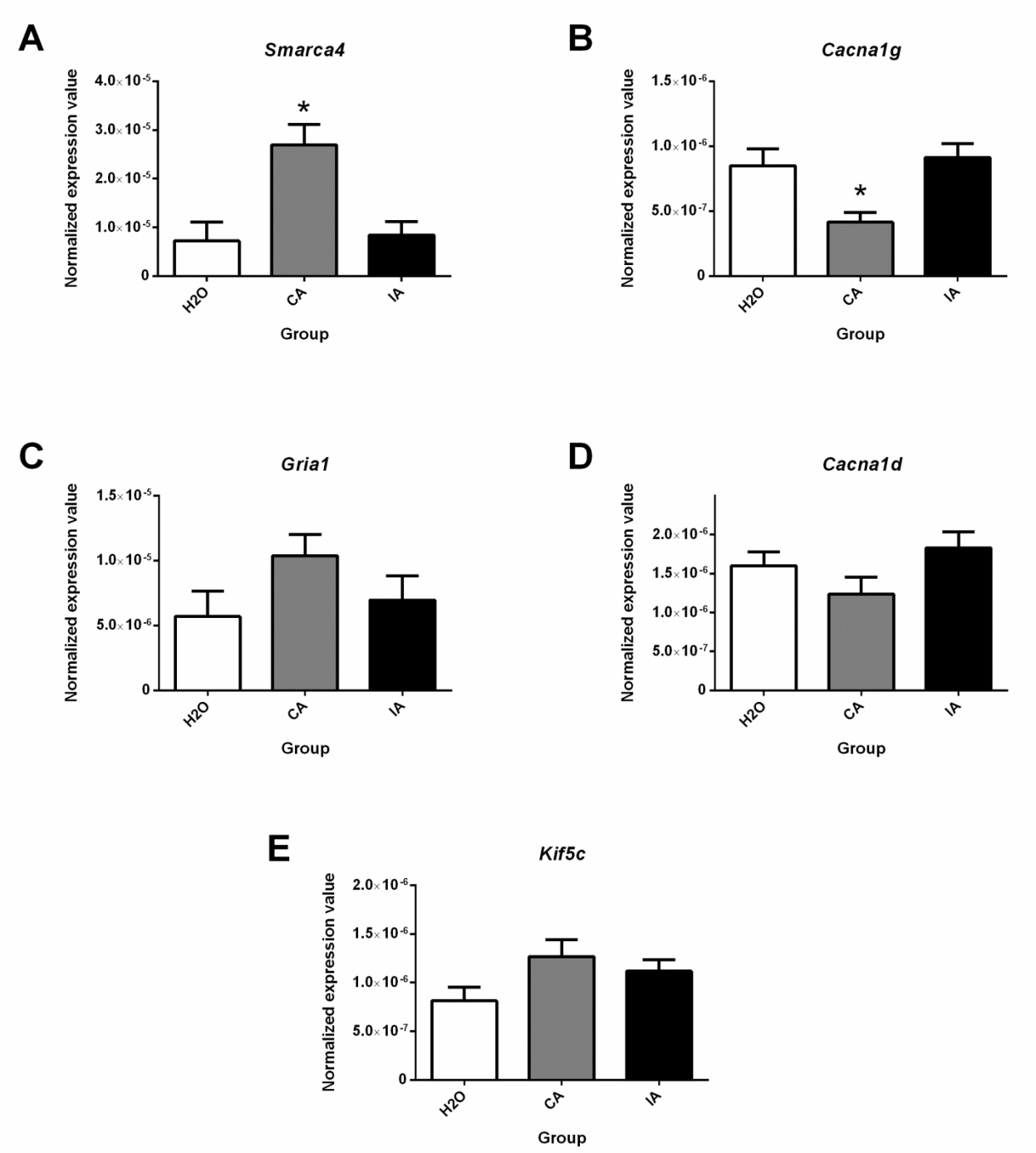


Figure 4.15. Gene expression determined by quantitative real-time PCR across groups of C57BL/6NCrl mice allowed access to water or to 15% alcohol on a continuous access (CA) or 1-day-per-week intermittent access (IA) schedule. Data were normalized to low-variance transcripts using Genorm, and analyzed using ANOVA with repeated measures followed by Tukey post-hoc test. * : $p < .05$ for significant difference compared to H2O and IA groups.

Discussion

Intermittent alcohol access with 15% alcohol induces significantly elevated consumption and preference for the drug over water in C57BL/6NCrl and C57BL/6J mice, in accordance with hypotheses. This is the first demonstration of IAA in the C57BL/6NCrl mouse, and establishes the efficacy of repeated scheduled abstinence to elevate consumption and preference in this strain. This result is important because conflicting evidence exists regarding the utility of alcohol deprivation in this strain for increasing alcohol intake; some studies show that single and repetitive 4-day deprivation periods have no effect (Tomie et al., 2013; Wolstenholme et al., 2011), and others have found significant increases in consumption and reference upon reinstatement (Khisti et al., 2006b).

Similarly, for the C57BL/6J strain this result confirms that IAA produces elevated consumption and preference, in agreement with previous studies. This result confirms the effect observed in two other publications reporting 1-day-per-week IAA in the C57BL/6J strain (Melendez et al., 2006a; Rosenwasser et al., 2013). All other IAA publications in mice and rats use every-other-day IAA (Adermark et al., 2011; Ahmadiantehrani et al., 2013; Barak et al., 2011; Carnicella et al., 2009a; Chappell et al., 2013; Cippitelli et al., 2012; Crabbe et al., 2012; Daoura et al., 2011; Daoura and Nylander, 2011; Dawson et al., 2013; Egecioglu et al., 2013; George et al., 2012; Hargreaves et al., 2009a; Hopf et al., 2011; Hwa et al., 2011; Hwa et al., 2013; Lee et al., 2013; Li et al., 2010a; Li et al., 2012a; Li et al., 2012b; Li et al., 2011b; Loi et al., 2010; Melendez, 2011; Moorman and Aston-Jones, 2009b; Nielsen et al., 2012; Obara et al., 2009a; Osterndorff-Kahanek et al., 2013; Palm et al., 2011; Sabino et al., 2013; Sajja and Rahman, 2013; Shirazi et al., 2013; Simms et al., 2010; Simms et al., 2013; Simms et al., 2008; Stuber et al., 2008; Wen et al., 2012; Yardley et al., 2012).

The magnitude and immediacy of increases in drinking behavior are similar within rodent strains across studies, but varied greatly between strains. Interestingly, the effects of IAA on consumption were similar in relative magnitude for the two strains in this study: the C57BL/6NCrl mice increased from approximately 3 g/kg/day to 6 g/kg/day, and the C57BL/6J increased from approximately 8 g/kg/day to 15 g/kg/day. This result suggests that IAA is useful in rodents regardless of initial ethanol consumption, and should allow for experimental dissection of factors that contribute to baseline drinking and development of maladaptive drinking, which may be distinct, at least within C57BL/6 strains. This type of study is important for understanding the consequences of binge-drinking and intermittent ethanol exposure in humans, which differ in initial sensitivity to and intake of alcohol, and in propensity to develop AUDs (King et al., 2013; Rohsenow et al., 2012; Schuckit et al., 2013).

Study of alcohol-related behavior in C57BL/6NCrl mice is relatively limited, and generally involves comparisons to the alcohol-preferring C57BL/6J strain, which shows greater alcohol consumption and preference, reduced effect of alcohol deprivation, and less ethanol-induced dopamine release in the ventral striatum (Khisti et al., 2006b; Mulligan et al., 2008b; Ramachandra et al., 2007a). In pair-housed C57BL/6NCrl mice alcohol deprivation produces no effects on drinking, but in mice housed alone the procedure produces increases of 50% or more, which suggests that increases in drinking produced by alcohol deprivation in this strain is dependent on stress induced by social isolation (Khisti et al., 2006b; Tomie et al., 2013).

Other direct behavioral comparisons between C57BL/6NCrl and C57BL/6J mice show behavioral differences with some relevance to alcohol drinking and other alcohol-related behaviors. In contextual fear-conditioning C57BL/6NCrl mice show more conditioned freezing, and this difference is maintained in an altered context (Bryant et al., 2008; Radulovic et al.,

1998). Stressors such as maternal separation also potentiate conditioned fear responses and alcohol drinking, and these differences are associated with altered mesocortical glutamate and GABA receptor expression (Romano-Lopez et al., 2012; Wilber et al., 2009). Interestingly, C57BL/6J mice show better motor coordination than C57BL/6NCrl mice in the Rota-rod task, which raises the possibility that ataxia due to alcohol intake influence consumption in these strains (Bryant et al., 2008). Mice are prey animals, and any action that increases likelihood of predation, such as reduced motor control, would be expected to be avoided by the animal.

Mice from the C57BL/6J strain significantly increase alcohol consumption and preference when offered multiple concentrations of alcohol, compared to a single concentration of alcohol. This observation is consistent with other studies that show that choice of alcohol concentrations elevates intake, although most such studies report results in rats, and mouse studies are rare (Bell et al., 2003; Bell et al., 2004c; Holter et al., 1998; Melendez et al., 2006a; Rodd-Henricks et al., 2001a; Serra et al., 2003b; Vengeliene et al., 2005; Wolffgramm and Heyne, 1995). Despite a doubling in consumption, this level of alcohol drinking does not represent an upper limit on consumption for the strain, because intermittent access significantly increases overall consumption and preference.

Interestingly, only consumption and preference for 15% alcohol increase over time in response to IAA in mice offered access to 7.5%, 15%, and 30% ethanol. Consumption of 30% ethanol is approximately equivalent to that of 15% ethanol at the beginning of IAA procedures, and it does not change over time. Consumption of 7.5% ethanol accounts for a trivial fraction (< 10%) of total intake and similarly does not change over time. While these results agree with others showing that choice of alcohol concentration greatly enhances drinking, they differ in that deprivation causes a shift to higher concentrations of alcohol (Siegmund et al., 2005a; Spanagel

and Holter, 1999). It is unclear why no such shift was seen in the current study, but other studies used slightly different access schedules. In rats multiple concentration drinking occured over a period of months, punctuated by 3-day deprivations, the first of which shifted concentration preference. In mice weekly access was begun only after an initial 2-week deprivation period, which delayed escalation. It therefore seems that the length of baseline drinking and of initial deprivation has lasting effects on the manifestation of the increased drinking, and further experimentation should address the neuromolecular correlates of these differences in the mesolimbocortical dopaminergic circuitry. Furthermore, these results indicate that offering a single alcohol concentration may skew results and mask significant effects of experimental manipulations, which may be specific to particular concentrations. Furthermore, multiple concentrations greatly enhance the validity of animal models for representing drinking in humans, who generally have some choice in the strength of alcohol they consume.

In the C57BL/6NCrl strain ethanol intake is at least partially determined by epigenetic influences on gene expression, and thus changes in gene expression associated with altered drinking behavior produced by IAA can illuminate the particular neuromolecular mediators of the behavior. The nucleus accumbens is of particular interest in the context of alcohol craving and addition, because it is known that changes in functioning and gene expression in this region contribute to changes in motivated behavior for reward, including drugs (Barrot et al., 2002; Bauer et al., 2013; Carlezonjr and Thomas, 2009; Li et al., 2011c; McBride et al., 2009). Results for transcriptional regulation in nucleus accumbens of IAA mice compared to water drinking mice do not resemble results for single-deprivation mice compared to water drinking mice, and this result is unexpected. For the transcripts measured in C57BL/6NCrl mice regulation in nucleus accumbens due to continuous alcohol access is similar to that observed in abstinent mice

after a single 4-day deprivation period, when compared to water drinking controls. In contrast, no significant differences are apparent between IA and H2O groups for any measured transcript. It is therefore hypothesized that gene expression differences observed after alcohol deprivation are a result of previous alcohol exposure that are in the process of diminishing, and thus are apparent after 4 days, but not 6 days, of abstinence.

These results show that alcohol exposure regulates gene expression in a transient manner that is no longer apparent after several days of abstinence, when no differences are observed between IA and H2O mice. This is intriguing given the divergent drinking behavior observed in ethanol-naïve water drinkers and IAA mice when exposed to alcohol, and may indicate that alcohol-induced transcriptional changes have propagated to the functional protein level, where they exert control over neuronal function and drinking behavior. It is therefore important to study acute transcriptional regulation induced by alcohol drinking as a control group in any studies examining gene expression associated with abstinence and relapse behavior, because changes that are important mediators of behavior may no longer be apparent in deprived mice. To confirm this hypothesis, future studies will address the correlations between alcohol-induced transcription and protein translation and abundance over time.

These results establish the utility of the one-day-per-week IAA model to produce significantly elevated alcohol consumption and preference in C57BL/6NCrl mice, which drink less alcohol than the commonly-used C57BL/6J strain, for which the effectiveness of one-day-per-week IAA was also confirmed. Furthermore, the use of multiple alcohol concentrations (7.5%, 15%, 30% v/v) produces significantly elevated consumption and preference under continuous access and intermittent access schedules. In the multiple concentration model escalation due to intermittent access occurs for consumption and preference of 15% alcohol, while intake of 7.5% alcohol and

30% alcohol remain unchanged. After six cycles of IAA and six days of abstinence, transcriptional regulation in nucleus accumbens of C57BL/6NCrl mice compared to water-drinking control mice does not resemble that observed in 4-day-abstinent mice compared to their own controls. Future studies will determine the persistence of alcohol-responsive regional gene expression in abstinent animals, and the contribution of this transcriptional regulation to binge-like drinking behavior. The results reported herein provide important direction for the study of the development of binge-like drinking in rodent strains with dissimilar genetic backgrounds and alcohol-related behaviors, which is a powerful tool for deciphering the neurobiology of alcohol craving, and the genetic and environmental factors that influence heavy drinking behavior and the transition to alcoholism.

Chapter 5. Nucleus accumbens transcriptional regulation in the intermittent alcohol access model: differential expression and weighted gene co-expression networks

Introduction

Current therapeutic strategies are not effective in the long term for the majority of alcoholics, because the neurobiology that mediates the development and maintenance of maladaptive drinking behavior characteristic of the disease is not well understood. Both the transition to alcoholism and attempts at abstinence are characterized by episodes of binge-like drinking, in which blood alcohol concentrations of greater than 80 mg/dl are achieved through consumption of several drinks in a short period of time (Fillmore and Jude, 2011). In general, alcoholics consume greater amounts of ethanol than non-alcoholics, and in those trying to quit drinking a pattern of cyclic withdrawal and relapse is often observed. Animal models that produce this type of binge-like relapse drinking are crucial to the understanding of alcohol-related behavior, but progress in this area has been slowed by the dearth of preclinical models that produce such intake.

In the past decade models have been developed that produce elevated alcohol consumption and/or binge-like drinking in rodents, with or without induction of dependence (Becker, 2013; Crabbe et al., 2011). These models vary in effectiveness and validity, but the most successful have been the chronic intermittent ethanol (CIE) vapor chamber model, the drinking-in-the-dark (DID) model, and the intermittent alcohol access (IAA) model (Crabbe et al., 2009; Iancu et al.,

2013; Lopez et al., 2011; Rhodes et al., 2005; Simms et al., 2008; Sparta et al., 2008). Of these models the IAA procedure appears to be the most valid for comparison to human alcoholism, in that it requires the least intrusive experimental manipulation and is the only procedure in which all alcohol exposure is voluntary (Wise, 1973).

The CIE model makes use of the alcohol dehydrogenase inhibitor pyrazole and extensive exposures to stressful vapor chambers for daily sessions of 14 hours or more (Knapp and Breese, 2012). In addition to its actions on alcohol dehydrogenase, which cause greatly elevate blood ethanol concentrations, pyrazole is an NMDA receptor agonist and has some agonist activity at acetylcholine receptors (Pereira et al., 1992). The DID model elevates drinking through limited access of two to four hours, in which only solutions containing alcohol are offered (Rhodes et al., 2005). While the DID and CIE models produce binge-like drinking behavior, human alcoholics do not generally use pyrazole in conjunction with drinking, or drink in limited-access scenarios in which water is unavailable. Although inhaled alcohol, for example the "vapor-tini" has increased in popularity in recent years, it remains a relatively rare method of administration.

Thus, while the CIE and DID models reliably produce binge-like drinking behavior, limitations in validity undermine the meaningfulness of conclusions drawn through their use. In contrast, the IAA model uses only repeated scheduled abstinence periods of 1 to 6 days to produce elevated alcohol consumption and binge-like drinking in rodents (Melendez, 2011; Simms et al., 2008). IAA drinking occurs in 24h voluntary access periods in the home cage, in which food and water are always available, and gradual escalation in drinking occurs over time, eventually leading to binge-like consumption and near-total preference (Hwa et al., 2011). While the IAA model is not thought of as one that produces dependence *per se*, extended cycles of IAA over weeks and months produce inflexible drinking behavior and handling-induced convulsions, which are

thought to be signs of addiction and dependence, and it is therefore thought to model the transition from controlled to compulsive behavior (Hopf et al., 2010; Loi et al., 2010).

The validity of the model to represent human alcohol-related behavior is further supported by pharmacological studies that have identified the molecular mediators of its effects on drinking behavior, which are similar to results from human studies. IAA drinking and drinking in humans are attenuated by acamprosate, naltrexone, and drugs that interfere with the functioning of the corticotropin releasing hormone (CRH) system (Daoura and Nylander, 2011; Hwa et al., 2013; Sabino et al., 2013; Simms et al., 2008).

Because IAA is a valid and reliable model of binge-drinking and the transition to alcoholism, much study in recent years has been devoted to understanding the behavior. However, relatively few studies have examined regional changes in transcription in the brain produced by IAA, and their contribution to escalation in drinking. Consistent findings have been that gene expression associated with IAA is distinct from that induced by continuous access drinking and other models of binge-drinking, and that transcriptional regulation is necessary for induction of binge-like drinking (Ahmadiantehrani et al., 2013; Lee et al., 2013; Obara et al., 2009a; Osterndorff-Kahanek et al., 2013). IAA drinking is associated with subregion-specific accumulation of ΔFosB in nucleus accumbens, striatum, and prefrontal cortex, which indicates that it shares some common molecular mechanisms with motivated behavior for other drugs of abuse (Li et al., 2010a; Sajja and Rahman, 2013). Crucially, ΔFosB accumulation and binge-like drinking produced by IAA are attenuated by naltrexone and nAChR antagonist cytisine, showing that changes in gene expression associated with the procedure are directly related to the changes in behavior it produces (Li et al., 2010a; Sajja and Rahman, 2013).

The role of transcriptional regulation in binge-drinking produced by IAA is further supported by studies showing that transcript coding glial cell line-derived neurotrophic factor (GDNF) in the VTA is increased by alcohol drinking, and decreased by 24h of abstinence during IAA procedures. Neuronal GDNF signaling activates CREB-mediated gene transcription, and plays a role in the differentiation and maintenance of dopaminergic neurons (Hayashi et al., 2000; Jongen et al., 2005). Intra-VTA GDNF reverses the reduction of NAc dopamine associated with 24h withdrawal, and attenuates binge-like alcohol consumption (Ahmadiantehrani et al., 2013; Barak et al., 2011; Carnicella et al., 2009a). Furthermore, increased cAMP signaling reduces IAA drinking, likely through activation of CREB-mediated transcription, which is known to modulate addictive behavior (Wen et al., 2012). Retinoic acid signaling is deficient in the brain of alcoholics, and this transcriptional pathway is also implicated in PFC dopamine receptor regulation associated with binge-like drinking in IAA, but not in the DID model (Osterndorff-Kahanek et al., 2013).

Finally, epigenetic regulation of gene expression contributing to changes in drinking behavior is suggested by the strong influence of early-life environment and experience on escalation of intake. Maternal separation and adolescent social isolation produce stress and persistent changes in gene expression through modulation of epigenetic processes, and these manipulations also increase drinking in the IAA model. In addition to increasing drinking behavior, these early life stressors increase the efficacy of naltrexone to attenuate binge-like drinking, and induce anxiety-like behavior (Chappell et al., 2013; Daoura and Nylander, 2011). Abundance of genes involved in epigenetic processes is correlated with alcohol intake across individual mice within the C57BL/NCrl inbred strain, and histone deacetylase inhibitor Trichostatin A induces gradual increases in drinking over time (Wolstenholme et al., 2011).

Because IAA is a valid model for binge-drinking and the transition to alcoholism, and changes in gene expression in mesolimbic dopaminergic pathway brain regions are crucial to its effects, genomic investigation was performed to examine the nucleus accumbens transcriptome of C57BL/6J mice allowed intermittent or continuous access to alcohol, with the goal of identifying important genes and gene networks involved in maladaptive alcohol-related behavior. Along with water-drinking control mice, alcohol-drinking mice were subjected to microarray analysis to identify differentially-expressed genes across group, and weighted gene co-expression analysis (WGCNA) was used to generate gene networks associated with alcohol drinking behavior across individuals within each group. Network topology was compared across group networks to identify global changes in gene co-expression relationships, and expression of network clusters (modules) were correlated with drinking behavior. Regulated transcripts and modules were interrogated for functional roles in the cell and published evidence for involvement in ethanol-related behavioral phenotypes. Finally, behaviorally relevant genes and modules were compared to published literature for association with alcohol-related gene sets and phenotypes.

It was hypothesized that differentially expressed genes would be identified via LIMMA for all group-wise comparisons, and that WGCNA would identify group differences in global transcriptome connectivity (network-level differences), as well as local co-expression networks (module-level differences). Furthermore, it was hypothesized that modules significantly associated with drinking behavior would not be preserved between IAA and CA mice. Finally, it was hypothesized that gene ontology and transcription factor binding site analysis would implicate regulation of gene expression via deltaFosB, CREB, and epigenetic processes in the control of drinking behavior.

Materials and Methods

Animals

Subjects were the same cohort of C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) used for IAA behavioral studies in Chapter 4. For microarray studies only the 24 mice offered multiple concentrations of alcohol concurrently (7.5, 15, 30%) and the water-drinking (H2O) control mice were used. All mice were individually housed and allowed 30 days to habituate to the animal facility. Intermittent access (IA) mice drank continuously for 17 days as a baseline access period, followed by 7 cycles of IAA, in which 24h alcohol access periods were separated by 6 days of abstinence. IA mice were sacrificed in the afternoon on the 7th day following the 7th and final 24h alcohol access period, to correspond to the period just before ethanol would have been reintroduced for the 8th access session. Continuous access (CA) mice were sacrificed in the afternoon on the 25th consecutive day of alcohol access, to closely match total days of access to the IA mice, who received 24 days of access. For LIMMA and WGCNA analyses consumption and preference measurements for total, 15%, and 30% alcohol intake were considered. Intake of 7.5% alcohol was not considered due to trivial consumption and lack of changes over time.

Dissection and tissue preparation

Brains were dissected according to published procedures ((Kerns, 2005), (see supplementary materials) and nucleus accumbens was collected and frozen in 1.5 mL sterile microcentrifuge tubes in liquid nitrogen. Following collection tissue was stored at -80° C until RNA was extracted.

RNA extraction and quality control

Frozen tissue was homogenized in STAT-60 (Tel-test, Inc., Friendswood, TX) and processed by chloroform extraction and column purification using the Qiagen RNeasy kit (Qiagen N.V., Hilden, Germany) (see supplementary materials).

Extracted total RNA was subjected to quality control measures for purity and degradation using UV spectrophotometry and Bio-Rad Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). RNA samples were accepted for further analysis conditional on 260/280 ratio greater than 2.0, and RNA Quality Index (RQI) greater than 8.0.

