BEHAVIORAL AND NEUROBIOLOGICAL CORRELATES OF GENETIC RISK FOR BINGE-LIKE ALCOHOL DRINKING

By

Amanda M. Barkley-Levenson

A DISSERTATION

Presented to the Department of Behavioral Neuroscience

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

April 2015

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Amanda M. Barkley-Levenson

has been approved

John C. Crabbe, Mentor

Christopher L. Cunningham, Member

Aaron J. Janowsky, Member

Tamara J. Phillips, Member

Andrey E. Ryabinin, Member

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES..... iii
LIST OF ABBREVIATIONS..... vi
ACKNOWLEDGEMENTS..... viii
ABSTRACT..... x
CHAPTER 1: General Introduction..... 1
Alcohol Use Disorders and Binge Drinking..... 1
Motivation to Consume Alcohol..... 4
Rewarding and aversive effects of alcohol..... 5
Self-medication hypothesis of alcohol use..... 6
Behavioral Genetics Strategies for Alcohol Research..... 7
Inbred strains..... 9
Single-gene manipulations..... 10
Selective breeding..... 12
Advantages and disadvantages of selected lines and inbred strains..... 14
Modeling Binge Drinking in Rodents..... 15
Limited access vs. continuous access drinking procedures..... 15
High Drinking in the Dark selection..... 18
Alcohol drinking microstructure..... 20
Measuring Other Alcohol-Related Behaviors in Animal Models..... 21
Neurobiology of Binge Alcohol Drinking..... 23
Neuropeptide Y and Binge Drinking..... 24
Dissertation Goals..... 27
CHAPTER 2: Distinct ethanol drinking microstructures in two replicate lines of mice selected for drinking to intoxication..... 29
Abstract..... 30
Introduction..... 32
Methods..... 34
Results..... 40
Discussion..... 46
Figures..... 54
CHAPTER 3: Rewarding and aversive effects of ethanol in High Drinking in the Dark selectively bred mice 65
Abstract..... 66
Introduction..... 67
Methods..... 69
Results..... 76
Discussion..... 80
Figures..... 88

CHAPTER 4: Genotypic and sex differences in anxiety-like behavior and alcohol-induced anxiolysis in mice selectively bred for drinking intoxication..... 95
Abstract..... 96
Introduction..... 98
Methods..... 100
Results..... 105
Discussion..... 109
Figures..... 115
CHAPTER 5: Evaluation of neuropeptide Y system involvement in the binge-like drinking phenotype of the High Drinking in the Dark selected mice..... 123
Abstract..... 124
Introduction..... 125
Methods..... 128
Results..... 137
Discussion..... 140
Figures..... 146
CHAPTER 6: General Discussion..... 157
Summary of Findings..... 157
How Do HDID Mice Reach Intoxicating BECs?..... 157
What Motivates Alcohol intake in HDID mice?.....165
Neurobiology of HDID Mice and Binge-like Drinking.....171
The HDID Mouse Phenotype as a Model of Binge-Like Drinking..... 173
Strengths and weaknesses of the model..... 173
Future Directions..... 179
Relationship between feeding and alcohol consumption..... 179
Drinking microstructure as a predictor of risk..... 180
Binge drinking across the lifespan and developmental aspects of HDID mice..... 182
Binge-like drinking in a choice procedure..... 183
QTL mapping in the HDID and HS mice..... 184
Conclusions..... 185
REFERENCES..... 187

LIST OF TABLES AND FIGURES

2-1 Daily ethanol DID intake and Day 4 BECs in the lickometer for HDID-1 and HS male mice

2-2 Ethanol bout features on Day 4 of the lickometer DID test for HDID-1 and HS male mice

2-3 Daily ethanol intake and Day 4 blood ethanol concentrations during BioDAQ single-bottle ethanol DID test

2-4 Ethanol bout features on Day 4 of the BioDAQ single-bottle ethanol DID test

2-5 Cumulative record of g/kg ethanol intake on Day 4 of the BioDAQ single-bottle ethanol DID test for all three genotypes

2-6 Daily ethanol and water intake during the BioDAQ two-bottle choice DID test

2-7 Ethanol and water bout features on Day 4 of the BioDAQ two-bottle choice DID test

2-8 Daily saccharin DID intake for males and females of each genotype during the BioDAQ saccharin DID test

2-9 Saccharin bout features on Day 4 of the BioDAQ saccharin DID test

S2-1 Change scores for number of ethanol bouts in 2 h and average ethanol bout size

S2-2 Ethanol and water interbout intervals and average bout lengths on Day 4 of the BioDAQ two-bottle choice DID test

3-1 NaCl solution intake in ml/kg body weight across the 6 conditioning trials in HDID-1 and HS mice

3-2 NaCl solution intake in ml/kg body weight across the 6 conditioning trials in HDID-2 and HS mice

3-3 Ethanol conditioned place preference expression during the pretest and Tests 1-3

3-4 Average locomotor activity counts (beam breaks) per minute during the pretest and each place preference test (T1-3)

S3-1 CTA change scores for HDID-1 and HS mice of each drug group

S3-2 CTA change scores for HDID-2 and HS mice of each drug group

Table 4-1 Inbred strain panels tested for anxiety-like behavior included in Experiment 3

Table 4-2 Correlation matrix of alcohol DID and anxiety variables for inbred mouse strains

4-1 Day 2 ethanol intake in g/kg body weight and blood ethanol concentration for males and females of each genotype

4-2 Percent time spent in the open arms by males and females of each genotype and drinking group

4-3 Number of line crosses made by males and females of each genotype and drinking group

4-4 Percent time spent in the open arms displayed versus blood ethanol concentration

4-5 Percent time spent in the open arms across alcohol doses collapsed on sex

4-6 Number of line crosses made by each genotype across alcohol dose groups collapsed on sex

5-1 Alcohol intake across each day of the 4-day DID test and BECs after drinking on Day 4 for HDID-1 and HS in Experiment 1

5-2 NPY immunohistochemistry in the nucleus accumbens

5-3 Correlations between alcohol and water intake and NAc core NPY immunoreactivity in the HDID-1 and HS mice

5-4 NPY immunoreactivity in the central nucleus of the amygdala, bed nucleus of the stria terminalis, basolateral amygdala, and paraventricular nucleus of the hypothalamus

5-5 Schematic of bilateral nucleus accumbens cannula hit approximate locations in Experiment 2

5-6 Alcohol intake by HDID-1 male mice on Day 4 of a 4-day DID test and BECs after Day 4 drinking shown as an average for each drug tested in Experiment 2

5-7 Schematic of bilateral nucleus accumbens cannula hit approximate locations in Experiment 3

5-8 Alcohol intake across each day of a 4-day DID and BECs after Day 4 drinking in HDID-1 mice tested with the Y2R agonist NPY_{13-36}

5-9 Alcohol intake across each day of a 4-day DID and BECs after Day 4 drinking in HDID-1 mice tested with the Y1R antagonist BIBP 3226

5-10 HDID-1 male mice water intake during 4 h DID control sessions following intra-accumbal infusion with Y2R agonist or Y1R antagonist

LIST OF ABBREVIATIONS

AA –Alko Alcohol rat line

aCSF – artificial cerebral spinal fluid

ANA – Alko Non-Alcohol rat line

ANOVA – analysis of variance

AUD – alcohol use disorder

B6 – C57BL/6J inbred mouse strain

BEC – blood ethanol concentration

BLA – basolateral amygdala

BNST – bed nucleus of the stria terminalis

CeA – central nucleus of the amygdala

CDC – Centers for Disease Control

CPP – conditioned place preference

CTA – conditioned taste aversion

D2 – DBA/2J inbred mouse strain

DID – drinking in the dark

DSM – Diagnostic and Statistical Manual

EPM – elevated plus maze

EZM – elevated zero maze

GABA – γ-Aminobutyric acid

GWAS – genome wide association study

HAD – High Alcohol Drinking rat line

HAP – High Alcohol Preferring mouse line

HDID – High Drinking in the Dark

HS – heterogeneous stock; HS/Npt mouse line

IBI – interbout interval

IHC – immunohistochemistry

i.p. – intraperitoneal

LAD – Low Alcohol Drinking rat line

LAP – Low Alcohol Preferring mouse line

LDB – light-dark box

LiCl – lithium chloride

NAc – nucleus accumbens

NaCl – sodium chloride

NIAAA – National Institute on Alcohol Abuse and Alcoholism

NIH – National Institutes of Health

NP rat – alcohol Non-preferring rat line

NPY – neuropeptide Y

OF – open field

P rat – alcohol Preferring rat line

PBS – phosphate buffered saline

PFA – paraformaldehyde

PVN – paraventricular nucleus of the hypothalamus

QTL – quantitative trait locus

VTA – ventral tegmental area

Y1R – NPY Y1 receptor subtype

Y2R – NPY Y2 receptor subtype

ACKNOWLEDGMENTS

There are many people that I need to acknowledge for their contributions to this dissertation and my time at Oregon Health & Science University. First and foremost, I would like to thank my mentor, Dr. John Crabbe. I began working in John's lab as a summer intern during high school and I returned in various capacities each summer throughout college because of the incredible experiences that I had working for him. He has been unfailingly supportive of me throughout the years, and this work would not have been possible without his help. His mentorship and example have shaped the way I think about and practice science and I am so grateful for the training I've received as his student. Looking back on nearly a decade spent working with John, I cannot think of a more fortunate encounter than stumbling into his lab as an ASE student when I first thought to give research a try.

I would also like to thank the members of my dissertation advisory committee, Drs. Tamara Phillips, Andrey Ryabinin, and Aaron Janowsky. In addition to being the chair of my dissertation and exam committees, Tamara has been a wonderful collaborator and all-around excellent source of knowledge. These experiments would not have been possible without her graciously letting me use her lickometers, stereotaxic surgical setup, and cryostat. Andrey has not only been a member of my advisory committee, but also a co-sponsor of my NRSA and an incredibly generous collaborator. He welcomed me into his lab to learn immunohistochemistry and was always happy to answer any questions I had and to discuss my data and provide his own thoughtful analysis and expertise. Many thanks also to Aaron for thoughtful insights on my dissertation project throughout the past few years.

Thanks to Dr. Chris Cunningham who was my first rotation advisor and co-mentor on my second year paper, and who is now the ad-hoc member of my exam committee. Chris has been a wonderful collaborator and source of endless expertise during my time in graduate school. Thanks also to past Cunningham lab member Phoebe Smitasin for running all the HDID CPP experiments presented here.

Many, many thanks to all the members of the Crabbe lab, especially Dr. Pam Metten for her mentorship during my early days as a trainee. The lab would not run nearly so well without the expertise of our amazing members (past and present): Jason Schlumbohm, Stephanie Spence, Larry Huang, Wyatt Hack, Mark Rutledge-Gorman, Andy Cameron, and Lauren Brown. Thanks also to Drs. Angela Ozburn and Gian Greenberg for their help and insights during this past year.

So many people have graciously taken the time to help train me in various techniques throughout graduate school, and I especially need to thank Harue Baba in the Phillips lab and Dr. Caroline Hostetler in the Ryabinin lab. Harue very patiently trained me in all of the techniques for performing intra-cranial cannulation surgeries and microinjections, and Caroline taught me how to do immunohistochemistry and held my hand through my first bumbling attempt at an assay.

A big thank you to Kris Thomason, Nicole Ernst, Laura Young, and the rest of the Behavioral Neuroscience office staff for being so supportive and helpful and for answering a lot of dumb questions for me over the years. Thanks also to Dr. Suzanne Mitchell for her tireless efforts as Program Director.

I would like to acknowledge the Portland chapter of the Achievement Rewards for College Scientists Foundation, and especially my scholarship sponsors Diane and Dick Alexander who sponsored me for three years and who have remained incredibly supportive throughout my time at OHSU.

The support of my fellow graduate students and friends has been instrumental in reaching this point. I particularly need to acknowledge my classmates, Emily Eastwood, John Harkness, and Josh Kaplan for being the best cohort I could imagine. Your support and camaraderie made graduate school (even quals) not only survivable but enjoyable. I also want to thank my fellow members (past and present) of the We Heart Brains pub trivia team: Drs. Allison Stafford, Travis Moschak, Caroline Hostetler, and Trish Pruis. Thanks for your friendship and for giving me something to look forward to each week.

Finally, I need to acknowledge the immense amount of love and support I have received from my friends and family throughout the years. Thank you to my mother Jane Barkley and my father Charlie Levenson for cultivating in me a thirst for knowledge and a curiosity about the world, and for never asking me when I’m going to get a real job. Thanks also to my sister, Dr. Emily Barkley-Levenson, for doing it all first so that I knew it was possible and then turning right around and cheering me on. Thank you for getting excited about my data even when I can’t and for helping me to remember why I love what I do.

The work presented here has been supported by NIH-NIAAA grants AA13519, AA10760, AA07702, a grant from the Department of Veteran’s Affairs, and an APA Dissertation Award. I have been supported by NIH-NIAAA grants AA007468 and AA02009, an ARCS Foundation Scholarship, an N.L. Tartar Trust Fellowship, an Ashworth Award, and an OHSU Graduate Scholar Research Award.

ABSTRACT

Alcohol use disorders (AUDs) are complex, devastating disorders with a significant impact on individuals and society. AUDs arise from the interplay of genetic and environmental factors. Binge drinking is a particular form of excessive intake, defined as a pattern of drinking that results in intoxicating blood alcohol levels. Binge drinking is associated with AUDs, but is also a high-risk behavior separate from a diagnosis with an AUD. As with AUDs, binge drinking is presumed to have genetic and environmental risk factors that contribute to this behavior. The High Drinking in the Dark (HDID) mouse lines were selectively bred for drinking to intoxicating blood ethanol concentrations (BECs) in order to begin to address the question of what biological substrates underlie genetic risk for binge-like drinking. There are two ongoing, independent replicate selections for the HDID lines (HDID-1 and HDID-2), and these mice represent a genetic model of risk for drinking to intoxication. In the experiments in this dissertation, HDID mice were tested for behaviors and neurobiological changes that might be related to their binge-like drinking phenotype. Chapter 1 is an overview of the background data needed to provide context for the present studies. In Chapter 2, data are presented showing that HDID selection has resulted in replicate-specific alcohol drinking microstructures that both result in the same selection phenotype of high BECs. In Chapters 3 and 4, several possible motivational factors are assessed for their potential roles in driving HDID drinking to intoxication. In Chapter 5, neuropeptide Y is examined molecularly and behaviorally as a possible neurobiological candidate that may underlie part of the HDID-1 drinking phenotype. Finally, Chapter 6 provides a general discussion of the findings presented here and what they indicate about the HDID mice as a genetic model of binge-like drinking.

Chapter 1: General Introduction

Alcohol use disorders and binge drinking

Alcohol use disorders (AUDs) encompass both alcohol abuse and alcohol dependence. The DSM-V classifies AUDs as mild, moderate, or severe depending on the number of diagnostic criteria met. Criteria for a diagnosis of an AUD include such behaviors as drinking more than intended, trying unsuccessfully to reduce drinking, continuing alcohol use in spite of negative consequences from drinking, tolerance to alcohol, and withdrawal symptoms upon ceasing alcohol use (Diagnostic and Statistical Manual version 5, American Psychological Association, 2013). AUDs are a significant public health concern, with excessive alcohol consumption costing an estimated $223.5 billion in a given year in the United States due to alcohol-related accidents and injuries, health care expenses, and lost workplace productivity (Bouchery et al., 2006). Because of this major cost to affected indiviuals and society as a whole, AUDs are an area of intense interest and study. There are currently few treatments available for AUDs, and existing treatments have shown only modest efficacy at treating all affected individuals (for review, see Franck & Jayaram-Lindström, 2013). Consequently, it is of great importance that we continue to investigate novel treatment options in order to combat AUDs.

Though alcoholism was once seen as a social or moral issue, it is now widely accepted that AUDs arise from a complex interaction between genetic and environmental risk factors. By studying AUD rates in fraternal and identical twins, the heritability (i.e., the proportion of variability in AUD diagnosis that is attributable to genetics) of AUDs has been estimated to be approximately 50% (Goldman et al., 2005). This indicates that

genes play a significant role in the development of AUDs. Consequently, identifying what genetic factors promote alcohol use and abuse will be integral to understanding AUDs and developing better strategies for prevention and treatment.

Not all risky alcohol intake qualifies a person for a diagnosis with an AUD. However, this does not mean that these other forms of intake are harmless. Risky drinking has several definitions, but it generally refers to drinking and intake that are potentially harmful or that put a person at risk for developing an AUD. Binge drinking is a specific form of risky drinking. Binge drinking refers to a pattern of consumption that results in intoxicating blood alcohol (ethanol) concentrations (BECs). In humans, this is any BEC at or above 0.08 g% and is often thought of as drinking "too much, too fast." In general, this relates to four standard drinks in two hours for women, or five drinks in two hours for men (NIAAA, 2004). Binge drinking is associated with significant short- and long-term health risks. For example, binge drinking is associated with increased risk of traffic accidents, personal injury, and risky sexual decisions (Dawson et al., 2008; Flowers et al., 2008; Hutton et al., 2008). It is also associated with more protracted risks such as certain cancers and liver disease (Gupta et al., 2010; Zakhari & Li, 2007).

Binge drinking is extremely prevalent, with one in six adults in the U.S. reporting four or more binge drinking sessions per month (Centers for Disease Control [CDC], 2012). Furthermore, at least 50% of the total alcohol intake in the U.S. occurs during binge drinking sessions (U.S. Department of Justice, 2005) and almost 75% of the alcohol misuse-related costs are attributable to binge drinking (Bouchery et al., 2011). Although binge drinking is frequently thought of as a behavioral issue specific to adolescence and emerging adulthood, there is much evidence pointing to binge drinking

occurring throughout the lifespan. Binge drinking is most prevalent and has the greatest reported intensity (i.e., drinks per binge) in people ages 18-24, but people over the age of 65 actually report more frequent binge drinking than any other age group, with an average of 5-6 binge episodes per month (CDC, 2012). Due to its prevalence and associated risks, it is clear that binge drinking is a considerable public health concern on its own. However, binge drinking is also known to be associated with AUDs. Binge intake is related to future risk of problem drinking and alcohol abuse (e.g. Bobo et al., 2013; Chassin et al., 2002). Additionally, individuals with AUDs have been shown to have alcohol intake that exceeds the criterion for binge drinking, and drinking to intoxication is undoubtedly a frequent experience in alcoholism (e.g. Mello & Mendelson, 1970).

Alcohol consumption survey data are most commonly collected and reported as intake over a given period of time, such as drinks per day or week (Leeman et al., 2010). However, this tells us little about the presence or frequency of binge drinking unless binge episodes are specifically reported. Additionally, the patterning of intake within a drinking session (e.g. time between drinks, total length of drinking session, etc.) affects BEC and subsequently whether alcohol intake produces significant intoxication. The specifics of within-session drinking pattern should therefore be of great interest to researchers studying binge drinking. Different components of the drinking pattern may also be differentially predictive of various outcome measures such as risk of AUDs or alcohol-related problems. For example, a high number of total drinks per day is positively associated with AUD diagnosis (Saccone et al., 2000), and even with substance use and abuse in an individual's offspring (Malone et al., 2002). Similarly, maximum dose per

drinking session may be a better predictor of alcohol related problems than drinking frequency or annual intake (Bobak et al., 2004). Drinking pattern also represents a potential target for limiting excessive alcohol intake. Behavioral interventions aimed at preventing harmful drinking have shown successful reductions in total intake by promoting decreases in drinks per session, even if drinking session frequency does not change (Baer et al., 2001).

Motivation to consume alcohol

In human research, self-reported information about drinking motivation can be readily collected from participants, most commonly using variations of the Drinking Motives Questionnaire (Cooper et al., 1992, 2014; Stewart et al., 1996). This inventory includes numerous potential motivating factors for alcohol drinking and differentiates between classes of motives such as social vs. coping determinants of drinking (e.g. Gire, 2002; Hussong, 2003; Kuntsche et al., 2008; Stewart & Chambers, 2000). However, self-reports always come with some risk of unreliability. Using both self-reported data and tasks with measurable behavioral outcomes, such as willingness to work to obtain alcohol or preference for alcohol over a placebo, provides a more objective and complete measure of reward. Such assessments can be achieved through laboratory studies of self-administration and through the incorporation of behavioral economic analyses (e.g. Amlung & MacKillop, 2015; Leeman et al., 2013; Skidmore et al., 2014). Drinking determinants can also be assessed more indirectly by measuring sensitivity to the motivational effects of alcohol, as described below.

Rewarding and aversive effects of alcohol

Like many drugs of abuse, alcohol use can produce both rewarding and aversive effects (Verendeev & Riley, 2013). In general, the rewarding or positive effects of alcohol are associated with ratings of drug liking, euphoria, and stimulation, whereas aversive or negative effects are associated with drug disliking, dysphoria, and sedation (Martin et al., 1993; Morean et al., 2013). Sensitivity to the subjective effects of alcohol has been widely studied in current drinkers and those with a family history of AUD. In individuals with a family history of AUDs, assessments of sensitivity to alcohol's subjective effects have shown mixed results; some studies have reported a lower level of response in family history-positive individuals, whereas others have found an increased alcohol response (e.g. McCaul et al., 1990; Schuckit 1984). These differences in findings appear to be due to when assessments are made in relation to alcohol intake (i.e., during the ascending or descending limb of the BEC curve) and which measures are used (e.g. body sway, sedation, stimulation, subjective rating of intoxication). When taking timing factors into consideration, a consistent relationship emerges wherein a family history of AUDs appears to produce increased sensitivity to alcohol effects as BECs rise and decreased sensitivity as BECs fall (Newlin & Thomson, 1990). There is also evidence for a similar pattern of sensitivity in heavy current drinkers, but not light drinkers (King et al., 2002; 2011; Rueger et al., 2015). Alcohol effects experienced during the ascending limb of the BEC curve tend to include those associated with reward, and descending limb alcohol effects are those associated with aversion (Martin et al., 1993). Thus, both genetic risk and current alcohol experience appear to influence sensitivity to alcohol's subjective effects and therefore the balance between perceived reward and aversion. This

combination of enhanced rewarding sensitivity and impaired aversive sensitivity seems to result in higher motivation to consume alcohol and relates to binge drinking and AUD diagnosis (King et al., 2011).

Self-medication hypothesis of alcohol use

In addition to its positive rewarding effects, alcohol can also have negative reinforcing effects. That is, alcohol intake can produce reward through the alleviation of an existing negative affective state such as anxiety or depression. Alcohol is a potent anxiolytic (e.g. Gilman et al., 2008), and AUDs and anxiety disorders are frequently comorbid (Blanco et al., 2011; Kessler et al., 1997; Kushner et al., 2000). One potential explanation for this relationship is that high-anxiety individuals self-medicate through alcohol use, which can subsequently develop into problem drinking. There is some evidence to suggest a causal relationship between anxiety and alcohol consumption (Bolton et al., 2006; DeMartini & Carey, 2011). This relationship may hold particularly true for individuals with social anxiety disorders, wherein alcohol is used to counteract the high anxiety state produced by social situations (Buckner & Heimberg, 2010; Miller et al., 2014; Terlecki et al., 2014). It is also possible that anxiety disorders and AUDs share similar underlying genetic contributions, and that this is the reason for the observed comorbidity. Family and twin studies suggest possible common transmission of anxiety disorders and AUDs, and this may reflect shared genetic risk (Tambs et al., 1997).

Similarly, depression and AUDs also show a high degree of comorbidity (e.g. Grant & Harford, 1995; Hasin et al., 2005). As with anxiety disorders, there is evidence that depression may precede AUDs and that drinking may be motivated at least in part by an attempt to alleviate depression symptoms (Abraham & Fava, 1999; Gilman &

Abraham, 2001; Holahan et al., 2003). There is also evidence that depression and AUDs may have shared genetic liability. Twin studies suggest that there might also be a genetic association between major depression and AUDs (Lyons et al., 2006; Prescott et al., 2000), and this could explain in part the frequent comorbidity of these disorders. This pattern of findings is similar to those seen for anxiety disorders, and the negative reinforcing effects of alcohol should therefore be considered alongside more traditional rewarding/aversive effects as potential motivators of alcohol consumption.

In addition, alcohol use itself may lead to an increase in negative affective states. In one theory of addiction known as "allostasis," repeated excessive alcohol use leads to a shift over time from drinking that is primarily motivated by the positive reinforcing effects of alcohol to drinking that is motivated by the negative reinforcing effects (for review, see Koob & Le Moal, 2001). This model postulates that neuroadaptations in brain stress and reward systems occur with chronic drinking and alcohol withdrawal, and that these adaptations may contribute to feelings of anxiety and depression experienced with alcohol dependence. Alcohol consumption may then be continued in an attempt to alleviate these increases in negative affect, and this could reflect a mechanism by which alcohol use is maintained. Thus, the relationship between affective state and motivation to drink is complex and includes potential shared genetic contributions as well as a bidirectional relationship between AUDs and mood disorders.

Behavioral genetics strategies for alcohol research

Given the significant costs of AUDs and binge drinking, there is much interest in trying to understand better the underlying causes of these behaviors as well as potential

treatments. There has been much productive research done in human populations, both with active alcohol users and those at risk for future AUDs (i.e., individuals with a positive family history for AUDs). However, there is still much we do not know about the complex etiology of risky drinking. Genome-wide association studies (GWAS) have helped to identify some potential genetic variants related to AUD diagnosis and various drinking behaviors (e.g. mutations in the alcohol dehydrogenase genes) (Kapoor et al., 2013; Park et al., 2013; Treutlein et al., 2009). However, these variants likely represent a small percentage of the genetic risk for AUDs. This is due to such factors as limitations in the current ability of GWAS to detect rare variants and epistatic effects (i.e., gene-by-gene interactions), as well the contributions of epigenetics and gene-by-environment interactions (for review, see Eichler et al., 2010 and Parker & Palmer, 2011). Additionally, alcohol use is extremely prevalent in many societies and it can therefore be difficult to find research participants who are alcohol-naïve. Thus, alcohol research using human subjects is frequently confounded by prior alcohol exposure and it is challenging to determine whether behavioral and neurobiological differences are due to genetic susceptibility to alcoholism, or are consequences of alcohol use itself.