Microarray hybridization and scanning

Affymetrix Mouse Gene 1.0 ST microarrays were used for all samples (Affymetrix, Santa Clara, CA). For hybridization and scanning steps processing groups were subjected to supervised randomization to minimize batch effects that act as confounding variables when batches and experimental groups correspond. Total RNA was processed according to manufacturer's instructions using the Ambion WT expression kit (Life Technologies, Carlsbad, CA), followed by the Affymetrix GeneChip WT Terminal Labeling and Controls Kit, and the Affymetrix GeneChip Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara, CA).All samples were hybridized to oligonucleotide arrays overnight for 16 hours, and scanned according to manufacturer's instructions.

Expression data processing

Raw data was analyzed using Affymetrix Expression Console software and transformed using the Robust Multichip Average (RMA) method to summarize probeset-level expression data. RMA expression data was exported as tab-delimited text files and processed using Microsoft Excel software to exclude probesets for which no sample registered RMA > 3 (Microsoft,

Redmond, WA). This step was performed to exclude probesets for which expression was not in the reliable linear range for measurement. Filtered data was further adjusted using the ComBat method to reduce the influence of hybridization batch effects that may have been introduced as a consequence of processing order. The resulting expression-filtered and ComBat adjusted expression data was used for all further analysis.

Differential expression analysis with LIMMA

Filtered and adjusted RMA expression data was subjected to multi-class LIMMA analysis across the IA, CA, and H2O groups to identify probesets with significantly different abundance across groups. LIMMA fits a linear model to the expression of each gene, and compares models between groups of samples to identify significantly different genes. Multi-class LIMMA returns an F-statistic for each probeset that is similar in meaning to that returned by ANOVA; it indicates whether any of the group-wise differences are significant. Multi-class LIMMA results across groups were filtered for $F < 0.05$. Also returned are p-values for significant differences in probeset expression for each pair-wise group comparison, and FDR-adjusted p-values to correct for false-positive results due to multiple comparisons. The FDR-adjusted p-value is computed using the method of Benjamini and Hochberg, and indicates the expected proportion of false discoveries in the group of genes filtered for adjusted p-value below that threshold. For example, in the group of genes selected for adjusted p-value < .05 for IAA vs CA differential expression it is expected that 5% of results will be false-positives. This adjustment is strict, but necessary given that tests will be performed for 35, 466 probesets. However, to reveal small differences that may inform hypothesis generation, but not be apparent with FDR adjustment, raw p-values may be analyzed to determine differential expression, with the caveat that expected false-positive ratio for a given p-value threshold is equal to that proportion of the total probesets analyzed. For

example if 35,466 probesets are tested for differential expression using a raw p-value threshold of $p < .01$, then 1% or 355 of the identified probesets are expected to be false-positive results. Differential expression testing between groups using FDR-adjusted p-value < 0.05 was used to obtain groups of significantly regulated genes for IA vs.CA and CA vs. H2O, but not for IA vs. H2O. A raw p-value filter of $p < 0.01$ was applied to IA vs. H2O LIMMA results to identify differential expression.

Weighted Gene Correlation Network Analysis (WGCNA)

WGCNA is used to analyze correlation patterns in genes across microarray samples, and to identify clusters of highly correlated genes, known as modules, which are gene co-expression networks. Modules can be associated with phenotypic data to provide powerful insight into the connections between gene expression and behavior. WGCNA produces networks that are approximately scale-free, which are characterized by highly connected nodes at multiple levels, and a high level of fault tolerance that preserves network function despite node failure or removal. Another relevant characteristic of scale-free networks is that clustering coefficient decreases following a power law as the node degree increases; that is, low-degree nodes tend to be clustered in tightly interconnected "neighborhoods" that are connected to one another by network hubs. Network analysis methods such as WGCNA that assume scale independence are particularly useful, because many complex biological, social, and technological networks have characteristics of scale-free networks, including yeast protein interactions and metabolic networks (Albert, 2005).

Expression in particular modules can be summarized numerically by a single value called a module eigengene (ME, or first principal component), which allows for module expression to be

correlated with phenotypic data such as behavior to give an association score and eigengene significance (ES). Within each module individual probesets can also be analyzed for correlation with phenotypic data, which yields a gene score (GS), and for intra-modular connectivity via intra-modular expression correlation, which is represented by a module membership score (MM). Values for ES, GS, and MM range from -1 to 1 and are derived by Pearson correlation analysis, which also gives a p-value for significance of relationship. While connectivity varies across modules and hub genes, genes with the highest MM scores within each module are considered to be highly connected hub genes for those modules. Each probeset can be assigned to one module only, although MM for other modules may also reach significance ($p < .05$).

The meaningfulness of a module is indicated by the correlation between GS and MM; that is, modular genes with the most intra-modular connectivity are also the most well-correlated with drinking behavior, showing that module expression is likely associated with behavior. Determining whether GS and ES relationships are causal, consequential, or merely correlational can be inferred somewhat through study of module genes in the context of published literature, but only determined with confidence through *in vivo* functional verification. There is no standard accepted criterion for GS/MM correlation threshold for meaningfulness, but in general the majority of modules are significantly correlated at $p < 0.2$ (Pearson correlation). For all module sets generated herein 4 network module definitions were considered at deepSplit values of 0 through 3, and the value that gave the greatest percentage of modules with GS/MM Pearson correlations was chosen. The IA and H2O network module sets were generated with deepSplit = 1, and the CA network was generated with deepSplit = 2.

Modules can be analyzed for significant enrichment for any list of genes, which allows for interrogation of factors relevant to biological function. Of particular interest is module over-

representation of genes that are differentially expressed between groups according to LIMMA analysis in modules associated with drinking, because these genes are likely primary mediators of the effects of IAA on behavior. Enrichment analysis also allows for the examination of module overlap across group networks, to study module preservation under different experimental conditions, and differences in the relationship between preserved modules and drinking behavior. Module enrichments were determined by hypergeometric test for probeset-level overlap, with resulting p-values subjected to Bonferroni correction for multiple comparisons.

Filtered and adjusted RMA expression data was subjected to WGCNA analysis in the IA, CA, and H2O groups to identify gene co-expression networks associated with drinking behavior in each group (for detailed methods see supplementary materials). Expression and phenotype data for each group was analyzed separately to produce gene co-expression networks and modules, and to correlate modules with behavior. In IA and CA mice modules were correlated with alcohol drinking behavior, and with 5-day average water consumption in H2O mice. Modules in each group were analyzed for statistically significant over-representation of modules in other groups, differentially expressed genes from LIMMA, cell-type specific markers (Cahoy et al., 2008), gene ontology term members, miRNA targets (microRNA.org database, August 2010 release), and transcription factor binding sites. For phenotype analysis BXD nucleus accumbens mRNA expression was correlated with BXD published phenotypes.

The cell-type marker list was converted to Affymetrix Mouse Gene 1.0 ST probeIDs, and trimmed to remove genes with no such ID, to ensure proper matching and a valid background gene set for statistical comparison. The final cell-type marker list contained 5382 total entries.

Gene ontology analysis

Gene sets obtained by LIMMA and WGCNA were subjected to gene ontology (GO) analysis to illuminate biological relevance and regulation mechanisms. Analysis was performed using the GOrilla Gene Ontology Enrichment Analysis And Visualization Tool (http://cbl-gorilla.cs.technion.ac.il/) to identify over-represented GO terms for biological process, molecular function, and cellular component (Eden et al., 2009). All p-values were corrected for multiple comparisons within the software using a specialized method developed by the authors for use with the Gorilla tool (Eden et al., 2007). Gene symbol lists for annotated probesets were input as test sets, and the background set was the gene symbol list from the subset of annotated probesets in the 35, 467 probesets used for LIMMA and WGCNA analyses. For LIMMA sets, Hierarchical charts with terms shaded by significance were output to visualize relationships between over-represented terms.

Transcription factor binding site analysis

Transcription factor binding site analysis was performed using the Promoter Analysis and Interaction Network Toolset (PAINT) (Vadigepalli et al., 2003). PAINT was used to search for consensus binding sites for transcription factors in promoter regions of gene sets, and to identify significantly over- or under-represented sites, which provide mechanistic information about transcriptional regulation. Gene sets were input using Affymetrix Mouse Gene 1.0 ST gene-level probeset IDs, and regions within 2000 base pairs of the transcription start site were considered for analysis. The TRANSFAC Pro v 2009.4 database was used for transcription factor binding site identification. For all analyses the false discovery rate (FDR) threshold was 0.30.

Results

Differences in gene expression between IA, CA, and H2O groups

Differential gene expression among groups was determined using multi-class LIMMA procedures and Benjamini-Hochberg FDR multiple comparison correction applied to RMA expression data. The set of 3521 probesets with $F < .05$ across groups (referred to as the AcrossGroup gene set) were filtered for FDR-adjusted p-value < .05 for pairwise comparisons between groups (see Table 1, Supplementary Table 1). The group-wise comparison process identified 683 probesets (521 upregulated in IA vs. CA, 162 downregulated in IA vs. CA) differentially expressed between IAA and CA mice, and 343 probesets (106 upregulated in CA vs. H2O, 237 downregulated in CA vs. H2O) differentially expressed between CA and H2O mice (see Table 1, Supplementary Tables 2 and 3). No probesets were identified as significantly different in abundance between IAA and H2O mice using FDR-adjusted p-values, but 202 probesets were identified using a raw p-value filter of $p < .01$ (161 upregulated in IA vs. H2O, 42 downregulated in IA vs. H2O) (see Table 1). Of the set of 3521 probesets with $F < .05$, 2817 were annotated and represented a known gene. Of the 683 probesets significantly different between IAA and CA 661 were annotated, and of the 343 probesets significantly different between CA and H2O groups 325 were annotated (see Table 1).

Table 5.1. Differential gene expression determined by multi-class LIMMA. Analysis was performed using an F-statistic threshold of $F < .05$ to derive genes regulated across groups, and FDR-adjusted p-value < .05 for comparisons between groups.

Comparison	Significant probesets	Annotated probesets	Upregulated	Downregulated
IA vs. CA	683	661	521	162
CA vs. H2O	343	325	106	237

IA vs. H2O	0	0	N/A	N/A
Across groups	3521	2817	N/A	N/A

Sets of differentially expressed genes were subjected to gene ontology analysis to identify over-represented GO terms for biological process (BP), molecular function (MF), and cellular compartment (CC). Only annotated probesets with FDR-adjusted p-value < .05 were subjected to gene ontology analysis. For the set of 2817 annotated probesets identified as significantly regulated across groups GO analysis identified 214 BP terms, 60 MF terms, and 60 CC terms (see Supplementary Tables 4, 5, and 6). For the set of 661 annotated probesets identified as significantly regulated between IA and CA mice GO analysis identified 110 BP terms, 36 MF terms, and 28 CC terms (see Supplementary Table 7). For the set of 325 annotated probesets identified as significantly regulated between CA and H2O mice GO analysis identified 5 BP terms, 18 MF terms, and 7 CC terms (see Supplementary Table 8).

For each gene set GO results across function, process, and compartment converged on key areas that provide insight into function. For the AcrossGroup gene set terms converged on areas related to glutamatergic and GABAergic neurotransmission, and neuroplasticity through axonal/dendritic remodeling and control of neurogenesis, differentiation, and cell death. Regulation of MAPK cascade, Wnt signaling, syntaxin binding, cadherin binding, and ion homeostasis for calcium, sodium, and potassium are also implicated by over-represented GO terms in the AcrossGroup set (see supplementary Tables 4, 5, and 6, Supplementary Figure 1). For the gene set regulated between IA and CA mice terms were related primarily to calcium and potassium ion transport and homeostasis, as well as regulation of cell-cell adhesion and synaptic

plasticity (see Supplementary Table 7, Supplementary Figure 2). For the gene set regulated between CA and H2O mice terms were related primarily to potassium ion channel activity, calcium ion binding, and cell adhesion (see Supplementary Table 8, Supplementary Figure 3).

Gene sets with FDR-adjusted p-values < .05 for IAA vs. CA and CA vs. H2O were subjected to transcription factor binding site analysis to identify mechanisms of transcriptional regulation for genes induced by continuous alcohol access and intermittent alcohol access. After multiple comparison correction 40 significantly over-represented transcription factor binding sites were identified in genes differentially expressed between IAA and CA mice, and no factors were identified for genes significant for CA compared to H2O mice (see Table 5.2). Interrogation of significant gene lists from LIMMA identified several differentially regulated transcripts coding for over-represented transcription factors, transcription factor binding proteins, or regulators of transcription factor activation (see Table 5.3).

Table 5.2. Transcription factor binding site analysis in genes differentially expressed between IAA and CA nucleus accumbens. Binding sites are reported with FDR-adjusted p-value.

TRE/External Group	Corrected p-value
AP-2/V$AP2_Q6	p < .0001
AP-2/V$AP2_Q6_01	0.0148
AP-2alpha/V$AP2ALPHA_01	0.0011
AP-4/V$AP4_01	0.0633
ATF6/V$ATF6_01	0.0867
Bach2/V$BACH2_01	0.1419
CKROX/V$CKROX_Q2	p < .0001
CP2/LBP-1c/LSF/V$CP2_02	p < .0001
CREB/V$CREB_02	0.0015
E2F/V$E2F_03	p < .0001
E2F/V$E2F_Q6_01	0.0009
ER/V$ER_Q6	0.1100
ETF/V$ETF_Q6	p < .0001
Hand1:E47/V$HAND1E47_01	0.0645
HIC1/V$HIC1_02	p < .0001
HIF1/V$HIF1_Q3	0.0867
Ikaros/V$IK_Q5	0.1419
Kid3/V$KID3_01	0.0224
KROX/V$KROX_Q6	p < .0001
LRF/V$LRF_Q2	0.0466
MAZ/V$MAZ_Q6	p < .0001
MyoD/V$MYOD_Q6_01	0.0442
myogenin / NF-1/V$MYOGNF1_01	0.0001
NF-Y/V$NFY_Q6_01	0.2314
Pax-3/V$PAX3_B	0.0301

PPARalpha:RXRalpha/V$PPARA_01	0.1409
RFX/V$RFX_Q6	0.0184
RFX1/V$RFX1_02	0.1409
SF1/V$SF1_Q6_01	0.0141
Sp1/V$SP1_Q2_01	p < .0001
Sp3/V$SP3_Q3	0.2247
SREBP/V$SREBP_Q3	0.0531
SREBP/V$SREBP_Q6	0.0184
STAT1/V$STAT1_01	0.0689
Tax/CREB/V$TAXCREB_01	0.2342
VDR/V$VDR_Q3	0.0689
v-Myb/V$VMYB_02	p < .0001
WT1/V$WT1_Q6	p < .0001
ZF5/V$ZF5_B	p < .0001
ZNF219/V$ZNF219_01	p < .0001

Table 5.3. Significantly over-represented transcription factors with factor, binding protein, or direct mediator of action regulated across groups (LIMMA, F < .05 and/or adjusted p-value < .05)

TF	Related genes	F-value	Adj. p-value, IAA vs. CA	log fold change, IAA vs. CA	adj-p-value, CA vs. H2O	log fold change, CA vs. H2O
CP2	*Tcfcp2l1*	1.46E-03	0.039	-0.13	0.234	0.12
CKROX	*Zbtb7b*	7.78E-03	0.920	0.02	0.124	-0.13
CREB	*Creb5*	2.43E-02	0.528	0.07	0.247	-0.12
CREB	*Creb3l2*	1.71E-02	0.680	0.04	0.176	-0.10
E2F	*E2f1*	1.11E-04	0.008	0.19	0.085	-0.17
ER	*Esrrg*	5.28E-03	0.294	0.07	0.120	-0.12
PPARα:RXRα	*Rdh10*	1.99E-04	0.057	-0.15	0.025	0.22
PPARα:RXRα	*Rbp1*	4.93E-04	0.030	-0.17	0.097	0.18

WGCNA network properties

For each group expression data was analyzed for network scale independence, and mean, median, and maximum probeset connectivity was calculated for a range of soft-thresholding powers (see Table 5.4). This step is performed to determine the power to be used in the adjacency function for network construction (see supplementary materials for details). Scale independence is a measure of the degree to which the network resembles an ideal scale-free network, and ranges from 0 to 1. Connectivity (k) measures the degree of correlation of probeset gene expression values with other probeset expression values, or the degree to which expression patterns are correlated across probesets.

By examining these properties across a range of soft-thresholding powers, conclusions can be drawn regarding changes in network topology and connectivity associated with experimental treatment. For each network soft-thresholding powers were chosen based on the range of scale independence produced. For the IA and CA networks the lowest soft-thresholding power was chosen that achieved scale independence greater than 0.7, and for the H2O network the lowest lowest soft-thresholding power greater than or equal to 6 was chosen that achieved scale independence greater than 0.8. These criteria were adapted from the procedure of (Zhang and Horvath, 2005).

Some conclusions were drawn from group differences in the relationships among soft-thresholding power, scale independence, and connectivity measurements. For all soft-thresholding powers the H2O network, which represents an alcohol-naïve NAc transcriptome, was more scale-independent than IA or CA networks, and had greater mean, median, and maximum gene connectivity. Comparison of CA and H2O networks at the default soft-

thresholding power value of 6 showed that alcohol drinking fragmented gene co-expression networks and altered clustering topology, as indicated by decreased connectivity measures and scale independence at corresponding soft-thresholding powers. Abstinence during intermittent access partially rescued overall connectivity deficits induced by alcohol drinking, but did not restore scale independence (see Table 5.4).

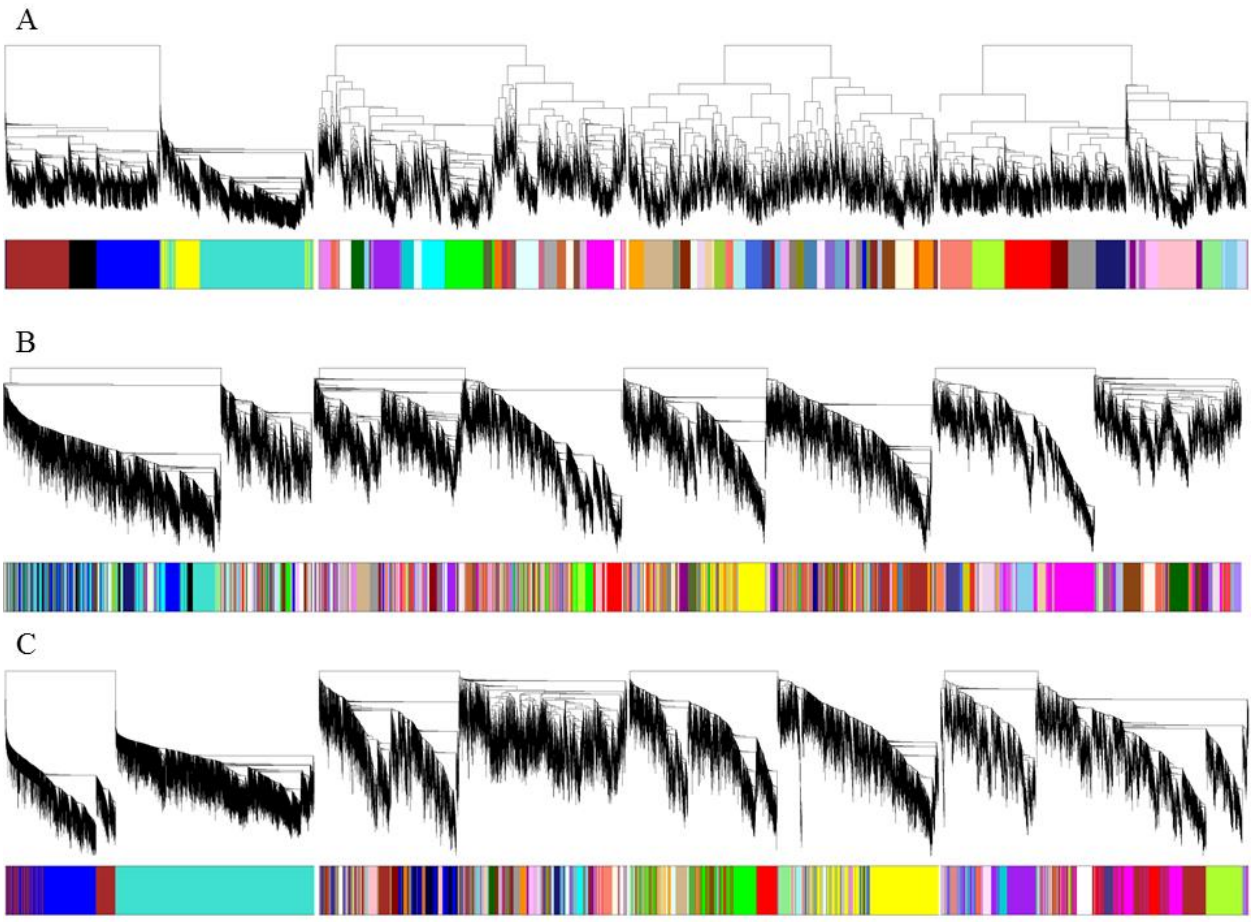
Table 5.4. Scale independence and connectivity (k) for networks across soft-thresholding powers. Underlined values are those chosen for network construction in each group.

	Intermittent access network				**Constant access network**				**H2O network**			
Power	Scale ind.	Mean k	Median k	Max k	Scale ind.	Mean k	Median k	Max k	Scale ind.	Mean k	Medi an k	Max k
1	0.154	12300	12400	18600	0.0151	10200	10000	14600	0.214	14700	14500	19200
2	0.134	6060	5800	12200	0.207	4310	4140	8020	0.658	8400	8090	13100
3	0.476	3490	3110	8830	0.404	2180	2040	5010	0.795	5540	5170	9850
4	0.591	2200	1820	6710	0.531	1240	1120	3360	0.848	3960	3580	7820
5	0.651	1480	1120	5280	0.618	756	664	2370	0.87	2990	2630	6430
6	0.668	1040	728	4260	0.672	489	415	1740	***0.886***	***2340***	***2000***	***5420***
7	0.686	762	489	3500	***0.713***	***331***	***272***	***1310***	0.895	1890	1580	4650
8	0.695	571	340	2920	0.741	232	185	1010	0.902	1560	1270	4050
9	0.697	438	242	2470	0.765	167	129	788	0.905	1310	1050	3580
10	***0.716***	***343***	***176***	***2110***	0.784	124	92.3	628	0.908	1110	877	3190
12	0.718	219	98.9	1570	0.804	71.7	50.5	424	0.907	839	640	2600
14	0.714	148	58.9	1210	0.815	44.4	29.6	299	0.908	656	487	2170
16	0.735	103	36.9	948	0.819	28.9	18.3	218	0.914	527	383	1840
18	0.744	74.4	24.1	757	0.829	19.6	11.7	164	0.914	433	309	1590
20	0.758	55	16.3	613	0.820	13.7	7.84	126	0.913	362	254	1390

Network construction and module eigengene significance

Network construction proceeded with the chosen soft-thresholding power, and for each network a set of modules was selected from 4 candidate sets of modules that were derived through adjustment of dynamic tree cut procedures (see supplementary methods). The IA network produced 82 modules, the CA network produced 182 modules, and the H2O network produced 84 modules. Next eigengene significance for each module with behavior was determined via Pearson correlation of drinking behavior with module eigengenes, with significance taken as $p < 0.15$. For the IA network Pearson correlation of module eigengenes with ethanol consumption and preference (total, 15%, and 30%) at Test Day 7 was performed to generate ES values for each module (see Supplementary Table 9). For the CA network Pearson correlation of module eigengenes with ethanol consumption and preference (total, 15%, and 30%) on the penultimate day of alcohol access (corresponding to IA Test Day 7) was performed to generate ES values for each module (see Supplementary Table 10). For the H2O network Pearson correlation of module eigengenes with 5-day average water intake was performed to generate ES values for each module (see Supplementary Table 11). Calculation of ES yielded modules associated with drinking behavior: 27 modules in the IAA network, 28 modules in the CA network, and 7 modules in the H2O network. Modules within each network were assigned a color label that was unique within that network and used for identification in other analyses (see Figure 5.1).