Because of the issues faced in human research, animal models of alcohol use and related behaviors are a useful alternative and complementary method. Many human behaviors such as voluntary alcohol drinking and sensitivity to alcohol (e.g. stimulation and sedation) can be readily modeled in laboratory animals ranging from invertebrates like *Drosophila* and *C. elegans,* to non-human primates (e.g. Barr, 2013; Cowmeadow et al., 2005; Davies et al., 2004; Kaun et al., 2011). Animal models therefore provide an excellent way to replicate discrete aspects of AUDs to examine the determinants of

specific behaviors in a controlled environment. Mouse models are particularly valuable for genetic studies of alcohol consumption and related behaviors. The mouse genome has been completely sequenced and shares a high degree of homology with the human genome, meaning that genetic risk factors identified in mouse studies are likely to be informative for human research. Mice are also an ideal system for generating specific genetic manipulations like gene knockouts or overexpressing transgenic animals. Though the technology to produce these changes in other species such as rats has increased rapidly in recent years, it is still most readily accomplished in mice. Large-scale selective breeding efforts are also more attractive with mice due to the lower monetary and space requirements needed to house a large number of animals. However, of the three broad behavioral genetics strategies described below, all have been successfully implemented with rats and mice and data from both species will be discussed.

Inbred strains

Inbred mouse and rat strains have been developed over repeated generations through brother–sister matings so that all same-sex animals within a strain are assumed to be genetically identical. Genetic variation is therefore held constant across subjects, providing an excellent means of examining environmental contributions to alcohol-related traits and gene by environment interactions. Panels of inbred strains can also be tested for a given trait to determine whether that trait shows genetic variation (e.g. Crawley et al., 1997; Yoneyama et al., 2008). Greater variation in the trait across strains than within strains indicates that there is a significant genetic component, and heritability values for a particular trait in a panel of strains can be calculated from the data. Similarly, inbred strain panels can be given a battery of behavioral and physiological tests to

determine genetic correlations between traits. Genetic correlation refers to the proportion of variance shared between two traits that is due to common genetic factors. With inbred strain panel data, the existence of shared genetic contributions between traits can be inferred from similar strain rankings in performance on the traits. With this method, each genotype (strain) functions essentially as an individual data point, and a correlation between two traits can be calculated using these strain means. Given the presumed complexity of genetic contributions to alcoholism, it is preferable to use a large number of inbred strains to include more genetic variation and to provide a greater ability to detect a statistically significant genetic correlation between traits. Inbred strains also provide an opportunity for studying differences in brain morphology and neurochemistry between strains with innate differences for alcohol-related traits, and this approach has been used to gain greater insight into biological factors promoting AUDs. For example, the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains represent opposite ends of the spectrum with regard to voluntary oral consumption of alcohol, with B6 mice readily drinking large quantities and D2 mice consuming very little (e.g., Lê et al., 1994). Many studies have utilized these two strains to study biological and behavioral factors that might explain the difference in alcohol consumption. However, this approach is inherently weaker than using a panel of inbred strains because it is essentially looking for a correlation between only two data points, and it does not necessarily follow that any traits differing between B6 and D2 are related to their drinking phenotypes.

Single gene manipulations

Specific genes of interest can also be studied in animal models (particularly invertebrates and mice) by targeted manipulation of the gene (e.g. Pich & Epping-Jordan,

1998; Schumann et al., 2003; Sora et al., 2010). This can include knockout or knockdown studies in which the gene is removed or disabled, or its expression is reduced, respectively. In another technique, transgenic experiments use increased gene expression or the insertion of a particular polymorphism or mutated version of the gene to determine the effect on the phenotype of study. Candidate genes for transgenic and knockout studies in animals can be identified from human gene-expression and linkage studies, and this is one approach for translating genetic findings between species (for review, see Foroud et al., 2010). Studies with knockout animal models must be interpreted with care, however, because the total absence of genes can have profound effects on development and may result in unanticipated compensations by other systems (e.g. Sora et al., 1998). Increasingly, techniques are available that allow for a greater degree of spatial and temporal control of genetic manipulations (e.g., inducible knockout systems, short-interfering RNA, CRISPR/Cas systems). For example, Cre/lox inducible knockout mice can be developed so that gene knockout is limited to a specific tissue, or can be turned on and off at a chosen time in development (for review, see Sauer, 1998). Short-interfering RNA can produce selective knockdown of gene expression in targeted brain areas by using the existing cellular RNA interference pathways to repress mRNA translation (e.g. Bahi & Dreyer, 2012). CRISPR/Cas systems involve the use of engineered nucleases to produce efficient targeted genome editing and can be used to develop transgenic and knockout models in a variety of species and genetic backgrounds (for review, see Hsu et al., 2014). As technology continues to improve, these methods should provide a way to bypass the limitations of conventional knockout strategies and increase our understanding of specific gene influences on alcohol behaviors.

Selective breeding

Selective breeding of rodent lines is another method used for studying genetic contributions to alcoholism. Beginning from a genetically diverse population, a behavior or physiological trait of interest is identified and animals are tested for this trait and bred on the basis of their level of response (e.g. Phillips et al., 1989; Morato & Morato, 1993). Two divergent lines of animals can be produced using bidirectional selection, wherein high responders are bred with high responders and low responders with low responders across repeated generations. This strategy typically results in two distinct lines of animals that differ significantly for the selection trait. Sometimes a control line is developed as well, consisting of animals from the founding population that are bred and maintained without selection, but using the same population restrictions as the selected lines (e.g. number of families). In unidirectional selection, a single selected line of animals is produced by selectively breeding for only high or low response. In this type of selection, unselected animals from the founding population or a control line are typically used for comparison. Selective breeding can be either short-term or long-term. Short term selection generally involves few breeder pairs and individual selection (highest/lowest responders chosen for breeder pairs without regard to family or relatedness). This approach is desirable for quick turn-around on heritability estimates or quantitative trait loci (QTL) mapping experiments, as this procedure can produce a significant selection phenotype more quickly than long-term selections (e.g. Belknap et al., 1997). However, this maximization of selection pressure is at the expense of increased genetic drift, and short-term selections are therefore only viable for a limited number of selection generations (e.g. Hitzemann et al., 2008). Long-term selection involves significantly

more breeder pairs and is used to produce selectively bred lines of animals that can be maintained over numerous generations of selection and which can serve as long-lasting animal models (e.g. Crabbe et al., 2009; Grahame et al., 1999). Long-term selection may use within-family selection (highest or lowest responders within each family are chosen for breeders), individual selection, or a combination.

Selective breeding strategies can be used to demonstrate the heritability of the selection trait, and also to identify the genetic relationship between the selection trait and other behaviors. If selection results in changes to another behavior or trait other than the selection phenotype of interest, this is referred to as a correlated response to selection and it suggests a shared genetic component between the selection trait and the correlated response. Testing lines bred for divergence on a given trait (such as alcohol intake) for performance on other measures, therefore, is a useful technique for identifying the genetic relatedness of various behaviors. For example, the numerous pairs of alcohol preferring/nonpreferring and high-alcohol–drinking/low-alcohol–drinking selected rat lines show selection-related differences on such traits as locomotor stimulation in response to alcohol and an alcohol-conditioned taste aversion, as well as on biological factors such as endogenous neurotransmitter levels (Bell et al., 2006; McBride et al., 2014; Stewart & Li 1997). The development of replicate lines can be another strength of selective breeding. Replicate lines refer to independent repeated selections using the same selection procedure and phenotype. The resulting replicate pairs of lines can then be tested for correlated responses to selection. If both replicates show similar divergence in related behaviors or neurobiological changes, this provides strong evidence for the genetic relationship between these features and the selection phenotype.

Although correlated responses to selection suggest similar underlying genetic factors between traits, spurious correlations can be seen as well. Selection trait-irrelevant genes may become fixed and give rise to what appears to be a correlated response to selection. These correlations are unlikely to occur by chance multiple times, and therefore can potentially be identified by their presence in only a single replicate line. Correlated responses can also be due to linkage disequilibrium, wherein alleles are transmitted together more often than would be expected by random assortment (for review, see Slatkin, 2008). Linkage disequilibrium can arise from physical proximity of alleles on a chromosome. Therefore, what may appear to be a shared genetic factor between two traits could actually be two separate loci that are closely situated and thus inherited together.

Advantages and disadvantages of selected lines and inbred strains

Selective breeding can be advantageous over the use of individual inbred strains because selected animals have a greater degree of genetic diversity. Founding populations for selected lines are by definition heterogeneous stocks (HS), ranging in genetic diversity from two-way inbred strain crosses (e.g. a B6 x D2 F2 generation) to HS populations derived from eight or more inbred strains. Some of these HS populations included wild-derived progenitor strains (Iancu et al., 2010), which further increases the genetic diversity available. This genetic variation is important for identifying novel risk alleles for the behaviors of interest that may not be present in the more widely studied inbred strains. Because all animals of an inbred strain are presumed to be genetically identical, the extensive alcohol research that has been conducted using B6 mice has essentially involved the study of a single individual. This is extremely useful if our

interest is in understanding why the B6 mouse drinks alcohol, but is of less utility for identifying the determinants of alcohol intake in broader populations. Consequently, selected lines provide an excellent option for examining genes and behaviors associated with alcohol phenotypes of interest. Inbred strains, however, are advantageous when wanting to study specific gene by environment interactions, or when doing targeted genetic manipulations. The lack of genetic diversity is a strength in this type of study, because only the effects of the experimental manipulations will vary systematically across animals. This allows for enhanced ability to detect an effect of the treatment. Thus, the choice to use selected lines or inbred strains will be best determined by the specific question being asked and whether genetic variation will be a help or a hindrance.

Modeling binge drinking in rodents

Limited access vs. continuous access drinking procedures

A majority of the alcohol research done in rodent models has used 24 h continuous access, two-bottle choice drinking procedures where animals are offered the choice between water and an alcohol solution (frequently 10%). This procedure has been used to select multiple lines of rats (P/NP, HAD/LAD, AA/ANA) and mice (HAP/LAP) for high preference ratio for alcohol (i.e. alcohol consumed divided by the total fluid intake) (for review, see Crabbe, 2008). A strength of this procedure is that it mimics the situation of human drinking in that alcohol is consumed during concurrent access to water and the animals can drink as little or as much they choose. Additionally, intermittent access in this procedure has been shown to produce escalations in intake (Hwa et al., 2011; Melendez, 2011; Simms et al., 2008), which is another frequent feature

of alcohol use in humans. These procedures therefore appear to be able to model some of the neuroadaptations that occur with chronic alcohol drinking. However, two-bottle choice, continuous access drinking has several limitations as well. For example, induction of drinking in this procedure sometimes requires sucrose fading in order to establish alcohol intake, particularly in rats (e.g. Samson 2000). Additionally, there is evidence that rodents drinking in this paradigm may pattern their drinking in such a way that intoxication is experienced only as transient episodes (Dole & Gentry, 1984; Murphy et al., 1986). This suggests that the high alcohol preference exhibited by some of these high-preferring mice and rats may not be motivated by alcohol's pharmacological effects, but rather by other factors such as taste. In B6 and D2 mice, for example, the significant genotypic difference in alcohol intake can be partially attenuated when animals self-administer alcohol either intravenously or intragastrically, thereby largely bypassing any influence of olfaction or taste (Fidler et al., 2012; Grahame & Cunningham, 1997). B6 and D2 mice differ in their sensitivity to bitter and sweet tastants, and this genotypic difference in pre-ingestive sensitivity may account in part for their markedly different preferences for alcohol (Blizard, 2007; Boughter et al., 2005). Recently there has been evidence from a high alcohol-preferring selected mouse line to suggest that these specific animals do reach consistently high BECs during a two-bottle choice test (Matson & Grahame, 2013). Rat lines selected for high alcohol preference have also shown evidence of intoxicating levels of consumption in continuous drinking procedures (McBride et al., 2014).

Because of the likelihood that significant intoxicating BECs may not be reached by most mice in two-bottle choice procedures, other drinking tasks have been developed

to model a more binge-like pattern of intake. These procedures are designed to provide limited access to alcohol under conditions that promote high intake. One example is scheduled access to alcohol drinking. In this procedure, animals are given daily short (30 min) limited access alcohol sessions coupled with 21-hour fluid restriction (Cronise et al., 2005). B6 mice in this test drink sufficient quantities of alcohol to demonstrate behavioral intoxication as measured by ataxia on the rotorod at the end of the drinking session. However, the translational relevance of this procedure is somewhat limited due to the fluid restriction required to produce the binge-like phenotype.

Probably the most frequently used test of binge-like drinking for rodents is the drinking in the dark, or DID test (Rhodes et al., 2005). This is a limited access procedure wherein mice are given a single tube of 20% alcohol during the period of the dark cycle when consummatory behaviors are highest. During the initial development of this test, a variety of drinking session lengths (2 or 4 h) and time points (0, 1, 2, 3, or 4 h after lights off) were systematically evaluated (Rhodes et al., 2005) in order to determine a limited access window during the consummatory (dark) phase during which mice would drink to intoxicating BECs. Binge-like intake was seen most reliably when access to alcohol was provided for two hours on the first three days, and then for four hours on the final day. Using this protocol, B6 mice have been shown to reach intoxicating BECs following the four-hour drinking session (Rhodes et al., 2005; 2007), and this has become the standard DID procedure. The DID test models the human definition of binge drinking, wherein there are both temporal and intake components. This test has been widely used and shows similarly high intake in B6 mice across laboratories (for review, see Thiele & Navarro, 2014). Additionally, it provides an efficient means of testing potential therapeutic agents

for their ability to reduce intake. Several drugs shown clinically to reduce alcohol consumption and also two-bottle choice drinking have been shown to reduce DID as well (e.g. Gupta et al., 2008; Kamdar et al., 2007). This indicates that the DID test is a viable alternative to two-bottle choice procedures for high-throughput screening of compounds with possible clinical relevance.

Although the DID test does successfully promote binge-like alcohol intake in high-drinking mice, there are several limitations as well. These are discussed in detail elsewhere (Chapter 6). Briefly, the primary limitation is that only alcohol is provided, meaning that animals have no fluid choice available. Because DID takes place during the period of greatest consummatory activity, offering only alcohol means that intake may reflect general prandial drinking patterns rather than alcohol-specific motivations for consumption. Additionally, intake in the DID test across days shows a strong correlation between Days 2-4, but no relationship between intake on Day 1 with intake on subsequent days (Rhodes et al., 2005). Initiation of drinking in this model (i.e., Day 1 intake), therefore, does not seem to be a useful predictor of subsequent consumption even over the limited number of days of drinking.

High Drinking in the Dark selection

Because intoxicating BECs were a feature of previous high drinking rodent models that had been lacking, we sought to selectively breed mice that drink to high BECs. To do this, mice were selected based on their BECs at the end of a modified two-day DID test where alcohol access was given for two hours on the first day and four hours on the second day. The starting population for this selection was a genetically heterogeneous stock of mice developed from a systematic 8-way inbred strain cross

(progenitor strains: A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, CBA/J, DBA/2J, and LP/J) known as HS/Npt or HS (see Crabbe et al., 2009 for details). Selection was unidirectional, with mice that reached high BECs being bred together over successive generations. For the first five generations, selection used a within-family breeding approach, but due to slow response to selection, individual selection was initiated with the selection of breeders for generation S6 (Crabbe et al., 2009). These selection procedures resulted in a High Drinking in the Dark (HDID) line of mice that readily drinks to intoxicating BECs in the DID test. The unselected HS mice have shown consistently modest DID intake and BECs (Crabbe et al., 2009; 2014), and these mice are used as a comparator control line. Two independent replicate lines were developed successively using the same breeding procedure, with the second replicate initiated after seven generations of selection had been completed for the first replicate. The selection procedure for the second replicate line was the same as that used for the first, except that individual selection was used for all selection generations (Crabbe et al., 2009). We now have two replicates of HDID mice (HDID-1 and HDID-2), which allow us better to probe potentially correlated responses to selection. The realized heritability of high BECs after DID is relatively low (h^2 = 0.08–0.09), but selection has successfully increased BECs across generations (Crabbe et al., 2009). A recent report of this selective breeding effort (Crabbe et al., 2014) shows that BECs have increased 4.7-fold across 27 selected generations in HDID-1 mice (average BEC = 1.4 mg/mL; 80% of mice reach BECs > 1.0 mg/mL) and 4-fold in HDID-2 mice after 19 selected generations (average BEC = 1.1 mg/mL; 50% of mice reach BECs > 1.0 mg/mL). The rate of elimination of an acute injection of alcohol has been assessed in these lines and has been found not to differ

between the HDID lines and the HS mice (Crabbe et al., 2009; 2014). This indicates that the higher BECs of the HDID animals are not principally due to differences in alcohol metabolism. HDID mice also drink significantly more alcohol than HS mice (g/kg of body weight) and their intake is highly correlated with BEC. This provides further evidence that the high BECs reached are primarily the result of their binge-like alcohol intake. Additionally, HDID mice have been shown to be behaviorally intoxicated after the DID test, as assessed by a measure of ataxia (Crabbe et al., 2009). Given this strong phenotype in both HDID replicates, we believe that the HDID mice now represent a useful model of genetic risk for binge-like alcohol drinking

Alcohol drinking microstructure

Because we are interested in alcohol drinking that results in intoxication, the timing and patterning of drinking during the DID session is particularly important for understanding the phenotype. Binge drinking itself has a temporally specific definition, but the rate of intake during a binge session can significantly affect the BEC reached. Rodents drink fluids in discrete bursts, with clusters of sustained and rapid licking separated from each other in time (Johnson et al., 2010; Rushing et al., 1997; Smith, 2001). In order to quantify and analyze this drinking pattern, a drinking bout can be defined as some minimum number of licks separated by no more than a given amount of time. This allows for the quantification and analysis of the bout features or bout 'microstructure' of drinking. Drinking microstructure has numerous components (size and number of drinking bouts, time between bouts, rate of intake during a bout, etc.) and these factors all influence overall rate of intake during an alcohol drinking session and ultimately the BEC reached (e.g. Barkley-Levenson & Crabbe, 2012; Rhodes et al.,

2007). In addition, studies in pairs of rat lines selectively bred for high and low alcohol preference show that the number and size of drinking bouts in a session is generally greater in the high preferring line (Samson, 2000). This suggests some degree of genetic relationship between these bout features and high intake. Research from the non-human primate literature has also shown a relationship between large, quick bouts (a 'gulping' phenotype) and subsequent heavy drinking (Grant et al., 2008). It has also been postulated that animal microstructural elements might map onto more human constructs such as loss of control of drinking and alcohol craving.

Measuring other alcohol-related behaviors in animal models

Although alcohol intake is obviously one relevant behavior to model when studying AUDs, there are many other alcohol-related behaviors that are important for understanding alcohol consumption and disordered drinking. As mentioned above, a large area of research is dedicated to investigating the motivational factors driving alcohol intake. Producing animals that drink significant quantities is an important first step, but the true utility of these models lies in identifying what drives their intake and finding ways to reduce the motivation to drink excessively. In animal research, we do not have the luxury of self-reported reasons for drinking, and motivation to drink must be inferred from behavior. Willingness to work to obtain alcohol can be measured using operant self-administration tasks. Access to alcohol has been shown to maintain operant responding in rats and mice (e.g. Lopez & Becker, 2014; Samson et al., 2000). With operant conditioning procedures, sensitivity to the rewarding effects of alcohol can be assessed by changing response requirements (e.g. number of lever presses or nose pokes) so that

the “cost” of alcohol changes, and then determining the effect on responding. Similarly, a progressive ratio procedure can be used in which the response requirement increases with each reinforcer given until the animal is no longer willing to work to obtain alcohol. This is known as the breakpoint, and changes in breakpoint can be used as a measure of changes in the perceived rewarding strength of alcohol (e.g. Czachowski & Samson, 1999).

Sensitivity to alcohol reward and aversion can be readily assessed in rodent models using procedures such as place and taste conditioning (for review, see Cunningham et al., 2000). These procedures use Pavlovian conditioning techniques to pair alcohol exposure with a conditioned stimulus such as a novel physical cue or tastant. If alcohol is rewarding to the animal, we would expect a preference for the alcohol-paired cue. In contrast, alcohol aversion is shown by an avoidance of the alcohol-paired cue. In this way, it is possible to determine differences between groups (e.g. genotypes) in sensitivity to the motivational effects of alcohol. Alcohol conditioned behaviors have been studied across inbred strains and selected lines of animals to determine if there are genetic relationships between these behaviors and alcohol intake. A review of rodent studies found that conditioned taste aversion (CTA) is significantly negatively correlated with home cage alcohol consumption (Green & Grahame, 2008). In contrast, conditioned place preference showed a less consistent genetic relationship, with some studies showing a positive genetic correlation with intake, and some showing a negative correlation or no relationship. A study using a panel of 15 inbred strains found a significant negative genetic correlation between alcohol conditioned place preference and sweetened alcohol consumption (Cunningham, 2014). One possibility for the mixed findings across studies

is that the genetic relationship between conditioned place preference and alcohol intake may be affected by procedural variations (e.g. alcohol sweetening). In contrast, the more consistent findings for conditioned taste aversion suggest that environmental factors may play a lesser role in mediating the relationship between intake and conditioned taste aversion.

As noted before, it is important to keep in mind that 'reward' actually may represent a multifaceted sensation. That is, the experience of euphoria could produce the same subjective sense of pleasure as the alleviation of anxiety, but these two outcomes may represent different actions of alcohol at the level of the brain. Therefore when testing animal models of alcohol intake, it is necessary to assess potential differences in alcohol's ability to alleviate negative states, like anxiety and depression, when identifying contributions to drinking. In this thesis, I chose to focus on anxiety-like behavior, but depression-like behavior is equally relevant and can also be measured in rodent models. Anxiety-like behavior can be assessed in rodents in a number of ways, but the most common tasks involve measuring the willingness of an animal to enter an anxiogenic region of a test apparatus. Mice and rats tend to prefer dark, enclosed areas, so more time spent in bright or exposed regions suggests lower anxiety. Common tasks for assessing anxiety-like behavior include the elevated zero and plus mazes, the light-dark box, and the open field test.

Neurobiology of binge alcohol drinking

Limited access binge-like alcohol drinking and continuous access two-bottle choice drinking undoubtedly have shared genetic components, but there is evidence that

these traits have distinct genetic contributions as well. Specifically, QTL mapping efforts have identified only minimal chromosomal overlap between QTLs implicated in continuous access two-bottle choice drinking and DID (Iancu et al., 2013; Phillips et al., 2010). Although the genetic and neurobiological factors that underlie binge drinking are not yet as widely studied as those involved in preference drinking, there have been significant efforts made to assess various neurotransmitter systems for their involvement in alcohol DID. There is considerable evidence for glutamatergic, GABAergic, dopaminergic, and opioidergic system modulation of DID intake (for review, see Sprow and Thiele, 2012). Additionally, there is increasing evidence for involvement of other neurotransmitter systems in DID. The endocannabinoid system, for example, has been implicated in DID (Linsenbardt & Boehm, 2009). There is also evidence for a role of stress-related peptides like corticotropin-releasing factor and urocortin (Lowery et al., 2010; Ryabinin et al., 2008), and feeding-associated peptides like ghrelin and melanocortins (Kaur & Ryabinin, 2010; Navarro et al., 2009). As with continuous access drinking studies, much of this work has been done only in B6 mice. It therefore remains to be seen what other genotypes may tell us about the neurobiology of binge-like intake. Many of these neurotransmitter systems have also been implicated in continuous access two-bottle choice drinking, and it is not yet clear which neurobiological features are specific to binge-like alcohol drinking.

Neuropeptide Y and binge drinking

One neuropeptide that has been implicated in DID and is of particular interest in the HDID mice is neuropeptide Y (NPY). NPY is a 36 amino acid peptide that is

expressed widely throughout the brain and the periphery. Identified as behaviorally relevant first as a pro-feeding peptide (Levine & Morley, 1984), NPY is produced in the arcuate nucleus of the hypothalamus (Morris, 1989). NPY goes through several steps of posttranslational modifications, beginning as a large precursor molecule (pre-pro-peptide). NPY can also be further processed by cleavage at various points, which results in altered affinity at the NPY receptors (Grandt et al., 1996). The NPY receptors are G protein coupled receptors, each with seven transmembrane domains and coupled to $G_{i/o}$. There are six known receptor subtypes (Y1-Y6), with the Y1, Y2, and Y5 subtypes believed to be the major receptors relevant for signaling in the brain (for review, see Michel et al., 1998). The Y1 and Y5 receptors (Y1R and Y5R, respectively) are thought to be post-synaptic receptors, whereas the Y2 receptor (Y2R) is found presynaptically. Y1R and Y5R have a greater affinity for intact NPY than the cleaved forms, and Y2R has greater affinity for C-terminal fragments than whole NPY (Michel et al., 1998). Y2R is primarily thought of as an autoreceptor (i.e., ligand binding decreases NPY release from the cell), but there is evidence that it can also function as a heteroreceptor and influence the release of other neurotransmitters (e.g. Gilpin et al., 2011).