Figure 5.1. Gene co-expression networks generated by WGCNA for H2O (A), CA (B), and IA (C) mice. For each network the expression dendrogram produced by hierarchical clustering is presented above module definitions corresponding to dendrogram branches, which are indicated by color. From left to right across dendrograms and module color graphs every pixel represents a single probeset.



Module preservation across networks

Modules within the IA, CA, and H2O networks were examined for significant over-representation of any modules from other networks using the hypergeometric test on probeset level module overlaps, with p-values subjected to Bonferroni corrections for multiple comparisons. Analysis found 69 of 182 CA modules significantly enriched for H2O modules (38%), and 100 of 182 CA modules significantly enriched for IAA modules (55%). There were 31 of 82 IAA modules significantly enriched for H2O modules (38%), and 38 of 82 IAA modules significantly enriched for CA modules (46%). In the H2O group 45 of 84 modules were significantly enriched for CA modules (54%), and 41 of 84 modules were significantly enriched for IAA modules (49%). Thus module preservation was greater between alcohol-exposed groups than between either alcohol-exposed group and the H2O group, but only about half of the modules in the IAA group correspond with modules in the CA group, and vice-versa.

When only modules with ES for drinking behavior were considered two significant overlaps were identified for all group-wise module comparisons: the CA lightslateblue and IA salmon4 modules, and the IAA darkviolet and H2O palevioletred2 modules (see Tables 5.5 and 5.6). The CA lightslateblue module is positively correlated with total consumption for the CA group, and the IA salmon4 module is negatively correlated with total preference for the IA group. These modules share six probesets, three of which code are for the same gene. Only *Ptp4a3* (protein tyrosine phosphatase 4a3) is significantly correlated with behavior in both groups, and that correlation is both strong and in the opposite direction for each group: for IAA it is -0.74 (p=0.005), and for CA it is .68 (p=0.015). The IAA darkviolet and H palevioletred2 modules share only *Tmem49*, which is significantly correlated with RE7_15c for the IAA group, but not

with water consumption in the H2O group. No modules with ES for drinking behavior were significantly preserved between H2O and CA networks (see Table 5.7).

Table 5.5. Module preservation between IA and CA networks for modules with behavioral eigengene significance calculated by hypergeometric test of probeset level overlaps with Bonferroni corrected p-values ($p < .05$).

IA module	CA module	Corrected p-value
turquoise	bisque4	2.63E-08
salmon4	brown2	4.96E-13
yellow	brown3	1.54E-04
paleturquoise	darkolivegreen	6.57E-51
darkviolet	darkseagreen3	2.71E-02
lightyellow	lavenderblush1	5.30E-06
darkgreen	lightcyan	1.68E-02
salmon4	lightslateblue	7.45E-04
blue	lightsteelblue1	3.42E-14
brown	lightsteelblue1	2.05E-10
green	lightsteelblue1	3.05E-02
red	lightsteelblue1	4.33E-03
salmon4	magenta	3.90E-06
blue	pink	3.24E-64
brown	pink	8.60E-77
red	pink	4.26E-05
blue	royalblue	1.52E-12
brown	royalblue	4.16E-02
green	royalblue	3.89E-08
plum	royalblue2	2.55E-09
greenyellow	saddlebrown	8.16E-05
skyblue	saddlebrown	8.55E-10
pink	salmon1	1.99E-03
turquoise	skyblue2	7.24E-05
orangered4	yellow	1.77E-04

Table 5.6. Module preservation between IA and H2O networks for modules with behavioral eigengene significance calculated by hypergeometric test of probeset level overlaps with Bonferroni corrected p-values ($p < .05$).

IA module	H2O module	Corrected p-value
darkviolet	palevioletred2	1.94E-02
lightyellow	blue	1.44E-02
midnightblue	thistle2	2.94E-02
paleturquoise	coral1	1.67E-12
paleturquoise	grey	2.04E-04
paleturquoise	paleturquoise	3.48E-03
salmon4	coral1	2.10E-13
salmon4	paleturquoise	1.16E-02
sienna3	turquoise	1.35E-02
turquoise	darkturquoise	2.23E-10
yellow	darkturquoise	5.27E-08

Table 5.7. Module preservation between CA and H2O networks for modules with behavioral eigengene significance calculated by hypergeometric test of probeset level overlaps with Bonferroni corrected p-values ($p < .05$).

CA module	H2O module	Corrected p-value
blue	darkturquoise	2.28E-02
coral1	greenyellow	3.09E-02
lightsteelblue1	lightsteelblue	1.33E-14
lightsteelblue1	turquoise	1.47E-17
orangered1	coral1	3.07E-03
pink	lightgreen	5.35E-04
pink	magenta	1.33E-06
pink	pink	2.53E-16
pink	turquoise	1.12E-115
powderblue	grey60	3.35E-02
royalblue	lightgreen	5.75E-05
royalblue	turquoise	1.55E-42
tan	navajowhite2	1.57E-02

Module enrichment for cell-type specific markers

All modules were examined for enrichment for cell-type specific markers for neurons, astrocytes, and microglia, to determine likely biological context for module functioning. Results for neuron (8 IA modules, 8 CA modules, 11 H2O modules, see Table 5.8) and astrocyte (5 IA modules, 7 CA modules, 6 H2O modules, see Table 5.9) marker enrichment were similar across networks, but for oligodendrocyte markers there were 7 enriched IA modules, 12 enriched CA modules, and only 1 enriched H2O module (see Table 5.10). Few modules enriched for cell-type specific markers had ES for drinking behavior. The IA salmon4 module was enriched for neuron markers, and no other IA module with ES for behavior was enriched for any cell-type. In the CA network the saddlebrown and royalblue modules were enriched for neuron markers, royalblue, lightsteelblue1, and pink were enriched for oligodendrocyte markers, and lightsteelblue1 was enriched for astrocyte markers. No H2O modules with ES were enriched in cell-type specific markers.

Table 5.8. Neuron cell-type specific marker enrichment in modules identified in IA, CA, and H2O gene networks (hypergeometric test, Bonferroni correction, $p < .05$). Modules with eigengene significance for drinking behavior are indicated with * (Pearson correlation, $p < .10$).

Module	**Cell type**	**Corrected p-value**	**Network**
tan	Neuron	7.89E-60	IA
lightcyan	Neuron	3.31E-20	IA
brown	Neuron	7.58E-12	IA
blue	Neuron	1.04E-11	IA
salmon4*	Neuron	1.81E-05	IA
skyblue	Neuron	0.002	IA
greenyellow	Neuron	0.0029	IA
darkred	Neuron	0.0438	IA
magenta	Neuron	1.61E-86	CA
tan	Neuron	1.55E-21	CA
lightpink4	Neuron	1.77E-08	CA
saddlebrown*	Neuron	1.86E-07	CA
navajowhite2	Neuron	2.16E-06	CA
skyblue	Neuron	0.0002	CA
darkgoldenrod4	Neuron	0.0315	CA
royalblue*	Neuron	0.0423	CA
darkgrey	Neuron	2.78E-16	H2O
turquoise	Neuron	2.90E-15	H2O
floralwhite	Neuron	7.46E-07	H2O
royalblue	Neuron	8.91E-05	H2O
lightcyan	Neuron	0.0002	H2O
yellow	Neuron	0.0002	H2O
salmon4	Neuron	0.0016	H2O
magenta	Neuron	0.0043	H2O
firebrick4	Neuron	0.0076	H2O
grey60	Neuron	0.0149	H2O
darkslateblue	Neuron	0.0202	H2O

Table 5.9. Oligodendrocyte cell-type specific marker enrichment in modules identified in IA, CA, and H2O gene networks (hypergeometric test, Bonferroni correction, $p < .05$). Modules with eigengene significance for drinking behavior are indiciated with * (Pearson correlation, $p < .10$).

Module	**Cell type**	**Corrected p-value**	**Network**
blue	Oligodendrocyte	6.65E-17	IA
brown	Oligodendrocyte	6.68E-12	IA
green	Oligodendrocyte	2.76E-11	IA
red	Oligodendrocyte	4.36E-05	IA
lightgreen	Oligodendrocyte	0.0003	IA
antiquewhite4	Oligodendrocyte	0.0035	IA
coral1	Oligodendrocyte	0.0168	IA
tan	Oligodendrocyte	5.26E-11	CA
green	Oligodendrocyte	7.43E-11	CA
royalblue*	Oligodendrocyte	9.57E-07	CA
yellow	Oligodendrocyte	1.06E-06	CA
skyblue3	Oligodendrocyte	6.28E-06	CA
skyblue	Oligodendrocyte	6.87E-06	CA
yellow3	Oligodendrocyte	8.86E-05	CA
lightsteelblue1*	Oligodendrocyte	0.0004	CA
firebrick4	Oligodendrocyte	0.0015	CA
pink*	Oligodendrocyte	0.0113	CA
magenta	Oligodendrocyte	0.0128	CA
orangered4	Oligodendrocyte	0.0137	CA
turquoise	Oligodendrocyte	1.02E-17	H2O

Table 5.10. Astrocyte cell-type specific marker enrichment in modules identified in IA, CA, and H2O gene networks (hypergeometric test, Bonferroni correction, $p < .05$). Modules with eigengene significance for drinking behavior are indiciated with * (Pearson correlation, $p < .10$).

Module	**Cell type**	**Corrected p-value**	**Network**
brown	Astrocyte	6.52E-18	IA
darkslateblue	Astrocyte	4.09E-10	IA
red	Astrocyte	0.0009	IA
greenyellow	Astrocyte	0.0269	IA
saddlebrown	Astrocyte	0.0276	IA
yellow	Astrocyte	2.24E-09	CA
green	Astrocyte	0.001	CA
lightpink4	Astrocyte	0.006	CA
lightsteelblue1*	Astrocyte	0.006	CA
lightblue3	Astrocyte	0.019	CA
plum2	Astrocyte	0.044	CA
darkgrey	Astrocyte	0.047	CA
lightsteelblue1	Astrocyte	0.0002	H2O
turquoise	Astrocyte	0.0006	H2O
darkorange	Astrocyte	0.0154	H2O
darkgreen	Astrocyte	0.0188	H2O
brown2	Astrocyte	0.0317	H2O
skyblue	Astrocyte	0.0453	H2O

ES module enrichment for differentially expressed genes

Modules with eigengene significance for drinking behavior were examined for enrichment in differentially expressed genes from multi-class LIMMA. Analyses were performed with the set of regulated genes across groups with $F < .05$ (AcrossGroup), and the sets of genes with group-wise differences for IAA vs. CA, and CA vs. H2O. Among the IA modules with ES the grey module was enriched for genes in the AcrossGroup set, and the salmon4 module was enriched for genes in the AcrossGroup set and the and CA vs. H2O set (Bonferroni-corrected $p < .05$). Among the CA modules with ES only the saddlebrown module was enriched for differentially expressed genes, and the IAA vs. CA set and AcrossGroup set were over-represented (Bonferroni-corrected $p < .05$).

ES module gene ontology analysis

Modules with eigengene significance for alcohol drinking behavior were examined for enrichment in gene ontology terms for biological process (BP), molecular function (MF), and cellular compartment (CC). GO terms were considered significant only after correction for multiple comparisons (corrected p-value < .10).

Four IA network modules with ES for drinking behavior were significantly enriched for GO terms: sienna3 (ES for total preference, Pearson: 0.565, $p = 0.021$), midnightblue (ES for 15% consumption, Pearson: -0.741, $p = 0.006$), darkorange (ES for 15% consumption, Pearson: -0.652, $p = 0.022$), and lightyellow (ES for 15% consumption, Pearson: -0.601, $p = 0.039$) (see Supplementary Table 12). For sienna3 only CC terms were identified, and indicated that genes in cytoplasm, nucleus, and non-membrane-bound organelles were over-represented. For midnightblue and lightyellow modules several BP and MF terms were identified, primarily due

to the presence of several olfactory receptors and trace-amine-associated receptors in the modules. As such, terms identified were involved in sensory perception of smell and G-protein coupled receptor activity. For the darkorange module terms were involved in cellular response to interferon-beta and defense response to virus, which indicated potential module involvement in neuroimmune and neuroinflammatory processes.

Four CA network modules with ES for drinking behavior were significantly enriched for GO terms: pink (ES for total consumption, Pearson: -0.524, p = 0.08), bisque4 (ES for total consumption, Pearson: 0.488, p = 0.107), saddlebrown (ES for total preference, Pearson: -0.502, p = 0.096), and blue4 (ES for 30% consumption, Pearson: 0.528, p = 0.078) (see Supplementary Table 13). For the pink module terms were related to cellular metabolic processes: ubiquitin-related protein metabolism and mRNA processing and splicing. GO terms for CC in the pink module were consistent with cellular metabolic functioning, and indicated enrichment for genes functioning in spliceosomal complex, transcription factor complexes TFTC and TFIID, and ribonucleoprotein complex. For the bisque4 module terms were related to G-protein coupled signaling, again primarily due to the presence of olfactory receptors. The saddlebrown module was enriched for CC terms related to neuronal excitability and action potential transmission: axolemma and voltage-gated potassium channel complex. Two significant terms in the blue4 module were related to fertilization processes, and thus likely not directly relevant to network functioning in the nucleus accumbens.

ES module miRNA target enrichment

Modules with eigengene significance for drinking behavior were examined for enrichment in miRNA targets to identify potential module regulation mechanisms (see supplementary Table

14). Among the IA modules with ES for alcohol drinking behavior only sienna3 was enriched for miRNA targets (see Table 5.11).

Table 5.11. miRNA target enrichment in sienna3 module from IA network. Hypergeometric test with Bonferroni-corrected p-value < .05.

Target-enriched miRNA	Corrected p-values
miR-590-3p	3.55E-05
miR-27b	3.57E-04
miR-27a	6.04E-04
miR-23b	4.17E-03
miR-186	4.22E-03
miR-494	9.26E-03
miR-340-5p	9.94E-03
miR-129-5p	1.50E-02
miR-23a	1.70E-02
miR-592	3.96E-02
miR-9	4.68E-02

Among the CA modules with ES for alcohol drinking behavior the pink, royalblue, and lightsteelblue1 modules were enriched for miRNA targets. The pink module was enriched for targets of 100 miRNAs, the royalblue model was enriched for 65 miRNAs, and the lightsteelblue1 module was enriched for 10 miRNAs (see supplementary Table 14). Targets for 61 miRNAs were found to be enriched in more than one CA module (see supplementary Table 14). Targets for nine miRNAs were found to be enriched in the IA sienna3 module and one or more CA modules (see Table 5.12).

Table 5.12. miRNA target enrichment in modules with IAA and CA ES.

miRNA	Network	Module	Corrected p-value
mmu-miR-129-5p__miRNA	IAA	sienna3	1.50E-02
	CA	pink	2.43E-09
	CA	royalblue	2.79E-06
mmu-miR-186__miRNA	IAA	sienna3	4.22E-03
	CA	pink	2.11E-13
	CA	royalblue	8.73E-09
mmu-miR-23a__miRNA	IAA	sienna3	1.70E-02
	CA	pink	3.02E-07
	CA	royalblue	2.26E-03
mmu-miR-23b__miRNA	IAA	sienna3	4.17E-03
	CA	pink	4.34E-08
	CA	royalblue	8.21E-03
mmu-miR-27a__miRNA	IAA	sienna3	6.04E-04
	CA	pink	1.43E-02
	CA	lightsteelblue1	1.32E-02
mmu-miR-27b__miRNA	IAA	sienna3	3.57E-04
	CA	pink	1.26E-02
mmu-miR-340-5p__miRNA	IAA	sienna3	9.94E-03
	CA	pink	4.44E-15
	CA	royalblue	1.13E-07
	CA	lightsteelblue1	8.17E-03
mmu-miR-494__miRNA	IAA	sienna3	9.26E-03
	CA	pink	2.31E-12
	CA	royalblue	1.33E-09
mmu-miR-590-3p__miRNA	IAA	sienna3	3.55E-05
	CA	pink	5.79E-23
	CA	royalblue	2.85E-12
	CA	lightsteelblue1	2.11E-03

Transcription factor binding site analysis

Modules with eigengene significance for drinking behavior were examined for enrichment in transcription factor binding sites (TFBS) in promoter regions to identify potential module regulation mechanisms. TFBS results were combined with results from LIMMA for IAA vs. CA differential gene expression to identify significantly regulated transcription factors in modules with eigengene significance for drinking behavior. Consumption and preference ES modules were considered together within each network and for each alcohol concentration (total, 15%, 30%). Targets for regulated transcription factors tended to be over-represented in modules for with ES in either the IA network or the CA network, with the exceptions of targets with the Pbx/V$PBX_Q3, Pbx-1/V$PBX1_03, and POU1F1/V$POU1F1_Q6 TFBS, which were enriched in modules in both networks (see Table 5.13).

Table 5.13. Over-represented TFBS in modules with ES for drinking behavior across networks, and for which transcription factor genes are differentially expressed between IA and CA mice (multi-class LIMMA, F < .05, adjusted p-value < .05).

TFBS	TF gene	Log FC IAA from CA	Network (ES behaviors)
Pbx/V$PBX_Q3	*Pknox2*	0.22	IA and CA (total/15%/30%)
Pbx-1/V$PBX1_03	*Pbx1*	-0.12	IA and CA (total/15%/30%)
TBP/V$TBP_Q6	*Tbpl1*	-0.06	IA only (15%/30%)
BRCA1:USF2/V$BRCA_01	*Brca1*	-0.04	IA only (15%/30%)
POU1F1/V$POU1F1_Q6	*Pou1f1*	-0.09	IA (15%/30%), CA (15%)
E2F/V$E2F_01	*E2f1*	0.19	CA only (total)
c-Maf/V$CMAF_01	*Maf*	0.06	CA only (15%)
PPARgamma:RXRalpha/V$PPARG_01	*Pparg*	0.14	CA only (30%)
	Ppargc1a	0.09	

Discussion

In contrast to expected results, differential gene expression analysis with LIMMA identified significant differences in expression between the IA and CA groups, and the CA and H2O groups, but not between the IA and H2O groups. Transcription factor binding site analysis implicated CREB-mediated transcription and retinoic acid signaling, among other mechanisms, in the regulation of genes differentially expressed between IA and CA groups. Furthermore, transcription factors or factor binding proteins for six over-represented sites, including CREB and PPARalpha:RXRalpha, were differentially expressed among groups. Gene ontology analysis of differentially expressed genes identified significantly over-represented terms in each set that provide insight into function and cellular localization.

The WGCNA identified global differences in network topology and connectivity within the nucleus accumbens transcriptome across groups. Gene co-expression modules were only moderately preserved across groups, with the alcohol exposed groups more similar to each other than to the H2O group. Only one set of preserved gene co-expression modules was associated with ethanol drinking behavior in both alcohol exposed groups, and modules associated with drinking behavior did not tend to be enriched in differentially expressed genes. Transcription factor binding sites for AP-1 were over-represented among gene co-expression modules with ES for drinking behavior in intermittent access mice, which implicated deltaFosB in the regulation of those networks. Enrichment (gene ontology, cell-type specific marker) and regulation (TFBS, miRNA) analysis identified several modules of particular interest with eigengene significance for drinking behavior. In the IA network modules of interest were salmon4, sienna3, darkorange, grey, midnightblue, and lightyellow. In the CA network modules of interest were saddlebrown, pink, lightsteelblue1, royalblue, bisque4, and blue4 (see Table 5.14). The study of alcohol-related

behavior will be informed by hub genes in modules of interest, differentially expressed transcription factors, over-represented miRNAs, and GO processes/pathways, which represent targets for future experimentation.

Table 5.14. Modules of interest from IA and CA WGCNA networks with eigengene significance for drinking behavior (Pearson correlation, $p < .10$).

Network	Module	Eigengene/behavior correlation	Cell-type marker enrichment	DE gene enrichment (LIMMA)	GO term enrichment	mIRNA target enriched
IA	salmon4	Total preference (-.61)	neuron	AcrossGroup, CA vs. H2O	N/A	no
IA	grey	15% consumption (-.60), 15% preference (-.64), 30% consumption (.75), 30% preference (.77)	N/A	AcrossGroup	N/A	no
IA	sienna3	Total preference (.66), 15% preference (.54)	N/A	N/A	CC (cytoplasm, nucleus, non-membrane-bound organelle)	yes
IA	midnightblue	15% consumption (-.74), 15% preference (-.60), 30% consumption (.63), 30% preference (.68)	N/A	N/A	MF (perception of smell)	no
IA	darkorange	15% consumption (-.65), 15% preference (-.65)	N/A	N/A	BP (neuroimmune/neuroinflammation)	no
IA	lightyellow	15% consumption (-.60), 15% preference (-.54), 30% consumption (.52), 30% preference (.55)	N/A	N/A	BP/MF (perception of smell)	no
CA	saddlebrown	Total preference (-.50)	neuron	N/A	CC (axolemma, voltage-gated potassium channel complex)	no
CA	royalblue	Total consumption (-.49)	neuron, oligodendrocyte	N/A	N/A	yes
CA	lightsteelblue1	Total preference (-.51), 15% consumption (-.52), 15% preference (-.52)	oligodendrocyte, astrocyte	N/A	N/A	yes
CA	pink	Total consumption (-.52), total preference (-.50),	oligodendrocyte	N/A	BP/MF (protein metabolism/mRNA processing), CC (spliceosomal complex, transcription factor complexes TFTC/TFIID, ribonucleoprotein complex)	yes
CA	bisque4	Total consumption (.49)	N/A	N/A	BP/MF (GPCR signaling)	no
CA	blue4	30% consumption (.53), 30% preference (.59)	N/A	N/A	BP (fertilization)	no

Approximately 1% of measured transcripts in the nucleus accumbens (343 probesets) are differentially expressed between continuous alcohol access mice and water drinking mice. Mice had been drinking approximately 15 to 20 g/kg/day of alcohol for 25 days, so differentially expressed genes represent long-term ethanol-responsive genes. Interestingly, these genes were mostly downregulated (237 of 343), which differs from other studies, which have reported either predominant upregulation or no bias (Bell et al., 2009; Kerns, 2005; Rodd et al., 2008). (Bell et al., 2009; Kerns, 2005; Rodd et al., 2008). This disagreement could be caused by differences in methodology; other NAc-specific studies have used experimenter administered alcohol or rats as the animal model. An alternative explanation is that genes are transiently induced by alcohol upon initial exposure, but that persistent exposure blunts the transcriptional response to alcohol, such that fewer upregulated genes are observed. Furthermore, increased numbers of downregulated genes may be due to homeostatic mechanisms triggered at the mRNA or protein level by increased abundance after drinking. These homeostatic mechanisms would act to restore normal functioning despite continued alcohol exposure by altering gene expression, through processes such as regulation of miRNAs or transcription factors.