NPY has been implicated in a wide array of behaviors including pain sensitivity, circadian rhythms, feeding, stress and anxiety response, and drug and alcohol abuse (e.g. Ciccocioppo et al., 2009; Cleary et al., 2014; Roseboom et al., 2014; Wiater et al., 2011). Studies have shown a variety of effects of NPY on alcohol intake, with the literature containing examples of NPY increasing, decreasing, and not affecting alcohol drinking (for review see Thiele et al., 2004) Studies on the roles of various NPY receptor subtypes in alcohol drinking have been similarly conflicting. However, much of the work done

examining NPY effects on alcohol consumption has used knockout and transgenic animals in which NPY or a given NPY receptor subtype is absent everywhere. Similarly, pharmacological studies have predominately used intracerebroventricular (i.c.v.) administration of NPY and receptor agonists and antagonists. Because of the widespread expression of NPY and its variety of functions, these broad approaches may contribute to the lack of conclusive answers about the role of NPY in alcohol drinking. Brain region-specific targeting with NPY and its receptor ligands will likely be more useful for determining the role of discrete aspects of this system in modulating alcohol intake. A further complication is that NPY effects have also been shown to vary based on genetic background and alcohol experience. NPY's effects on alcohol intake are more pronounced in animals genetically-predisposed to drink (e.g. selected rodent lines, B6 mice) and animals with a history of dependence than in low-drinking or alcohol-naïve animals (Badia-Elder et al., 2007).

NPY has been less extensively characterized for its role in binge-like alcohol drinking than in continuous access drinking procedures. However, there is evidence that central administration of NPY can reduce intake and BECs in B6 mice when given before a DID test (Sparrow et al., 2012). In the same study, Y2R and Y1R antagonists were found to have opposite effects on DID intake and BEC, with Y2R antagonism decreasing DID and Y1R antagonism increasing it. However, NPY infusions into the amygdala of non-dependent Long-Evans rats showed no effect on binge-like drinking (Henderson & Czachowski, 2011). Thus, NPY effects on binge-like drinking appear to be similar to those seen with continuous access preference drinking and may be contingent upon a history of dependence or genetic risk for excessive intake. Despite this variation in

results, NPY does appear to play a role in some instances of alcohol intake, and continued exploration of the region-specific contributions to drinking in various genetic and procedural models is needed. NPY is of particular interest in the HDID lines because a gene expression study in naïve animals found significantly higher *Npy* mRNA levels in the extended amygdala of HDID-1 than HS male mice (Zhang et al., 2011). This expression difference, coupled with the previously described evidence from the literature, led to the identification of *Npy* as a candidate gene for study in the HDID mice.

Dissertation goals

The HDID mice are a novel genetic model for studying binge-like drinking to intoxication. Now that both replicate lines show a strong established selection phenotype, the next critical step is to develop a more complete picture of what these mice are actually modeling—what behavioral and neurobiological traits have been altered through selection and what is the ultimate utility of the HDID mice? This dissertation represents one of the first major efforts to produce a comprehensive study of the HDID lines. It focuses on three key questions related to the HDID phenotype: (1) how do the HDID mice drink to intoxicating BECs, (2) what are the motivational factors that drive their drinking to intoxication, and (3) can a candidate gene approach be used to identify one specific neurobiological effect of selection and potential therapeutic targets in these mice? Chapter 2 addresses the first question by examining the drinking bout characteristics and drinking patterns of both HDID replicates and HS control mice during variations of the DID test. In Chapters 3 and 4, the motivational drive behind HDID drinking is explored in relation to the rewarding and aversive effects of ethanol in these

lines (Chapter 3), and their basal and alcohol-related anxiety phenotypes (Chapter 4). Chapter 5 provides an example of a candidate gene approach for identifying specific neurobiological differences in the HDID mice that may underlie binge-like drinking by describing studies examining the relevance of NPY to the HDID phenotype. Finally, the dissertation concludes with a general discussion in Chapter 6 of the larger implications of the behavioral phenotype of the HDID mice and how best to utilize them as a model as we continue moving forward in alcohol research.

Chapter 2: Distinct ethanol drinking microstructures in two replicate lines of mice selected for drinking to intoxication

Amanda M. Barkley-Levenson and John C. Crabbe

This chapter has been published in a revised form in *Genes, Brain and Behavior* (2015), 14(5): 398-410

Abstract

The High Drinking in the Dark (HDID) mice have been selectively bred for high blood ethanol concentrations (BECs) following the limited access Drinking in the Dark (DID) test. We have shown previously that mice from the first HDID replicate line (HDID-1) drink in larger, but not longer, ethanol drinking bouts than the low-drinking HS/Npt control mice when both genotypes are consuming modest amounts in the DID test (Barkley-Levenson & Crabbe, 2012). In the present studies, we investigated whether this bout size difference persists during excessive binge-like intake, and whether it can be explained by changes in either lick rate or lick volume. We also tested HDID mice from both replicates (HDID-1, -2) and HS mice for drinking microstructure in three different DID tests to determine whether there are selection-dependent changes in drinking bout features. In Experiment 1, HDID-1 and HS male mice were given sequential water and alcohol DID tests in lickometer chambers. In Experiment 2, HDID-1, HDID-2, and HS mice of both sexes were housed in shoebox cages connected to the BioDAQ Episodic Intake Monitor system (Research Diets Inc.), which continuously records fluid consumption. Mice were tested sequentially on three 4-day DID procedures: single-bottle ethanol drinking (20%), two-bottle choice preference drinking for ethanol (20%) and water, and single-bottle saccharin drinking (3.2mM). HDID-1 in the lickometer chambers had larger ethanol bouts than HS mice, due to a larger lick volume in the HDID-1 mice than the HS. In Experiment 2, HDID-1 and HDID-2 mice had different drinking microstructures that resulted in both high intake and high BECs. HDID-1 mice drank in larger ethanol bouts than HS, whereas HDID-2 mice had smaller bouts but drank more frequently. This pattern was the same for two-bottle choice drinking as well, although the

group differences were more modest. HDID-2 mice had a high bout frequency for all fluid types tested (ethanol, water, and saccharin). In contrast, the large bout size phenotype of the HDID-1 mice was specific to alcohol. These findings suggest that selection for drinking to intoxication has resulted in two distinct drinking microstructures, both of which lead to high BECs and high ethanol intake.

Introduction

Binge drinking as defined by the National Institutes of Health is a pattern of alcohol (ethanol) intake that results in intoxicating blood ethanol concentration (BECs). In humans this is generally considered to be 4 standard drinks in 2hr for women and 5 for men (NIAAA, 2004). Binge drinking is a frequent component of alcohol use disorders (Esser et al., 2014), but it is also a dangerous behavior because it is associated with significant short- and long-term risks such as motor vehicle accidents, liver disease, and risky sexual behaviors (e.g. Dawson et al., 2008; Flowers et al., 2008). One aspect of binge drinking that can affect the BEC reached (and thus the degree of risk associated with the drinking session) is the patterning of intake during the drinking period. Furthermore, there is evidence from the animal and human literature that drinking is episodic, and the size and number of bouts during a drinking session (i.e., the drinking 'microstructure') is related to overall intake and risk of heavy drinking (Bobak et al., 2004; Grant et al., 2008; Samson, 2000).

We have selectively bred the High Drinking in the Dark (HDID) lines of mice for reaching high BECs after limited access drinking. These mice readily drink to intoxicating blood levels when offered access to a single tube of 20% ethanol during a 4 h period. Alcohol intake during DID and the more commonly used 24 h, two-bottle choice preference drinking procedures show only a moderate correlation and likely have some distinct underlying genetic contributions (Iancu et al., 2013; Phillips et al., 2010). HDID mice represent a novel genetic model of risk for binge-like drinking. There are two replicate lines – the HDID-1 and the HDID-2, and these genotypes were produced in independent experiments using the same foundation breeding stock and selection

criterion (Crabbe et al., 2009; 2014). Initiation was staggered, and HDID-1 are currently in generation S30 and HDID-2 in Generation S24. Although these lines show similarly high BECs and ethanol intake in the DID test, it is not known whether these animals pattern their intake during the DID test in the same way, nor whether they differ in any aspect of their drinking microstructure. We have shown previously that male mice from Generations S19-21 of the HDID-1 line differ from low drinking control HS mice in several bout features, most notably in having a larger bout size (Barkley-Levenson & Crabbe, 2012). However, HDID-1 mice in that study only showed a moderate level of intake with few animals reaching actual binge levels of BEC. Thus, it is not clear what ethanol drinking microstructure might look like in these mice under more binge-like conditions.

In the present studies, we were interested in several different aspects of the drinking patterns and structures of the HDID and HS lines. Since we have previously reported microstructural differences in ethanol intake between the HDID-1 and HS mice when HDID-1 mice had only moderate levels of consumption, the first goal was to determine whether these line differences extended to the characteristic binge intake of the HDID-1 mice. HDID-1 and HS mice were tested for ethanol DID using lickometer chambers to acquire more temporally and volumetrically sensitive measures such as lick rate and lick volume during binge-like drinking. A second goal was to determine whether the ethanol drinking structure of the HDID-1 male mice would extend to the HDID-2s (i.e., if enhanced bout size is a correlated response to HDID selection) and to female mice. Mice of both sexes and all three genotypes were tested for 4-day single-bottle ethanol DID in continuous consumption recording BioDAQ cages. We were also

interested in how ethanol drinking microstructure and pattern might change across and within DID sessions, and we compared microstructure variables on Day 1 and Day 4 of the DID test to address this question. The final goal of these studies was to assess microstructure during binge-like exposures to two-bottle choice ethanol drinking and to another palatable rewarding substance (saccharin) to determine which microstructure features may generalize across drinking solutions and procedures.

Materials and Methods

Animals and husbandry

Male and female mice of the HDID-1, HDID-2, and HS lines were bred and housed in the Veterinary Medical Unit of the Veterans Affairs Portland Health Care System (Portland, OR). Mice were weaned at 3 weeks of age and were reared with both dam and sire until weaning. After weaning, mice were housed in groups of 2-5 with same-sex littermates or with mice of the same genotype when necessary to avoid single housing. All mice were between the ages of 56 and 91 days old at the start of testing and were experimentally naïve. Mice were housed in standard polycarbonate shoebox cages on Bed-o-cob bedding and received *ad libitum* access to food (Purina 5001 chow, LabDiet, St. Louis, MO) and water unless otherwise specified. Experiment 1 used HDID-1 male mice from selection generation S23. Experiment 2 used HDID-1 male mice from selection generations S25 and S26, HDID-1 female mice from generation S27, HDID-2 male mice from generation S18, and HDID-2 female mice from generations S20 and S21. HS mice are the starting population from which the HDID-1 mice were selected and are the product of a systematic 8-way cross of inbred strains described in detail elsewhere

(Crabbe et al., 2009). HS mice are maintained without selection and are used as a control comparator line for the HDID animals because HDID selection was unidirectional and a corresponding low-drinking line does not exist. HS mice had a low level of ethanol intake when tested at Generation S0 for the initiation of the HDID selection, and drinking and BECs remained low in these mice when tested concurrently with selection of Generation S14 of the HDID-1 mice (Crabbe et al., 2009; 2014). Mice were kept on a reverse 12 h/12 h light/dark cycle with lights on at 21:30 for all experiments. At the beginning of each experiment, mice were singly-housed either in lickometer chambers placed inside standard shoebox cages with Bed-o-cob bedding (Experiment 1) or in BioDAQ-compatible polycarbonate shoebox cages with Bed-o-cob bedding (28.5 x 17.5 x 12 cm) (Experiment 2) and allowed to acclimate for 5-7 days. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Continuous fluid consumption recording

Two different apparatus were used in these experiments to record fluid intake continuously during the DID tests. Lickometers from Med Associates (St. Albans, VT) were used to record intake in Experiment 1. Lickometer chambers consisted of plastic boxes with ventilated lids (dimensions: 17.8 cm x 10.2 cm x 10.2 cm) that rested on top of stainless steel grid floors. Each chamber had a hole in the rear wall through which a sipper tube was inserted. The grid floors were each connected to ground and an electrode was connected to the portion of the sipper tube outside of the plastic chamber such that a mouse drinking from the tube closed an electrical circuit. Each circuit closure (i.e. lick) was time-stamped and recorded by Med Associates software.

In Experiment 2, fluid intake was measured using the BioDAQ Episodic Intake Monitoring System (Research Diets Inc., New Brunswick, NJ). Details of use of this system to record fluid intake have been reported elsewhere (Barkley-Levenson & Crabbe, 2012). Briefly, the BioDAQ consists of hoppers resting on force and weight sensors that continuously record the weight of the fluid hopper and register changes in weight to a sensitivity of 0.01 g. Drinking solutions were provided in 10 ml drinking tubes with volumetric markings readable to the 0.05 ml. These tubes were a modified version of our laboratory's standardly used 10 ml drinking tubes and were designed to eliminate the reduced intake in the BioDAQ system that we previously reported (Barkley-Levenson & Crabbe, 2012). The drinking tubes were fitted with 3 inch curved stainless steel ball bearing sipper tubes and situated in the hoppers such that the spouts projected into the cage. Pilot studies with these sipper tubes showed ethanol intake consistent with levels seen in our standard home cage DID tests.

DID procedure

For these experiments, a 4-day version of the DID test was used. In this procedure, animals are weighed on each experiment day approximately 1 h before the start of testing. Testing begins at 3 h into the dark cycle (12:30PM) at which point water bottles are removed and replaced with 10 ml drinking tubes. The fluid levels of these tubes are read, the recording system is started (BioDAQ or lickometer depending on experiment), and the tubes are left in place for the duration of the drinking session. At the end of the session, fluid levels are read again and the continuous recording is stopped. Drinking tubes are removed and water bottles are returned to each cage. On Days 1-3, drinking sessions are 2 h long. On Day 4, the drinking session is 4 h long, with an

additional fluid level reading taken at the 2 h point. For DID tests where ethanol is being consumed, a 20 μl blood sample is taken from the peri-orbital sinus at the end of the 4 h drinking session in order to determine BEC. Blood samples are processed according to standard lab protocol and are analyzed by gas chromatography as described elsewhere (Rustay & Crabbe, 2004).

Experiment 1: lickometer assessment of water and ethanol DID microstructures

This experiment used 24 HDID-1 and HS male mice (n=12/genotype). Only HDID-1 mice were tested here because this experiment was intended to follow-up bout size differences found between the first replicate and the HS mice in the previous paper (Barkley-Levenson & Crabbe, 2012). In this study, mice were housed in the lickometer chambers and allowed to acclimate for one week. Mice then received a 4-day DID test with a single bottle of tap water. Mice received two days off and then were tested on a standard 4-day single bottle ethanol DID test with 20% ethanol.

Experiment 2: drinking microstructure of ethanol DID, 2-bottle choice DID, and saccharin DID

One hundred and nine HDID-1, HDID-2, and HS mice of both sexes were used in this experiment (n=16-31 /sex/genotype). Mice were tested in cohorts of 15-16. Due to animal availability, male mice of each HDID replicate were tested in separate cohorts (HDID-1 vs. HS and HDID-2 vs. HS), whereas all three genotypes of mice were tested in each cohort for female mice. Animals were tested sequentially on the three DID tests, with at least one week off between tests. The ethanol DID consisted of the presentation of a single tube containing 20% ethanol using the standard 4-day DID protocol as described above. For the two-bottle choice DID, technical limitations of our BioDAQ system

allowed for only a subset of 12 mice (n=4-6/genotype) to be tested at a time. The mice not given this test received no experimental manipulation during the two-bottle choice DID phase of the experiment. For those being tested, mice were given access to two tubes concurrently – one containing 20% ethanol and one containing tap water. Tube positions were counterbalanced across animals such that half of the animals received water on the left and ethanol on the right each day, and half the animals received the fluids in the inverse positions. In the final DID phase, all 109 animals were given a 4-day single bottle DID test with 3.2 mM saccharin solution.

Drugs

Ethanol (200 proof; Deacon Laboratories, King of Prussia, PA) was diluted in tap water (20% v/v). Saccharin (Sigma-Aldrich, St. Louis, MO) was diluted in tap water to a concentration of 3.2mM (0.066%). This concentration of saccharin has been previously shown to be preferred by all three genotypes in a 24 h two-bottle choice procedure (Crabbe et al., 2010).

Drinking microstructure analysis

In Experiment 1, a bout was defined as at least 20 licks, with no more than a minute between licks. This bout definition has been used for ethanol drinking bout analysis in previous studies (e.g. Ford et al., 2008; Rhodes et al., 2007). Total intake was determined from volumetric fluid level readings taken from the drinking tubes. Bout variables analyzed were the number of bouts in the drinking session, the average interbout interval (IBI), the average bout size (number of licks), the average bout length, and the average lick rate during bouts (licks/min). We also analyzed the total number of licks per drinking session and used this number to calculate the average lick volume in

ml/lick (total session intake/total session licks). In instances of group differences in lick volume, the average bout size was also converted to a g/kg or ml/kg measurement using the average lick volume.

In Experiment 2, a bout was defined as at least a 0.02 g change of weight in the fluid tube and no more than a minute between recorded weight changes. This was consistent with the bout definition used previously (Barkley-Levenson & Crabbe, 2012). Bout variables analyzed were the number of bouts in the drinking session, the average IBI in minutes, the average bout size (g/kg body weight dose for ethanol, or ml/kg body weight for water and saccharin), and the average bout length in seconds. For the two-bottle choice DID, bout features were determined for each fluid type separately.

Statistical analyses

Drinking volume data were converted to g/kg dose for ethanol and ml/kg intake for water and saccharin. Because we were interested in patterning of drinking during the initial ethanol exposure and the 4hr test session, intake on Days 1 and 4 was also separated into 20 min bins. Exploratory analysis analyzed smaller (10 min) and larger (30 min) bin sizes and found no change in results due to bin size, so the 20 min bin size was arbitrarily chosen for ease of data presentation. The binned data were analyzed by repeated measures analysis of variance (ANOVA) using a within-subjects factor of time bin and between-subjects factors of sex and genotype. Our primary interest, however, was in drinking on Day 4 of the DID tests because these drinking sessions are expected to result in binge levels of intake. While intake data for Days 1-3 are presented graphically, in most instances these data were not analyzed and discussion will focus only on Day 4 intake and drinking variables. Day 4 intake, BEC, and each bout feature were analyzed

using two-way ANOVA with factors of genotype and sex. Significant genotype x sex interactions were followed up using one-way ANOVAs for genotype within each sex. Main effects of genotype were followed up using the Tukey HSD post-hoc test. For all tests α=0.05. Statistical trends are discussed for p values $\geq$ 0.05 and $\leq$ 0.1.

Results

In some instances, equipment malfunction resulted in unusable data for an animal on a given drinking day. These instances were identified by the presence of physically or physiologically improbable data points (e.g. increases in recorded BioDAQ bottle weight or excessive number of total session licks) and in these cases, only the affected data were excluded from analysis while the remaining valid data points for the animal were included. Excluded data made up less than 2% of all data points on any given day. Figures show data collapsed on sex except for instances where there were significant interactions between sex and another variable.

Experiment 1

Figure 2-1 shows ethanol consumption and BECs in the lickometer DID study. HDID-1 mice showed significantly greater ethanol intake on Day 4 ($F_{1,22}$=16.973, p<0.001) and higher BECs ($F_{1,22}$=75.911, p<0.001) than HS mice. Figure 2-2 shows ethanol bout features for Day 4 of the lickometer DID test. The number of bouts did not differ between the genotypes while the HDID-1 mice had significantly larger average bout size (licks per bout) than the HS mice ($F_{1,20}$=4.388, p=0.049). There was also a statistical trend toward a significant effect of genotype on total number of licks ($F_{1,21}$=4.017, p=0.058), with HDID-1 mice having more licks than HS. There was no

significant difference between the genotypes for IBI, bout length, or lick rate ($p>0.1$ for all, data not shown). HDID-1 and HS mice differed significantly in average lick volume, with HDID-1 mice having a larger lick volume (ml/lick) than HS mice ($F_{1,22}=8.393$, $p=0.008$). Analysis of water intake and bout features showed no significant differences between the lines for any of the variables ($p>0.1$ for all, data not shown).

Experiment 2

Ethanol intake and Day 4 BEC for the single-bottle DID test are shown in Figure 2-3. Analysis of Day 4 intake showed the expected significant main effect of genotype ($F_{2,103}=28.193$, $p<0.001$), with HDID-1 and HDID-2 mice consuming significantly more ethanol than HS mice. There was no significant main effect of sex and no significant genotype x sex interaction ($p>0.1$ for both). Analysis of BEC also showed a significant main effect of genotype ($F_{2,87}=28.204$, $p<0.001$), with HDID-1 and HDID-2 mice having significantly higher BECs than HS mice ($p<0.001$ for both). There was a trend toward higher BECs in males ($F_{1,87}=2.940$, $p=0.09$), but no significant genotype x sex interaction ($p>0.1$).

Figure 2-4 shows the bout features of ethanol intake on Day 4 of the single-bottle DID test. Analysis of the number of drinking bouts found significant effects of both genotype ($F_{2,101}=15.347$, $p<0.001$) and sex ($F_{1,101}=4.112$, $p=0.045$), but no significant genotype x sex interaction ($p>0.1$). Male mice had a greater number of drinking bouts than female mice, and HDID-2 mice had a greater number of drinking bouts than HDID-1 and HS mice ($p\leq0.001$). Analysis of IBI also showed significant main effects of genotype ($F_{2,88}=3.932$, $p=0.023$) and sex ($F_{1,88}=5.846$, $p=0.018$). Male mice had a longer average IBI than female mice, and post-hoc analyses showed HDID-2 mice had

significantly shorter IBIs than HS mice (p=0.025), but HDID-1s did not (p=0.110). Analysis of bout size showed only a significant main effect of genotype ($F_{2,98}$=5.513, p=0.005), with post-hoc analyses showing that HDID-1 mice had significantly larger bouts than both HDID-2 and HS mice ($p \leq 0.035$). For bout length, there was only a significant main effect of sex ($F_{1,96}$=37.779, p<0.001) with female mice having significantly longer bouts than male mice.

We were also interested in patterning of intake during the Day 4 drinking session and possible changes in bout structure across the 4 day DID test. Figure 2-5 shows the cumulative record of ethanol intake for all three genotypes during the 4 hr test. Repeated measures ANOVA was used to analyze Day 4 intake in 20-min time bins. There were no significant interactions of time and sex or time and genotype, and no significant 3-way interaction. There was a significant effect of time ($F_{11,1122}$=2.710, p=0.003 [Greenhouse-Geisser correction]). Bonferroni-corrected post-hoc comparisons of intake during each time bin showed that intake in the first 20-min bin was significantly lower than the intake during bins 4, 5, 6, 7, and 12 ($p \leq 0.031$ for all).

Bout number and bout size change scores are shown in supplemental figure S2-1. We computed a change score for bout number by subtracting the number of bouts on Day 1 from the number of bouts in the first 2 h of drinking on Day 4. The average change score for each genotype was negative, indicating that the number of bouts in 2 h decreased from Day 1 to Day 4. Analysis of change scores showed statistical trends toward main effects of line ($F_{2,103}$=2.824, p=0.064) and sex ($F_{1,103}$=2.816, p=0.096), but no statistically significant effects or interactions. Bout size also changed between Day 1 and Day 4, with all genotypes increasing bout size across the DID test. Analysis of bout

size change score showed significant main effects of genotype ($F_{2,89}$=6.609, p=0.002) and sex ($F_{1,89}$=4.588, p=0.035). Male mice had greater change scores (i.e., larger increase in bout size) than female mice, and post-hoc analyses showed that HDID-1 mice had greater change scores than either HDID-2 or HS ($p \leq 0.021$ for both).

Two-bottle choice ethanol intake and water intake are shown in Figure 2-6. Total ethanol intake analysis on Day 4 revealed main effects of genotype and sex ($F_{1-2,64} \geq 3.805$, $p \leq 0.027$), and no significant interaction. Tukey post-hoc analyses showed that HDID-1 mice drank significantly more ethanol than HS mice (p=0.026) and male mice drank more ethanol than female mice (p=0.009). For water intake during the two-bottle choice test there was a significant main effect of genotype ($F_{2,63}$=13.051, p<0.001), with HDID-2 mice consuming more water than either HDID-1 or HS ($p \leq 0.014$ for both). There was no significant effect of sex and no significant genotype x sex interaction (p>0.1 for both). Analysis of ethanol preference ratio (ethanol intake/total intake) yielded a significant main effect of sex ($F_{1,63}$=41.877, p<0.001) and a significant main effect of line ($F_{2,63}$=7.252, p=0.001). Male mice had a greater ethanol preference ratio than female mice (means±SEM: 33.695±4.367 and 25.744±3.923, respectively). Tukey post-hoc analyses showed that HDID-1 mice had a greater preference ratio than both HDID-2 and HS mice ($p \leq 0.01$ for both). A combination of low g/kg ethanol intake and technical issues with the gas chromatography assay during the two-bottle choice DID resulted in only a negligible number of animals registering non-zero BECs and BEC was therefore not analyzed for this test.

Figure 2-7 shows ethanol and water bout features for the two-bottle choice DID test. There was a significant main effect of sex ($F_{1,64}$=5.319, p=0.024) and a trend toward

an effect of genotype ($F_{2,64}$=2.831, p=0.066) for the number of ethanol bouts. Male mice had more ethanol bouts than female mice (mean±SEM: 5.806±0.718 and 3.676±0.534, respectively), and HDID-2 mice tended to have more bouts than the other genotypes. For the number of water bouts, there was a significant main effect of line ($F_{2,64}$=15.588, p<0.001); HDID-2 mice had significantly more water bouts than HDID-1 and HS mice (p≤0.003), and HS mice had more water bouts than HDID-1 mice (p=0.024). Analysis of ethanol bout size found a significant main effect of genotype ($F_{2,65}$=3.49, p=0.036), with HDID-1 mice having larger ethanol bouts than HS mice (p=0.047) and a trend toward larger bouts than HDID-2 mice (p=0.058). Analysis of water bout size found only a significant genotype x sex interaction ($F_{2,65}$=5.082, p=0.009). Follow-up ANOVAs showed that this was due to a significant main effect of genotype in the male mice only ($F_{2,33}$=4.575, p=0.018). HDID-2 male mice had significantly more water drinking bouts than HS mice (p=0.015). Supplemental figure S2-2 shows IBI and bout length data for ethanol and water. Analysis of average ethanol IBI found significant main effects of genotype ($F_{2,52}$=6.434, p=0.003) and sex ($F_{1,52}$=9.216, p=0.004). Female mice had longer IBIs than male mice (mean±SEM: 54.918±8.073 and 34.194±3.633, respectively), and post-hoc tests showed that HDID-1 mice had longer IBIs than HDID-2 mice (p=0.002). For average water IBI there was only a significant main effect of genotype ($F_{2,62}$=3.937, p=0.025), with HDID-1 mice again having longer IBIs than HDID-2 mice (p=0.021). Analysis of average bout length found no significant effects or interactions for either ethanol or water.

Saccharin intake is shown in Figure 2-8. Analysis of total intake on Day 4 showed a main effect of genotype ($F_{2,83}$=18.919, p<0.001) and a significant genotype x sex

interaction ($F_{2,83}$=6.996, p=0.002). Follow-up one-way ANOVAs for genotype were run for each sex. For males, there was a statistical trend toward a main effect of genotype ($F_{2,55}$=3.005, p=0.058). For female mice, there was a significant main effect of genotype ($F_{2,28}$=16.386, p<0.001) and post-hoc analyses showed greater saccharin intake by HDID-2 mice than either HDID-1 or HS (p<0.001 for both).

Figure 2-9 shows saccharin bout features for Day 4. For the number of drinking bouts, there were significant main effects of genotype ($F_{2,82}$=9.023, p<0.001) and sex ($F_{1,82}$=8.541, p=0.004). HDID-2 mice had more drinking bouts than either HDID-1 or HS mice (p≤0.018 for both), and male mice had more drinking bouts than female mice, with no significant interaction. Analysis of IBI showed a significant main effect of genotype ($F_{2,82}$=12.15, p<0.001) and a trend toward a significant main effect of sex ($F_{1,82}$=3.34, p=0.071). Post-hoc analyses showed that HDID-2 mice had shorter IBIs than HDID-1 and HS mice, and HS mice had shorter IBIs than HDID-1 mice ($p \leq 0.048$ for all). There was no significant genotype x sex interaction. For bout size, there was a significant main effect of sex ($F_{1,82}$=17.046, p<0.001) and a significant genotype x sex interaction ($F_{2,82}$=11.313, p<0.001). Follow-up one-way ANOVAs were run for each sex with genotype as the factor. Male mice showed a significant main effect of genotype ($F_{2,54}$=4.634, p=0.014) with HDID-2 mice having significantly smaller bouts than HS (p=0.01). For female mice there was also a significant main effect of genotype ($F_{2,28}$=4.969, p=0.014). This effect differed from the pattern of the males, however, with HDID-2 female mice having larger bouts than HS (p=0.013) and a trend toward larger bouts than HDID-1 as well (p=0.07). Analysis of bout length found significant main effects of genotype ($F_{2,82}$=4.344, p=0.016) and sex ($F_{1,82}$=21.31, p<0.001), and a

significant genotype x sex interaction ($F_{2,82}$=4.636, p=0.012). Follow-up one-way ANOVAs for each sex found a significant effect of genotype for female mice ($F_{2,28}$=4.169, p=0.026), but no significant effect for male mice (p>0.1). Post-hoc analyses showed that female HDID-2 mice had longer bouts than HS mice (p=0.032) and a trend toward having longer bouts than HDID-1 mice as well (p=0.062).

Discussion

In these experiments, we identified specific features of the ethanol drinking microstructure that have changed as a result of HDID selection. Specifically, we found that the HDID-1 and HDID-2 replicate lines differ in their ethanol bout structures while not differing in total ethanol intake or BEC. The HDID-1 mice appear to achieve high intake and high BECs primarily through taking in a large ethanol dose per bout. These mice have a similar number of ethanol bouts compared with the HS mice, but their g/kg intake per bout is significantly larger. The increase in bout size, however, is not accompanied by a change in the length of the bout. This bout size phenotype is consistent with what was previously observed in these mice at sub-binge levels of intake (Barkley-Levenson & Crabbe, 2012), but the findings here show that their greater bout size is also present during binge-like drinking. Furthermore, the results from Experiment 1 shed light on how the HDID-1 mice achieve their greater g/kg intake per bout. While there was no significant difference between the HDID-1 and HS mice in their average drinking rate (licks/min), the HDID-1 mice were found to have a significantly larger lick volume, i.e. these mice consume more ethanol per each lick of the sipper tube. This greater lick volume in the HDID-1 mice was specific to ethanol, with no difference seen between the lines for water lick volume. Thus, HDID-1 mice appear to be more ‘efficient’ at drinking

ethanol than the HS mice, taking in a larger quantity of ethanol than HS mice with each lick. This difference in lick volume also probably explains the significant line difference in g/kg intake in the absence of a significant difference in total licks during the session. In contrast to the findings for HDID-1, Experiment 2 showed that HDID-2 mice do not significantly differ from HS mice in ethanol bout size. Instead, high ethanol intake and BECs in the HDID-2 mice appear to result from a greater number of drinking bouts during the session. Thus, HDID-2 drinking phenotype shows enhanced drinking frequency as compared with the enhanced drinking efficiency of the HDID-1 mice. Differences in the number of drinking bouts (8.3 ± 0.46 for HDID-2 vs. 4.2 ± 0.64 for HS) or drinking bout size (0.51 ± 0.04 for HDID-1 vs. 0.36 ± 0.04 for HS), therefore, cumulatively appear to explain significant differences in overall phenotype (g/kg intake and BEC) between the HDID and HS mice. While these differences appear relatively small in absolute magnitude, they represent a 50% reduction in number of drinking bouts and a 29% reduction in bout size. It has been suggested that drinking microstructure variables in animals such as bout size and IBI might serve as models for more complex behaviors such as loss of control of drinking or alcohol craving, respectively (Samson, 2000). The distinct drinking microstructures of the HDID replicates could consequently suggest underlying deficits in drinking termination (HDID-1s) or dysregulated appetitive mechanisms (HDID-2s), and these possibilities will need to be explored in future studies.

In the two-bottle choice DID test, a similar pattern of results was seen with regard to the ethanol bout structure differences between the lines. However, the genotypic differences were weaker, with several significant only at trend levels. This presumably reflects the attenuated HDID phenotype that is seen here and in previous studies during

two-bottle choice DID wherein ethanol intake and BEC are lower than that seen with single-bottle DID (Barkley-Levenson & Crabbe, 2012; Crabbe et al., 2012a, 2012b). Continuous access two-bottle choice preference drinking and DID have been shown to be largely genetically distinct traits (Crabbe et al., 2012a, 2012b; Phillips et al., 2010). However, the present results suggest that the bout structure of ethanol intake does appear to generalize across procedures. HDID-1 mice had a larger ethanol bout size than HS mice in both the single-bottle DID test and the two-bottle choice procedure, although the effect was more modest when water was also present. Interestingly, the HDID-2 enhanced drinking frequency was also observed for water bouts (and saccharin, see below). This indicates that a greater bout number may be a feature of HDID-2 drinking regardless of fluid type. In contrast, the HDID-1 mice did not have a larger water bout size than the other genotypes and their enhanced ethanol bout size appears to be fluid specific.

Saccharin DID showed that all three genotypes are capable of similarly large bout sizes when drinking a different palatable rewarding solution. The genetic differences in ethanol bout size are therefore not likely due to some sort of ingestive deficit in the HDID-2 and HS mice. The HDID-2 mice did have significantly more saccharin bouts than the other genotypes, however, providing further evidence that this drinking pattern is not specific to ethanol. Additionally, an interesting genotype x sex interaction was found for saccharin DID intake. Female HDID-2 mice consumed significantly more saccharin than the other genotypes and sexes. These mice do not differ in their saccharin preference during a 24-h two-bottle choice test (Crabbe et al., 2011), so this difference appears to be specific to binge-like saccharin exposure and/or the time period tested (i.e. the early part

of the dark cycle). It is not certain why this effect was seen only in the female HDID-2s. In rat lines selectively bred for saccharin preference, female rats consume more saccharin than male rats (Carroll et al., 2008), so it may be that this is an expected sex difference in high-saccharin consuming animals. Sucrose DID has not yet been tested in any of these genotypes, but it would be an interesting follow-up to determine whether the sex and genotypic differences in total intake and microstructure extend to caloric sweet solutions as well. Sweet preference and alcohol consumption have been shown to be genetically related in some rodent lines selected for high vs. low alcohol preference (Sinclair et al., 1992) as well as in human alcoholics (Kampov-Polevoy et al., 2003). The HDID-2 female mice may then provide a good model for this association between sweet intake and ethanol intake, but specific to a binge-like pattern of consumption. The presence of this phenotype in only one of the HDID replicate lines, however, suggests that binge-like saccharin consumption is not a correlated response to selection *per se* and that the genetic relationship between alcohol drinking and sweet preference is not a necessary component of the HDID selection phenotype.

Changes in bout microstructure were also evident across the 4 days of ethanol DID. Specifically, all genotypes showed an increase in bout size from Day 1 to Day 4, and this increase was significantly greater in the HDID-1 mice than HDID-2 or HS. The number of bouts in 2 h of drinking also decreased from Day 1 to the first 2 h of Day 4 in all animals, but the magnitude of this decrease was not significantly different between the genotypes. The higher number of bouts on Day 1 may be due to the novelty of the drinking solution, and thus declines over the DID test. We have shown previously in a 2-day DID test that HDID mice will drink to intoxicating BECs during the initial 2 h

exposure to ethanol on Day 1 (Crabbe et al., 2014). The decline in bout number across days could therefore also suggest the development of a conditioned taste aversion resulting from high ethanol intake on Day 1. Intake on the first day of a 4-day DID does not correlate with intake on subsequent days, although intake on Days 2–4 show significant correlations with each other (Rhodes et al., 2005). Therefore, the changes in microstructure between Days 1 and 4 seen here may simply reflect distinct drinking patterns inherent to each session (or to initial vs. repeated exposure to ethanol) that underlie the unrelated intake on these 2 days. It is not yet known how microstructure might continue to change with repeated ethanol exposures. The HDID mice have been shown to be largely resistant to escalation of drinking induced by offering ethanol intermittently during limited access (Crabbe et al., 2012b), but they do escalate their drinking in a two-bottle choice procedure with longer exposures (Rosenwasser et al., 2013), and HDID-1 mice showed increased two-bottle choice DID-like drinking over many weeks (Crabbe et al., 2011). Thus, future studies will be needed to determine how a more chronic drinking schedule may alter bout structure and patterning in these mice.

The patterning of ethanol intake during the DID session in the HDID mice shows an interesting contrast with previous reports of drinking timing in the inbred B6 mice. B6 mice have repeatedly been shown to drink with a characteristic ‘front-loading’ behavior wherein most of their intake occurs during the early part of the drinking session, and the rate of this early session drinking has been seen to increase with repeated ethanol experience (Griffin et al., 2009; Linsenbardt & Boehm, 2014; Rhodes et al., 2007; Wilcox et al., 2014). In Experiment 2, HDID and HS mice showed fairly consistent rate of intake throughout the initial 2-h drinking session on Day 1 of the single-bottle DID

test. On Day 4, intake rate remained consistent for much of the session, although mice of all three genotypes showed their lowest rate of consumption at the beginning of the session and drank significantly less ethanol in the first 20 min than in some of the later time points. This difference in drinking pattern between the HDID and B6 mice shows that multiple ethanol drinking patterns are related to high intake and high BECs during limited access drinking.

There are several procedural considerations that might have an effect on the results seen in these experiments. First, there is the potential issue of the within-subjects design of Experiment 2. In this experiment, animals were tested sequentially on two to three DID procedures. The testing procedure was designed to try to minimize any potential carry-over effects by including sufficient water-only washout periods between each DID procedure, and also by testing saccharin last so as to prevent confounds due to prior sweet tastant experience. However, we cannot be certain that repeated testing had no effects. Consequently, it may be beneficial to test preference drinking DID and saccharin DID again in separate experiments with naïve animals to ensure that the group differences do not change. Additionally, animals across both experiments were from a range of selection generations, and the HDID-2 mice in general are not as far along in the selection procedure as the HDID-1 mice. It is possible, therefore, that observed replicate differences are simply the result of less advanced selection in the HDID-2 mice. Genetic variance has been shown to be lower in the HDID-1 mice as compared with the HDID-2, presumably due to increased inbreeding and fixation of alleles from the greater number of selections (Iancu et al., 2013). Greater genetic variation in the HDID-2 mice could explain greater variation in behavior, such as the generalized enhanced bout frequency

across DID tests and fluid types. However, HDID-2 mice show a robust selection phenotype with over 50% of the mice in Generation S19 reaching BECs of 1.0 mg/ml or higher. It is therefore uncertain whether the drinking microstructures of the HDID-2 will change as selection continues. It may be necessary to repeat these experiments again in the future after the HDID-2 mice are farther along in selection.

The HDID selection for high BECs following binge-like drinking has resulted in two distinct drinking patterns that both lead to the selection phenotype and drinking to intoxication. This parallels findings from human research that suggest there are numerous ways to pattern intake to result in intoxication and harmful drinking. Consequently, the replicates of the HDID lines will be useful for teasing apart the specific genetic contributions to different aspects of drinking structure (drinking bout size and lick volume vs. drinking frequency) and how drinking microstructure might relate to other behaviors. Furthermore, we have recently undertaken the start of an additional HDID line by crossing the HDID-1 and HDID-2 replicates using the strategy successfully employed with the high and low alcohol preferring mice (Oberlin et al., 2011). This cross should result in an enhanced HDID phenotype by capturing the specific genetic contributions to the selection phenotype in each replicate line. As the HDID replicates arrive at high BECs via different bout structures, we might expect that the HDID cross mice will have a combination of these drinking structures with both an increased bout size and bout number. Future testing of the ethanol drinking microstructure of the HDID cross will show whether this is the case, and whether it promotes even higher intake and BECs during ethanol DID.

Acknowledgments

The authors would like to thank Stephanie Spence and Andy Jade Cameron for assistance with analysis of blood samples for BEC determination. We would also like to thank Drs. Matthew Ford and Marcia Ramaker for expert assistance with lickometer data collection and analysis, and Dr. Tamara Phillips for generous sharing of her lickometer equipment. These studies were supported by NIH-NIAAA grants AA13519, AA10760, and by a grant from the Department of Veterans Affairs (101BX000313). AMB-L was supported by NIH-NIAAA grants AA02009 and AA007468, the Achievement Rewards for College Scientists foundation, and an Oregon Health & Science University Graduate Research Scholar award.

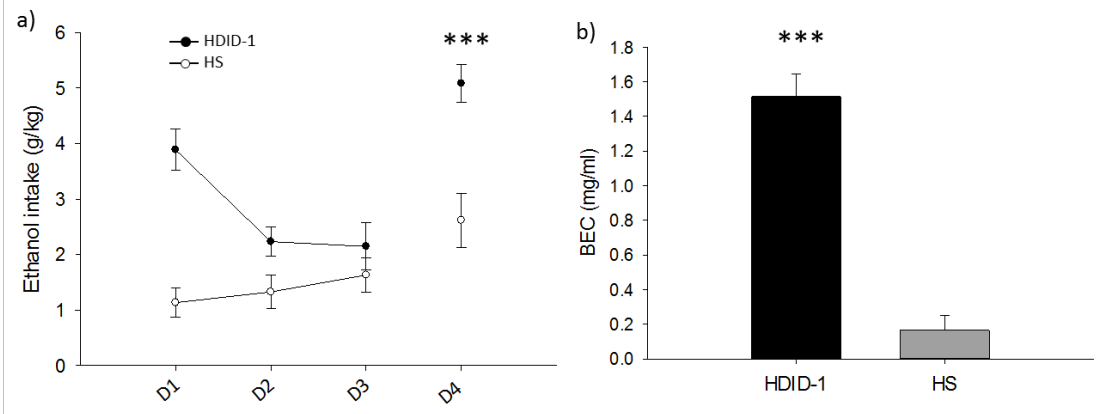


Figure 2-1. Daily ethanol DID intake (a) and Day 4 BECs (b) in the lickometer for HDID-1 and HS male mice in Experiment 1. Ethanol intake (g/kg) is shown for each 2 h drinking session (D1-D3) and for the final 4 h drinking session (D4). BEC (mg/ml) was determined after drinking on Day 4. Means ±SEM shown. N=12/line. *** indicates statistically significant difference from HS mice ($p \leq 0.001$).

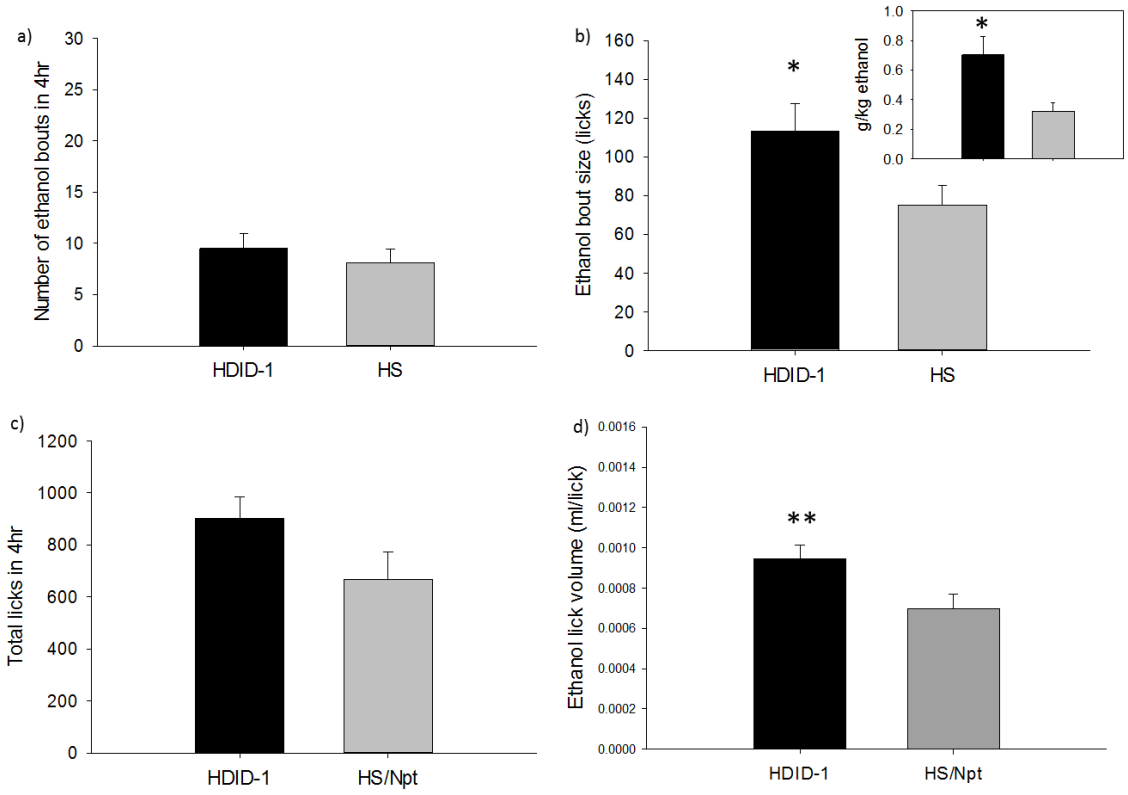


Figure 2-2. Ethanol bout features on Day 4 of the lickometer DID test for HDID-1 and HS male mice in Experiment 1. Panel a shows the number of bouts, panel b shows the bout size in licks (inset shows g/kg bout size), panel c shows the total number of licks in the session, and panel d shows the average lick volume. Means ±SEM shown. N=12/line. * indicates statistically significant difference from HS mice ($p<0.05$), ** indicates $p\leq0.01$.

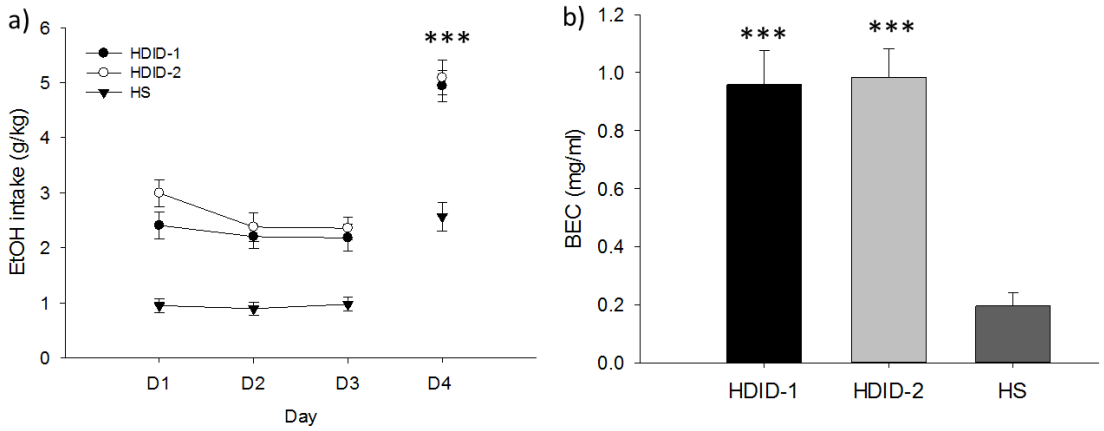


Figure 2-3. Daily ethanol intake (a) and Day 4 blood ethanol concentrations (b) during BioDAQ single-bottle ethanol DID test in Experiment 2. Ethanol intake (g/kg) is shown for each 2 h drinking session (D1-D3) and for the final 4 h drinking session (D4). BEC (mg/ml) was determined after drinking on Day 4. Means ±SEM shown. N=31-65/line. *** indicates statistically significant difference from HS mice ($p \leq 0.001$).

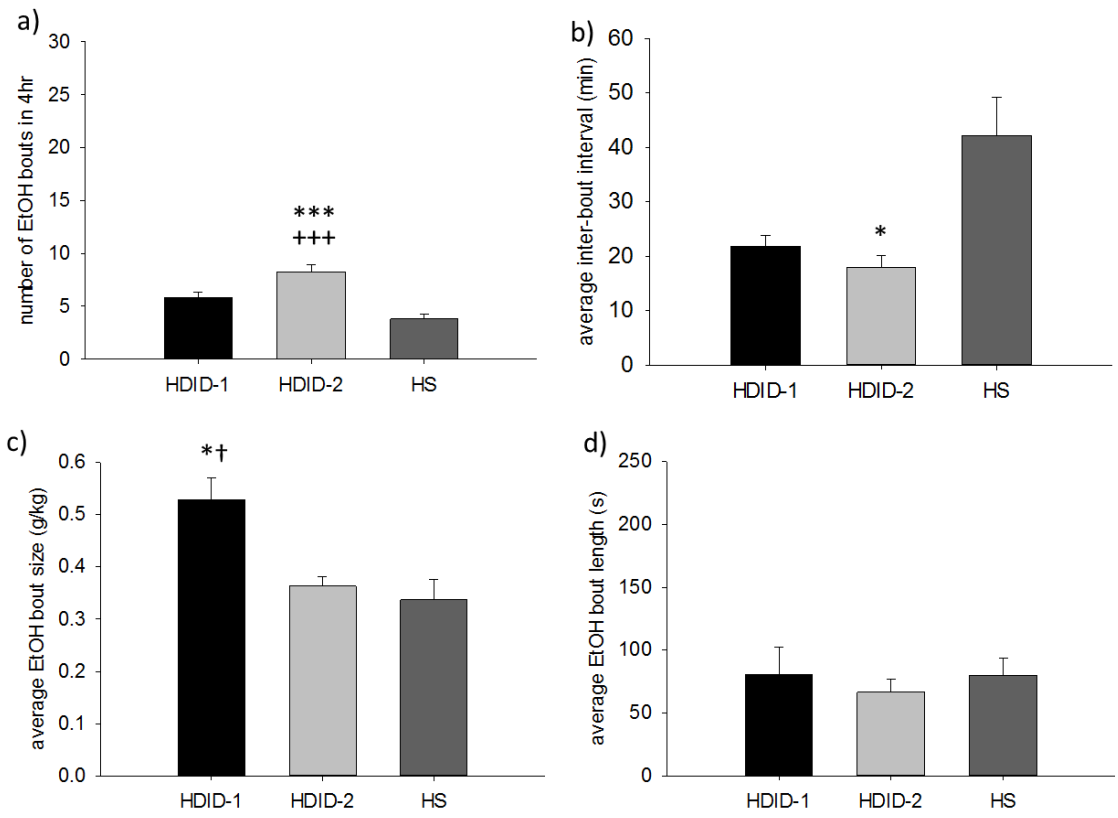


Figure 2-4. Ethanol bout features on Day 4 of the BioDAQ single-bottle ethanol DID test. Drinking structure variables recorded were the number of drinking bouts (a), the average inter-bout interval (b), average g/kg bout size (c), and average bout length (d). Means ±SEM shown. N=31-65/line. * indicates statistically significant difference from HS mice ($p<0.05$; *** indicates $p\leq0.001$). +++ indicates statistically significant difference from HDID-1 ($p\leq0.001$). † indicates statistically significant difference from HDID-2 ($p<0.05$).