Differential expression analysis between IA and CA mice supports the hypothesis that the state of the transcriptome during abstinence is partially, but not entirely, a product of the decay of alcohol-responsive gene expression. Approximately 2% of measured genes (683 probesets) were differentially expressed in IA mice compared to CA mice, and 76% of these were upregulated. Of these genes, 241 are ethanol-responsive transcripts identified as differentially expressed between H2O and CA groups, and every one of these genes is expressed in the opposite direction for the two comparisons (see Supplementary Table 17). Of these 241 genes, 178 are down in CA

mice compared to H2O mice, and up in IA mice compared to CA mice. Furthermore, the degree of change for these genes in the two comparisons is extremely well correlated (Spearman correlation: $r = -.95$, $p < .0001$) (see Figure 5.2). In other words, the majority (241 out of 343) of genes regulated in nucleus accumbens by 25 days of alcohol drinking are regulated in the opposite direction, and to nearly the same degree, after 6 cycles of IA and 6 days of abstinence. Future studies should examine whether these genes are altered after the first deprivation period, and whether alcohol drinking in IA mice restores expression of these genes to levels seen in CA drinkers. In addition, a time-course study of gene expression during CA drinking over a period of weeks would be useful, in that it would determine whether the unusual distribution of directional changes in the present study was aberrant, and whether gene expression changes over time in these mice, which show steady intake. A particularly useful study might be differential gene expression in the nucleus accumbens transcriptome after 24 hours of alcohol drinking in naïve mice compared IAA mice after several cycles of IA, because there is emerging evidence that alcohol-responsive gene expression is directly related to the development of binge-like drinking (Ahmadiantehrani et al., 2013).

Unexpectedly, there are no differentially expressed genes between the IA and H2O groups, despite the reasonable assumption that IA mice would drink far more alcohol than H2O mice if offered the opportunity. This drastic phenotypic difference likely indicates that changes in gene expression caused by cycles of IAA have propagated to the functional protein level, at which changes in neuronal function and behavior are mediated. It should also be mentioned that the fact that genes are different between H2O and CA groups, and genes are different between CA and IA groups, does not necessarily mean that one should expect differences between H2O and IA

groups. Instead, this expectation stems from the robust transcriptional effects of alcohol, combined with the high levels of intake displayed by IA mice on test days.

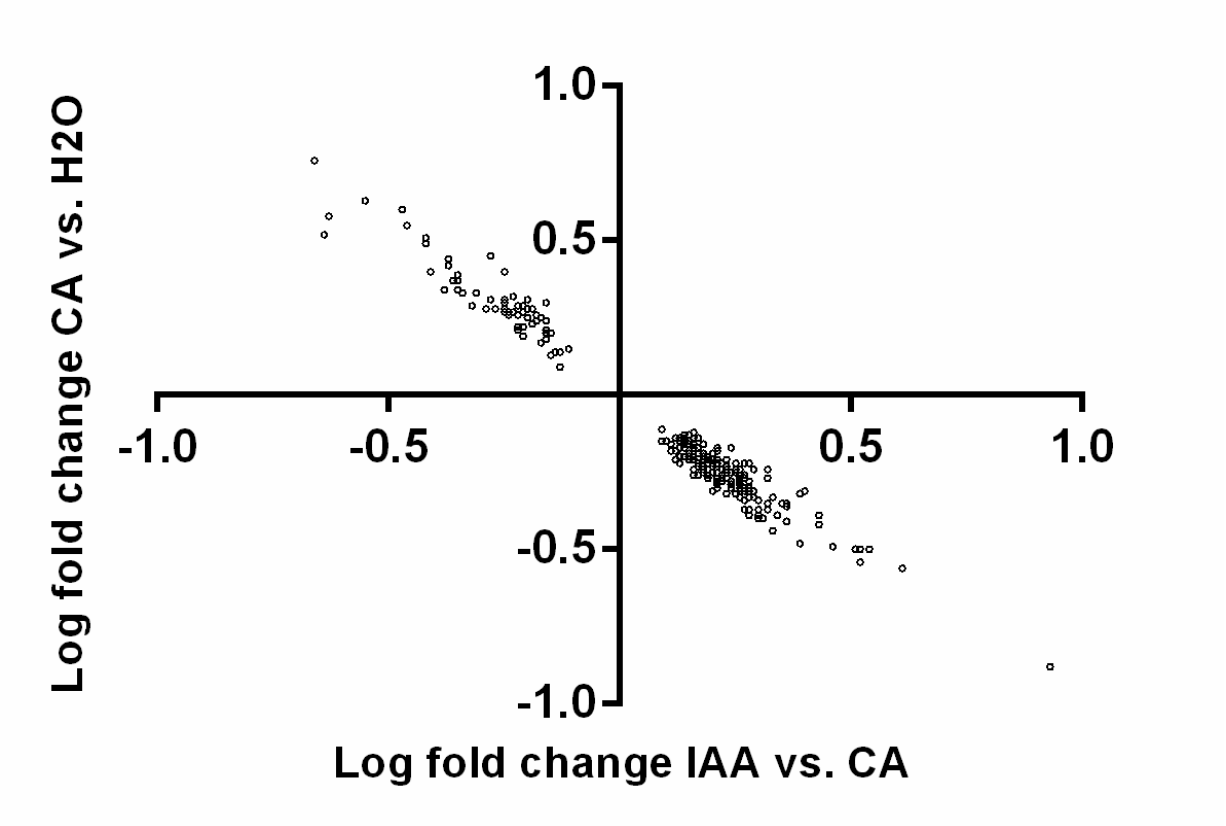


Figure 5.2. Correlation of changes in gene expression for 241 transcripts identified by LIMMA as differentially expressed for CA vs. H2O and IA vs. CA comparisons. (two-tailed Spearman correlation, r = 0.9492, p < .0001)

The lack of expression differences for IA vs. H2O could be due to the strict multiple comparison correction to which raw p-values for group-wise differences were subjected during LIMMA, and within-group variance that masks between-groups differences. By comparison, a raw p-value cutoff of .05 gives 631 probesets differentially expressed between IA and H2O groups, and it is therefore possible that subtle differences are present between H2O and IA groups that are below the threshold of significance used in this study. Other potential explanations for this result are that gene expression induced by a single day of alcohol drinking in nucleus accumbens is no longer apparent after 6 days of abstinence, that repeated cycles of IAA have attenuated the transcriptional response to ethanol, or a combination of these effects. Future studies will address these issues in several ways. Transcription should be measured in IA mice immediately after drinking, and during abstinence regulated genes should be compared to H2O and CA subjects at the mRNA and protein level. In CA mice that drink long-term, time-course studies will address the persistence of the transcriptional response to ethanol over time, and determine the utility of the group as genomic controls for comparison. Finally, increased sample size and alternative methods of assessing differential expression can be applied to further strengthen results and conclusions drawn from studies of this nature.

Gene ontology results for differentially expressed genes identified over-represented terns biological processes, molecular functions, and cellular compartment localization that provide some insight into the functional consequences of regulation. The differentially expressed gene sets are enriched in terms related to potassium ion channel activity, calcium ion binding, and cell adhesion. Several terms in the H2O vs. CA set contain several genes coding for proteins mediating the acute psychoactive effects of ethanol: GABA receptor subunits *Gabra2* and *Gabrg3*, glycine transporter *Slc6a9*, and ion channels for potassium, sodium, and calcium

(Spanagel, 2009; Vengeliene et al., 2009). The terms related to cell adhesion are notable for the presence of *Mag* and *Mog*, which are myelin-associated glycoproteins.

Alcohol is known to downregulate myelin-related genes, and to induce white-matter degeneration in alcoholics through neuroinflammatory processes (Alfonso-Loeches et al., 2012; Kril et al., 1997; Lewohl et al., 2005). Cell signaling via Wnt pathways and MAPK cascade are also implicated by gene ontology terms, and ethanol is known to interact with both pathways (Darlington et al., 2013; Vangipuram and Lyman, 2012).

The involvement of Wnt signaling is particularly intriguing, because it is upstream of other implicated processes: gene expression, regulation of calcium, and alterations in cell cytoskeleton and adhesion via MAPK cascade (Baarsma et al., 2013; Nusse, 2012). In addition, several Wnt-related genes, primarily frizzled-related proteins and frizzled homologs, are differentially expressed among groups, and *Frzb* (frizzled-related protein, aka. secreted frizzled-related protein 3) is the 12th ranked gene in the IAA vs. CA set as ranked by log fold change. The gene *Sfrp1* (secreted frizzled-related protein 1) is upregulated in CA vs. H2O, and downregulated in IAA vs. CA, while *Sfrp2* (secreted frizzled-related protein 2) is also down in IAA vs. CA. Frizzled homologs 1, 2, and 3 (*Fzd1, Fzd3, Fzd4)* are receptors for the Wnt ligand, and are significantly regulated in the AcrossGroup LIMMA set, being more abundant in CA mice than IA or H2O mice. However, pair-wise differences for these genes were not significant after multiple comparison correction. The secreted frizzled-related proteins (SFRPs) bind Wnt and decrease pathway signaling, which alters gene expression; downregulation of these genes is associated with development of several types of cancer (Finch et al., 1997; Zhao et al., 2007). Thus, Wnt signaling seems to be altered by CA alcohol drinking, and increased expression of SFRPs may represent homeostatic mechanisms acting to regulate this change. Discernment of the exact

manner of regulation, and the particular Wnt pathways involved, will require further study, including measurement of protein abundance.

Transcription factor binding site analysis identified regulation mechanisms for differentially expressed genes among groups. Factors or factor binding proteins for six over-represented transcription factors were differentially expressed. Of particular interest are genes related to retinoic acid signaling and CREB-mediated transcription. Alcohol interferes with retinoic acid synthesis and impairs retinoic acid signaling, which alters gene expression and is associated with development of hepatic cancer, through MAPK signaling and AP-1-mediated transcription (Poschl et al., 2004; Wang, 2003). Furthermore, genes associated with retinoic acid and MAPK signaling are regulated in hippocampus of C57BL/6J mice during ethanol withdrawal (Daniels and Buck, 2002). Gene expression mediated by the CREB (cAMP response element binding protein) transcription factor is extensively implicated in gene expression related to consumption of abused substances and stress, and relevant targets include stress hormone CRF/CRH, opioid peptide precursor dynorphin, and BDNF (brain derived neurotrophic factor) (Briand and Blendy, 2010; McCarthy et al., 2012; Pandey, 2004a; Robison and Nestler, 2011). Each of the over-represented transcription factors represents a target of interest to interfere with ethanol-responsive gene expression, with the goal of interfering with transcription associated with the development of binge-like drinking behavior. The congruence between the neurobiological effects of alcohol and the changes in gene expression revealed by LIMMA support the notion that genomic changes measured in the present study are a reliable basis on which to draw other conclusions.

Calculating scale independence and connectivity prior to WGCNA revealed differences in network topology and transcript co-expression across group networks. Continuous access alcohol

drinking for 25 days decreased scale-independence and gene connectivity, and thus fragmented gene co-expression networks operating in the nucleus accumbens of naïve mice. Consistent with recovery of normal transcriptome expression in IA shown by LIMMA, abstinence partially restores deficits in mean, median, and maximum gene connectivity associated with CA drinking, but did not restore scale independence, which is indicative of a less fault-tolerant network in which hub gene connectivity is particular reduced. These results may indicate less coordinated signaling and gene regulatory mechanisms operating in neurons in general during drinking, amd future study should examine particularly well-connected hub gene identity, and regulation of connectivity due to alcohol exposure over time. Of particular interest is the process of distinguishing those hub genes that do or do not recover connectivity during abstinence, which could lead to heretofore unknown players in alterations in cell signaling. Genes of this type would not necessarily show up in differential expression analyses; because while connectivity is altered, expression may not necessarily be changed as well. There is some recent supporting evidence for this approach, as well data showing that gene network fragmentation leads to maladaptive alcohol behavior, but this result comes from naïve mice bred for high drinking (HDID-1 and HDID-2 strains from the Crabbe laboratory), rather than alcohol-exposed animals (Iancu et al., 2013).

In general overlap across networks for modules from WGCNA is moderate, and supports the notion that alcohol exposure alters global patterns of gene expression. When analyzing these results it should be noted that the groups represent a progression of experimental conditions; all mice began the study as naïve mice with water only, then some subject were allowed continuous access drinking, and a subset of these were allowed to progress to intermittent drinking. In the CA network only 38% of modules (69 of 182) are enriched for H2O modules, indicating that

gene co-expression relationships are altered by drinking. Interestingly, 38% of IAA modules (31 of 82) are enriched for H2O modules, indicating that six days of abstinence has not restored connectivity. Future studies should examine if this is an effect of the previous six weekly cycles of IAA, by determining whether a single six day abstinence period after CA drinking is sufficient to restore connectivity. The IAA and CA networks were moderately preserved (CA enriched for IA: 55%, IA enriched for CA: 46%), indicating that module identities were more similar to each other than to the H2O network. This is fascinating in light of differential expression results, which show more differences for IA vs. CA than for CA vs. H2O, and no differences for IA vs. H2O. These results indicate that while abstinence certainly reverses some ethanol-responsive gene expression, connectivity deficits and hub gene function are not restored, and these processes should be further studied for specific hub genes and gene networks.

When only modules with eigengene significance for drinking behavior were considered, differences between groups were even more stark; only two pairs of preserved modules with behavioral significance were identified. For modules with ES for alcohol drinking, only the CA lightslateblue and IAA salmon4 modules were preserved across networks. The CA lightslateblue module expression is correlated with total consumption for the CA group, and the IAA salmon4 module is negatively correlated with total preference for the IAA group. These modules share 6 probesets, 3 of which code for the same gene. Interestingly, only protein tyrosine phosphatase 4a3 (*Ptp4a3*) is significantly correlated with behavior in both groups, and that correlation is both strong and in the opposite direction (IAA: $r = -0.74$, CA: $r = .68$; Pearson correlation, $p < .05$). The function of *Ptp4a3* is not particular well characterized, but it seems to be membrane-bound and involved in calcium mobilization and cell growth, and high levels are associated with risk for some types of cancer (Laurent et al., 2011; Matter et al., 2001; Wang et al., 2002).

The only other pair of modules with behavioral significance in both groups is the IAA darkviolet/H2O palevioletred2 set. These modules share only *Tmem49*, which is significantly correlated with 15% consumption for the IAA group, but not with water consumption in the H2O group. These results suggest that gene co-expression patterns associated with drinking behavior share almost no similarity between groups, and highlight the differences in the molecular basis of alcohol intake under continuous access and intermittent access conditions.

Within each group gene network modules of interest are identified based on eigengene significance, gene ontology, cell-type marker enrichment, differential gene expression enrichment, and miRNA enrichment, and genes in these modules are targets for future study (see Table 14). Of particular interest are highly connected hub genes identified on the basis of module membership, which is a "fuzzy" measurement of intramodular connectivity (Zhang and Horvath, 2005). For each module of interest, the top 15 hub genes are reported, several are known to be involved in alcohol-related behavior or directly related processes (see Supplementary Table 14, Supplementary Table 15).

Hub genes for the IA salmon4 module include *Htr1a* (serotonin receptor 1a), agonists at which increase ethanol intake, and *Grm2* (glutamate receptor, metabotropic 2), which participates in CREB/AP-1 mediated transcriptional regulation downstream of glutamate activity (Tomkins et al., 1994; Wang et al., 2007). In the IA sienna3 module ubiquitin ligase *Nedd4* (neural precursor cell expressed, developmentally down-regulated 4) is the 6th ranked hub gene, and is known to be involved in regulation of sodium, potassium, and calcium channels (Krzystanek et al., 2012; Snyder et al., 2002; Zhang et al., 2010). Our laboratory recently showed that ethanol upregulates *Sgk1 (*Serum/glucocorticoid regulated kinase 1) in PFC through activation of the hypothalamic-pituitary-adrenal (HPA) axis (Costin et al., 2013a). SGK1 phosphorylates NEDD4, which

decreases its activity and leads to increases in sodium and potassium channel abundance in the cell membrane (Schuetz et al., 2008). The sienna3module may thus be involved in regulation of ion channel abundance and nucleus accumbens excitability as a result of ethanol activation of the HPA axis. Involvement in neuroinflammatory and neurodegenerative processes, particularly regulation of myelin, is indicated for the IA midnightblue module due to the presence of *Tlr4* (toll-like receptor 4), which is known to be involved in demyelination in chronic alcohol abuse, and *Il6r* (interleukin 6 receptor), which is involved in immune regulation (Alfonso-Loeches et al., 2010; Alfonso-Loeches et al., 2012; Wang et al., 2008b).

In the CA network the lightsteelblue1 module is enriched in targets for several miRNAs, and is no notable for the presence of *Tgfbr1* (transforming growth factor , beta receptor 1), which may mediate the effects of chloride intracellular proteins (CLICs) on alcohol-related behaviors through phosphorylation of SMAD proteins, which can modulate miRNA- regulated gene expression (Bhandari et al., 2012; Davis et al., 2008). The lightsteelblue1 module also contains the hub gene *Rgs5* (regulator of G-protein signaling 5), which reduces signaling through G-alpha subunits (Zhou et al., 2001). RGS family proteins influence mu-opioid receptor signaling and morphine reward behaviors (Traynor, 2012). Furthermore, mRNAs coding for RGS proteins are decreased in brains of human alcoholics, and SNPs in the *RGS4* gene are associated with risk for alcoholism in European Caucasians (Ho et al., 2010). The CA saddlebrown module is particularly notable for being enriched in neuron-specific cell-type markers, and the presence of hub genes *Kcnip2* (Kv channel-interacting protein 2) and *Adora2a* (adenosine A2a receptor). Adenosine receptors are known to be involved in ethanol-related behaviors and the physiological effects of the drug, and in particular decreased ADORA2A function in the striatum is associated with ethanol-seeking behaviors, and decreased CREB-mediate transcription (Chiang et al., 2013;

Nam et al., 2013a; Nam et al., 2013b). The KCNIP2 protein acts as a calcium-sensing modulator of voltage-gated potassium channels, where it acts to modulate neuronal excitability (Wang et al., 2013). In the light of studies showing that SK potassium channel (a calcium-activated potassium channel) inhibitors reduce IA alcohol intake, the connection between intracellular calcium/potassium regulation in striatum and the development of binge-drinking should be further explored (Hopf et al., 2011).

The present study illuminates the transcriptional consequences of alcohol drinking on continuous access and intermittent access schedules in C57BL/6J mice, and provides several genes, pathways, and gene networks associated with alcohol-related behavior that represent targets for future study, with the goal of improving understanding alcoholism and therapeutic intervention. Some limitations should be considered when interpreting the reported results. Any conclusions drawn must take into account that only the nucleus accumbens was studied, and in only one inbred strain. Alcohol-responsive gene expression tends to be region-specific, so the study of more regions in the same manner reported herein will be particularly useful in deciphering the connection between gene expression and addictive behavior. The C57BL/6J is an inbred strain, and shows relatively high alcohol consumption compared to other strains. The effects of IAA vary from strain to strain in mice and rodents, and future studies should address whether transcription during CA and IA drinking similarly vary, and the connections between regulated transcripts and behavior.

The data reported also represents only one time point for both CA and IA mice, which limits the conclusions that can be drawn. Future studies should examine gene expression at other time points during access, for example to compare the first cycle of IA to the last, and IA gene expression directly following alcohol consumption. Nevertheless, the results reported herein

provide novel insight into the connection between escalations in alcohol drinking and transcriptional regulation in the nucleus accumbens, and provide direction for future study.

Chapter 6. Mu-opioid receptor-selective antagonist NAQ selectively reduces high concentration alcohol consumption and delays the effects of intermittent alcohol access

Introduction

Nearly 4% of people in the United States are estimated to be afflicted with alcohol use disorders (AUDs), such as alcohol abuse or dependence. In the United States alone, the total cost to society related to alcohol use and abuse amounted to 2.7% of yearly GDP (Rehm et al., 2009). However, despite this high prevalence and cost, only three medications have been approved by the FDA to treat alcohol dependence: disulfiram, acamprosate, and naltrexone. Although these drugs are modestly effective in some patients, none are effective in all patients, and long-term efficacy remains limited (Anton et al., 2006; Bouza et al., 2004; Yancey and Lumbad, 2011). Naltrexone is modestly effective at reduction of heavy relapse drinking, but only marginally effective at enhancing abstinence (Garbutt, 2010). Acamprosate is most effective for abstinent alcoholics for whom relief craving is a key factor in relapse, but seems to only be effective in the long-term for 20-30% of patients (Kiefer and Mann, 2010). Thus new treatment strategies for AUDs are needed, and several neurotransmitter systems are under active investigation as therapeutic vectors, including the GABAergic signaling, endogenous opioid signaling, the endocannabinoid system, the corticotropin releasing factor (CRF) system, nicotinic acetylcholine receptors, and others (Leggio et al., 2010; Litten et al., 2012; Rosner et al., 2010).

Studies have shown that various neurotransmitter systems, neuromodulators, and intracellular signaling pathways are involved in alcohol dependence (Vengeliene et al., 2008). The endogenous opioid system appears to be involved in the reinforcing effects of alcohol, through its interactions with mesolimbic dopaminergic reward pathways and glutamate, gamma-aminobutyric acid (GABA), serotonin, endocannabinoid, and glycine neurotransmitter systems (Johnson, 2008). There are three main subtypes of opioid receptors, designated as the μ opioid receptor (MOR), the κ opioid receptor (KOR), and the δ opioid receptor (DOR), respectively. Naltrexone is a nonselective opioid receptor antagonist with greatest affinity for MOR (Ki: 0.1 to 2.0 nm), but also blocks KOR (Ki: 0.5 nm) and DOR (Ki: 8.0 nm). The three main opioid receptors subtypes, as well as the nociceptin/orphanin FQ receptor and the sigma receptor, which are similar in structure to opioid receptors, have been studied in the context of alcohol-related behaviors and therapies for alcoholism (Murphy, 2010; Robson et al., 2012; Soyka and Rosner, 2010).

Animal and human studies suggest a crucial role for the MOR in alcoholism, as MOR agonists and antagonists oppositely modulate ethanol consumption (Hyytiä and Sinclair, 1993; Zhang and Kelley, 2002). Furthermore, MOR knockout mice consume less ethanol than wild-type littermates, and do not develop ethanol place preference (Hall et al., 2001; Roberts et al., 2000b). In humans naltrexone attenuates the stimulating and reinforcing effects of alcohol, and enhances the sedative effects.(Davidson et al., 1999; Drobes et al., 2004; McCaul et al., 2000; Swift et al., 1994). Naltrexone decreases alcohol-related cue activation of the ventral striatum in alcohol dependent subjects, and increases activity in cortical regions during decision making tasks in abstinent alcoholics (Boettiger et al., 2009; Myrick et al., 2008).

However, despite preclinical and clinical evidence for involvement of the endogenous opioid system in alcohol reward and addiction, meta-analyses have shown that naltrexone is only moderately effective at reducing heavy binge drinking, and effectiveness is extremely variable across studies (Garbutt, 2010; Johnson, 2008). Several factors have been shown to be predictive of good patient response to naltrexone: high craving for alcohol, use of other drugs, early age of onset of alcoholism, and a family history of alcoholism (Monterosso et al., 2001; Rubio et al., 2005). The latter two factors in particular indicate that genetic background is a primary influence on the role of the endogenous opioid system in ethanol-related behaviors. Consistent with this notion, poor compliance and treatment success rates have limited the clinical usefulness of oral naltrexone in many patients, with the notable exception of carriers particular single nucleotide polymorphisms (SNPs) in the mu opioid gene (Chamorro et al., 2012; Chen et al., 2012; Oslin et al., 2003; Ray et al., 2012). A different SNP in MOR has recently been shown to mediate some alcohol-related phenotypes in Rhesus macaques (Barr, 2013). Interestingly, psychological variables also play a role in patient response to naltrexone. Significant differences between naltrexone and placebo effectiveness were observed in Type A personalities, but not Type B personalities (Bogenschutz et al., 2009). Differences in hedonic processing indicated by preference for sweet substances have also been shown to be a predictive phenotype for naltrexone effectiveness (Garbutt et al., 2009).