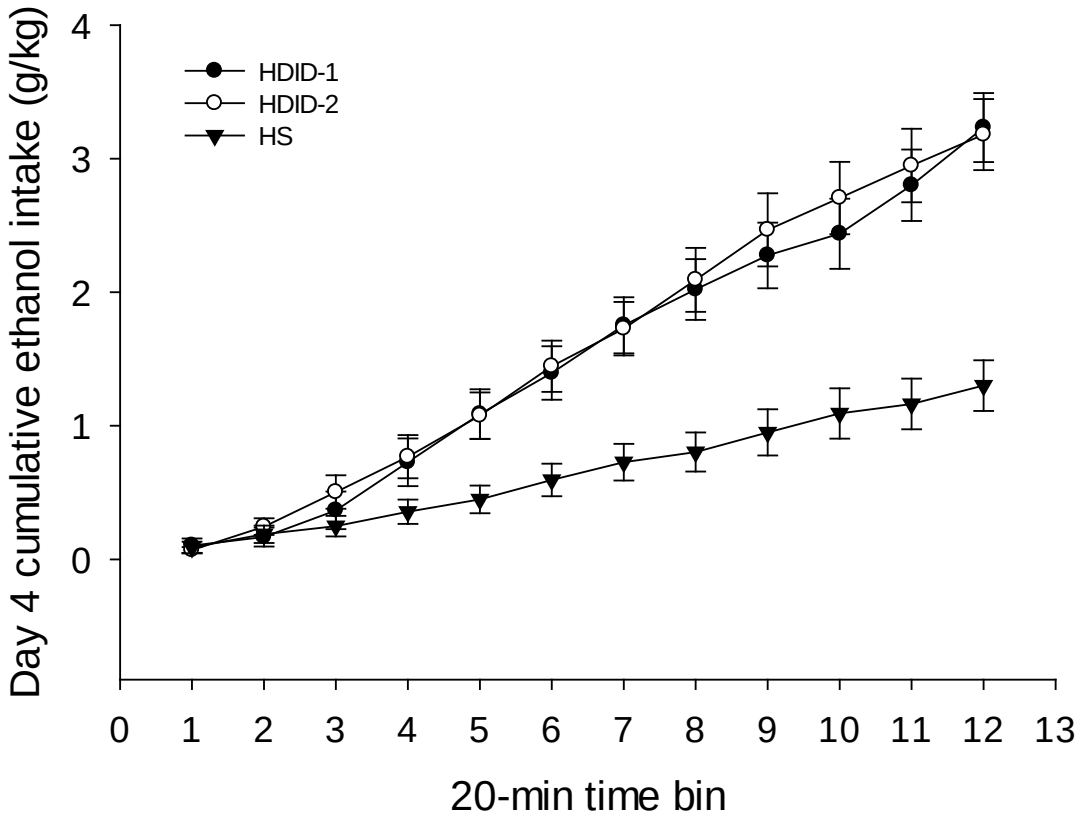


Figure 2-5. Cumulative record of g/kg ethanol intake on Day 4 of the BioDAQ single-bottle ethanol DID test for all three genotypes. N=31-65/line

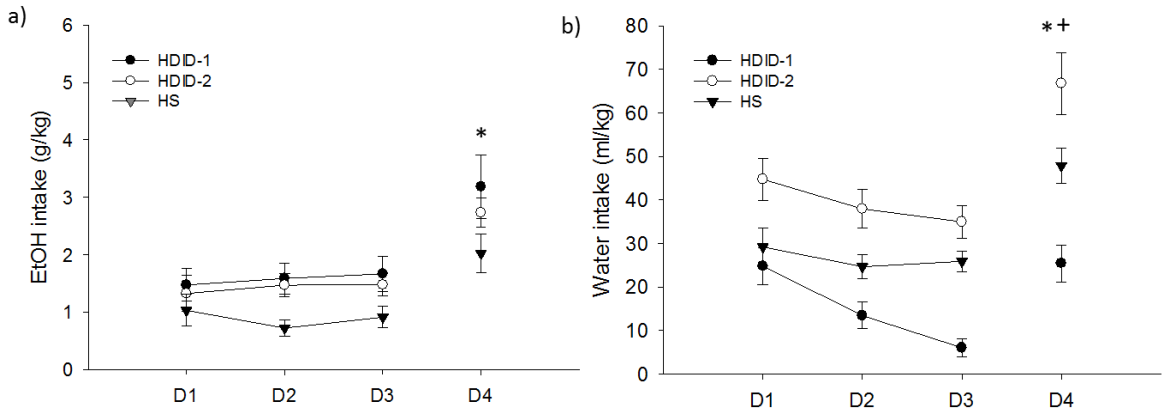


Figure 2-6. Daily ethanol (a) and water (b) intake during the BioDAQ two-bottle choice DID test in Experiment 2. Ethanol intake (g/kg) and water intake (ml/kg) are shown for each 2 h drinking session (D1-D3) and for the final 4 h drinking session (D4). Means ±SEM shown. N=17-30/line. * indicates statistically significant differences from HS ($p<0.05$). + indicates statistically significant difference from HDID-1 ($p<0.05$)

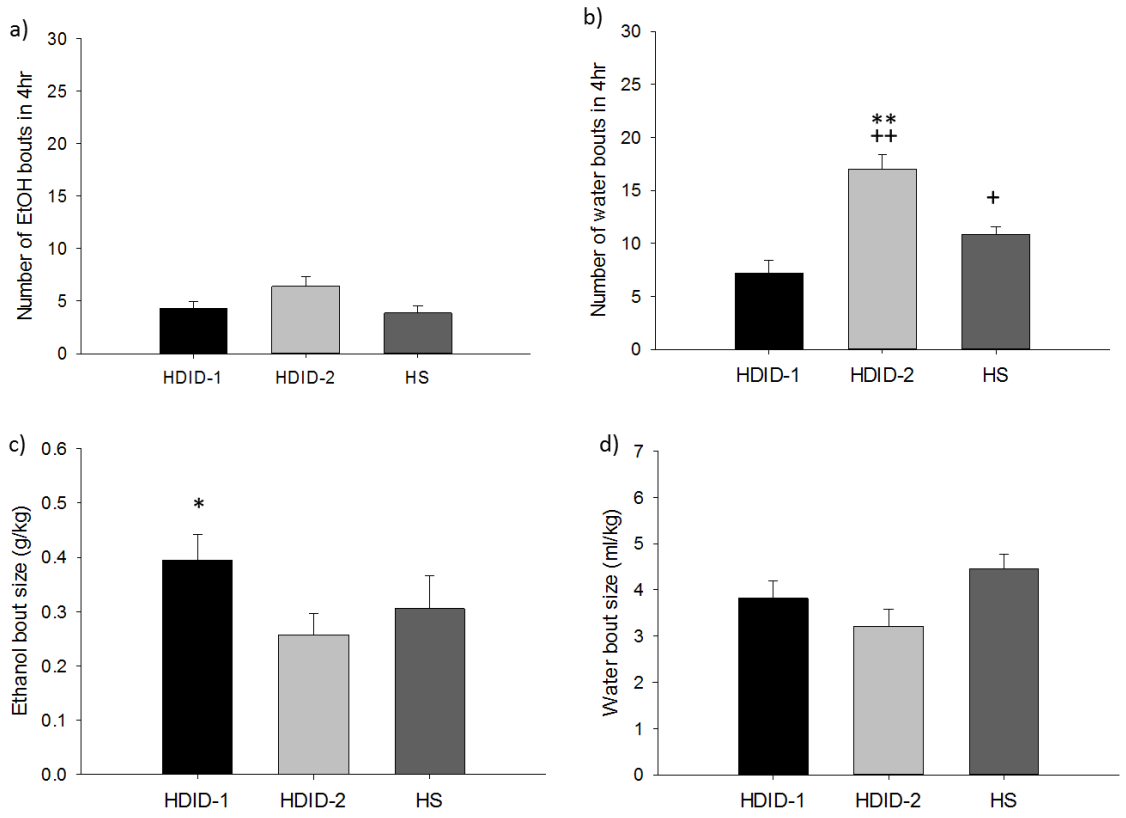


Figure 2-7. Ethanol and water bout features on Day 4 of the BioDAQ two-bottle choice DID test in Experiment 2. The number of drinking bouts are shown for ethanol (a) and water (b). Drinking bout size are shown as g/kg for ethanol (c) and ml/kg for water (d). Mean ±SEM shown. N= 17-30/line. * indicates statistically significant difference from HS ($p<0.05$; ** indicates $p\leq0.01$). + indicates statistically significant difference from HDID-1 ($p<0.05$; ++ indicates $p\leq0.01$).

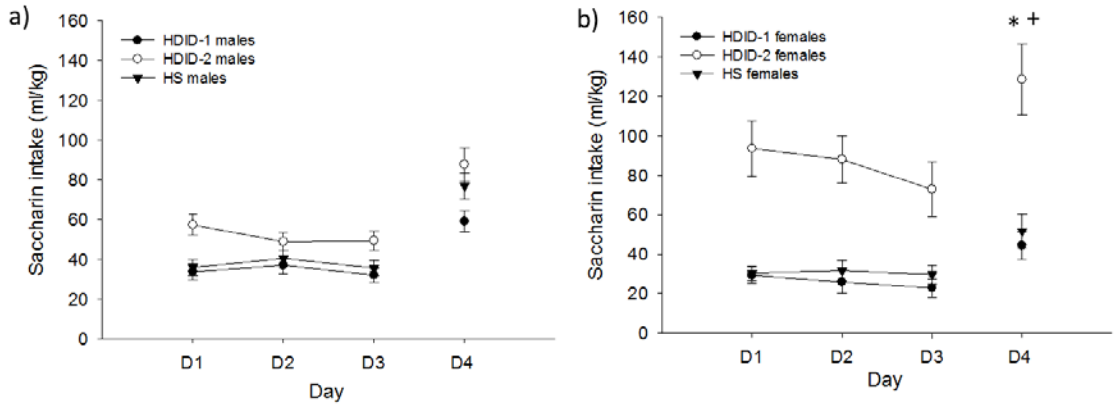


Figure 2-8. Daily saccharin DID intake for males (a) and females (b) of each genotype during the BioDAQ saccharin DID test in Experiment 2. Saccharin intake (ml/kg) is shown for each 2 h drinking session (D1-D3) and for the final 4 h drinking session (D4). Means ±SEM shown. N=10-31/sex/line. * indicates statistically significant difference from HS ($p<0.05$) and + indicates statistically significant difference from HDID-1 ($p<0.05$).

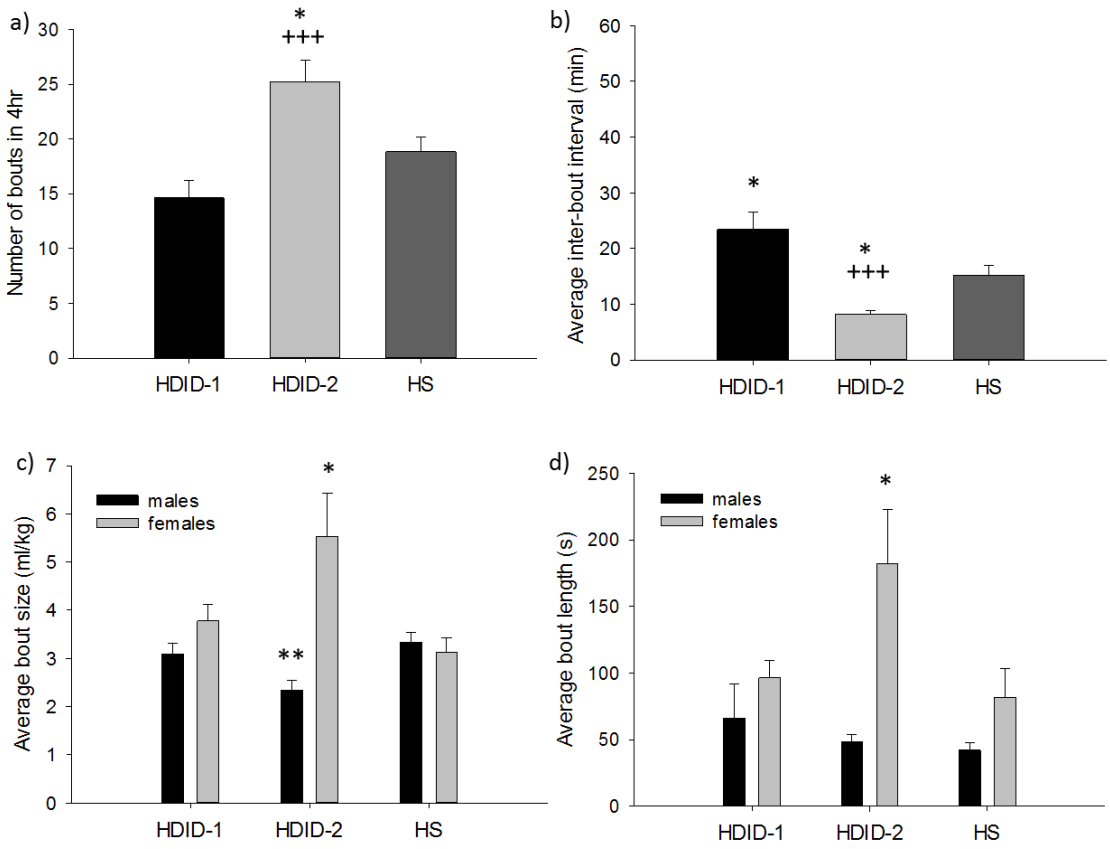


Figure 2-9. Saccharin bout features on Day 4 of the BioDAQ saccharin DID test. Drinking structure variables recorded were number of drinking bouts (a), average inter-bout interval (b), average ml/kg bout size (c), and average bout length (d). Bout size and bout length had significant sex x genotype interactions and are shown here broken down by males and females of each line. Means ±SEM shown. N=31-65/line in panels a and b and n=10-31/sex/line in panels c and d. In panels a and b, * indicates statistically significant difference from HS ($p<0.05$) and +++ indicates statistically significant difference from HDID-1 ($p\leq0.001$). In panels c and d, * indicates statistically significant difference from same-sex HS group ($p<0.05$; ** indicates $p\leq0.01$).

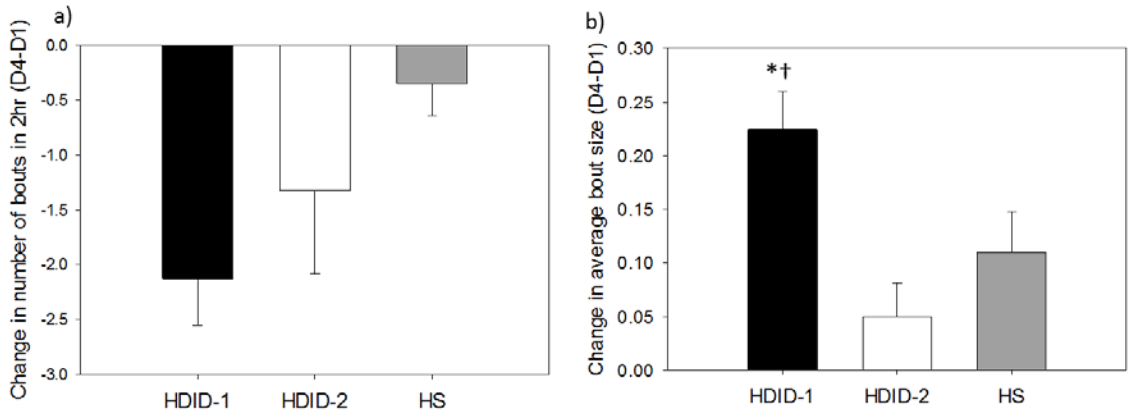


Figure S2-1. Change scores for number of ethanol bouts in 2 h (a) and average ethanol bout size (b). Change score for number of bouts represents the number of bouts on Day 1 of the BioDAQ single-bottle ethanol DID subtracted from the number of bouts in the first 2 h on Day 4. Bout size change score represents the average bout size on Day 1 subtracted from the average bout size on Day 4. Means ±SEM shown. N=31-65/line. * indicates statistically significant difference from HS and † indicates statistically significant difference from HDID-2 ($p<0.05$)

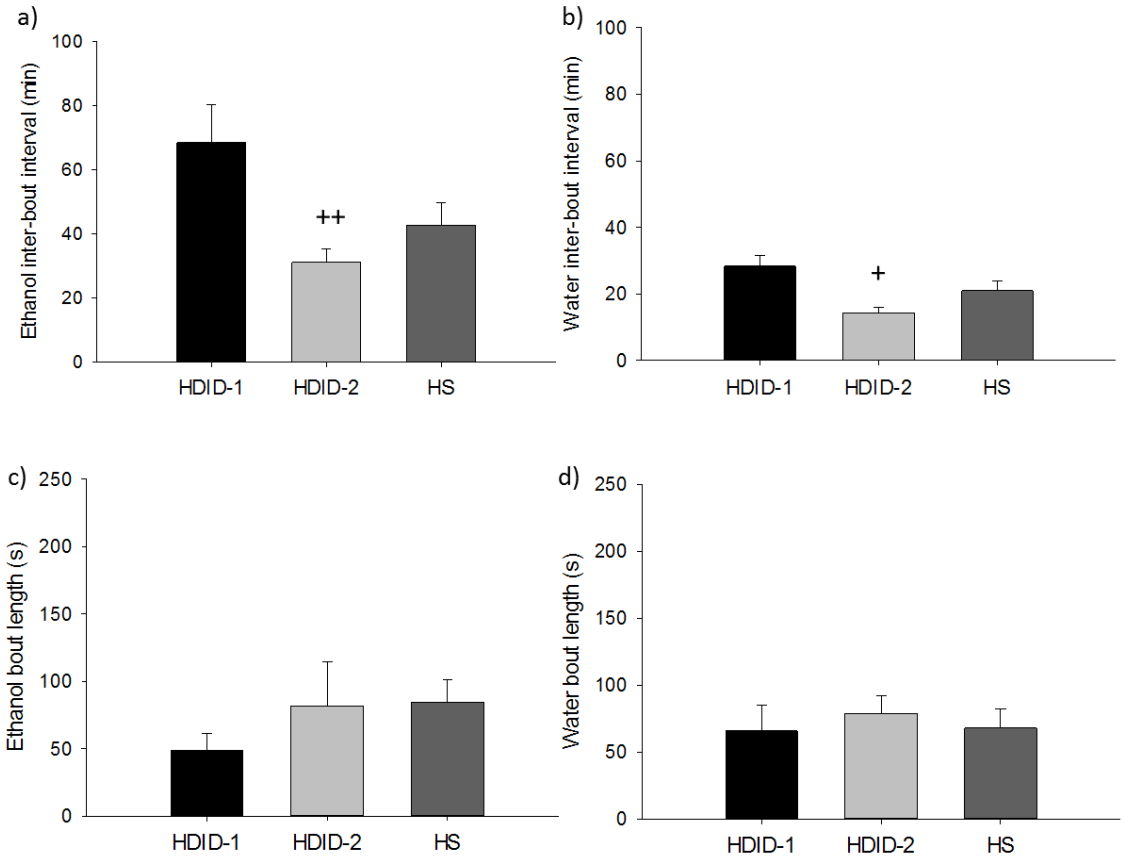


Figure S2-2. Ethanol and water interbout intervals (a and b) and average bout lengths (c and d) on Day 4 of the BioDAQ two-bottle choice DID test. Means ±SEM shown. N=17-30/line. + indicates statistically significant difference from HDID-1 ($p<0.05$; ++ indicates $p\leq0.01$).

Chapter 3: Rewarding and aversive effects of ethanol in High Drinking in the Dark selectively bred mice

Amanda M. Barkley-Levenson, Christopher L. Cunningham, Phoebe J. Smitasin, and John C. Crabbe

This chapter has been previously published in *Addiction Biology* (2015), 20(1): 80-90

Abstract

Both rewarding and aversive effects contribute to alcohol consumption. Animals genetically predisposed to be high drinkers show reduced sensitivity to the aversive effects of alcohol, and in some instances, increased sensitivity to alcohol's rewarding effects. The present studies tested the High Drinking in the Dark (HDID) selected lines, a genetic model of drinking to intoxication, to determine whether intake in these mice was genetically related to sensitivity to alcohol aversion or reward. Male HDID mice from the first and second replicate lines (HDID-1 and HDID-2, respectively) and mice from the heterogeneous progenitor control population (HS/Npt, or HS) were conditioned for a taste aversion to a salt solution using 2 doses of alcohol, and lithium chloride (LiCl) and saline controls. In separate experiments, male and female HDID-1, HDID-2, and HS mice were conditioned for place preference using alcohol. HDID mice were found to have an attenuated sensitivity to alcohol at a moderate (2 g/kg) dose compared to HS mice, but did not differ on conditioned taste aversion to a high (4 g/kg) dose or LiCl or saline injections. HDID and HS mice showed comparable development of alcohol-induced conditioned place preference. These results indicate that high blood alcohol levels after drinking in the HDID mice is genetically related to attenuated aversion to alcohol, while sensitivity to alcohol reward is not altered in these mice. Thus, HDID mice may find a moderate dose of alcohol to be less aversive than control mice and consequently may drink more because of this reduced aversive sensitivity.

Introduction

Alcohol (ethanol) has some seemingly paradoxical effects, as ethanol treatment can produce responses indicating both reward and aversion. In humans, ethanol's rewarding effects are frequently associated with euphoria, stimulation, and drug liking. In contrast, aversive effects are often associated with dysphoria and sedation (Martin et al., 1993; Morean et al., 2013). These rewarding (positive) and aversive (negative) stimulus effects of ethanol can be present at the same time (for review, see Verendeev & Riley, 2013). Thus, perception of the motivational value of ethanol is presumably determined by an individual's relative sensitivity to the rewarding versus aversive effects. In this manner, sensitivity to both ethanol reward and aversion can modulate ethanol intake.

In animal studies, these motivational effects of ethanol can be readily assessed using procedures based in Pavolvian conditioning such as conditioned place preference (CPP) and conditioned taste aversion (CTA). Previous research has shown that the degree of ethanol-CTA is negatively genetically correlated with home cage preference for ethanol in a panel of inbred strains (Broadbent et al., 2002). Similarly, short-term selected mouse lines bred for strength of ethanol CTA were found to differ in their ethanol consumption, with low CTA mice consuming more ethanol than high CTA mice (Phillips et al., 2005). Mouse lines selected for high and low alcohol preference also show this inverse relationship between drinking and strength of CTA (Chester et al., 2003). In all these studies, the correlated traits are tested in separate groups of naive animals, an advantage of inbred strains and selectively bred lines.

The genetic relationship between ethanol drinking and ethanol CPP has also been examined, but the results are more variable than those seen for CTA (Green & Grahame,

2008). Short-term selected mouse lines for high and low ethanol drinking show divergent place conditioning, with the high drinkers having a greater expression of ethanol CPP (Phillips et al., 2005). However, studies of other mouse and rat lines selected for ethanol preference have failed to show this line difference for CPP (Gauvin et al., 2000), and some have shown no difference or even the opposite relationship depending on the conditioning dose used (Grahame et al., 2001). More consistent results have been seen with relating single gene effects to drinking and CPP. For example, studies of mu opioid receptor (Hall et al., 2001), dopamine D2 receptor (Cunningham et al., 2000), and cannabinoid receptor 1 knockout mice (Naassila et al., 2004; Houchi et al., 2005) have shown that knockout mice of all genotypes have reduced intake and weaker CPP as compared to wild-type controls. Overall, there appears to be a modest, but inconsistent, positive relationship between home cage ethanol consumption and ethanol CPP.

Of the studies examining the genetic relationship between ethanol drinking and the motivational effects of ethanol, most have utilized a continuous access two-bottle choice test of ethanol versus water to measure ethanol consumption. Because ethanol intake is distributed across the entire day, this assay may not result in intoxication even in high drinking animals (Dole & Gentry 1984, but see Matson & Grahame 2013). Limited access binge-like drinking, which results in intoxicating blood ethanol concentrations (BECs), has been much less well studied in relation to ethanol reward and aversion. One study of inbred strains (Rhodes et al., 2007) showed a negative genetic correlation between drinking on the final day of a limited access Drinking in the Dark (DID) test and published strain data on ethanol conditioned taste aversion (Broadbent et al., 2002), consistent with the findings from two-bottle choice experiments. Very few studies have

examined both CPP and ethanol DID, but one study did find that histamine H3 receptor knockout mice have reduced DID intake and also diminished CPP as compared to wild-type mice, suggesting a positive genetic relationship (Nuutinen et al., 2011).

In the present studies, we sought to extend these findings by assessing ethanol-motivated behaviors in animals that have been specifically bred for drinking to intoxication. The High Drinking in the Dark (HDID) lines have been selected for reaching high BECs during the DID test and can be thought of as a genetic model for binge-like drinking (Crabbe et al., 2009). Mice of these genotypes regularly drink to intoxicating BECs in the DID test (i.e., above 0.8 mg/ml) and the high intake and BECs have been shown not to be due to underlying differences in ethanol metabolism (Crabbe et al., 2009; 2014). Here, we tested naïve mice of both replicate lines (HDID-1 and HDID-2) and the control heterogeneous progenitor line (HS/Npt, or HS) for ethanol CTA and ethanol CPP to determine whether changes in sensitivity to ethanol aversion or reward have arisen as correlated responses to selection. We predicted that reduced aversive sensitivity and/or increased ethanol reward sensitivity might be present in the HDID mice and might therefore be related to their high BEC and high drinking phenotype.