Thus it is clear that signaling by endogenous opioids, in particular through MOR, is involved in alcohol-related behavior and alcoholism, yet naltrexone shows limited effectiveness in a subset of alcoholics. It is therefore important to determine the relative importance of opioid receptor subtypes in alcohol-related behaviors, to determine what patients can benefit from naltrexone, and where to target novel and more selective therapeutic ligands. Study of the role of MOR in

alcohol-related behaviors has been limited because small molecule mu-selective antagonists have been unavailable, and studies using bioactive peptides are relatively difficult and expensive. Novel MOR selective antagonists are thus a high priority target in drug development, both for the treatment of AUDs and understanding of the role of signaling through the MOR in behavior.

NAQ, an isoquinoline substituted 6α-naltrexamine derivative, was recently reported to be a highly selective MOR ligand (Ki ratios $\delta/\mu \approx 241$, $\kappa/\mu \approx 48$) (Li et al., 2009b; Mitra et al., 2011; Yuan et al., 2013; Yuan et al., 2011; Zaidi et al., 2013). NAQ was determined to be a low efficacy competitive antagonist at MOR compared to DAMGO in the 35S-GTP[γS]-binding assay, and in the warm-water tail immersion test NAQ was found to be a potent blocker of morphine antinociception (AD50 = 0.45 mg/kg). Interestingly, NAQ showed much lower efficacy in morphine dependent mice, in which it failed to fully block the receptor even at doses up to 100 mg/kg, as measured by somatic signs of withdrawal. Naltrexone at 1 mg/kg produced significantly more escape jumps and wet dog shakes than vehicle, but NAQ at 10 mg/kg produced no escape jumps and only modest wet dog shakes (Yuan et al., 2011). NAQ has not been studied in the context of alcohol-related behavior, but its molecular selectivity and behavioral profile make it a useful tool for study of the role of MOR in alcohol-related behavior, and a candidate drug for the prevention of relapse in alcoholism.

Taken together this evidence suggested further exploration of the potential for NAQ to alter alcohol-related behaviors, and differences in the efficacy of NAQ compared to naltrexone. Thus, studies were conducted to evaluate the effects of NAQ compared to naltrexone on voluntary ethanol consumption in C57BL/6J mice using a multiple concentration 3-bottle choice model (water, 15% ethanol, and 30% ethanol v/v) under continuous access and every-other-day

intermittent access (IAA) conditions. NAQ was also evaluated for changes in taste preference for bitter (quinine) and sweet (saccharin) solutions.

Materials and Methods

Animals

For experiments 1 and 2, C57BL/6J mice (N = 40) were purchased from Jackson Laboratories (Bar Harbor, ME) at 8 to 9 weeks of age and housed 1 mouse per cage at 23 ± 1 °C on a 12 h light/dark cycle (lights off at 6 pm). Experiments 3 and 4 were performed on a new group of 32 C57BL/6J mice of the same age, from the same supplier, and housed under identical conditions. Food (Harlan Teklad #7912; Madison, WI) and water were available *ad libitum*. Cages and bedding were changed weekly. Mice were allowed to habituate to the animal facility for one week prior to experiments. Procedures adhered to the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

NAQ was synthesized as described by Li and Zhang (2009). For all studies NAQ was first dissolved in DI water to produce a stock solution that was diluted to the proper concentration as needed. Prior to administration sodium chloride was added for a final concentration of 0.9%. For all studies a 0.9% saline solution prepared from DI water and sodium chloride was used as vehicle. All injections were administered to the intra-peritoneal cavity at a volume of 1 mL per 100 g of animal weight. Ethanol solutions (15% and 30% v/v) were prepared by diluting 200-

proof absolute anhydrous ethanol (Pharmco-Aaper , Brookfield, CT) with tap water. Saccharin and quinine hydrochloride (Sigma-Aldrich, St. Louis, MO) were dissolved in tap water.

Three-bottle choice drinking

All mice were allowed access to tap water and ethanol in tap water at concentrations of 15% and 30% (v/v) for 24 hours/day on access days. Fluids were measured daily to the nearest 0.1 mL and replaced at 4pm, 2 hours before the onset of the dark cycle. All injections occurred once per day after drinking measurements were taken and before drinking tubes were replaced. Injections were prepared outside the drinking facility to minimize experimenter/animal interaction and abstinence time.

For experiment 1 (constant access with drug administration) mice were allowed to drink for 14 days without interruption to establish baseline levels of consumption and preference for water and each ethanol concentration. After this baseline period mice were assigned to 3 groups, balanced for total ethanol consumption and preference such that no significant group differences were present (1-way ANOVA). All mice were injected with saline for 2 days to habituate them to the stress of injections, followed by 3 days of vehicle, 1.0 mg/kg NAQ, or 1.0 mg/kg naltrexone, with alcohol and water consumption measured daily. Mice were then allowed access to water only for 14 days prior to experiment 2.

For experiment 2 (intermittent access) the 3-bottle choice model was again used, but in this experiment mice were only allowed access to ethanol for 24 hours on Monday, Wednesday, and Friday of each week for 4 weeks (12 days of access). No drugs were administered during this experiment.

Taste preference

For experiment 3 (NAQ effects on quinine and saccharin taste preference), 32 mice were allowed access to 30 µM quinine and tap water for 4 days. After a day of access to water only, mice were given 350 µM saccharin and tap water for 4 days. For both solutions consumption was measured to the nearest mL and expressed in µg/kg/day. Preference was calculated as mL of adulterated solution divided by total fluid intake. Saccharin and quinine were dissolved in the same tap water used for ethanol solutions.

Statistics

Data were expressed as mean ± SEM. Analysis was performed in Graphpad Prism version 6.01. Analysis of consumption and preference data was performed separately for 15% ethanol, 30% ethanol, and total ethanol (15% and 30% combined). For all experiments two-way analysis of variance (ANOVA) was conducted to independently compare ethanol consumption and preference across treatment groups over time, and repeated-measures ANOVA was conducted within each treatment group to identify significant effects of treatment over time. Dunnett's multiple comparisons tests were performed to identify significant group differences on particular days.

For experiment 1 subject #17 failed to habituate to the stress of injection as measured by recovery to pre-injection intake levels and was excluded from analysis after analysis of baseline consumption using Chauvenet's criterion determined it to be a statistical outlier (Gad and Weil, 1988). For experiment 2 only two-way ANOVA tests were conducted with repeated-measures. Significant effects from either test were subjected to Dunnett's test to compare individual test days to baseline for each treatment group. Tubes with no liquid remaining to measure after 24

hours were not included in calculations, and any mouse with data of this type was excluded from repeated-measures tests due to this missing data.

Results

Experiment 1: NAQ effects on continuous alcohol consumption

In experiment 1 two-way ANOVA did not identify significant effects of treatment on total consumption or total preference ($p = 0.10$, 0.89 respectively). Repeated-measures ANOVA within each group showed that NAQ significantly reduced total ethanol consumption on both test days at $p < .05$ compared to baseline [$F(2,24) = 5.150$, $p = 0.013$; Dunnett's post-test $p < 0.05$]. In contrast, a significant reduction in total ethanol consumption was seen only on test day 1 for naltrexone [$F(2,22) = 3.932$, $p = 0.034$; Dunnett's post-test $p < 0.05$]. NAQ failed to significantly decrease total preference compared to baseline on either test day [$F(2,24) = 1.338$), whereas naltrexone reduced total preference on test day 1 only [$F(2,22) = 5.805$, $p = 0.009$; Dunnett's post-test $p < 0.05$]. Saline treatment had no effect on total consumption or preference (see Figure 1). NAQ and naltrexone failed to significantly alter consumption or preference for 15% ethanol (see Figure 6.1).

For 30% ethanol consumption [$F(2,99) = 3.236$, $p = .043$] and preference [$F(2,99) = 3.676$, $p = .028$], two-way ANOVA identified a significant effect of treatment, but no interaction, and Dunnett's tests did not identify any means significantly different from baseline for any group on any test day . However, within-groups repeated-measures ANOVA revealed that NAQ treatment significantly decreased consumption [$F(2, 22) = 6.518$, $p = .006$] and preference [$F(2, 22) = 6.649$, $p = .005$] for 30% ethanol compared to baseline on both test days. NTX treatment did not

produce significant changes in consumption [$F(2, 22) = 2.620$, $p = .095$] or preference [$F(2,22) = 2.151$, $p = .140$] for 30% ethanol, but a trend towards significance was observed.

Water intake and total fluid intake were examined to determine whether observed decreases in ethanol consumption and preference were explained by non-selective decreases in total fluid intake. Two- way ANOVA across groups, repeated-measures ANOVA within groups, and ANOVA on each day were performed on both measurements, and no significant differences were found (data not shown).

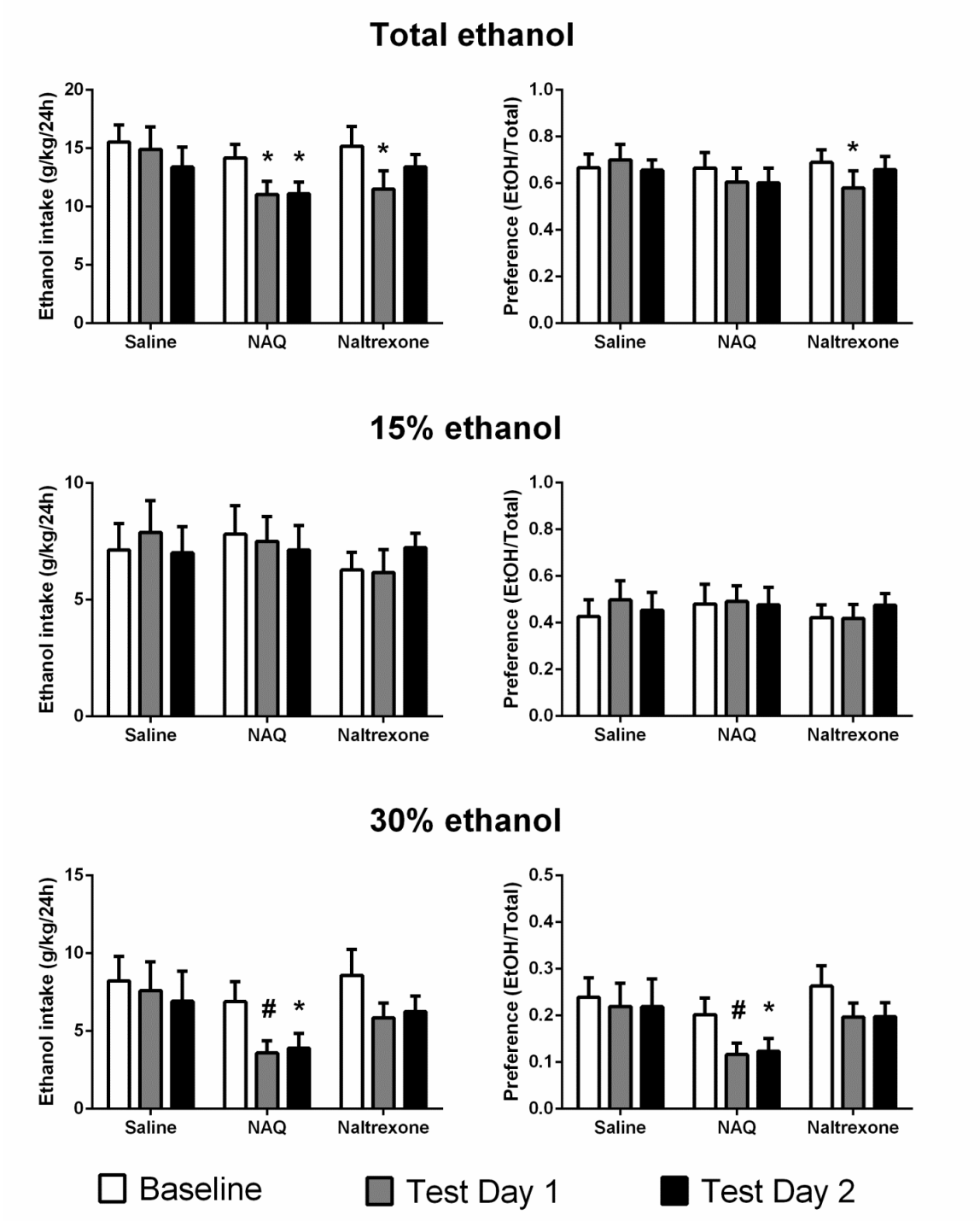


Figure 6.1. Consumption and preference for total ethanol, 15% ethanol, and 30% ethanol from experiment 1, with baseline in white, test day 1 in gray, test day 2 in black. Within-group repeated-measures ANOVA for total ethanol, two-way ANOVA between groups

Experiment 2: Effect of NAQ pre-treatment on drinking produced by IAA

In experiment 2 no drugs were administered and groups were identical to experiment 1. Following 14 days of abstinence mice were allowed access to 15% ethanol and 30% ethanol on Mondays, Wednesdays, and Fridays for 4 weeks. The baseline for all comparisons was day 1 of IAA, on which the groups did not differ significantly in any preference or consumption measurement (One-way ANOVA, data not shown).

For total ethanol consumption no significant effects were identified with two-way ANOVA with repeated measures. However, repeated-measures one-way ANOVA within groups revealed a significant effect of treatment over time for total ethanol consumption in the NAQ group [$F(11,143) = 4.340$, $p < .0001$], the NTX group [$F(11, 132) = 3.332$, $p = .0004$], and the saline group [$F(11, 132) = 4.602$, $p < .0001$]. For consumption data Dunnett's test indicated significant differences from baseline for the saline group at day 10 only (see Figure 6.2). No significant differences in consumption were identified using Dunnett's test for the NAQ and NTX group on any day compared to day 1 of IAA.

For total ethanol preference no significant effects were identified with two-way ANOVA with repeated measures, but one-way ANOVA with repeated measures identified significant changes over time for mice treated with NAQ [$F(11,143) = 6.393$, $p < .0001$], NTX [$F(11, 132) = 5.316$, $p < .0001$], and saline [$F(11,132) = 4.065$, $p < .0001$]. For total ethanol preference Dunnett's test with a significance threshold of $p < .05$ indicated a delayed effect of IAA in the NAQ- and NTX-treated groups compared to the saline-treated group. The saline-treated group showed significantly increased preference on day 2 compared to day 1, and preference was significantly elevated compared to day 1 on all subsequent access days, with the exception of day 5. In

contrast, total ethanol preference of the NAQ group did not differ significantly from day 1 until day 4 (Monday of week 2) and was elevated from days 6 through 12, while in the NTX group this measurement only differed significantly from baseline on days 6 through 12 (see Figure 6.2).

For 15% ethanol alone repeated-measures ANOVA showed significant changes in consumption and preference over time for all groups, although the patterns of intake differed markedly between groups (see Figure 6.3). In NAQ-treated mice consumption [$F(11,143) = 5.950$, $p < .0001$] and preference [$F(11,143) = 5.088$, $p < .0001$] were significantly elevated compared to day 1 by day 4, and remained so from days 6 through 12, with the exception of consumption on day 9. In NTX-treated mice consumption [$F(11, 132) = 4.316$, $p < .0001$] was significantly different from day 1 on days 8, 10 and 12, while preference [$F(11, 132) = 3.452$, $p = .0003$] was significantly different on days 8, 10 and 11. In saline-treated mice gradual increases in consumption [$F(11, 132) = 2.217$, $p = .0170$] led to significant differences on day 12 only, while preference [$F(11, 132) = 2.376$, $p = .0103$] was significantly increased from days 2 through 4 and on days 8, 11, and 12.

For 30% ethanol alone repeated-measures ANOVA showed significant changes in consumption of saline-treated mice [$F(11, 99) = 2.091$, $p = .0277$], but not for mice treated with NAQ [$F(11, 121) = 1.139$, $p = .3372$] or NTX [$F(11, 110) = 1.201$, $p = .2946$] (see Figure 6.4). For 30% ethanol preference saline mice showed a trend toward significant changes over time [$F(11, 99) = 1.698$, $p = .0848$], and no differences were observed for mice treated with NAQ [$F(11, 121) = .8457$, $p = .5951$] or NTX [$F(11, 110) = .6089$, $p = .8179$]. Dunnett's test did not identify significant differences between day 1 and any subsequent access day for any measurement in any group (data not shown).

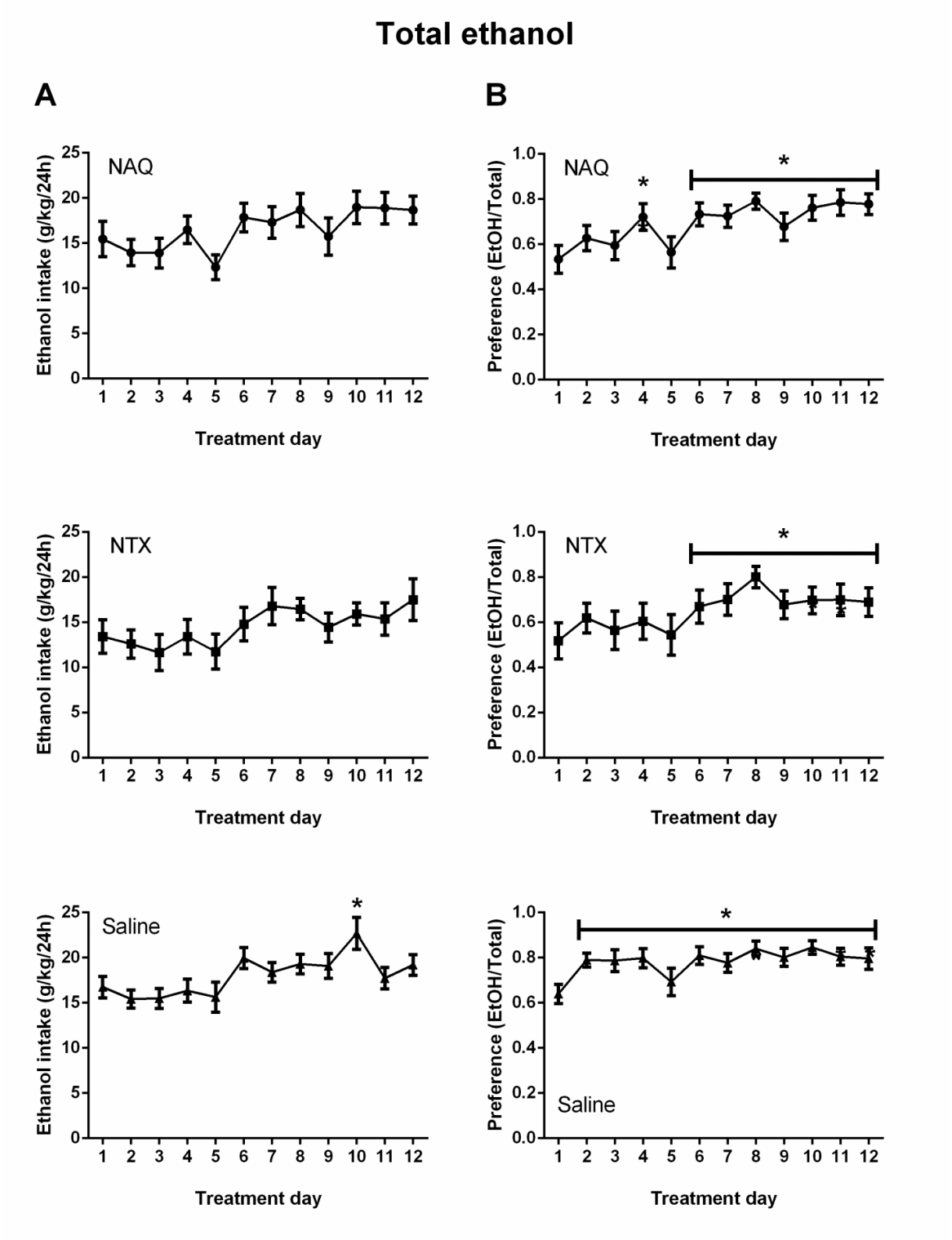


Figure 6.2. Consumption (A) and preference (B) for total ethanol from experiment 2, within-group repeated-measures ANOVA, Dunnett's test compared to IAA day 1: * $p < 0.05$.

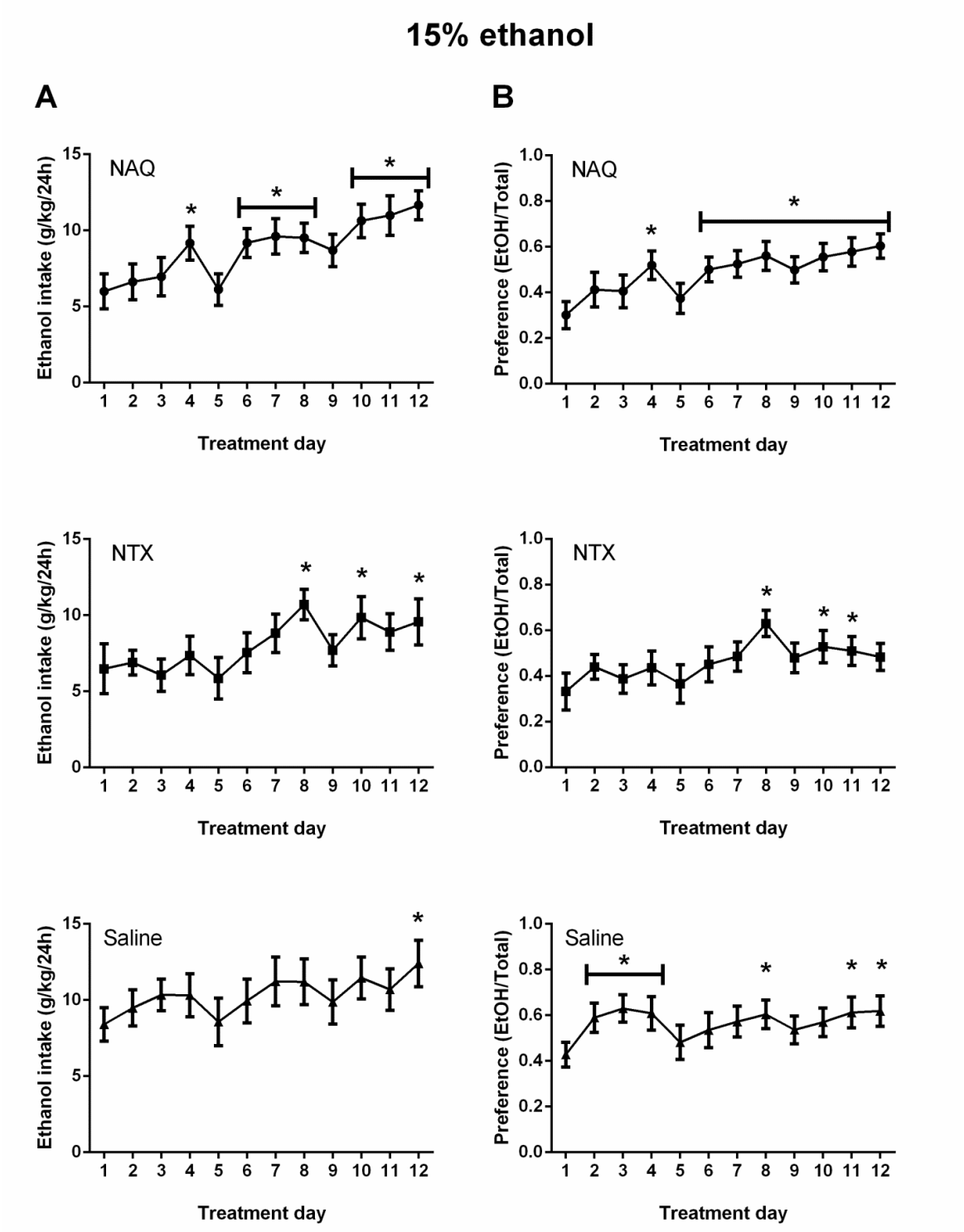


Figure 6.3. Consumption (A) and preference (B) for 15% ethanol from experiment 2, within-group repeated-measures ANOVA, Dunnett's test compared to IAA day 1: * $p < 0.05$.