Materials and Methods

Animals and Husbandry

Mice in the CPP study were bred and housed at Oregon Health & Science University (Portland, OR) and mice in the CTA studies were bred and housed in the Veterinary Medical Unit of the Veterans Affairs Portland Health Care System (Portland,

OR). Mice were weaned at 3 weeks of age and were reared with both dam and sire until weaning. After weaning, mice were housed in groups of 2-5 with same-sex littermates or with mice of the same genotype when necessary to avoid single housing. Mice were housed in standard polycarbonate shoebox cages on Bed-o-cob bedding and received *ad libitum* access to food (Purina 5001 chow, LabDiet, St. Louis, MO) and water unless otherwise specified. All mice were between 50 and 78 days of age and were experimentally naive at the start of testing. 50 day old mice were used in the CTA experiments because the habituation and fluid-restriction phases were relatively long and all mice were fully mature by the start of conditioning phase. HDID-1 mice from the 21st selection generation were used for the CTA experiment. The HDID-2 CTA experiment was run in 2 separate passes, with the first pass using mice from selection generation 14 and the second pass using mice from selection generation 17. The CPP experiment used offspring from breeder pairs taken from the 19th and 12th selection generations for HDID-1 and HDID-2 mice, respectively. HS/Npt mice were used as the control comparator line for the HDID mice in all experiments. HS/Npt mice are the starting population from which the HDID lines were selected and are the product of a systematic 8-way cross of inbred strains described in detail elsewhere (Crabbe et al., 2009). These mice can therefore be considered an outbred heterogeneous stock, and are bred with no selective pressure and thus maintain random assortment of genes. Mice were kept on a 12 h/12 h light/dark cycle with lights on at 07:00 for the CPP study, 08:00 for the HDID-1 CTA study, and 06:00 for the HDID-2 CTA studies. All procedures were approved by the local Institutional Animal Care and Use Committees and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

CTA procedure

The taste conditioning procedure was adapted from that used by Broadbent and colleagues (2002). Testing consisted of 3 phases: habituation, fluid restriction, and conditioning. At the start of the habituation phase (Days 1-7), mice were singly housed and provided with water via a 25 ml graduated cylinder fitted with a stainless steel drinking spout or a polycarbonate bottle with a stainless steel drinking spout fixed to the screw-top lid depending on experiment. Water consumption was monitored to ensure that mice learned how to drink from the sipper tube. During the fluid restriction phase (Days 8-14), water bottles were removed and access to water was provided during a 2 h period at the same time each day. Mice were weighed daily and water consumption was recorded for the fluid access period. On the first day of the conditioning phase (Days 15-27), mice were weighed and then provided with a drinking tube containing a 0.2 M sodium chloride (NaCl) solution for 1 h. After 1 h, fluid consumption was recorded and tubes were removed. Approximately 5 h after removal of the tube, mice received 30 min of access to water to prevent dehydration. On the second day of conditioning, mice received 2 h of water access as during the fluid restriction phase. This alternating pattern of NaCl conditioning trials and water access was continued such that NaCl was presented on Days 15, 17, 19, 21, 23, 25, and 27 and water was provided on the intervening days. NaCl was presented without a paired injection on Day 15 to allow familiarization of the animals with the novel drinking solution. On the remaining conditioning trials, removal of the NaCl solution after the hour of drinking was immediately followed by intraperitoneal (IP) injection with saline, 2 g/kg ethanol, 4 g/kg ethanol, or 3 mEq/kg lithium chloride (LiCl) as determined by drug group assignment. Saline injections were

used as a control for changes in NaCl consumption over time or due to handling. LiCl injections were designed to serve as a positive control to determine whether the genotypes differ in their ability to condition a taste aversion, as this LiCl dose has been shown to produce CTA in mice (Risinger & Cunningham, 2000). CTA was assessed by comparing NaCl solution consumption on the first injection-paired trial (Day 17) to consumption on the last conditioning trial (Day 27). A decrease in NaCl consumption across conditioning trials was considered to indicate the development of a taste aversion. This procedure is preferable to conditioning a taste aversion to ethanol itself because it eliminates the potential influence of pre-absorptive effects such as taste and olfaction.

HDID-1 and HDID-2 mice were tested in different experiments (see below) at different times due to animal availability and procedural necessity. Separate control groups of HS mice were consequently tested with each experiment, and the HDID-1 and -2 mice are only compared to the control group that was tested concurrent with their respective line.

Experiment 1: CTA in HDID-1 and HS

Eighty male mice were used (n=10/genotype/drug group) for this experiment. Due to the number of variable groups (four doses and two genotypes), testing both sexes would have resulted in a procedurally unfeasible number of animals. Consequently, only male mice were tested here and in Experiment 2, and female mice will need to be tested at a future date. The taste conditioning procedure was performed as described above. Drinking sessions took place between 12:30-14:30 on water days and between 12:30-13:30 on conditioning days.

Experiment 2: CTA in HDID-2 and HS

The first pass of this experiment used 79 male mice and the second pass used 77 male mice (n=9-10/genotype/drug group for each pass). Drinking sessions took place between 10:30-12:30 on water days and between 10:30-11:30 on conditioning days.

Conditioned Place Preference apparatus

The apparatus used is described in detail elsewhere (Cunningham et al., 2006). Briefly, twelve conditioning boxes (30 x 15 x 15 cm) made of acrylic and aluminum were each enclosed in a light- and sound-attenuating chamber (Coulbourn Instruments Model E10-20). General activity and the time spent on each side of the box was recorded by six sets of infrared photo emitters and detectors mounted 2.2 cm above the floor at 5-cm intervals along the wall. The experiment was conducted with no light in the chamber. The conditioning stimuli (CS) consisted of interchangeable grid and hole floor halves. The grid floor was made out of 2.3-mm stainless steel rods mounted 6.4 mm apart in acrylic rails. The hole floor was made out of perforated stainless steel sheets (16 gauge) consisting of 6.4-mm round holes with 9.5 mm staggered centers. These cues were chosen based on previous studies showing that naïve DBA/2J mice spend equal amounts of time on each cue (Cunningham et al., 2003).

Experiment 3: Ethanol CPP

One hundred and thirty-eight male and female HDID-1, HDID-2, and HS mice were used. Although sex differences were not anticipated for either CPP or CTA, female mice were readily available at the time of CPP testing and could be easily included in the experimental design and were therefore tested here in order to attain as complete a dataset as possible. All genotypes and sexes were tested at the same time. This experiment

consisted of three phases: pretest (one session), conditioning (eight ethanol sessions and eight saline sessions), and place preference testing (three sessions). Before each session, mice were placed into a sex-specific weigh bucket, weighed, and received an IP injection immediately before being placed in the apparatus. Each squad consisted of a single sex, and the order of squads was counterbalanced.

The pretest was conducted to confirm that there were no inherent biases to either floor cue. All mice were weighed, injected with saline IP, and placed in the center of the apparatus with both floors (half grid/half hole) for a single 30 min session. The position of each floor was counterbalanced and number of beam breaks and time spent on each floor were recorded.

An unbiased one-compartment place conditioning procedure was used (Cunningham et al., 2003, 2006). During conditioning sessions, mice were weighed and given an IP injection before being placed on a single floor cue for 5 min. Within the six groups, mice were assigned to either GRID+ or GRID- conditioning subgroups (n=9-12/genotype/sex/conditioning subgroup). The GRID+ subgroup received ethanol paired with the grid floor (CS+) and saline paired with the hole floor (CS-). The GRID- subgroup received ethanol paired with the hole floor (CS+) and saline paired with the grid floor (CS-). CS+ and CS- trials alternated over 16 days (counterbalanced order), with place preference tests performed after the fourth, sixth, and eighth pairs of conditioning sessions. Place preference tests were performed identically to the pretest and number of beam breaks and time spent on each floor were recorded. This procedure of multiple preference tests was used in order to allow us to capture possible genotypic differences in learning rate and asymptote during the acquisition of CPP.

Drugs

Ethanol (OHSU Research Stores, Portland, OR: 20% v/v) for the CPP study was prepared using a 95% stock solution diluted with 0.9% saline and was administered IP at a dose of 2g/kg. This dose and administration of ethanol has been successful in achieving CPP in DBA/2J and C57BL/6J mice (Gremel et al., 2006). Saline was administered IP at a volume of 12.5 ml/kg. Ethanol (Decon Laboratories Inc., King of Prussia, PA: 20% v/v) for the CTA studies was prepared using 100% ethanol diluted with saline and administered at a 2 or 4 g/kg dose. LiCl (0.15 M; Sigma-Aldrich, St. Louis, MO) was diluted in sterile distilled water and administered IP at a dose of 3 mEq/kg. Saline injections were given at a volume of 10 ml/kg. NaCl drinking solution (0.2M; Sigma-Aldrich, St. Louis, MO) was prepared by dissolving NaCl in tap water.

Statistical Analyses

Statistical analyses were carried out using multifactorial analyses of variance (ANOVAs) in Systat (version 13; Systat Software, Inc., Chicago, IL) or StatView (version 5.0, SAS Institute, Cary, NC). Lower order ANOVAs were used to follow up significant interactions. Significance was set at $\alpha=0.05$ for all tests unless otherwise specified and a Bonferroni correction for multiple comparisons was used for post-hoc analyses. Statistical trends are discussed for p values ≥ 0.05 and ≤ 0.1. For CTA experiments, daily intake was converted to ml per kg of body weight. CTA change scores were computed by subtracting intake at baseline (i.e., conditioning trial 1) from intake during the final conditioning trial. CTA daily intake data were analyzed by repeated measures ANOVA, with between subjects factors of genotype and drug group and the within subjects factor of conditioning trial. Baseline intake of the NaCl solution on

conditioning trial 1 (before any injections were given) was analyzed by two-way ANOVA with the factors of genotype and drug group to determine whether there were any initial group differences in consumption. Change scores were analyzed by two-way ANOVA with genotype and drug group as factors. CPP test data for time spent on the grid floor were analyzed by three-way ANOVA for the pretest and each preference test with genotype, sex, and conditioning subgroup as factors. A significant difference between conditioning subgroups was used to indicate the development of a place preference. A repeated measures ANOVA with between subjects factors of sex, genotype, and conditioning subgroup was also used to analyze potential changes across tests 1-3. Locomotor activity data were analyzed by two-way ANOVA for the pretest and each preference test with genotype and sex as factors.

Results

Experiment 1: CTA in HDID-1 and HS

Six animals (1 HDID-1 animal each from the saline, LiCl, and 2 g/kg ethanol groups, and 1 HS animal each from the saline, LiCl, and 4 g/kg groups) were excluded from analysis because they received unintentional water access during the night prior to conditioning trial 4. Figure 3-1 shows intake of the NaCl solution across conditioning trials in the HDID-1 (3-1a) and HS (3-1b) mice. Statistical analyses showed a significant main effect of genotype ($F_{1,66}$=4.9, p=0.030), drug group ($F_{3,66}$=37.2, p<0.001), and conditioning trial ($F_{5,330}$=119.7, p<0.001). Significant two-way interactions were found for trial x drug group ($F_{15,330}$=14.3, p<0.001) and genotype x drug group ($F_{3,66}$=4.9, p=0.004). A significant three-way interaction of conditioning trial x genotype x drug group ($F_{15,330}$=1.8, p=0.040) was also found. Follow-up ANOVAs examining each drug

group separately showed significant main effects of genotype ($F_{1,17}$=21.8, p<0.001) and trial ($F_{5,85}$=34.6, p<0.001), and a significant trial x genotype interaction ($F_{5,85}$=4.2, p=0.002) in the 2 g/kg ethanol treated animals. HDID-1 mice had significantly greater NaCl solution consumption than HS on conditioning trials 3-6 ($F_{1,17\text{-}18}\geq$16.4, p≤0.001), indicating that magnitude of CTA at this dose was smaller for HDID-1 than the HS animals. For the 4 g/kg ethanol group there was a significant main effect of trial ($F_{5,85}$=129.8, p<0.001), but only a trend toward a main effect of genotype ($F_{1,17}$=3.7, p=0.072) and no significant trial x genotype interaction. These results indicate the development of comparable CTA in both HDID-1 and HS mice at this dose. There was a significant main effect of conditioning trial ($F_{5,80}$=22.3, p<0.001) in the LiCl group, but no significant trial x genotype interaction , indicating that LiCl CTA was similar between genotypes. For vehicle-treated animals there was no significant main effect of genotype or significant trial x genotype interaction. There was a significant effect of trial ($F_{5,80}$=3.2, p=0.011), but post-hoc analyses showed this to be due solely to lower NaCl consumption on Trial 3 than Trial 1 (p=0.027). These results indicate that the handling and injection procedure had no systematic effect on NaCl intake over time.

Drinking on trial 1 was analyzed individually to determine whether there were any baseline group differences in consumption of the NaCl solution. There was a significant main effect of genotype ($F_{1,66}$=6.1, p=0.016), with HDID-1 mice (Fig. 3-1a) drinking more NaCl at baseline than HS mice (Fig. 3-1b). Because of this initial line difference in drinking, we decided to also analyze CTA change score as a way to minimize any potential impact of baseline variation. CTA change scores (intake on the final trial minus intake on the first trial) for each group are shown in Figure S3-1. A

significant interaction of genotype x drug group was found ($F_{3,66}$=4.5, p=0.006), as well as a significant main effect of drug group ($F_{3,66}$=36.3, p<0.001). Follow up ANOVAs showed that analysis of change scores yielded the same pattern of results as the repeated measures ANOVA, with HDID-1 mice showing a trend toward a smaller magnitude of CTA for 2 g/kg ethanol than the HS animals, though this difference failed to reach the Bonferroni-corrected significance threshold of α=0.0125 ($F_{1,17}$=6.7, p=0.020). HDID-1 and HS mice did not differ significantly on change score for any of the other conditioning groups, reinforcing the idea that HDID-1 mice are specifically resistant to a taste aversion conditioned with moderate dose ethanol.

Experiment 2: CTA in HDID-2 and HS

Three mice in Experiment 2 died during the course of the study and their data have been excluded from analysis. Figure 3-2 shows intake of the NaCl solution across conditioning trials in the HDID-2 (3-2a) and HS (3-2b) mice. Statistical analyses showed a pattern of results similar to that seen with the first replicate line in Experiment 1. There were significant main effects of genotype ($F_{1,141}$=36.8, p<0.001), drug group ($F_{3,141}$=26.8, p<0.001), and conditioning trial ($F_{5,705}$=54.1, p<0.001), as well as significant two-way interactions of genotype x trial ($F_{5,705}$=3.5, p=0.004) and drug group x trial ($F_{15,705}$=7.8, p<0.001). Although the three-way interaction of genotype x drug group x trial was not statistically significant, we chose to use the same data analysis strategy as for Experiment 1 due to our *a priori* interest in interpreting results across the replicate lines. Follow up ANOVAs of each drug group individually showed a significant main effect of genotype ($F_{1,36}$=6.2, p=0.018) and trial ($F_{5,180}$=11.3, p<0.001), and a significant genotype x trial interaction ($F_{5,180}$=2.8, p=0.019) in the 2 g/kg ethanol group. HDID-2 and HS mice did

not differ in intake on trials 1-4, but HDID-2 mice had significantly greater intake than HS mice on trials 5 and 6 ($F_{1,37} \geq 7.9$, $p \leq 0.008$), indicating a reduced magnitude of CTA at this dose. For the 4 g/kg ethanol-treated animals, there were significant main effects of genotype ($F_{1,34}=16.0$, $p<0.001$) and trial ($F_{5,170}=93.4$, $p<0.001$), but no significant interaction of genotype x trial, indicating the development of a similar CTA in both lines. The LiCl group showed significant main effects of genotype ($F_{1,35}=17.6$, $p<0.001$), trial ($F_{5,175}=11.3$, $p<0.001$), and a significant genotype x trial interaction ($F_{5,175}=3.2$, $p=0.009$). Follow-up ANOVAs showed that HDID-2 mice consumed significantly more NaCl solution than HS mice on trials 3, 4, and 6 ($F_{1,36} \geq 11.8$, $p \leq 0.002$; Bonferroni-corrected $\alpha=0.008$), indicating transiently weaker conditioning to LiCl in the HDID-2 mice. The vehicle group showed a significant main effect of genotype ($F_{1,36}=4.2$, $p=0.048$), but no significant effect of trial or significant genotype x trial interaction.

Analysis of baseline NaCl drinking on trial 1 showed no significant main effect of drug group, a trend toward a main effect of line ($p=0.076$), and no significant genotype x drug group interaction, indicating that groups were relatively well matched for baseline intake. CTA change from baseline scores for each group are shown in Figure S3-2. Analysis of CTA change scores showed significant main effects of genotype ($F_{1,144}=7.7$, $p=0.006$) and drug group ($F_{3,144}=18.5$, $p<0.001$), and a significant genotype x drug group interaction ($F_{3,144}=2.9$, $p=0.035$). Follow-up ANOVAs of each drug group individually showed that HDID-2 mice conditioned with 2 g/kg ethanol had a significantly smaller change score than HS mice ($F_{1,37}=7.8$, $p=0.008$). Mice in the 4 g/kg ethanol, LiCl, and saline groups did not differ significantly between the genotypes for the CTA change

score, although there was a statistical trend toward a greater change score for the HS mice in the vehicle-treated group (p=0.076).

Experiment 3: Ethanol CPP

Time spent on the grid floor during the pretest and each preference test is shown in Figure 3-3. Analysis of time spent on the grid floor during the pretest (Fig. 3-3a) showed no significant main effect of conditioning subgroup, sex, or genotype, and no significant interactions, indicating that animals of each group had no initial bias toward the tactile cues (All F < 1.7). Analysis of tests 1-3 (Fig. 3-3) showed significant main effects of conditioning subgroup ($F_{1,126}$>27.4, p<0.0001), but no significant effects of genotype or sex and no significant interactions. A repeated measures ANOVA of grid time across all CPP tests (tests 1-3) showed a significant 4-way interaction of test x conditioning subgroup x genotype x sex ($F_{4,252}$=2.4, p=0.047). A follow-up ANOVA showed a significant 3-way interaction of test x conditioning subgroup x genotype for males only ($F_{4,132}$=2.48, p=0.047). ANOVAs were run for each test for males only and showed a significant conditioning subgroup x genotype interaction only on test 3 ($F_{2,66}$=3.43, p=0.038), which was due to significant place conditioning in both HDID lines but not in the HS males. Place conditioning on tests 1 and 2 was comparable across genotypes. Analysis of locomotor activity during each test showed only a significant main effect of genotype ($F_{2,132}$≥7.5, p<0.001), with HDID-1 mice having lower activity than HDID-2 and HS mice on the pretest and all preference tests (Fig. 3-4).

Discussion

In the CTA experiments, both HDID replicates had weaker ethanol CTA to a 2 g/kg conditioning dose than HS mice. In contrast, all genotypes conditioned a strong

aversion to the 4 g/kg dose, indicating that HDID mice do not simply have deficits in the associative learning necessary to develop a CTA. Consequently, the attenuated CTA to a moderate dose of ethanol is presumably due to a difference in sensitivity to the aversive effects of that dose. This is further supported by the fact that HDID-1s, and to some extent HDID-2s, developed a CTA similar to HS mice when treated with LiCl. The specificity of the genotypic difference to the 2 g/kg ethanol dose is consistent with previous mouse studies showing that the inverse genetic relationship between ethanol preference drinking and ethanol conditioned taste aversion is more robust with a 2 g/kg dose than a 4 g/kg dose (e.g. Chester et al., 2003).

There is some evidence that the HDID-2 mice also had less LiCl CTA than the HS mice, an effect which was not seen in the HDID-1s. This could suggest that the second replicate is less sensitive to mildly aversive drugs in general; testing additional compounds would be necessary to resolve this. This potentially attenuated aversive sensitivity does not seem to reflect a deficit in learning, as the HDID-2 mice readily conditioned an aversion to the 4 g/kg ethanol dose and show no deficits in ethanol CPP. These mice also show no significant difference from HS mice in baseline consumption of the saline solution in the CTA test, suggesting that the present results cannot be explained by reduced exploration or increased novelty aversion in this line. That the HDID-2 mice show this more generalized attenuation of aversion than the HDID-1 mice is not entirely surprising: these two lines were independently selectively bred and thus it is possible that different combinations of genes were affected in the two iterations of the selection experiment. The broadly-defined similarity of relative insensitivity to moderate-dose ethanol CTA in the HDID-1 and HDID-2 lines argues strongly for the role of some of the

same genes that lead to high binge-like drinking. However, the HDID-1 and HDID-2 lines differ for many other genes as well, due to unavoidable chance effects. Because the genetic contributions to individual differences in ethanol CTA and high drinking in the dark are not completely the same, the pattern of ethanol CTA results is not expected to be identical in the two HDID lines. Additionally, linkage disequilibrium could play a role, if there is a difference between the replicates in a gene (or genes) that is linked to the ones influencing DID.

Alternatively, the lesser sensitivity of HDID-2 mice to LiCl on 3 of the 6 test trials could merely reflect transient differences. The HDID-2s (14^{th} and 17^{th} selected generations) are not as far along as the HDID-1s (21^{st} generation) in the selection process. Because the difference between HDID-1 and HS mice in the primary selected trait (DID-BEC) was greater than the difference between HDID-2 and HS, some putative correlated responses to selection that appear early (i.e., in HDID-2) might not survive as the intensity of selection increases (HDID-1). One possible explanation is that this is due to the disruption of haplotype blocks with increasing numbers of selection generations. We examined the data for the two passes of HDID-2 mice tested and saw a stronger tendency for reduced HDID-2 LiCl CTA in S14 than in S17 (data not shown), which is consistent with the hypothesis that LiCl sensitivity in HDID-2 was a "false positive" correlate. This is supported by a difference in the selection phenotype between these generations as well (average BECs for S14 and S17 plus/minus the standard deviation: 0.790±0.038 and 1.171±0.043 mg/ml, respectively; HDID-1 mice of S21 had a BEC of 1.22±0.057 mg/ml. Data are unpublished). Regardless, the consistent and significantly reduced sensitivity to the 2 g/kg ethanol dose in both replicates across trials is strong

evidence for a correlated response to selection. Thus, while it is possible that the random assortment of genes during the HDID-2 selection has given rise to attenuated aversive sensitivity to other drugs as well, the apparent shared genetic contributions to ethanol aversion and drinking to intoxication are likely present in both lines.

Mice of all genotypes and sexes developed a modest but significant ethanol CPP, indicating that ethanol is perceived as rewarding by these animals. The lack of any difference in magnitude between the lines on tests 2 and 3 suggests that initial ethanol reward sensitivity does not differ between HDID and HS mice. The finding of greater time on the drug-paired floor in the HDID males than the HS males on test 4 suggests possible subtle genotypic differences in ethanol CPP. Specifically, each CPP test essentially acts as an extinction trial since the conditioning cues are being presented in the absence of drug. Thus, the difference in CPP on test 4 could indicate a difference between HDID and HS males in sensitivity to ethanol extinction, with the HDID mice extinguishing more slowly than the HS. Future testing using an actual extinction procedure after CPP acquisition could be used to investigate this possibility further. Another possibility would be to perform a single CPP expression test after the full number of conditioning trials to assess whether a genotypic difference in CPP remains in the absence of repeated drug-free tests.

One limitation of the CPP study is that only a single dose of ethanol was tested. It is therefore possible that at a different dose we would have been able to distinguish genotypic differences in place conditioning, as were seen with the CTA experiments. The place conditioning seen here seemed to reach a performance ceiling by the first test and did not increase with additional conditioning trials, so it might be possible to see

differences between the lines at a lower dose. However, the magnitude of the CPP was relatively modest and CPP has been previously noted as often lacking a graded dose-response curve (e.g. Cunningham et al., 1992), so a lower dose might simply eliminate CPP expression entirely. It could prove useful in the future, though, to test these genotypes of mice using a more dose-sensitive paradigm such as the reference dose procedure (Groblewski et al., 2008) to see if we are able to observe any differences between the lines. It may also prove beneficial to test these genotypes for CPP using a different conditioning agent (e.g. a natural reinforcer or a different drug of abuse), to determine whether the present results generalize to other rewarding substances.

When examining CPP data, it is important to consider the fact that locomotor activity can affect the expression of a place preference, with higher levels of activity impairing the demonstration of CPP (Gremel & Cunningham, 2007). We did observe a genotypic difference in activity in the CPP tests (Fig. 3-4), with the HDID-1 mice showing lower activity than the other two genotypes. This difference in activity is consistent with previous findings from these lines (Crabbe et al., 2012b), but is unlikely to have affected the CPP results since even the most active lines had locomotor activity well below levels shown to interfere with CPP expression (Gremel & Cunningham, 2007). It should also be noted that CPP is not an entirely unambiguous measure of drug reward, and that additional testing (e.g. ethanol-reinforced responding on a progressive ratio) is needed in order to completely rule out possible differences in reward sensitivity. From the present results, however, it would appear that drinking to intoxication in the HDID lines does not reflect an alteration in perceived rewarding effects of ethanol. This is consistent with much of the previous work from rodent selected lines that also failed to

show alterations in ethanol CPP as a correlated response to selection for ethanol preference or intake. Single gene effects have been shown to influence ethanol drinking as well as CPP, and it is possible that future experiments examining individual gene contributions to the HDID phenotype will yield similar results, but an overall genetic relationship between drinking to intoxication and ethanol CPP does not appear to be present.

The results of the present experiments demonstrate that the genetic relationship between ethanol motivated behaviors and binge-like drinking is similar to that seen previously with two-bottle choice continuous access drinking. Specifically, these findings are consistent with the literature on the negative genetic correlation between preference drinking and ethanol CTA in mouse and rat selected lines (e.g. Broadbent et al., 2002; Brunetti et al., 2002; Chester et al., 2003; Froehlich et al., 1988; Phillips et al., 2005; see review by Cunningham et al., 2009) and extend this association to another model of ethanol intake. The consistency of findings for the binge-like and continuous access, preference drinking traits is consistent with data suggesting a moderate genetic correlation between these two traits. Although inbred strain correlations between DID and preference drinking are generally high (Rhodes et al., 2007; Crabbe et al., 2012c), HDID mice show only modestly greater preference drinking than HS (Crabbe et al., 2010; Rosenwasser et al., 2013). Our results suggest the existence of similar genetic contributions underlying sensitivity to ethanol aversion and drinking to intoxication (DID), but not ethanol reward as measured by CPP. Previous work has shown little evidence of genetic correlation between ethanol CTA and CPP (e.g. Phillips et al., 2005), and quantitative trait loci mapping suggests only limited chromosomal overlap

(Cunningham 1995; Risinger & Cunningham, 1998), so it is not surprising that HDID selection has resulted in line differences in one behavior and not the other.

It is important to note that the present results demonstrate a negative *genetic* correlation between the HDID phenotype and ethanol CTA. In order to determine whether drinking to high BECs and CTA are related within a given individual, it would be necessary to test CTA and ethanol DID in the same animals. It is possible to speculate, however, as to how changes in aversive sensitivity could produce increased intake. In humans, for example, heavy drinkers given ethanol in a laboratory setting report lower sedation and higher drug "liking" than light drinkers, and individual differences in these responses predict subsequent binge drinking frequency in the heavy drinking individuals (King et al., 2011). In our model, it could be that a reduced sensitivity to the aversive effects of ethanol at moderate doses such as those ingested during the DID test allows HDID mice to drink in a manner that produces higher BECs and greater intoxication than HS mice. We have shown previously that HDID-1 mice drink ethanol in significantly larger "bouts" (i.e., consume a larger g/kg dose of ethanol in the same amount of time) than HS mice during the DID test (Barkley-Levenson & Crabbe 2012). It might be that this pattern of intake is possible for the HDID-1 mice because they are less sensitive to the negative pharmacological effects of these large drinking bouts. Another possibility is that the reduced aversion of the HDID mice allows them to more readily drink enough ethanol to experience its rewarding effect. Thus, while we report here that experimenter-administered ethanol is equally rewarding to HDID and HS mice, in a home cage drinking situation where the mice determine the dose administered, HDID mice might be more likely to actually experience ethanol reward which may in turn enhance motivation

to continue drinking. The specific genetics underlying sensitivity to ethanol's aversive effects are not yet well understood, but the α2 subunit of GABA type-A receptors has been implicated in mediating ethanol CTA (Blednov et al., 2013). Consequently, these subunits could be an interesting target for future pharmacological and genetic studies in the HDID and HS lines. Overall it remains unknown exactly what drives the high intake and high BECs in the HDID mice, but the current studies provide strong evidence that reduced sensitivity to ethanol's aversive effects may be a significant contributor to the HDID phenotype.

Acknowledgments

The authors would like to thank Jason Schlumbohm, Lawrence Huang, and Tyne Brandon for assistance with the HDID-2 CTA experiment. These studies were supported by NIH-NIAAA grants AA13519, AA10760, AA07702, AA020245, and by a grant from the Department of Veterans Affairs. AMB-L was supported by NIH-NIAAA grant AA007468, the Achievement Rewards for College Scientists Foundation, and an OHSU Graduate Research Scholar fellowship.

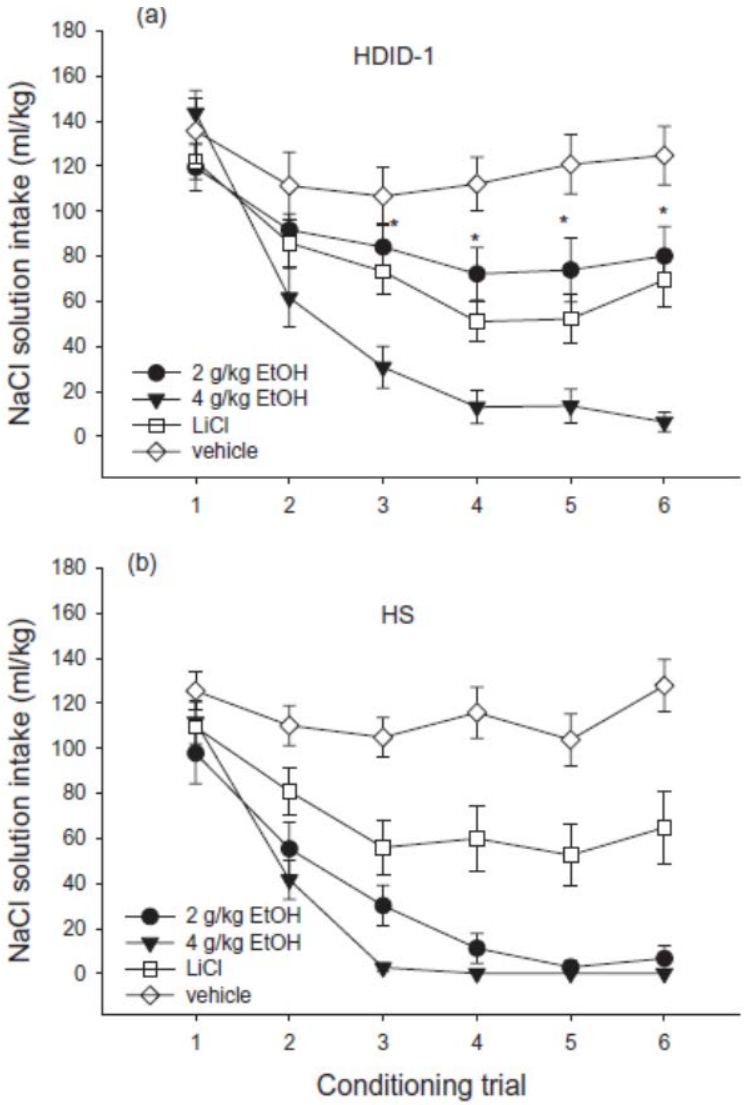


Figure 3-1. NaCl solution intake in ml/kg body weight across the 6 conditioning trials. Panel a shows intake for the HDID-1 mice and panel b shows intake for the HS mice. Ethanol conditioning groups (2 and 4 g/kg) are shown in filled symbols and control groups (vehicle and LiCl) are shown in open symbols. Means ±SEM shown. * indicates a statistically significant difference ($p \leq 0.001$) in NaCl solution intake from corresponding HS group.

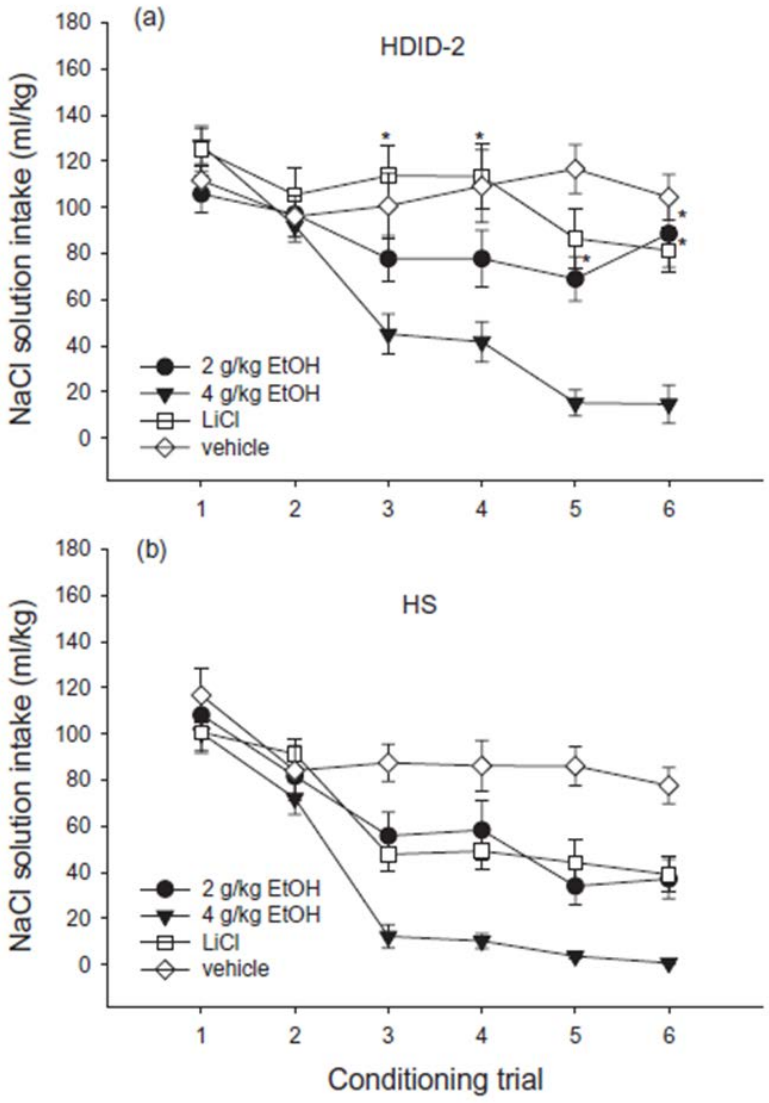


Figure 3-2. NaCl solution intake in ml/kg body weight across the 6 conditioning trials. Panel a shows intake for the HDID-2 mice and panel b shows intake for the HS mice. Ethanol conditioning groups (2 and 4 g/kg) are shown in filled symbols and control groups (vehicle and LiCl) are shown in open symbols. Means ±SEM shown. * indicates a statistically significant difference ($p \leq 0.01$) in NaCl solution intake from corresponding HS group.

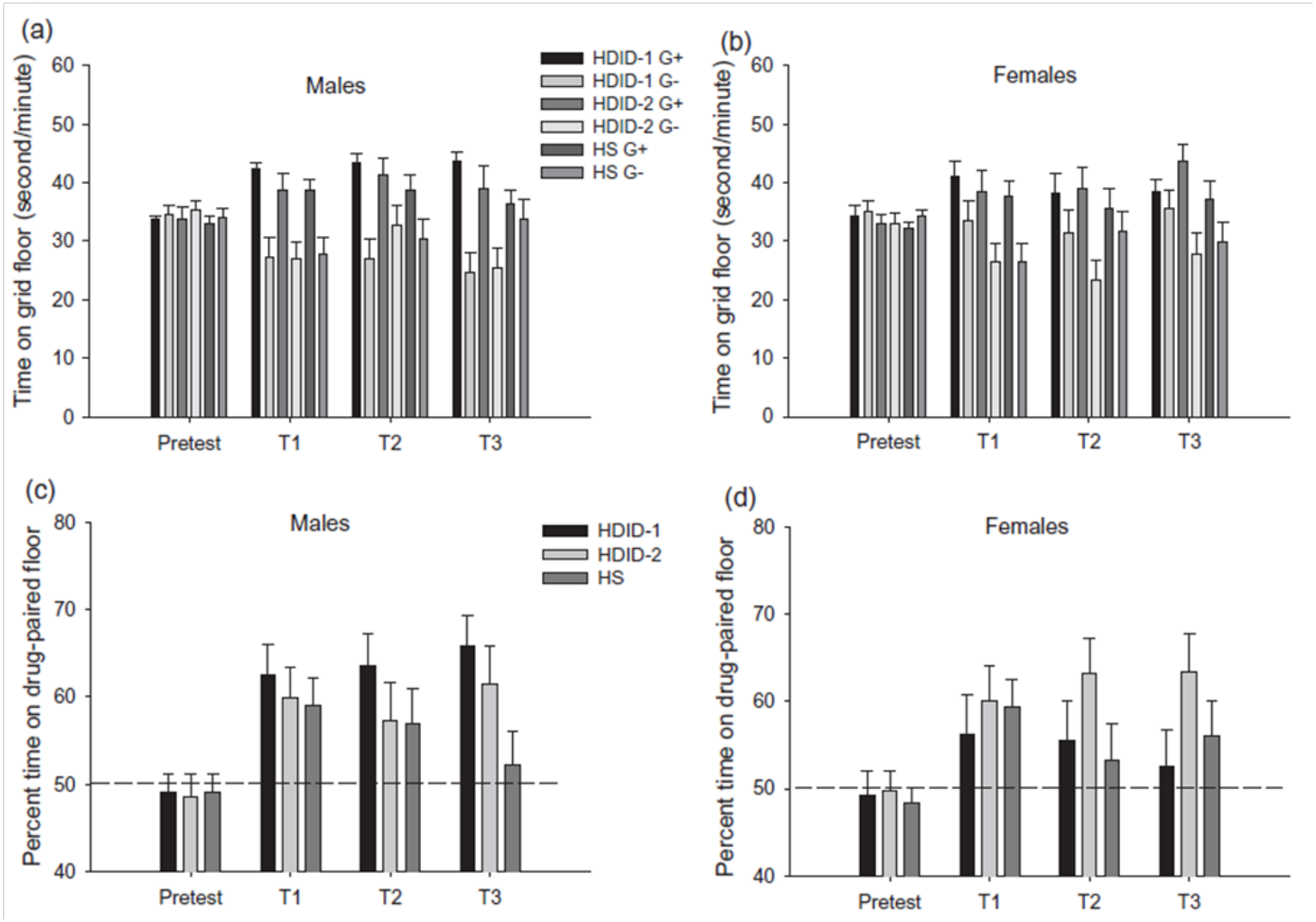

(a)
Males
Time on grid floor (second/minute)
HDID-1 G+
HDID-1 G-
HDID-2 G+
HDID-2 G-
HS G+
HS G-
Pretest
T1
T2
T3
(b)
Females
Time on grid floor (second/minute)
(c)
Males
Percent time on drug-paired floor
HDID-1
HDID-2
HS
(d)
Females
Percent time on drug-paired floor

Figure 3-3. Ethanol conditioned place preference expression during the pretest and Tests 1-3 (after 4, 6, or 8 pairings, respectively). Panels a and b show time spent on the grid floor by male (a) and female (b) animals of each genotype. G+ indicates mice that received 2 g/kg ethanol paired with the grid floor (GRID+ conditioning subgroup) and G- indicates mice that received 2 g/kg ethanol paired with the hole floor (GRID- conditioning subgroup). The difference between G+ and G- group scores reflects the magnitude of conditioned place preference. Panels c and d show percent time spent on the drug-paired floor (collapsed on conditioning subgroup) for males (c) and females (d) of each genotype. The dashed line at 50% indicates the point of no preference for either floor. Means ±SEM shown.

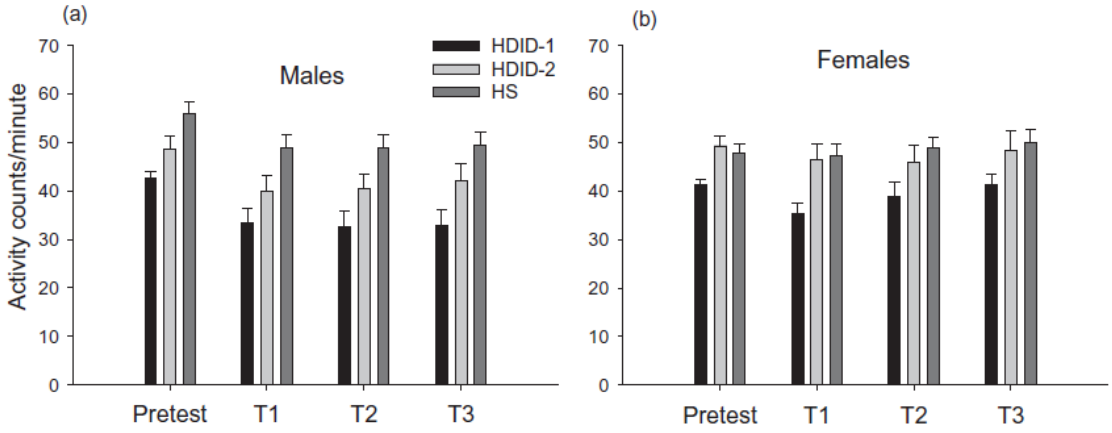


Figure 3-4. Average locomotor activity counts (beam breaks) per minute during the pretest and each place preference test (T1-3) for males (a) and females (b). Means ±SEM shown.

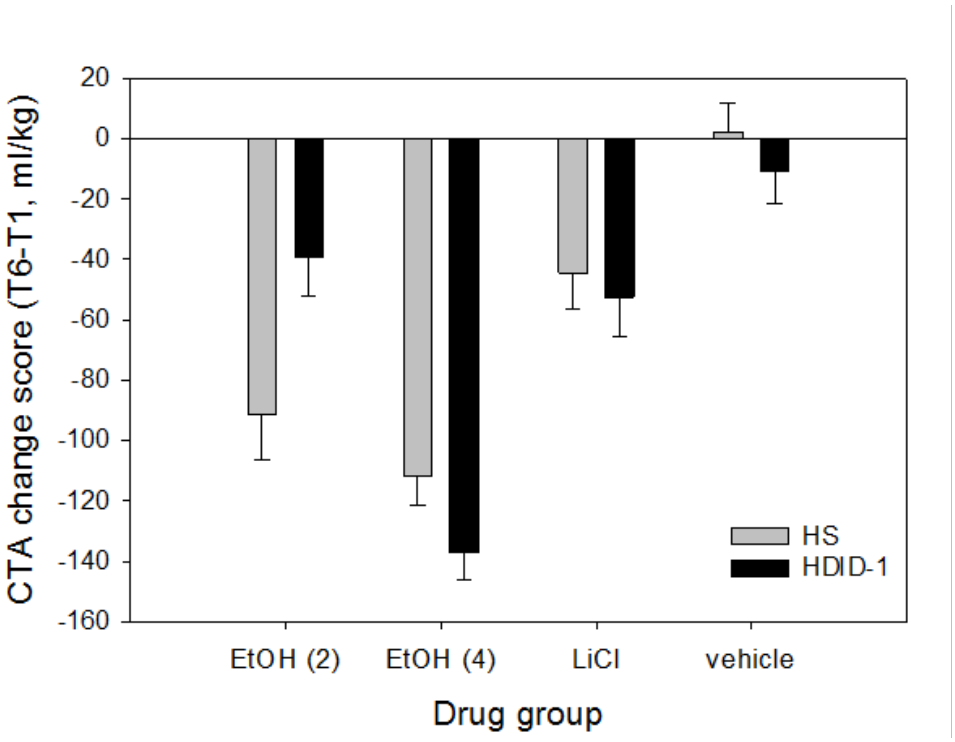


Figure S3-1. CTA change scores for HDID-1 and HS mice of each drug group. Change scores are the ml/kg NaCl solution intake at baseline (Trial 1) subtracted from the ml/kg NaCl solution intake on the final conditioning trial (Trial 6). A negative change score indicates a reduction in intake between the initial and final trials. Means ±SEM shown.

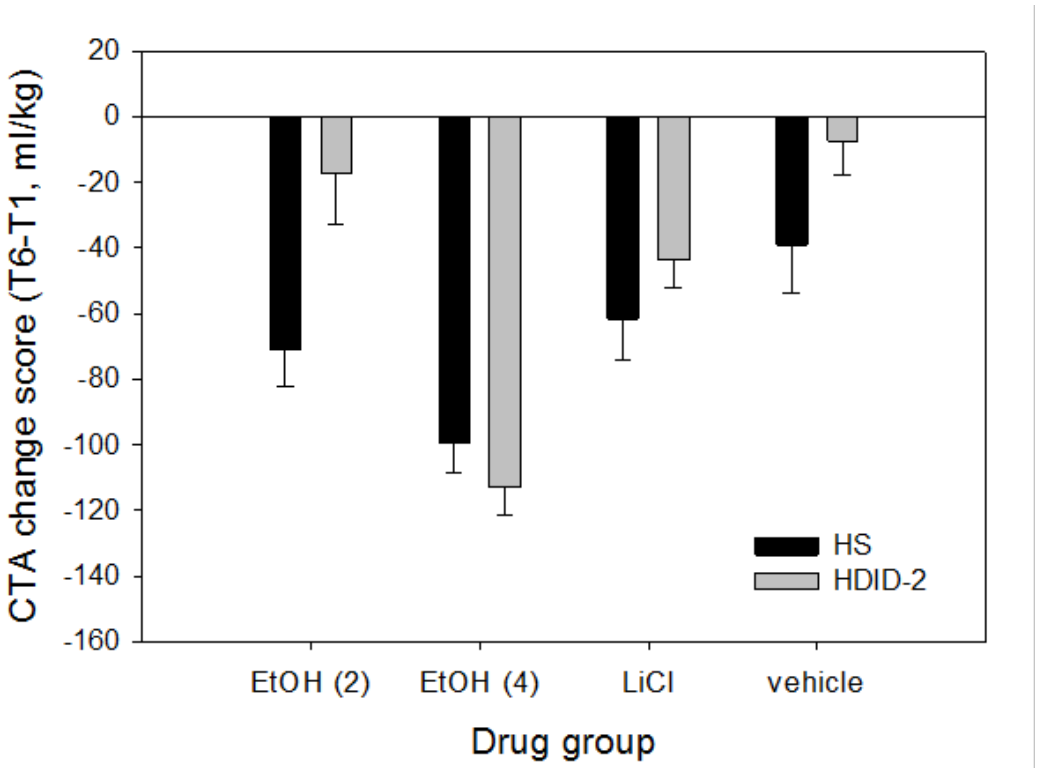


Figure S3-2. CTA change scores for HDID-2 and HS mice of each drug group. Change scores represent the ml/kg NaCl solution intake at baseline (Trial 1) subtracted from the ml/kg NaCl solution intake on the final conditioning trial (Trial 6). A negative change score indicates a reduction in intake between the initial and final trials. Means ±SEM shown.

Chapter 4: Genotypic and sex differences in anxiety-like behavior and alcohol-induced anxiolysis in High Drinking in the Dark selected mice

Amanda M. Barkley-Levenson and John C. Crabbe

This chapter has been previously published in *Alcohol* (2015), 49(1): 29-36

Abstract

Alcohol use disorders and anxiety disorders are highly comorbid in humans. In rodent lines selected for alcohol drinking, differences in anxiety-like behavior are also seen. The High Drinking in the Dark (HDID) lines of mice were selectively bred for drinking to intoxication during limited access to alcohol, and these mice represent a genetic model of risk for binge-like drinking. The present studies investigated whether these selected lines differ from control (HS) mice in basal anxiety-like behavior or in anxiolytic response to alcohol. We also assessed the genetic correlation between alcohol drinking in the dark (DID) and basal anxiety-like behavior using existing inbred strain data. Mice of both sexes and HDID replicates (HDID-1 and HDID-2) were tested on an elevated zero maze immediately following a DID test. In general, HDID mice showed more time spent in the open arms after drinking alcohol than HS mice, although a significant anxiolytic effect of alcohol drinking was only seen in the HDID-1 females and the HDID-2 males. When assessed collapsed across genotype, open-arm time was significantly correlated with blood alcohol concentration. HDID-1 male mice also showed less anxiety-like behavior at baseline (water-drinking controls). In a separate experiment, HDID-1 and HS mice were tested for anxiolytic dose-response to acute alcohol injections. Both genotypes showed increasing time spent in the open arms with increasing alcohol doses, and HDID-1 and female mice had greater open-arm time across all drug groups, including saline. HDID-1 control males showed lower anxiety-like behavior than the HS control males. Inbred strain data analysis showed no significant genetic relationship between alcohol DID and anxiety. These findings suggest that HDID selection has not produced consistent changes in anxiety-like behavior or sensitivity to

alcohol-induced anxiolysis, although reduced basal anxiety-like behavior was found to be a trait of the male HDID-1 mice. Given the lack of consistent findings across replicates and sexes, however, is does not appear that anxiety state and sensitivity to alcohol's anxiolytic effects contribute significantly to the high drinking behavior of the HDID mice.

Introduction

Alcohol use disorders (AUDs) and anxiety disorders have been shown to have a high degree of comorbidity (for review, see Kushner et al., 2000 and Smith & Randall, 2012). Two broad hypotheses that are not mutually exclusive might account for this. Alcohol has significant anxiolytic effects (e.g., Gilman et al., 2008), and there is evidence that higher basal anxiety may promote greater alcohol intake which can lead to abuse (Bolton et al., 2006). Another possibility is that anxiety disorders and AUDs may share some of the same underlying genetic risk factors. Family and twin studies have suggested possible common transmission of anxiety disorders and AUDs, which may represent shared genetic risk (e.g., Merikangas et al., 1994; Tambs et al., 1997).

There is also evidence from the animal literature to suggest a relationship between anxiety-like behavior and alcohol consumption. Rats classified as anxious by performance on an elevated plus maze (EPM) voluntarily drink more alcohol in a subsequent test than those classified as non-anxious (Spanagel et al., 1995). Similarly, some rodent lines selected for high vs. low alcohol preference also show innate differences in basal anxiety-like behavior and sensitivity to alcohol-induced anxiolysis, with high preferring animals generally having greater basal anxiety-like behavior and/or greater sensitivity to alcohol's anxiolytic effects (Colombo et al., 1995; Stewart et al., 1993). These anxiety-related behaviors appear to be correlated responses to selection for high alcohol intake in these lines. However, this relationship is not seen for all rodent lines selected for high vs. low drinking, with some lines showing an opposite relationship or no relationship with drinking (Can et al., 2012; Sandbak et al., 1998). Another way of examining this relationship is by measuring alcohol drinking in animals selectively bred

for anxiety-like behavior. Rats selectively bred for high (HAB) or low (LAB) anxiety-related behaviors on an EPM show differences in alcohol preference in a 2-bottle choice test (Henniger et al., 2002). However, LAB rats have a greater rather than lesser alcohol preference than HAB rats. The anxiolytic effect of an injection of alcohol is greater in the HAB rats than LAB rats, which is the direction of effect that would be expected for an anxiety-medication hypothesis of drinking. Consequently, the possible genetic relationship between alcohol intake and anxiety-like behavior appears to be complex for both alcohol- and anxiety-related selection phenotypes.