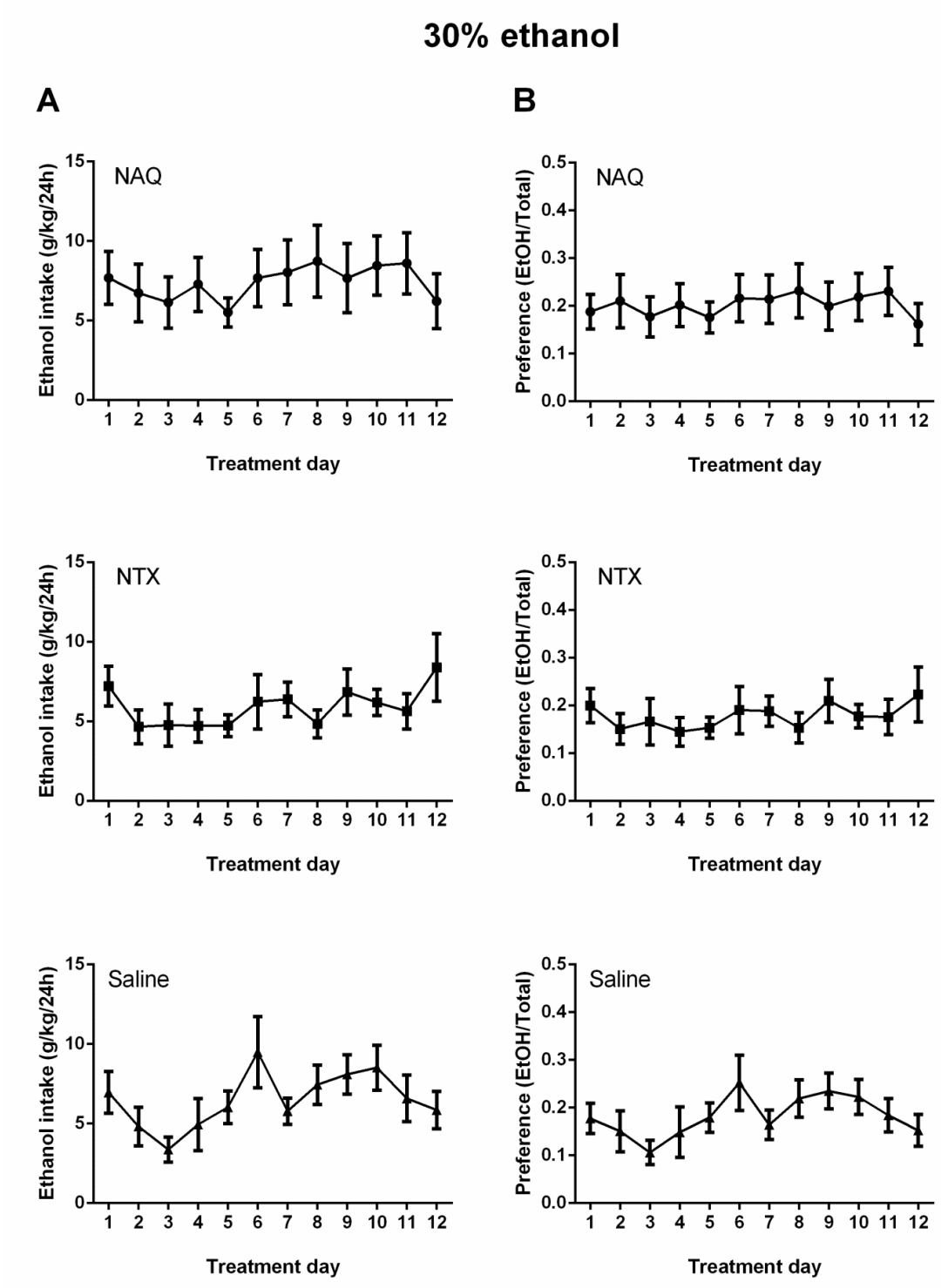


Figure 6.4. Consumption (A) and preference (B) for 30% ethanol from experiment 2, within-group repeated-measures ANOVA, Dunnett's test compared to IAA day 1: * $p < 0.05$.

Experiment 3: Effects of NAQ on consumption and preference of quinine and saccharin

No group differences in quinine consumption [treatment: $F(1, 119) = .2745$, $p = .6013$] or preference [treatment: $F(1, 119) = .9495$, $p = .3318$] were observed with 2-way ANOVA (see Figure 5). For saccharin consumption 2-way ANOVA identified a significant effect of treatment [$F(1, 120) = 12.74$, $p = 0.0005$] and time [$F(3, 120) = 15.70$, $p < 0.0001$], but no interaction [$F(3, 120) = 0.5491$, $p = 0.6497$]. Dunnett's test identified a significant difference on saccharin consumption between saline-treated and NAQ-treated mice at day 1 only ($p = .0364$) (see Figure 6.4). For saccharin preference 2-way ANOVA identified a significant effect of treatment [$F(1, 120) = 9.865$, $p = 0.0021$], but not of time [$F(3, 120) = .7542$, $p = .5220$], and no interaction was observed [$F(3, 120) = 0.2063$, $p = 0.8919$] (see Figure 6.5).

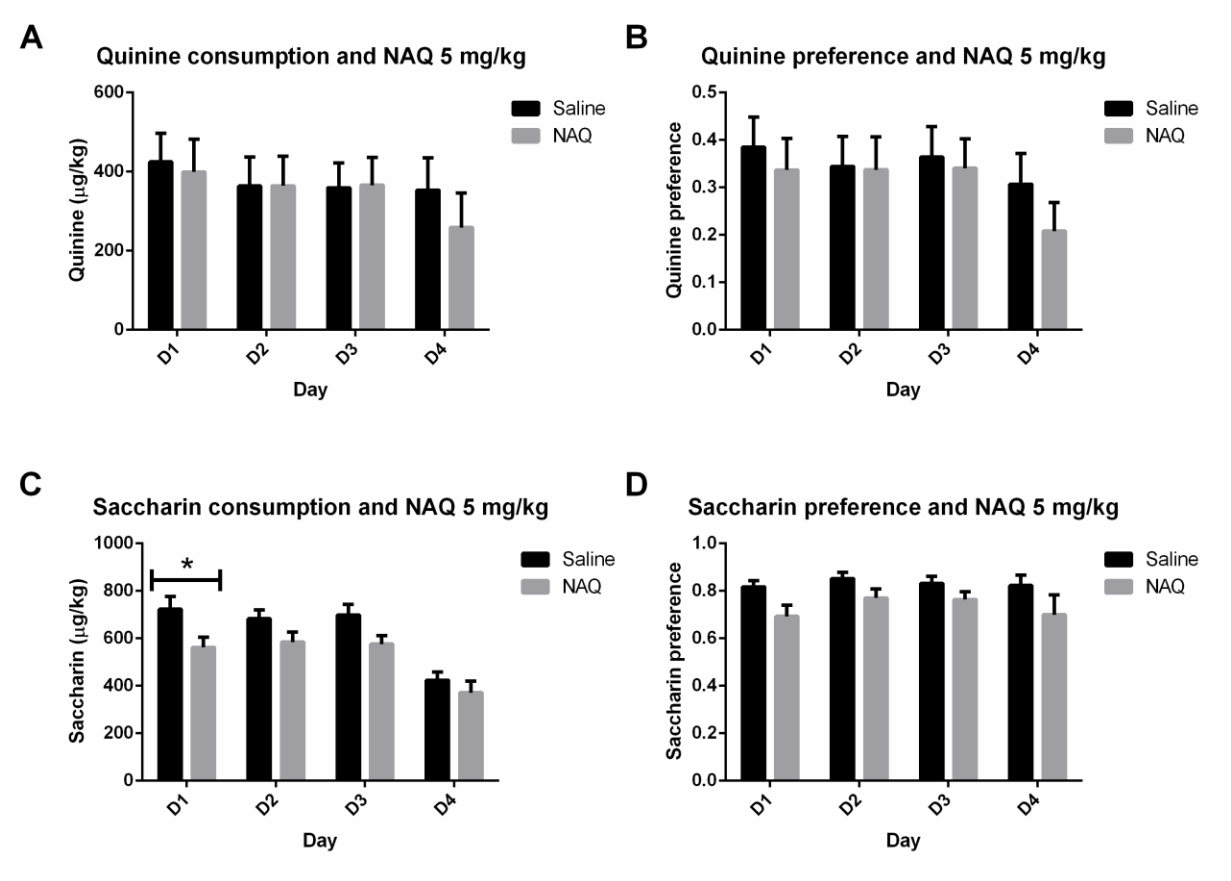


Figure 6.5. Effects of 5 mg/kg NAQ on 30 µM quinine consumption (A) and preference (B), and 350 µM saccharin consumption (C) and preference (D). Dunnett's test for saline vs NAQ: * $p < 0.05$.

Discussion

NAQ and naltrexone decrease continuous access ethanol consumption primarily by decreasing consumption of 30% ethanol, while leaving consumption of 15% ethanol unchanged. The effects of the two drugs on total consumption and preference are similar in magnitude, but not in duration, which indicates that tolerance to NAQ may develop more slowly than tolerance to naltrexone. However, only one dose of each drug was used, and both dose and treatment regimen would need to be optimized for particular behaviors to determine maximal effects and any advantages of one over the other. Thus, further study is needed to characterize tolerance to NAQ: drinking experiments to examine behavioral tolerance for different dosing regimens over time, and molecular experiments to determine the degree to which chronic exposure induces MOR desensitization and downregulation. Slower development of tolerance to NAQ would be a clear advantage over naltrexone in the clinical setting.

The selectivity of NAQ for high concentration alcohol compared to naltrexone is also an intriguing result, though difficult to interpret. While NAQ produces a significant decrease in consumption and preference of 30% ethanol, NTX does not, which indicates that selective MOR antagonism is more selective for reducing intake of high concentration ethanol. In this context high concentration is relative only to the other alcohol concentration offered, but few studies in the literature have offered concentrations greater than 30% (v/v), and this approaches the 40% alcohol content (80 proof) of most hard liquor consumed by humans. While some humans sip alcohol at this concentration in a controlled manner, it is also the quickest way to achieve high blood alcohol concentrations and the psychoactive effects of alcohol. As in humans, it seems probable that the decision to consume high concentration alcohol in mice reflects greater motivation for the psychoactive effects of ethanol than does consumption of low concentrations,

because these effects are reached more quickly, yet require drinking a more bitter and aversive substance. More study is needed to determine the motivational correlates of high concentration vs. medium concentration drinking in animals, but attenuation of this kind of drinking in mice may indicate particular effectiveness in control of heavy drinking in humans.

Furthermore, MOR antagonism is sufficient to selectively decrease 30% intake, while nonselective opioid receptor antagonism by naltrexone produces only a trend in this direction. This suggests the possibility that naltrexone action at other opioid receptor subtypes may be counter-productive to the reduction of consumption, although this effect could be due to an insufficient number of subjects to identify significance, or use of a dose of naltrexone that does not produce optimal attenuation of 30% alcohol intake. Future studies will address these methodological issues, as well as use DOR and KOR selective opioid receptor ligands in conjunction with naltrexone and NAQ to determine the role of each receptor subtype.

In the intermittent access model, scheduled abstinence produced an immediate significant increase in total ethanol preference in saline-treated mice, but in NAQ- and naltrexone-treated mice this effect did not manifest until the second week of intermittent access. Thus nonselective and mu-selective opioid antagonists selectively decreased intake of high concentration alcohol, and effects on intake persisted through 2 weeks of abstinence, after which the induction of binge-like drinking by intermittent access was delayed compared to saline-treated mice. These effects are consistent with the effects of naltrexone in humans, in which the drug is modestly effective at reducing heavy relapse binge drinking (Garbutt, 2010). The examination of this effect of pre-treatment was not the stated goal of the study in which it was discovered, and the 2 week abstinence period was intended to be a "wash-out" period, after which IAA would be used to escalate drinking, and NAQ and naltrexone would be tested for reduction of binge-like drinking.

Naltrexone and NAQ did not acutely alter binge-drinking after establishment, but when segregated by treatment from the previous experiment significant differences were observed between saline- and antagonist-treated groups. The nature of the molecular mediators of this effect of opioid antagonist pre-treatment are not apparent from the studies reported here-in, and future experiments will determine whether MOR receptor regulation or some downstream mechanism is responsible. Dopaminergic signaling in the mesolimbic reward pathways are controlled partially by opioidergic tone, and long-term antagonism at MOR may induce secondary adaptations in dopaminergic signaling or other related processes that lead to altered behavior. The persistence of antagonist treatment in attenuating drinking behavior is surprising, but encouraging as a factor in clinical treatment of alcoholism, where patient compliance can have a negative impact on treatment success.

NAQ does not alter taste preference for bitter solutions, which showed that its negative effects on alcohol intake are not produced through sensitization to bitter tastes. This control is particularly important because NAQ was selective for 30% alcohol over 15% alcohol, which is an effect that would also be produced by sensitization to bitter tastes. Changes in saccharin intake were minimal and transient, but may indicate that NAQ temporarily decreased consumption of sweetened solutions, either through decreased novelty-seeking or reduced reinforcing effects of sweet tastes. Given the persistent nature of attenuation in drinking by NAQ shown in the IAA experiments, this is likely an effect with minimal clinical relevance. However, further study is needed to explore the selectivity of NAQ for ethanol compared to other reinforcers, and to further dissect the effect of the drug on alcohol reward. Further preclinical study toward this end using operant self-administration with progressive ratio breakpoint measurement and conditioned place preference will measure the reinforcing and rewarding properties of the drug, respectively.

Evidence for effects of NAQ on cue- and stress-induced reinstatement will also be crucial support for the advancement of the drug to clinical trials in the future.

Taken together these results indicate that selective MOR antagonism is sufficient to reduce consumption of high concentration alcohol and attenuate relapse drinking behavior, and that NAQ represents a potential drug for use in the prevention of relapse in alcoholics that may have advantages over naltrexone.

The use of concurrent multiple ethanol concentrations in this study is an extension of the work of several laboratories in mice and rats that have shown that this type of access produces greater intake and allows for the expression of individual subject preference in concentrations, which are masked by the presentation of a single concentration of ethanol only (Melendez et al., 2006b; Rodd-Henricks et al., 2001b; Wolffgramm and Heyne, 1995). This model also allows for discernment of drug effects not possible in a binary-choice, single-concentration model, such as the selectivity for the higher-concentration alcohol solution seen in this study. However, interpretation of this type of behavior is limited, and more study is needed to determine the factors that cause a mouse to choose high or low alcohol concentrations. Because naltrexone also showed this property, albeit in a non-significant trend, it represents a factor that may be predictive for success in treating alcoholism, to which acamprosate, baclofen, and other drugs thought to have this potential should be subjected.

The authors are aware of only one other study using concurrent multiple ethanol concentrations in an IAA model with mice, and in that study C57BL/6J mice were allowed access to 7.5%, 15%, and 30% alcohol on only one day per week, after a baseline drinking period and 2 weeks of abstinence (Melendez et al., 2006b). In that study the effects of intermittent access differed

markedly from those reported here, despite similar baseline consumption of 15% and 30% ethanol. By the end of IAA procedures mice showed greatly elevated consumption of 30% ethanol, but not 15% ethanol, while in the present study IAA primarily increased intake of 15% ethanol. This effect is seen across groups, and may be a result of stress due to injection, a shorter baseline access period (2 weeks vs. 6 weeks), the absence of 7.5% ethanol solution, or general environmental differences. Whatever the cause, the results of the present study do not support the notion that binge-like drinking produced by IAA is selective for higher concentrations of alcohol.

The apparent delay in the effects of IAA in mice treated with NAQ and naltrexone compared to saline-treated mice shows that these drugs have long-lasting behavioral effects that may persist for weeks after cessation of treatment. Experiments of this type may represent a useful test for preclinical testing of drugs with potential in treating alcoholism. The analogous situation in human alcoholism treatment is that of a patient taking naltrexone while drinking and successfully transitioning to abstinence, followed by cessation of naltrexone treatment and re-exposure to alcohol. Further study should examine this effect in alcohol-dependent mice during withdrawal, and aim to determine whether it is a result of pairing the drug with alcohol, or if treatment in the absence of alcohol is sufficient to reduce future consumption. These results can inform the manner in which drug treatment and behavioral therapy are managed in abstinent alcoholics and those trying to cease drinking.

The similar, but not identical, effects of NAQ and naltrexone on alcohol intake may be explained in part by the differing pharmacodynamic and pharmacokinetic properties of the drugs. Interestingly, while effects on alcohol drinking are similar between the two drugs, drastic differences in withdrawal-induced behaviors are observed in chronic morphine-exposed mice administered NAQ and naltrexone at the same doses used in this study (Yuan et al., 2011). Even

at a dose of 100 mg/kg NAQ fails to produce half of the number of escape jumps or wet-dog shakes as 1 mg/kg naltrexone. These differences may be explained by dissimilarities between NAQ and naltrexone in metabolism and in action at the three primary opioid receptor subtypes.

In vitro studies show the absorption of the two drugs to be very similar, but hepatic clearance of naltrexone is approximately 50-fold greater than that of NAQ in human liver microsomes, and the unbound fraction of NAQ in human plasma is less than 3%, compared to 72-79% for naltrexone. NAQ lacks the ketone moiety exploited by aldo-keto reductase enzymes for naltrexone metabolism to 6β-naltrexol, which is followed by glucuronidation, and thus NAQ is not found to be detectably glucuronidated, and its primary oxidative metabolite is the N-dealkylated product (Mitra et al., 2011). Interestingly, this sets NAQ apart from naltrexone and other structurally similar compounds. Morphine and buprenorphine undergo N-dealkylation, although their primary route of metabolism is glucuronidation (Kobayashi et al., 1998; Projean et al., 2003). In contrast, naltrexone does not undergo N-dealkylation in vivo. More research into the pharmacological properties of NAQ and its metabolites is needed to identify whether these factors contribute to the differences seen in the behavioral effects produced by NAQ and naltrexone (Yuan et al., 2011).

In addition to MOR, evidence suggests that DOR is also involved in alcohol consumption (Roberts et al., 2001). Recently published work by van Rijn et al. demonstrates that DOR-1 selective agonist TAN-67 reduces ethanol consumption by enhancing the rewarding properties of lower doses of ethanol , and chronic ethanol exposure increases the potency of some DOR selective ligands in the spinal cord of mice (van Rijn et al., 2012a, b). NAQ is a partial DOR agonist with moderate efficacy (% Emax of SNC80 = 53.4 ± 5.4 %) while naltrexone produces little activation at the DOR (% Emax of SNC80 = 3.99 ± 1.32 %), and this difference may

partially underlie the selectivity of NAQ for high-concentration ethanol consumption compared to naltrexone (Yuan et al., 2011).

Differing action at the KOR likely also contributes to the differences in effect seen in this study between NAQ and naltrexone. The KOR antagonist norBNI selectively attenuates ethanol self-administration in ethanol-dependent rats (Nealey et al., 2011). In addition, KOR antagonist JDTic decreases alcohol self-administration and cue-induced reinstatement of alcohol seeking when administered 2 h prior to testing (Schank et al., 2012). In contrast, KOR agonist CI-977 potentiates ethanol intake and preference in long-term ethanol-experienced rats (Hölter et al., 2000). Naltrexone binds to the KOR with 5 times the affinity of NAQ and also produces greater KOR activation (NAQ, % Emax of U50,488H = 13.1 ± 2.0 %, EC50 = 10.9 ± 7.9 nM; naltrexone, % Emax of U50,488H = 17.6 ± 1.0 %, EC50 = 4.0 ± 1.6 nM), which could produce behavioral effects in opposition to those produced by MOR antagonism/partial agonism on ethanol consumption (Li et al., 2009a).

In these studies NAQ shows similar efficacy to naltrexone in the reduction of ethanol intake and in the delay of escalated intake due to intermittent access, and is thus a promising candidate for therapeutic intervention in alcoholism and deserving of further study. Some potential advantages over naltrexone were identified that should be explored: greater selectivity for high-concentration ethanol and slower development of tolerance. Some of these effects are likely due to differences in pharmacodynamics and pharmacokinetics, and may represent advantages over naltrexone in a clinical setting. Studies examining NAQ in morphine withdrawal have shown striking differences compared to naltrexone, but the drug has only begun to be examined in the context of alcohol-related behaviors, and its effects in the IAA model, other models of increased craving such as drinking-in-the-dark, and other related behaviors should be further explored.

Chapter 7. General discussion, limitations, and future directions

The studies herein report observations on the behavioral and genomic aspects of scheduled alcohol abstinence procedures in C57BL/6 mice, which produce binge-like and inflexible ethanol drinking behavior. These results are important because scheduled alcohol deprivation methods, which include the intermittent alcohol access (IAA) and alcohol deprivation effect (ADE) models, are thought to produce behavioral and neuromolecular adaptations that are similar to those occurring with binge drinking and the transition to alcoholism in humans (REF). Thus, insights gained through the preclinical study of the IAA and ADE models, including environmental factors that modulate their effects, and changes in brain gene expression associated with changes in behavior they produce, inform the study of motivated behavior for abused substances and the search for better treatments for alcoholism.

Accordingly, within are provided genes, cell signaling pathways, and gene networks acting in midbrain, striatum, and prefrontal cortex, that merit further preclinical study to determine relevance to the development of binge-like drinking behavior due to scheduled abstinence. Furthermore, methodological recommendations are made to maximize the utility of such preclinical models for representing maladaptive drinking behavior characteristic of alcohol use disorders. Finally, specific research goals and plans are provided to confirm the results reported and further understanding of binge-like drinking and the development of alcoholism.

Alcohol deprivation effect studies

Genomic analysis of transcriptional regulation induced by the ethanol deprivation effect reveals that differences in gene expression produced by abstinence are most severe in the nucleus accumbens (NAc), compared to the prefrontal cortex (PFC) and ventral midbrain (VMB). Interestingly, the directionality of changes induced by the four-day deprivation period also varies among regions: more mRNA transcripts are downregulated than upregulated in NAc and PFC, but in VMB all regulated transcripts are reduced in abundance. Thus the NAc seems to be a primary site of neuroplasticity occurring with alcohol deprivation.

There is little overlap in regulated transcripts between any two of the three regions studied, and no genes are altered by alcohol deprivation in all three. Despite the lack of overlapping genes, some biological themes were common to more than one region, and represent areas for further study: PI3K signaling, hormone signaling, regulation of cell fate, regulation of neuronal connectivity, and mRNA transcription and processing. In the NAc in particular, several signaling pathways and biological processes with known relevance to alcoholism and alcohol-related behavior are over-represented in regulated genes: myelination, CRF/CRH signaling, regulation of protein ubiquitination and degradation, extensive regulation of ion channels for sodium, calcium, and potassium. In addition, regulation of glutamate and GABA receptor populations and ligand transport are implicated by genes regulated during alcohol deprivation in nucleus accumbens.

During alcohol deprivation some gene regulation mechanisms are common across regions, but the nucleus accumbens gene set contains the majority of over-represented transcription factor binding sites in gene promoter regions. In all regions studied epigenetic processes involving histone acetylation and chromatin remodeling are clearly implicated in gene regulation during deprivation. In the NAc the functioning of *Smarca4*, also known as *Brg1*, is of particular interest,

because it is a calcium-responsive helicase that acts in several chromatin modifying complexes, regulates Fos gene transcription, and is increased nearly 2-fold by alcohol deprivation (Murphy et al., 1999; Qiu and Ghosh, 2008; Trotter, 2007). Interference with BRG1 function or associated complexes represents a direct mechanism for interference in ethanol-responsive transcription, which could interfere with neuroplasticity and changes in behavior. Future studies should attempt to address the involvement of BRG1 in transcriptional regulation in NAc during alcohol drinking. In particular, conditional BRG1 knockout mice would allow for the inactivation of the gene at particular time-points during drinking behavior, to determine the connection between gene regulation and alcohol intake.

It is not surprising that CREB binding sites are over-represented in genes regulated by alcohol deprivation, considering the well-established role of CREB-mediated gene expression in the regulation of addictive behavior, but this does indicate some similarity in underlying regulation mechanisms for gene regulation induced by alcohol deprivation and craving for other drugs of abuse. Interestingly, regulation of transcripts through miRNA is also implicated in the deprivation-regulated genes, which lends to support to the notion that alcohol deprivation is a valid model for the development of alcoholism, because miRNA alterations are a signature characteristic of the alcoholic brain (Bahi and Dreyer, 2013; Lewohl et al., 2011; Nunez and Mayfield, 2012).

Ion channel regulation seems to play a role in neuroplasticity occurring with scheduled alcohol deprivation. Transcripts coding for six voltage-gated calcium channel subunits (*Cacna1a, Cacna1d, Cacna1g, Cacna2d1*, *Cacnb2,* and *Cacnb4*) are upregulated by ethanol deprivation, and thus calcium channel blockers should be explored for efficacy in reducing maladaptive drinking produced by alcohol deprivation. Differential expression results from IAA mice also

contain an unusually large number of calcium channels, as indicated by significant over-representation of calcium ion-related gene ontology terms. Ethanol inhibits N- and P/Q-type calcium channels, and there is some evidence for efficacy of T-type and N-type calcium channel blockers to alter other alcohol-related behaviors, but not in the context of scheduled abstinence (McMahon et al., 2000; Newton et al., 2008; Solem et al., 1997). This finding is particularly intriguing in light of the recent landmark study showing that the anti-craving effects of acamprosate are mediated by increased calcium, and that in patients taking the drug positive outcomes such as time to relapse are correlated with plasma calcium levels (Spanagel et al., 2013).

Taken together with the efficacy of acamprosate in the ADE and IAA models, and the regulation of calcium channels and related genes in the ADE and IAA results, calcium deficits are implicated as a primary mediator of ethanol intake in scheduled deprivation models. Further studies should examine whether dietary calcium supplementation is sufficient to reduce binge-like drinking in the intermittent access model, i.e. are calcium carbonate antacids a treatment for alcoholism? It is also not yet clear by what mechanisms plasma calcium regulates drinking behavior, and whether this effect is CNS-mediated. These questions will be answered by measuring calcium levels in plasma and in brain regions associated with ethanol drinking behavior at several time points before, during, and after drinking on different access schedules. Calcium levels should be correlated with ethanol drinking and dopamine release, and changes in gene expression and protein abundance for voltage-gated calcium channels and other calcium-related genes measured.

Intermittent alcohol access

While the alcohol deprivation effect is a well-characterized model, it does not produce binge-like drinking in which blood ethanol concentrations reach levels greater than 80 mg/dl, and furthermore proved to be an extremely unreliable model in the Miles laboratory. With the publication of two seminal papers in 2006 and 2008, it became clear that cycles of abstinence on the proper schedule, in the form of repeated deprivations or intermittent access, was a better model for increasing alcohol intake, and a more valid model of maladaptive drinking in humans (Melendez et al., 2006a; Simms et al., 2008). Thus all other studies reported herein make use of the intermittent alcohol access (IAA) model, either in the form of once-per-week or every-other-day 24-hour access periods.

The transition from the Alcohol Deprivation Effect (ADE) model to the Intermittent Alcohol Access (IAA) model merits some explanation. The project assigned to me upon my arrival in the Miles Laboratory in 2007 was concerned with regional transcriptional regulation induced by the ADE. The laboratory had recently published on characterization of ADE behavior in C57BL/6J and C57BL/6NCrl mouse strains, and had determined that the C57BL/6NCrl strain was more amenable to the production of the ADE, despite drinking less alcohol than the closely-related C57BL/6J strain (Khisti, 2006). The first task I performed was to attempt to repeat the ADE procedure, with the objective of obtaining tissue samples for measurement of mRNA abundance using microarray and PCR methods. Unfortunately, I was unable to reproduce the effect using C57BL/6NCrl mice. Meanwhile, Melendez showed a result (2006) in which repeated one-day-per-week deprivation periods with multiple alcohol concentrations were used to induce an ADE in C57BL/6J mice. Our technician, Nathan Bruce, achieved significantly elevated intake using the Melendez procedure in a pilot study with C57BL/6NCrl mice (data not shown), and I adopted

this method for all further scheduled deprivation studies: first with C57BL/6NCrl mice, and later with C57BL/6J mice.

Goals for these studies are to determine the utility of the commonly used C57BL/6J and C57BL/6NCrl inbred strains in the IAA model, to determine the effect of providing a choice in alcohol concentrations on alcohol intake, to identify changes in the nucleus accumbens transcriptome associated with the development of binge-like drinking behavior, and to interfere with that development via targeted pharmacological manipulation. For all but the final pharmacological studies the one-per-week model was used, because at the time they were performed the utility of this model had been demonstrated in mice, but the every-other-day model had not (Melendez, 2011). With the confirmation of the usefulness of the every-other-day IAA model, which produces binge-like drinking and had been well-characterized in rats, this procedure was adopted, and used for studies with the mu-opioid selective antagonist NAQ. Furthermore, the every-other-day IAA model produces handling-induced seizures during withdrawal, which has not been demonstrated for the more sporadic version (Hwa et al., 2011). By comparison, the once-per-week procedure has been far less studied (Melendez et al., 2006a; Rosenwasser et al., 2013).

Initial IAA experiments establish for the first time the effectiveness of the IAA procedure in C57BL/6NCrl mice from Charles River Laboratories, which drink less ethanol than the closely related C57BL/6J strain, but do not show differences in initial alcohol sensitivity, acute functional tolerance to alcohol, or ethanol-induced loss of righting reflex (Mulligan et al., 2008a; Mulligan et al., 2005). These strains show differences in the effectiveness of a 4-day alcohol deprivation period to induce escalation in intake; the C57BL/6NCrl strain shows significant increases in consumption and preference upon reinstatement of access, while the C57BL/6J mice

do not (Khisti et al., 2006b). However, these results show that intermittent access on a once-per-week or every-other-day schedule increases alcohol intake in the C57BL/6J strain, and thus the lack of an alcohol deprivation effect was not a result of a "ceiling effect", as posited in the mentioned study. The result reported herein confirms the utility of scheduled abstinence for inducing increased consumption in C57BL/6NCrl mice, and further use of these mice in this context would be useful as a contrast to the well-characterized C57BL/6J strain.

There are several potential explanations for the differences in effectiveness of each scheduled deprivation model across the C57BL/6 sub-strains, and further experimentation in this area could be useful because the relative similarity of the strains makes it easier to highlight those differences which may be especially important for determining relapse behavior. The length of the deprivation period could be a crucial factor in determining efficacy of IAA in C57BL/6J mice; abstinence of one to two days, or six days, increases consumption and preference in these mice, but abstinence of four days does not. The C57BL/6NCrl strain shows less ethanol-induced dopamine release in ventral striatum than the C57BL/6J strain, which suggests differing effects of ethanol on the function of dopaminergic neurons in the ventral tegmental area, where it stimulates dopamine release in the nucleus accumbens (Ramachandra et al., 2007b). The use of *in vivo* microdialysis in both strains to measure dopamine levels in the NAc at different time-points during drinking would be informative, particularly in light of the conflicting relationship between ethanol preference and dopamine release in the literature (Spanagel, 2009).

There is evidence that these strains differ in expression of dozens of genes across brain regions, some of which are associated with alcohol preference and alcohol phenotypes (Mulligan et al., 2008a). Thus measurement of mRNA and protein abundance of primary ethanol targets and ethanol-responsive genes in the VTA and NAc, particularly at the 4-day abstinence time-point

when relapse behavior differs, could identify targets with particular behavioral relevance for further study. Because expression differences between the strains are minimal, there is a greater chance of finding a significant association between expression and behavior than with more distantly-related strains (Mulligan et al., 2008a).

There is also some evidence that C57BL/6NCrl mice are more fearful than C57BL/6J mice, and more susceptible to environmental stressors, and that the effects of alcohol deprivation may be dependent on social isolation stress (Tomie et al., 2013). These strains thus offer an opportunity to gain insight into the role of anxiety and stress in escalating drinking behavior (Bryant et al., 2008; Radulovic et al., 1998). Different stressors, such as foot-shock, yohimbine, or social stress will likely produce differing effects on drinking in these strains, and the effects of treatments such as naltrexone to reduce intake could inform the management of alcoholism in humans, in which the disease is often comorbid with PTSD or other anxiety disorders (Brady and Back, 2012; McCarthy and Petrakis, 2010). It would also be informative to study the development of binge-like drinking in the C57BL/6J mouse under group-housed conditions, to determine if the effects of IAA in these mice are similarly dependent on social stress. If the effects of scheduled alcohol deprivation are dependent on social stress, the scope of validity of the model could be narrowed, in that results might only be relevant to the development of alcoholism when there is a component of social stress involved.

Several other experimental parameters, such as the alcohol concentration used, could be responsible for the differences in the two strains across experiments. For example, it may be that a 4-day deprivation period is sufficient to escalate drinking in C57BL/6J mice if a choice in ethanol concentrations is offered, so more direct comparison is needed to establish that differences in the effects of abstinence are not a result of intended or unintended environmental

differences. Our laboratory has observed that voluntary ethanol drinking behavior is extremely sensitive to environmental change; and unintended environmental differences that alter drinking behavior can be as subtle as changes in bedding from wood-chip to corn cob based products. It is therefore crucial to measure drinking behavior in both strains at the same time, and in the same facility.

After establishing that IAA increases ethanol consumption and preference in C57BL/6NCrl mice, studies focused on the C57BL/6J mouse as an experimental model, because it drinks more alcohol, and this is also a characteristic of humans with a predisposition for development of alcohol use disorders, compared to the general population (Schuckit et al., 2006; Schuckit et al., 2013). Consistent with published literature, the presentation of multiple ethanol concentrations led to greatly increased consumption and preference (Rodd-Henricks et al., 2001a; Vengeliene et al., 2005; Wolffgramm, 1990). Interestingly, IAA did not cause a shift to high concentration alcohol, as seen in other studies, and this may be due to subtle differences in baseline drinking and abstinence schedule. Future studies should address the effect of a longer baseline access period, potentially on the order of months, on the subsequent escalation of drinking behavior. Study of the neuromolecular correlates of these differences in behavior will illuminate the mechanisms by which different aspects of maladaptive drinking are mediated.

The statistical methodology used to analyze repeated deprivation drinking data merits some discussion with regard to rationale. The most proper and stringent method for establishing significant differences between experimental groups (to reject the null hypothesis that the groups do not differ) is the 2-way ANOVA with repeated measures, which takes into account the continuous access (CA) and intermittent access (IA) data, as well as the persistence of subject identity over time. As with any ANOVA, post-hoc tests are used to establish significant

differences between any two group means, for example between CA and IA groups on reinstatment day 7. However, with a 2-way ANOVA of this type there is a concern that the test will fail to reject a null hypothesis which is in fact false, i.e. type II error or false negative. This concern arises from the relatively high degree of individual variation in drinking data within a group of mice of a size that is feasible for experimentation (typically 6 to 20 mice). The variance in such data, which is remarkable even within a single subject from day to day, leads to high standard deviation relative to the group mean, and it is thus difficult to establish significant differences in means between two groups of mice.

A suboptimal, yet valid, solution to this difficulty is to use one-way ANOVA with repeated measures within the IA and CA groups, followed by post-hoc tests, to examine differences between baseline drinking and subsequent access days. For the IA mice the intermittent access procedure is expected to produce significantly elevated consumption and preference, as inidicated by positive post-hoc results, and for the CA mice it is expected that no differences from baseline will be observed. These methods establish the efficacy of intermittent alcohol access to elevate alcohol intake, the lack of change over time that is typically observed with continuous access. To establish groupwise differences between IA and CA groups using 2-way ANOVA it is expected that groups sizes larger than those used in the present studies (N = 12) will be necessary, although differences from group to group and experiment to experiment preclude effective power analysis to determine ideal group size. Preliminary power analyses imply that groups of 20-25 mice may be required, which are at the limits of feasability given current techniques (data not shown). To remedy this problem, new methods for collecting drinking data may be needed, such as automated data collection using tube weight and

lickometers, which also provide advantages in temporal resolution and minimized experimenter-subject interaction.

Gene expression in the nucleus accumbens associated with intermittent access over a period of 7 weeks differs markedly from that observed after a single 4-day deprivation, and the magnitude of differences makes it unlikely that basal transcriptional differences between the C57BL/6J mice used for IAA and the C57BL/6NCrl mice used for EDE are responsible. In C57BL/NCrl EDE mice more than 500 transcripts are different between deprived mice and water mice after 4 days of abstinence, whereas no differences were observed with the same comparison in C57BL/6J IAA mice after 6 days of abstinence. This discrepancy could be a result of the length of abstinence or of different transcriptional responses to ethanol between the strains, which drink markedly different amounts of alcohol. Future studies should address whether time of deprivation or ethanol-responsive gene expression differences are responsible.

The nucleus accumbens was chosen as the first region to examine in the IAA genomics experiments because the EDE results showed far more transcript regulation in this region than in prefrontal cortex or ventral midbrain. Furthermore, this region is known to be of central importance in drug-related behavior, and multiple neurotransmitter systems are involved at different stages of drug use. Dopamine release from the VTA signals drug availability and triggers initial consumption behavior, while dysregulation of glutamatergic signaling during abstinence leads to elevated signaling to the region from the PFC, and this is thought to contribute to relapse behavior (Kalivas and Volkow, 2011; Quintero, 2013). Changes in the NAc regional transcriptome are likely to signal alterations in function that underlie alcohol use during relapse, and detailed knowledge of these changes will inform the study of the molecular mechanisms of addictive behavior, and the search for intervention strategies. For these reasons

the nucleus accumbens was chosen as the first region to examine using microarrays, but data from other regions, such as PFC, VTA, and amygdala are also of interest. As methods for dissection improve it will also become more feasible to examine subregions in these areas, for example to distinguish the nucleus accumbens core from shell, which will allow for even finer understanding down to the level of small neuronal populations.

Further insight into the nature of neuromolecular changes important for escalation of drinking behavior during IAA is offered by the studies with NAQ and naltrexone, in which the drug attenuates escalation two weeks after the cessation of treatment. This effect may potentially be explained by long-lasting changes in endogenous opioid neurotransmission engendered by repeated administration of mu-opioid selective and non-selective antagonists (Madia et al., 2012; Virk and Williams, 2008). The transient efficacy of the drugs to reduce drinking behavior hints that daily administration may not be the ideal treatment regimen for these drugs, at least at the doses tested. By the third day of naltrexone treatment the drug no longer reduced drinking behavior, and in other studies a similar effect was seen with NAQ after approximately 5 days (data not shown). It seems likely that repeated administration of the drug has altered abundance and expression of opioid receptors or downstream cell signaling components; mu-opioid receptor in the case of NAQ, and potentially mu-, delta-, and kappa-opioid receptors for naltrexone.

The choice to test a selective mu-opioid antagonist (NAQ) in the context of IAA merits some brief discussion. In fact the choice was not made from the genomic results from the IAA or ADE models, but instead was a natural avenue of research stemming from the availablity of a novel compound that shared a mechanism of action with naltrexone, which is approved by the FDA for

treatment of relapse in alcohol use disorders. This compound was produced by Dr. Yan Zhang in the Department of Medicinal Chemistry in the VCU School of Pharmacy, and some preliminary behavioral work had been done with regard to somatic signs of morphine withdrawal, but the effects of the compound on alcohol drinking were unknown. This compound was of particular interest because non-peptidyl mu-opioid receptor selective antagonists had not been previously synthesized, and being restricted to peptides greatly impairs the feasability of pharmacological experimentation, due to the impermeability of the blood brain barrier. Given that naltrexone is the only compound approved by the FDA for alcoholism with a clearly understood mechanism of action (relatively nonselective opioid receptor antagonism), it followed that a selective small molecule mu-opioid receptor antagonist such as NAQ represented a high priority target for preclinical experimentation in the context of alcohol drinking and relapse-like behavior. Thus, the studies with NAQ were initiated, and eventually lead to the results reported herein.

A role for mu-opioid receptor regulation in the intermittent access model is supported by the observation the *Oprm1* transcript is upregulated by continuous access drinking, and downregulated during intermittent access. Protein measurements will determine if these changes are translated into alterations in receptor abundance, and thus illuminate the precise implications of changes in mu opioid receptor in NAc on drinking behavior. Interestingly, the severity of alcohol craving in alcohol-dependent human beings is correlated to the degree to which abstinence increases mu-opioid receptor abundance in the NAc, and thus persistent downregulation due to antagonist treatment may be responsible for the attenuation in escalation due to IAA in treated mice (Heinz et al., 2005).

Beyond craving, altered mu-opioid receptor populations in NAc could also alter consumption after initiation, because ethanol and other drugs of abuse elevate extracellular levels of

endorphins in the NAc, the function of which would be impaired by a reduced receptor population (Olive et al., 2001). Furthermore, mu-opioid receptor knockout mice do not self-administer alcohol, which suggests that it may be more important than delta- or kappa-opioid receptors for determining intake, and lends support to the notion that NAQ may be a better drug than the non-selective antagonist naltrexone for reducing relapse (Mendez and Morales-Mulia, 2008). Given the clear potential of NAQ as a therapy for alcoholism, extensive future study should be devoted to further exploring its potential in preclinical models.

After effective dosing and treatment regimens are established in voluntary drinking models, the effects of the drug on operant self-administration should be examined; in particular it should be determined whether NAQ treatment of pretreatment alters progressive ratio breakpoints and reinstatement of responding due to cues or stressors. A hybrid IAA self-administration model has been demonstrated that would be particularly useful, in addition to non-deprived operant self-administration studies (Hopf et al., 2010). Behavioral studies of this type should be conducted in concert with neuromolecular studies to determine the efficacy of NAQ compared to naltrexone. For example, it should be determined whether NAQ directly reduces extracellular dopamine in the nucleus accumbens, and whether it produces desensitization or downregulation of opioid receptor populations.

Limitations

Several factors limit the scope and generalizability of the results reported herein to the human disease of human alcoholism, but offer opportunities for future study. First, results are reported in only two strains of mice, and although this progenitor separation occurred more than 60 years ago, the strains are still relatively similar in alcohol-related behavior and gene expression. While this offers the opportunity to home in on important neuromolecular and transcriptional mediators

of behavior in the absence of noise, the degree to which results can be generalized to other rodent strains, and to humans, is unclear. Some effects of alcohol, such as dopamine release in the NAc, are fairly stable across genetic backgrounds, while others, such as ethanol-responsive gene expression, are not. While the IAA model has been studied in several rodent strains, in most only the sub-optimal single concentration model has been applied. Of particular interest are the ways in which multiple alcohol concentrations effect baseline drinking, and how IAA alters preference among these concentrations. Even within a single inbred strain intermittent access results vary across laboratories, so the efficacy of the model in different rodent strains with divergent alcohol-related behavior must be confirmed (Crabbe et al., 2012; Hwa et al., 2011).

Second, in all reported studies mice are housed alone, and there is some evidence that in C57BL/6NCrl mice escalation due to IAA is dependent on stress produced by social isolation (Khisti et al., 2006b; Tomie et al., 2013). If this holds true for other strains it would limit the generalizability of results to human alcoholics; and may limit the scope to those who drink alone, or in which alcoholism is comorbid with anxiety disorders, such as PTSD. While this would reduce the validity of the model as a general representation of alcoholism, it would still be useful for study of drinking due to, or despite, anxiety and stress. A simple method for reducing stress due to social isolation is the use of clear plastic dividers to separate the home-cage into areas in which the drinking of a single mouse can be measured, but still allow for some minimal level of interaction. More sophisticated methods are possible, but expense limits their utility. For example, group-housed mice can be tagged with radio transmitters that identify each mouse as it approaches the drinking apparatus.

Third, drinking under intermittent access schedules is generally measured over the entire 24 hour access period, which may not provide the most useful description of drinking behavior. Under

normal conditions rodents, which are nocturnal, tend to drink during the dark cycle, and in particular immediately after lights off for a short period, which is followed by food consumption, and then a longer period of drinking (Boyle et al., 1997; Gill et al., 1986). Intake decreases as the night progresses, and in the second half of the dark cycle little alcohol is consumed. In rats in which maladaptive drinking has been produced through alcohol deprivation, temporal patterns of drinking are markedly altered; most rats show high levels of consumption during the light cycle, with little difference from the dark cycle (Holter et al., 1998). These differences likely reflect alterations in circadian rhythm that are at least partially mediated by changes in clock genes, which are posited to play a role in addiction processes and depression (Perreau-Lenz et al., 2007).

Therefore measurements of drinking behavior and gene expression at a finer temporal resolution would allow for finer dissection of the connection between transcription and behavior, and for the study of expression of immediate early genes, which mediate later gene expression. The use of lickometers that directly measure when a rodent is drinking is a fairly inexpensive solution to the measurement of drinking behavior on a sub-day time scale, and all future intermittent access studies should make use of this modification, if possible (Griffin et al., 2007). The measurement of global gene expression on this scale is more difficult, primarily because of the high cost of microarrays, but quantitative real-time PCR can be used to measure transcript abundance of specific genes based on genomic results from 24-hour drinker samples.

Fourth, gene expression results are limited by the use of mice as an experimental model, and the dissection methods utilized. In mice the consistent and accurate dissection of the nucleus accumbens from whole brain is difficult, and the separation of the core and shell subregions is not feasible. Thus, reported gene expression results collapse expression in the nucleus

accumbens core and shell, but expression changes associated with intermittent access can be subregion specific within the NAc, prefrontal cortex, and amygdala (Gilpin et al., 2012; Li et al., 2010b; Li et al., 2012b). Improved dissection techniques, including laser capture microdissection, will allow for more detailed study of changes in gene expression associated with continuous access and intermittent access drinking using genomic methods (Chimge et al., 2007).

As with all gene expression studies in animal models, the generalizability of results to humans is limited to a degree because of differences in the genome. While 99% of mouse genes have human homologues, non-coding putative regulatory regions show a far lower level of conservation (Mouse Genome Sequencing et al., 2002). Although expensive, the use of non-human primates for studies such as these would allow for better comparisons with the human condition. In particular, results can be compared to the growing body of data describing neuromolecular adaptations in post-mortem brain tissue of alcohol dependent humans, and these results would support the use of the model in rodents.

Fifth, the results for intermittent alcohol access behavior and transcription reported here were obtained using the once-per-week access model. While this model produces immediate significant increases in alcohol consumption and preference, it is not the form of the model which has begun to emerge as the consensus procedure in the literature. The every-other-day model has seen substantially more research effort in recent years, and the degree to which results are generalizable between models is unclear. No studies have directly compared the every-other-day, no baseline, single concentration model to the once-per-week, extended baseline, multiple concentration model, although the behavioral changes produced by the two schedules seem to be similar across laboratories. The degree to which continuous access prior to deprivation

contributes to subsequent escalation of intake is unclear, and it may be that each model is useful for describing a particular aspect of alcohol-related behavior. It should be noted that in our laboratory the initial presentation of alcohol is accompanied by what is believed to be a "novelty effect", in which mice drink markedly more alcohol on the first access day than on subsequent days, and stable intake is often not reached for several days, or even weeks. It is unclear whether this initial, transient increase in drinking has a correlate in the no-baseline model, because subjects are never allowed continuous access under which drinking can stabilize.

It may be that every-other-day drinking maintains and exacerbates this otherwise transient increase in drinking, and that once-per-week access mediates its effects through the same mechanisms. Because the state of the brain differs greatly upon the start of intermittent access (naïve mouse vs. alcohol-drinking mouse), it is an intriguing possibility that the neuromolecular substrates involved in the escalation of drinking behavior differ between the two models. Future studies should address the utility of the every-other-day schedule after a continuous access baseline period, and of the once-per-week schedule in alcohol-naïve animals, and compare the transcriptional response in these mice to naïve mice on the first alcohol access day.

An issue not addressed in the literature is that with scheduled deprivation procedures, such as the IAA and EDE models, abstinence is involuntary, and if the transcriptional response to alcohol differs between voluntary and involuntary drug taking, then it is likely that voluntary and involuntary abstinence also produce differing effects on neurobiology and subsequent drinking behavior (Fernandez-Castillo et al., 2012; Tapocik et al., 2013). In humans abstinence is often, but not always, voluntary, as negative social, economic, and physiological consequences of use mount, and people attempt to quit drinking (A.P.A., 2000; Haeny et al., 2013). It may be that wanting to stop drinking modulates the effects of abstinence on the brain in ways distinct from

involuntary situations, and that IAA is only a valid model for involuntary abstinence, such as occurs upon hospitalization, imprisonment, lack of funds to buy alcohol, or other uncontrollable environmental influence. Unfortunately, rodents do not have social and economic concerns that modulate abuse behavior, and their limited cognition limits possibilities for inducing abstinence.

It is unclear how to convince a rodent that alcohol consumption is not in its best interests, without the use of directly aversive stimuli such as foot-shock, or noxious tastes and smells, which do not have a clear correlate in the human condition. One imperfect, yet seemingly valid, method is the introduction of an alternative reinforcer concurrently with ethanol on reinstatement test days, with the correlate to the human condition being positive life activities that are neglected due to the desire to seek and consume alcohol instead. This behavior is a known characteristic of abuse across psychoactive substances, but has not been explored in the context of IAA. It is known that IAA produces an ethanol-selective increase in drinking that is inflexible in the face of aversive influences on intake, but not whether it increases the relative reinforcing value of alcohol compared to other rewarding stimuli. Potential alternative reinforcers include sweetened solutions and foods, social interaction, wheel running, and sex. This reinforcer can be offered concurrently with alcohol reinstatement following deprivation, using a design tailored to the reinforcer. For something like sex, the option to drink alcohol would be removed upon the animal choosing the alternate reinforcer, and the decision can be monitored over the course of weekly cycles, which may eventually result in alcohol being chosen. For a rewarding activity like social interaction or wheel running, it would be informative to measure the amount of time that the animal devotes to either activity during ethanol access, which would be expected to decrease with repeated cycles of deprivation.

A further limitation of the drinking studies reported herein is the lack of measurement of blood ethanol content (BEC), which is a direct measurement of alcohol intoxication, and be more meaningful in the context of drinking behavior than once-per-day measurements of volume consumed. Individual differences in consumption patterns and metabolism could lead to differences in BEC, and thus to ethanol intoxication, that would be not be apparent from simple 24-hour measurements. Future studies of this nature should aim to include BEC measurements when reporting drinking data, but care must be taken in the implementation of these procedures. Blood collection from mice is stressful for the animal, and reduces subsequent drinking behavior. In a repeated deprivation or intermittent access model this is a confounding influence, and it may only be practical to collect blood on the final day of alcohol access, or to use a previously implanted catheter system for easy access. This type of collection would likely be more feasible in rats than in mice, due to the size of the blood vessels. A further difficulty to consider is the timing of the blood collections. Rodents do not drink at a steady rate throughout the day, and the collection of blood would be expected to preclude further drinking by the animal for that day at least. Therefore to collect multiple time points and generate a meaningful picture of alcohol intoxication during reinstatement days it would be necessary to drastically increase the number of animals used; each time point would require an entirely separate group of continuous access and intermittent access mice. Comparisons from reinstatement days to baseline drinking are also possible, but necessitate the collection of blood during the baseline access period, which will certainly interfere with drinking and prolong the time required for drinking to stabilize, and for intermittent access to begin. Nevertheless, these measurements, along with other improvements to drinking data collection, such as lickometers and remote data collection, should be

implemented in future studies, to allow for better understanding of binge-like drinking behavior induced by scheduled deprivation and its relevance to alcoholism.

Finally, with any comparison of measurements of protein or nucleotides in a tissue sample collected from an animal, one must consider the influence of cell death caused by experimental treatments. Tissue collection is based on a regional perimeter, and the cellular content of the delineated area determines the results of abundance assays. For example, if continuous access alcohol drinking is killing oligodendrocytes in the nucleus accumbens, then it would be expected that expression of myelin-related genes decrease. However, without knowledge of the cell death that may be occurring, it might instead be hypothesized that decreases in gene expression instead indicate attenuated function of the myelin sheath, rather than outright destruction. In the same way death of particular neuronal populations might skew results of particular neuronal constituents. For example, if neurons containing D2 dopamine receptors are preferentially destroyed by alcohol exposure the microarray results may show a decrease in transcript coding for the receptor, which could otherwise be interpreted as a functional change in living cells. To complicate matters further, a cellular loss may have secondary repercussions in other neurons and glia that interfere with interpretation of results. For example, a preferential loss of GABAergic interneurons may cause neurons they modulate to increase activity, and then to compensate with homeostatic mechanisms that alter mRNA and protein abundance. These changes may simply be a result of cell death, and not relevant to alcohol-related behavior under conditions that do not destroy brain cells. To further explore these issues methods are available to assay the health state of isolated cells, which should be applied to mice after continuous access and intermittent access drinking, and will determine whether cell death plays a role in changes in mRNA abundance associated with alcohol drinking and abstinence.

Several avenues of molecular research will be useful for further characterizing the neuromolecular correlates of alcohol drinking before and after scheduled deprivation, and the intermittent alcohol access procedures described herein provide important methodological guidelines for further study of the effects of scheduled alcohol deprivation in rodents as a model of binge-drinking.

Transcriptional regulation mechanisms in NAc during drinking and scheduled deprivation

Despite the limitations outlined above, and although further study is needed to determine the nature of alcohol-responsive gene expression on intermittent access and continuous access schedules over time, some molecular mediators of transcriptional regulation that are likely to be important can be identified for experimentation based on the present results.

The acquisition of alcohol reinforcement appears to be mediated by mesolimbic A10 dopaminergic neurons projecting from the ventral tegmental area to the nucleus accumbens, and the degree of subsequent intake and preference is correlated with ethanol-induced increases in extracellular dopamine in the NAc (Katner and Weiss, 2001). Furthermore, in humans ethanol-evoked dopamine release in NAc is directly related to the degree of psychostimulation produced by the drug (Boileau et al., 2003). Therefore ethanol-evoked dopamine release in the nucleus accumbens, and changes in gene expression that alter the functioning of this region, are likely important mediators of the development of maladaptive drinking in the intermittent access model.

Modulation of gene expression by dopamine receptor activation is clearly involved in transcriptional regulation occurring with continuous access and intermittent access alcohol drinking. Dopamine receptors are G-protein coupled receptors that modulate gene expression

through the action of Gα subunits on adenylyl cyclase, which alter cAMP concentrations and the function of cAMP-dependent protein kinase (PKA). PKA and other kinases, such as Ca2+/calmodulin-dependent protein kinase IV (CaMKIV), phosphorylate the transcription factor cAMP response-element binding protein (CREB), which activates transcription of genes with cAMP response elements in promoter regions. Dozens of CREB-mediated genes have been identified, and include corticotrophin-releasing hormone (CRH), neuropeptide Y (NPY), prodynorphin (PDYN), and brain-derived neurotropic factor (BDNF). Alcohol reduces CREB function in the nucleus accumbens at least partially through activation of D2-like dopamine receptors (D2-D4), which inhibit PKA function (Spanagel, 2009).

In the nucleus accumbens reduced CREB function seems to promote drug intake associated with positive affective states, signaling mediators upstream of CREB are differentially expressed (multi-class LIMMA, $F < .05$, BH-adjusted p-value < .30) between intermittent access mice and continuous access mice from the cell membrane to the nucleus (Pandey, 2004a, b). For all of these enzymes transcriptional regulation means that mechanistic roles can only be loosely hypothesized; alterations in transcript abundance must be confirmed by quantitative PCR and protein measurement, and function confirmed *in vivo*.

Transcript coding for the D3 dopamine receptor (*Drd3),* which inhibits adenylyl cyclase, is more abundant in nucleus accumbens of CA mice than in IA or H2O mice, and this receptor is known to play a role in alcohol intake following deprivation. In several lines of rats undergoing alcohol drinking with repeated deprivations, DRD3 is upregulated in the striatum, and DRD3 antagonists dose-dependently reduce drinking during alcohol reinstatement (Vengeliene et al., 2006). Measurement of protein abundance will determine if changes in *Drd3* mRNA in the present experiments reflect changes in protein at the cell membrane, but it seems likely that continuous

access drinking increased DRD3 abundance in nucleus accumbens, and that during deprivation this upregulation in dopamine signaling promotes alcohol seeking through adenylyl cyclase inhibition and reduced CREB-mediated transcription.

In support of this hypothesis, transcript for *Adcy8* (adenylyl cyclase 8), a calcium/calmodulin-sensitive form of the enzyme, is reduced by continuous access drinking, and recovered by abstinence. The recovery of this gene expression may be promoted by homeostatic mechanisms acting to restore adenylyl cyclase function in the face of inhibition by alcohol-induced extracellular dopamine increases. In a similar vein, the downregulation of *Ddc* (dopa decarboxylase) in intermittent access mice may be a result of decreased monoamine signaling through dopamine and serotonin in the nucleus accumbens (Hodgetts and O'Keefe, 2006) . In general, repeated activation of neuromolecular processes by alcohol induce changes that gradually push the cell out of the normal boundaries of functioning, and thus promote alcohol intake to restore normality.

Furthermore, several kinases downstream of calcium and cAMP with CREB phosphorylation activity are regulated during intermittent access procedures (Pandey, 2004a). The classical CREB-activation pathways are represented by apparent PKA subunit regulation, as transcripts coding for *Prkacb* (protein kinase, cAMP dependent, catalytic, beta) and *Prkar2b* (protein kinase, cAMP dependent regulatory, type II beta), are upregulated by continuous access drinking. The regulation of *Camk4* (calcium/calmodulin-dependent protein kinase IV) and *Camk1d* (calcium/calmodulin-dependent protein kinase ID) are of particular interest, because they are not altered by continuous access drinking, but are more abundant in abstinent mice after intermittent access. The function of CAMK4 and CAMK1D could be particularly important for CREB-mediated gene expression during abstinence that contributes to elevated drinking during

relapse-like behavior. Furthermore, mRNA coding for *Camk2b, Camk2n1, Camkk2*, and *Rps6ka2* are all upregulated in intermittent access mice compared to continuous access mice, and all phosphorylate CREB. Finally, *Creb3l2* (cAMP responsive element binding protein 3-like 2) and *Creb5* (cAMP responsive element binding protein 5) are upregulated by continuous access drinking, while *Crem* (cAMP responsive element modulator) is increased during abstinence from intermittent access, as compared to both other groups.

The involvement of deltaFosB signaling in plasticity occurring with intermittent access is well-established, and so will not be mentioned in depth here, other than to mention that expression of dozens of putative targets of deltaFosB transcriptional regulation are altered (McClung and Nestler, 2003; Nestler et al., 2001; Robison and Nestler, 2011). These targets include AMPA1 glutamate receptor (*Gria1)*, cyclin-dependent kinase 5 and regulatory subunits (*Cdk5, Cdk5r1, Cdk5r2*), synaptotagmins (I, II, III, IV, V, VI, VII, XI), kinesins (1B, 1C, 3C, 5A, 5C, 7, 9, 13B), and microtubule-associated proteins (1A, 1 light chain 3 alpha, 1 light chain 3 beta, 1B, 4, tau). Interestingly, AP-1 mediated transcription (a dimer of Jun and Fos family proteins) is downstream of retinoic acid signaling by way of the MAPK cascade, and genes involved in retinoic acid are regulated among drinking groups in IAA studies. Regulated retinoic acid-related genes include *Rarb* (retinoic acid receptor, beta), *Rora* (RAR-related orphan receptor alpha), *Rorb* (RAR-related orphan receptor beta), *Rarres2* (retinoic acid receptor responder (tazarotene induced) 2), (*Stra6*), stimulated by retinoic acid gene 6, *Rdh10* (retinol dehydrogenase 10 (all-trans)), and *Rdh18* (retinol dehydrogenase 18). Regulated MAPK genes (*Map3k10, Map3k11, Mapk10, Mapk10, Mapk6,* and *Mapk8ip*) are uniformly downregulated in abstinent intermittent access mice compared to continuous access mice. Retinoic signaling is impaired in the brains of alcoholics, but the connection to altered behavior through gene regulation has not been explored,

and the intermittent access model represents a useful paradigm within which to study this connection (Clugston and Blaner, 2012).

Of particular interest is the observation that Wnt signaling in the nucleus accumbens may play a role in regulation of gene expression associated with alcohol drinking. Although alcohol modulation of Wnt signaling has been described in brain (PFC, hippocampus, neural stem cells), bone, and liver, alterations in Wnt signaling in the brain have not been directly associated with regulation of drinking behavior. There are three well-characterized Wnt signaling pathways, in addition to several that are less studied, through which Wnt ligand binding to Frizzled receptor regulates cellular processes (Inoki et al., 2006; Ishitani et al., 2003; Nusse, 2012). The canonical Wnt pathway modulates gene expression through β-catenin signaling, the noncanonical planar call pathway regulates cytoskeleton structure, and the noncanonical Wnt/calcium pathway regulates ER calcium release, which activates PKC, calcineurin, and CamKII. Given the apparent roles of transcriptional regulation, calcium regulation, and axonal/dendritic remodeling in neuroplasticity in the nucleus accumbens occurring with ethanol drinking and abstinence, the Wnt pathways represent a high-priority target for future study in alcohol drinking behavior, and binge-like drinking in particular.

Some evidence for a role of Wnt signaling in alcoholism comes from genetic studies in humans and mice. A SNP in DKK2 (dickkopf 2 homolog), an antagonist of canonical Wnt signaling, is significantly associated with alcohol-related harm in alcoholics (Kim, 2013). Furthermore, some risk for alcoholism is found to be conferred by haplotypic variation in exon 3 of TTC12, a gene about which very little is known, but which is similar to beta-catenin and may function in the Wnt pathway (Yang, 2007). By analyzing hippocampal miRNA abundance across BXD strains, it was found that microRNA miR-31 is associated with alcohol-related behaviors, and the

expression correlates of this gene are enriched in genes involved in Wnt signaling, axon guidance, and MAPK signaling (Parsons, 2012).

The most well-characterized effects of ethanol on Wnt signaling are in bone, where alcohol suppresses the canonical Wnt/ β-catenin signaling pathway and disrupts gene expression (Lauing, 2012). Serum levels of sclerostin, an endogenous Wnt antagonist, are raised in alcohol patients, but sclerostin levels seem to be related to liver function, and are not correlated with ethanol intake (Gonzalez-Reimers, 2013). In Sprague-Dawley rats experimenter-administered chronic binge alcohol (i.p, 3 g/kg, 3 days/week, for 4 weeks) modulated canonical Wnt signaling gene expression, as indicated by gene ontology analysis of microarray results from lumbar vertebrae (Himes, 2008). In genes regulated by ethanol exposure in two models of alcohol liver disease, genes related to Wnt signaling were upregulated (Bardag-Gorce, 2006).

The effects of ethanol on Wnt signaling are also well characterized with regard to fetal neurodegeneration. There is clear evidence that the teratogenic effects of ethanol on neurodevelopment in fetal alcohol syndrome are at least partially mediated through Wnt signaling, both upstream and downstream of Wnt/Frizzled binding. Elevated calcium induced by ethanol exposure activates CamKII, which phosphorylates β-catenin and prevents its transcriptional activation, preventing trophic support of neural stem cells. Blocking calcium increases prevents CamKII activation and prevents cell death (Flentke, 2013). Furthermore, in human neural stem cells ethanol exposure regulates the expression of Wnt proteins, Wnt receptor complex proteins, and cytoplasmic mediators of Wnt signaling (pGSK3β and β-catenin), but there is conflicting evidence as to the direction of regulation that may reflect methodological differences (Vangipuram, 2012; Choi, 2011).

Analysis of differential expression in nucleus accumbens during the IAA model revealed that Gsk3β is upregulated in intermittent access mice, compared to continuous access mice. The identification of GSK3B as a hub gene in an ethanol-responsive gene co-expression network in PFC of BXD mice offers further support for a role of Wnt signaling in alcohol-related behaviors (Wolen, 2012), as does its identification as a gene with influence on alcohol dependence in a recent multi-species gene ranking study (Zhao, 2012). Noncanonical Wnt signaling involving GSK3B and other signaling mechanisms could also mediate gene regulation in the nucleus accumbens. Wnt is capable of activating protein kinase mTOR through GSK3β inhibition via a pathway that does not involve β-catenin (Inoki, 2006).

Wnt signaling in nucleus accumbens occurring in the IAA model may also involve the noncanonical Wnt/calcium pathway, through which Wnt controls calcium release from the endoplasmic reticulum. Calcium release activates CamKII, which phosphorylates nemo-like kinase (NLK), which modulates gene expression (Ishitani, 2003). Transcripts coding for NLK, as well as for CamKII regulatory and catalytic subunits, are downregulated in mice during continuous access drinking, and upregulated in intermittent access mice during abstinence. Activation of NLK acts as a negative feedback loop that attenuates canonical Wnt signaling via β-catenin (Sugimura, 2010). Furthermore, in the nucleus accumbens Wnt signaling could be connected to dopamine release through regulation of the activity of PKA, which phosphorylates β-catenin, preventing its ubiquitination and degradation, and activating gene expression of canonical Wnt targets (Hino, 2005).

Future studies should examine the role of Wnt signaling in nucleus accumbens for the development of binge-like drinking behavior using the IAA model. Several approaches are available to perturb the signaling pathway at multiple levels, including ligands, RNAi, and viral-

vector mediated over-expression. Studies of this type may point the way to the development of more effective drugs for the prevention of relapse drinking in patients with alcohol use disorders.

Other likely nucleus accumbens adaptations of note for future study

Some other likely neuromolecular adaptations deserve mention. Abundance of *Fa2hv* (fatty acid 2-hydroxylase), the enzyme responsible for catabolism of the endogenous cannabinoid receptor ligand anandamide, is significantly downregulated by continuous access drinking, and upregulated during abstinence from intermittent access (Pacher and Kunos, 2013). This evidence hints at alterations in anandamide in the nucleus accumbens, and thus altered cannabinoid receptor activation. Thus there is some support for adaptation of the endocannabinoid system, which has been postulated to play a role in alcohol drinking behavior, during intermittent access (Pava and Woodward, 2012). Other endocannabinoid related genes coding for cannabinoid receptors 1 and 2, cannabinoid-receptor interacting protein 1, and monoacylglycerol lipase, were not regulated. These results are deserving of further exploration, because ethanol drinking increases levels of the endocannabinoid 2-arachidonoylglycerol (2-AG) in the nucleus accumbens, but does not alter anandamide levels. Furthermore, infusion of cannabinoid receptor antagonist rimonabant reduces self-administration of ethanol, but not of cocaine (Colombo et al., 2007). Therefore alterations in endocannabinoid function play a role in ethanol seeking behavior, and this connection should be explored in the intermittent access model, with a particular aim toward exploring the potential for cannabinoid-based treatments for relapse. In particular, interference with endocannabinoid metabolism represents a method for perturbing the system without producing marijauana-like psychoactive effects (Blankman and Cravatt, 2013).

In both models of scheduled deprivation reported herein, gene regulation clearly points to alterations in nucleus accumbens myelination, which would alter neuronal signaling and

excitability within the region. Interestingly, in the IAA model significantly regulated myelin-related genes are uniformly decreased by continuous access drinking, and all show significant recovery after six cycles of intermittent access, to the point that no differences are seen between IA mice and H2O mice. Regulation of seven myelin-related genes (*Mag*, *Mbp*, *Mobp*, *Mog*, *Omg*, *Opalin*, and *Pmp22*) are significantly decreased by continuous access, and recovered by intermittent access. Future study should examine myelin in the nucleus accumbens in IA and CA mice, as well as the expression of myelin-related genes in IA mice immediately following binge-like alcohol drinking, to determine whether regulation of these genes is altered by scheduled abstinence. It is currently unclear whether myelin deficits are recovered or exacerbated by intermittent access, but this information will illuminate the connection between myelination and the development of addictive behavior towards alcohol. Experimentation with the demyelinating agent Cuprizone in the context of intermittent access would show the effects of progressive degeneration of myelin in alcohol-seeking behaviors over time.

Neuroplasticity induced by ethanol over time is not well understood, but detailed study of the nucleus accumbens and other interconnected reward related regions using scheduled deprivation methods has revealed the involvement of molecular mediators with established roles in drug addiction processes. In addition to identifying mechanisms of gene regulation, genomic methodology identified differentially expressed genes and gene co-expression networks that are associated with maladaptive changes in drinking behavior over time, and these represent targets for preclinical study as potential therapeutic vectors for the treatment of relapse in alcohol-dependent patients. Although studies of mRNA transcription are not perfect representations of the functional changes occurring in neurons and glia, identification of functions and processes of regulated genes provides important clues to the functional implications for regions in the

mesolimbocortical reward related pathways, and directions for future study of neuroplasticity that leads to addiction. The weekly intermittent alcohol access procedure was shown to be an effective and valid preclinical model in C57BL/6 mice that likely produces binge-like drinking through mechanisms known to be involved in the pathology of alcoholism in humans, including CREB- and deltaFosB-mediated transcription, miRNA regulation, and epigenetic modifications. Finally, the compound NAQ, a novel small molecule mu-opioid receptor-selective antagonist, was shown to be effective at reducing high concentration alcohol drinking and escalation of intake due to intermittent access. NAQ shows similar efficacy to naltrexone, while showing some evidence for advantages in tolerance and behavioral selectivity, and thus represents a potential treatment for relapse in alcoholism that is deserving of further study. Future studies will follow the guidelines and directions laid out within, and further examine the role of gene expression in the nucleus accumbens and connected regions during intermittent and continuous access to multiple alcohol concentrations. This line of research will advance basic understanding of addictive behavior and of alcohol addiction.

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Appendix 1. Supplementary Materials Location

Supplementary materials for chapters 3 and 5, as well as annotated R code for WGCNA, can be found at http://jonathanandrewwarner.com and on the Miles Laboratory Wiki, which can be accessed through the VCU website or by contacting Dr. Michael Miles at mfmiles@vcu.edu.

Vita

Jonathan Andrew Warner was born on March 16th, 1983, in Durham, North Carolina, to Dr. Steve Warner and Mary Warner. He graduated from W.T. Woodson High School in Fairfax, Virginia, in 2001 and received his Bachelor of Science in Psychology from Virginia Commonwealth University in 2007. As an undergraduate he worked under Dr. Rob Vann and Dr. Jenny Wiley, and in 2008 he joined Dr. Miles' laboratory, where he has worked for the past 6 years to complete his doctorate.