With the exception of the study by Can and colleagues using mice, most of the previous work involved rat lines, and all previous studies have used animals either selectively bred for, or tested on, 2-bottle choice alcohol preference drinking. In the present experiments, we sought to extend these findings to another model animal species and a test of binge-like drinking by determining the relationship between anxiety-like behavior and alcohol drinking in mice selectively bred for blood ethanol concentration (BEC) after drinking to intoxication. The HDID lines of mice were selectively bred for BECs after drinking in the dark (DID), and routinely drink to intoxicating blood levels in a limited-access test (Crabbe et al., 2009, 2014). These mice have been extensively behaviorally phenotyped to determine correlated responses to selection and possible factors promoting their high drinking (for review, see Barkley-Levenson & Crabbe, 2014). Here, we tested whether drinking during the DID test is sufficient to produce alcohol-induced anxiolysis, and whether differences in anxiolytic response to alcohol or basal anxiety-like behavior may underlie the high drinking phenotype of HDID mice. We also used existing inbred mouse strain data sets to assess the genetic relationship between

anxiety-like behavior and alcohol DID.

Materials and methods

Animals and husbandry

Male and female mice of the HDID-1, HDID-2, and HS lines were bred and housed in the Veterinary Medical Unit of the Veterans Affairs Portland Health Care System (Portland, OR). Mice were weaned at 3 weeks of age and were reared with both dam and sire until weaning. After weaning, mice were housed in groups of 2-5 with same-sex littermates or with mice of the same genotype when necessary to avoid single housing. All mice were between 51 and 80 days of age and were experimentally naïve at the start of testing. Mice were housed in standard polycarbonate shoebox cages on Bed-o-cob bedding and received *ad libitum* access to food (Purina 5001 chow, LabDiet, St. Louis, MO) and water unless otherwise specified. HDID-1 mice from the 22nd and 27th selection generations were used in Experiment 1 and mice from the 23rd and 28th selection generation were used in Experiment 2. HDID-2 mice from the 19th selection generation were used in Experiment 1. HS/Npt (HS) mice are the starting population from which the HDID lines were selected and are the product of a systematic 8-way inbred strain cross (see Crabbe et al., 2009 for details). These mice are not subjected to selection pressure and represent a genetically heterogeneous population used as a comparator control for the HDID lines. For both Experiments 1 and 2, mice were tested in multiple passes (replicate experiments), with some or all of the sexes and genotypes included in each pass. For Experiment 1, all mice were kept on a 12 h/12 h reverse light/dark cycle with lights off at 09:30. For Experiment 2, one pass of mice was kept on a 12 h/12 h forward light/dark cycle with lights on at 06:00, and a second pass of mice

was kept on a reverse light/dark cycle with lights off at 10:30. Both groups were tested at approximately the same time during their circadian light phase, as our laboratory and most others routinely test anxiety-like behavior during the light cycle. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experiment 1: anxiety-like behavior after DID

Seventy-nine male and female mice of the HDID-1, HDID-2, and HS lines were used in this study (n = 6–9/line/sex/group). Mice were tested in 4 passes, with mice of all sexes and genotypes used in each pass except that only female HDID-1 mice were tested in the first pass. At the beginning of the experiment, mice were singly housed and habituated to reverse light/dark for 2 weeks. During this time, mice were given water from polycarbonate bottles with stainless steel sipper tubes attached. After the acclimation period, mice were given a modified version of our standard 2-day DID test. The 2-day DID was chosen because this is the test used in our selection procedure and we were interested in whether alcohol-induced anxiolysis is experienced by these mice under conditions comparable to HDID selection. The DID test is described in detail elsewhere (Crabbe et al., 2009). Briefly, 2–3.5 h after lights off, water bottles were removed and replaced with 10 ml graduated cylinders fitted with stainless steel ball-bearing sipper tubes containing either 20% alcohol or water depending on group assignment. Start times were staggered by 10-min intervals for every 2 mice to allow for testing on the elevated zero maze (EZM) immediately after drinking on the second day. At the start of the drinking session, fluid levels were recorded and tubes were left in place for 2 h. After 2 h,

fluid levels were recorded again and tubes were removed and water bottles were returned to the cages. The next day, the procedure was repeated identically except that tubes were left in place for 4 h. At the end of the 4 h, mice were tested on the EZM in squads of 2 (one mouse per maze) for 5 min. Immediately following the EZM test, a 20 µL blood sample was taken from the retro-orbital sinus of each alcohol-group mouse to determine BEC.

Experiment 2: dose-response to alcohol-induced anxiolysis

One hundred thirty-three male and female HDID-1 and HS mice (n = 5–10/line/sex/dose) were used in this experiment. Male mice were tested in two passes, and female mice were tested in a single pass. Prior to the start of testing, mice were pseudorandomly assigned to a dose group (saline, 0.5, 1, or 1.5 g/kg alcohol). Behavioral testing in Experiment 2 started at approximately 3 h after lights-on for both passes. On the day of testing, mice were moved into the procedure room, weighed, and allowed to habituate for 1 h. Mice were then injected intraperitoneally (i.p.) in squads of 2 with saline or the appropriate dose of alcohol as determined by group assignment. Mice were placed in individual holding cages for 10 min and then given a 5-min test on the EZM.

Experiment 3: genetic correlation of alcohol DID and basal anxiety-like behavior in inbred strains

Our laboratory has previously published 4-day DID consumption data for 23 inbred mouse strains (Crabbe et al., 2012c). Intake data for 2-day DID are not presently available for all of these strains, so the 4-day DID data were used for the correlational analysis. In order to correlate DID intake with anxiety-like behavior, inbred strain data were mined from extant data sets in the lab and those available on the Mouse Phenome

Database (MPD, The Jackson Laboratory, http://phenome.jax.org/). Criteria for selection of data for anxiety-like behavior were: at least 10 strains in common with the DID data set, inclusion of males and females, and a measure of anxiety-like behavior based on time spent in the anxiogenic region of a test apparatus (e.g., open arms, center region, light compartment). EPM data sets were excluded if the data were reported as time spent in the closed arms, as this could not be converted to a percent of total time in the open arms without knowing center time. Using these criteria, we selected one previously published study from our lab (Milner & Crabbe, 2008) and two data sets from the MPD (Brown et al., 2004, and Wahlsten & Crabbe, 2003). The anxiety measures included were open field (OF) center time (Milner & Crabbe, 2008; Wahlsten & Crabbe, 2003), light-dark box (LDB) light compartment time (Brown et al., 2004; Milner & Crabbe, 2008), EZM open arm time (Brown et al., 2004; Milner & Crabbe, 2008), and EPM open arm time (Wahlsten & Crabbe, 2003). All measures of anxiety-like behavior were converted as needed to percent of total time spent in the anxiogenic region. Table 4-1 summarizes the data sets included in the analysis.

EZM apparatus and testing

Two EZM apparatuses were used, each consisting of a ring-shaped plastic walkway divided into four sections. The diameter of each EZM was 45 cm, and the width of the walkway was 5.5 cm. Two sections of the maze were enclosed on either side by clear plastic walls 11 cm tall (closed arms) and two sections had only a 2-mm tall lip (open arms). Mazes stood 45 cm high and were placed in plastic tubs containing pine chip bedding to prevent injury should an animal fall. Testing was conducted under dim lighting conditions with light levels of approximately 15 lux in the center of each EZM.

Mice were tested on both mazes concurrently, with an opaque barrier placed between the mazes so that the mice could not see each other. Mazes were videotaped from above and video was scored by experimenters blinded to treatment and genotype. Videos for each experiment were scored by only one experimenter. Variables recorded were the time spent in the open arms as a percent of the total test time and number of line crosses. Eight lines were overlaid onto the video image of the maze such that each arm was divided into three equal segments by the lines. A line crossing was recorded whenever all four feet of an animal crossed the line and this number was used as a measure of locomotor activity. Open arm time was recorded starting when all four feet of a mouse had crossed into an open arm and ending when all four feet of the mouse had crossed back into a closed arm. Percent time spent in the open arms was used as a measure of anxiety-like behavior, with more time in the open arms indicating less anxiety. Mice were placed in the EZM at the start of the test in an open arm facing a closed arm. Between each subject, the floor and walls of the mazes were sprayed with 10% isopropyl alcohol and wiped down to eliminate any odor cues.

Drugs

Alcohol (Decon Laboratories Inc., King of Prussia, PA, USA: 20% v/v) for Experiment 1 was prepared using 100% alcohol diluted with tap water. Alcohol for Experiment 2 was diluted with 0.9% saline (20% v/v) and administered at a 0.5, 1.0, or 1.5 g/kg dose. These doses were chosen as they were expected to yield BECs in the range of those reached during the DID test used for HDID selection. Saline injections were given at a volume of 10 ml/kg.

Statistical analyses

Statistical analyses were carried out in Systat (version 13; Systat Software, Inc., Chicago, IL, USA). EZM variables were analyzed using multifactorial analysis of variance (ANOVA) with between-group factors of sex, genotype, and fluid group/dose group depending on experiment. Statistically significant interactions were followed up with lower-order ANOVAs, and significant main effects were followed up with Tukey HSD *post hoc* analyses when appropriate. Alcohol drinking data were converted to a g/kg body weight dose and water drinking data were converted to ml/kg. Day 2 intake data were analyzed separately by fluid type by two-way ANOVAs with factors of genotype and sex, as were BEC data. Linear regression was used to correlate BEC with open arm time and line crossings in Experiment 1. In Experiment 3, strain means were computed for each measure of anxiety-like behavior and Day 4 DID alcohol intake. Pearson correlations were used to assess the relationship between alcohol DID and each of the anxiety-like behaviors, and between anxiety behaviors within and between data sets. The significance level was set at $\alpha = 0.05$. Statistical trends are discussed for p values ≥ 0.05 and ≤ 0.1.

Results

In Experiment 2, one female HS mouse from the 0.5 g/kg group was given an incorrect dose of alcohol and was excluded from the analysis.

Experiment 1: anxiety-like behavior after DID

Figure 4-1 shows the Day 2 alcohol intake and BEC for each line and sex. Alcohol intake showed only a significant main effect of line [$F(2,34) = 3.976$, $p = 0.028$],

and *post hoc* analyses showed significantly greater intake by the HDID-2 mice than the HS mice ($p = 0.025$). For all other main effects and interactions, $F \leq 1.521$. Analysis of BEC data also revealed only a significant main effect of line [$F(2,34) = 19.669$, $p < 0.001$] and *post hoc* analyses showed that HDID-1 and HDID-2 mice had significantly greater BECs than HS mice ($p < 0.001$ for both). There was no significant main effect of sex or significant interaction with sex for either intake or BEC. From the EZM, we first analyzed the percent of total time spent in the open arms. There was a significant main effect of line [$F(2,66) = 4.583$, $p = 0.014$], and a significant line × sex × treatment interaction [$F(2,66) = 3.548$, $p = 0.034$]. Because of our interest in assessing basal anxiety-like behavior, as well as anxiolytic response to alcohol, we next analyzed the water-drinking group alone by two-way ANOVA. This showed a significant line × sex interaction [$F(2,31) = 3.709$, $p = 0.036$]. Results are shown separately for males and females in Figure 4-2. One-way ANOVA and subsequent *post hoc* analyses for each sex showed that this interaction was due to a significant main effect of line only in the male mice [$F(2,14) = 4.907$, $p = 0.024$]. HDID-1 water-drinking male mice spent significantly more time in the open arms than HS mice ($p = 0.037$; Fig. 4-2, left), and showed a statistical trend toward more time in the open arms than HDID-2 mice as well ($p = 0.052$). Water-drinking female mice (Fig. 4-2, right) did not show any significant differences in open-arm time across genotypes.

Next, to determine the anxiolytic effect of alcohol in each group, we performed one-way ANOVAs with the factor of drinking group for each genotype and sex. A significant anxiolytic effect of drinking was found only in the HDID-1 females [$F(1,16) = 5.734$, $p = 0.029$] and HDID-2 males [$F(1,9) = 6.09$, $p = 0.036$], with both

groups showing more time spent in the open arms by alcohol-drinking mice than water-drinking mice. All other groups did not have a significant difference in open-arm time between the water and alcohol animals ($F \leq 0.331$ for all; see Fig. 4-2).

For line crossings, analysis showed significant main effects of treatment [$F(1,67) = 14.637$, $p < 0.001$] and sex [$F(1,67) = 4.376$, $p = 0.04$], and a significant treatment × line interaction [$F(2,67) = 3.532$, $p = 0.035$]. In order to facilitate comparison of group differences in alcohol-induced locomotor stimulation with anxiety-like responses, we employed the same strategy as the anxiolysis analysis above and used one-way ANOVA with a factor of drinking group for males and females of each genotype. HDID-2 males (Fig. 4-3, left) and females (Fig. 4-3, right) and HDID-1 females (Fig. 4-3, right) showed significant alcohol-induced locomotor stimulation [$F(1,9\text{-}17) \geq 7.091$, $p \leq 0.016$]. Both sexes of HS mice and HDID-1 male mice did not show significant alcohol effects on locomotor activity ($F \leq 1.751$).

Regression analysis of the alcohol-drinking groups combined across all genotypes and sexes showed a moderately strong positive correlation between BEC and percent time in the open arms ($r = 0.556$, $n = 40$, $p < 0.001$; see Fig. 4-4, left), and also number of line crosses ($r = 0.623$, $n = 40$, $p < 0.001$; see Fig. 4-4, right).

Experiment 2: dose-response to alcohol-induced anxiolysis

Significant main effects were found for dose group [$F(3,11) = 10.835$, $p < 0.001$], line [$F(1,111) = 12.246$, $p = 0.001$], and sex [$F(1,111) = 11.270$, $p = 0.001$], and there were no significant interactions. Due to the lack of significant interactions with sex ($F \leq 1.284$ for all), Figure 4-5 shows the percent time spent in the open arms collapsed on sex. *Post hoc* analyses showed that 1.5 g/kg-treated animals spent significantly more time

in the open arms than all other groups ($p \leq 0.001$–0.029), and that 1 g/kg-treated animals spent more time in the open arms than the saline group ($p = 0.04$). HDID-1 mice spent more time in the open arms than HS mice ($p = 0.001$), and female mice spent more time in the open arms than male mice ($p = 0.001$). Due to the results from the water group in Experiment 1, we had an *a priori* interest in whether HDID-1 and HS male mice in the saline group would also show a similar genotypic difference in baseline anxiety-like behavior. Thus, we decided to also analyze open arm time in just the male saline-treated animals. Analysis showed a significant main effect of line, with HDID-1 male mice spending more time in the open arms than male HS mice [$F(1,17) = 12.557$, $p = 0.002$] (Fig. 4-5 inset).

For line crossings, there was a significant main effect of dose group [$F(3,111) = 10.63$, $p < 0.001$] and a statistical trend toward a main effect of line [$F(1,111) = 3.024$, $p = 0.085$]. There were no significant interactions, and Figure 4-6 shows the number of line crosses made by each group collapsed on sex. *Post hoc* analyses found that the 1.5 g/kg group made significantly more line crosses than the saline and 0.5 g/kg groups, and that the 1 g/kg group made more line crosses than the saline group ($p \leq 0.009$ for all).

Experiment 3: genetic correlation of alcohol DID and basal anxiety-like behavior in inbred strains

Table 4-2 shows the correlation matrix for g/kg alcohol intake during DID and all measures of anxiety-like behavior from the selected data sets. No anxiety variable showed a significant correlation with alcohol DID across strains (Table 4-2, first column). Several anxiety measures showed significant strain correlations with each other, both

within data sets and between data sets. These correlations are shown in boldface in Table 4-2.

Discussion

In these experiments, we found that selective breeding for high BECs after limited-access drinking has resulted in replicate- and sex-specific effects on basal anxiety-like behavior and alcohol-induced anxiolysis. In Experiment 1, HDID-1 female mice and HDID-2 male mice showed reductions in anxiety-like behavior after a 4-h alcohol DID session compared to water-drinking controls. These differences appear to be due to the pharmacological effects of alcohol since BEC showed a positive correlation with measure of anxiety-like behavior used. That is, the higher the BEC achieved following DID, the more open arm time an animal showed. Thus, the line differences in anxiety-like behavior seen following alcohol DID are presumably due to the difference in intake between the HDID and HS lines. We have shown previously that alcohol intake during DID is sufficient to produce behavioral intoxication as assessed by impairment on the balance beam test (Crabbe et al., 2009), but this is the first study to show DID-induced anxiolysis in these mice. The absence of an apparent anxiolytic effect of alcohol in the HDID-1 male mice is presumably the result of a ceiling effect due to their relatively non-anxious state at baseline (see below). It is less clear, however, why there was no apparent effect of alcohol in the HDID-2 females, as these mice did not show low anxiety-like behavior at baseline and had high alcohol intake. One possibility is that female mice of this genotype have a reduced sensitivity to the anxiolytic effects of alcohol compared to HDID-1 females and HDID-2 males. The HDID-2 mice have not yet

been tested for anxiolytic response to acute alcohol injections, and repeating Experiment 2 using the HDID-2 mice might help determine whether females of this line truly show blunted anxiolytic sensitivity.

HDID-1 females and HDID-2 mice of both sexes made more line crosses after alcohol drinking than did the HS mice, indicating that in HDID mice, alcohol intake during DID results in significant locomotor stimulation as well. Because performance on the EZM is activity-dependent, locomotor behavior and anxiety-like behavior can be closely related in this task (Kliethermes, 2005). Consequently, an increase in locomotor activity could potentially explain an apparent change in anxiety-like behavior. In this experiment, however, the line differences in anxiety-like behavior do not consistently parallel the activity differences, and therefore are unlikely to be solely a product of changes in locomotion. For example, some groups showing alcohol stimulation (e.g., HDID-2 females) did not also show alcohol-induced anxiolysis. This is supported by the results from Experiment 2, where genotypic differences in anxiety level were seen in the absence of a line difference in activity.

Results from the water-drinking control group in Experiment 1 showed genotypic differences in basal anxiety-like behavior in the males only. Specifically, HDID-1 males showed lower anxiety-like behavior than the HS mice and trended toward a difference from the HDID-2 male mice as well. HDID-2 and HS male mice, and female mice of all three genotypes, did not differ in baseline anxiety-like behavior. In Experiment 2, saline-treated HDID-1 male mice also spent more time in the open arms than saline-treated HS male mice. It appears, therefore, that HDID-1 male mice are unique among these groups in their relatively non-anxious basal state. However, all of the groups spent, on average,

at least 30% of the time in the open arms, suggesting that none of the sexes/genotypes have high baseline anxiety as assessed by this task. Previous studies with rats and mice have shown that sex differences in baseline anxiety-like behavior are influenced by both task and genotype (e.g., Johnston & File, 1991; O'Leary et al., 2013; Wilson et al., 2004), and a different anxiety test could therefore produce different results. Because of the relatively low basal levels of anxiety-like behavior, testing the animals on a more anxiogenic procedure or on a related behavior such as stress reactivity could better elucidate the replicate- and sex-specific differences among these lines. It should be noted that in these studies we did not control for estrous state in the female mice. Anxiety-like behavior has been shown to fluctuate throughout the estrous cycle in rats (Frye et al., 2000), and it is possible that if we were to match females for estrous, we would see a different pattern of sex and genotype effects.

In Experiment 2, HDID-1 mice showed generally lower anxiety-like behavior on the EZM than HS mice regardless of alcohol dose, and both genotypes showed increasing anxiolysis with higher doses of alcohol. There is the possibility of a ceiling effect in the HDID-1s, as they started at a relatively low level of anxiety-like behavior and had little room to show any further anxiolytic effect of alcohol. Due to the nature of the EZM, 50% of time spent in the open arms is the maximum measure for anxiolysis, as this indicates equal preference for (or an inability to distinguish between) the open and closed arms. Consequently, it is difficult to determine whether there were genotypic differences in alcohol-induced anxiolysis between HDID-1 and HS mice from these data. It might be possible to manipulate the parameters of the EZM test (e.g., brighter lighting, shorter open arm lip height) to make the test more anxiogenic in order to produce greater

baseline anxiety-like behavior in the HDID-1 mice (Wahlsten et al., 2003). With genotypes equated at baseline, it could then be determined more conclusively if there are differences in anxiolytic response to alcohol. From the current study, however, it appears that HDID-1 and HS mice do show similar dose-dependent decreases in anxiety-like behavior with increasing doses of alcohol.

HDID-1 mice, particularly males, may be less sensitive to the inherent anxiogenic characteristics of the EZM than HS mice. These mice have also been shown to be less sensitive to the aversive effects of alcohol than HS mice in a conditioned taste aversion test that employed only males (Barkley-Levenson et al., 2015), and could potentially have a general deficit in sensitivity to aversive stimuli. Rather than a high anxiety/stress reactive and high drinking phenotype, the HDID-1 mice may instead show more similarity to novelty-seeking or sensation-seeking at-risk human drinkers. Novelty seeking and sensation seeking have been demonstrated to be associated with risk for AUDs and excessive drinking (Lange et al., 2010; Manzo et al., 2014; Noël et al., 2011), and it would be interesting to test the HDID-1 mice on a measure of novelty seeking to determine whether this behavior is associated with binge-like drinking in these animals.

Work from other rodent lines selected for alcohol intake measures has shown varied relationships with anxiety-like behavior and alcohol-induced anxiolysis. Some lines, such as the Sardinian and Indiana alcohol-preferring rats, show higher baseline anxiety-like behavior and greater anxiolytic effect of alcohol than the non-preferring lines (Colombo et al., 1995; Roman et al., 2012; Stewart et al., 1993). In other selected lines, such as the high- and low-alcohol drinking rats and the high- and low-alcohol preferring mice, differences in anxiety-like behavior are not readily apparent or show replicate- and

task-dependent differences only (Badia-Elder, Stewart, Powrozek, Murphy, & Li, 2003; Can et al., 2012). Most similar to our findings with the HDID-1 male mice are the Finnish Alko Alcohol rats. Reduced anxiety-like behavior and enhanced exploratory behavior and risk taking was seen in the high-drinking rats compared to the low-drinking, Alko Non-Alcohol rats (Roman et al., 2012). The HDID-1 male finding is also consistent with the results of the drinking experiment with anxiety-selected lines, where LAB rats drank more alcohol than HAB rats, but showed no significant alcohol anxiolysis (Henniger et al., 2002). Our data appear to continue the trend in the literature that selection for high-drinking behaviors does not reliably produce consistent changes in anxiety-like behavior.

Analysis of the data from inbred strains in Experiment 3 also supports a lack of genetic association between basal anxiety-like behavior and DID. None of the anxiety variables from the data sets analyzed showed a significant correlation with alcohol DID across strains. Anxiety data used in these analyses included three different tasks and were collected in three different laboratories, and the failure of any of these variables to correlate significantly with DID provides strong support for different genetic factors underlying alcohol DID and basal anxiety-like behavior in mice. However, the anxiety measures were all based on avoidance of an anxiogenic region, and a different result may be found with other putative anxiety-related behaviors that are not captured by these variables (e.g., fecal boli count, defensive burying, stretch attend positions).

It should be noted that different assays of anxiety-like behavior may not always produce similar results and that a genotype's assessed basal anxiety is likely task-dependent (e.g., Bouwknecht & Paylor, 2002; Griebel et al. , 2000; Milner & Crabbe,

2008). Consequently, future experiments could look at the HDID lines on a different test of anxiety-like behavior, such as an open field apparatus, to determine whether the reduced basal anxiety-like behavior of HDID-1 male mice generalizes across tests and whether anxiolytic response to alcohol still does not differ from HS. From the present data, however, it seems that selection for drinking to intoxication has not produced systematic changes in alcohol-induced anxiolysis, and that decreases in basal anxiety-like behavior may only be related to selection in a sex- and replicate-specific way. Furthermore, alcohol dependence can itself produce increases in anxiety (e.g., Breese et al., 2005; Schuckit & Hesselbrock, 1994) and it remains to be seen whether post-dependent anxiety-like behavior is elevated differentially between the HDID and HS lines.

Acknowledgments

The authors would like to thank Jason Schlumbohm, Wyatt Hack, and Marissa Patterson for their assistance with data collection for Experiment 1, and Andy Cameron for analyzing the blood samples for BEC determination. These studies were supported by NIH-NIAAA grants AA13519, AA10760, AA020245, a grant from the US Department of Veterans Affairs, and the Saturday Academy Apprenticeships in Science and Engineering program. A.M.B.-L. was supported by NIH-NIAAA grants AA007468 and AA02009, the Achievement Rewards for College Scientists Foundation, and an OHSU Graduate Research Scholar fellowship.

Table 4-1: Inbred strain panels tested for anxiety-like behavior included in Experiment 3

Anxiety dataset	Anxiety variable	Number of strains in common with DID data	Strains included for correlation analysis
Brown et al. (2004), EZM	Percent of total time spent in open arms	10	129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, BALB/cJ, CAST/EiJ, C57BL/6J, DBA/2J, FVB/NJ, SJL/J
Brown et al. (2004), LDB	Percent of total time spent in light	11	129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, BALB/cJ, C3H/HeJ, CAST/EiJ, C57BL/6J, DBA/2J, FVB/NJ, SJL/J
Milner & Crabbe (2008), EZM	Percent of total time spent in open arms	14	129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, C3H/HeJ C57BL/6J, C57L/J, CBA/J, DBA/2J, FVB/NJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Milner & Crabbe (2008), LDB	Percent of total time spent in light	14	129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, C3H/HeJ C57BL/6J, C57L/J, CBA/J, DBA/2J, FVB/NJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Milner & Crabbe (2008), OF	Percent of total time spent in center (>7.7cm from wall)	14	129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, C3H/HeJ C57BL/6J, C57L/J, CBA/J, DBA/2J, FVB/NJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Wahlsten & Crabbe (2003), EPM	Percent of total time spent in open arms	16	129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, BTBR T+tf/J, C3H/HeJ, CAST/EiJ, C57BL/6J, C57L/J, DBA/2J, FVB/NJ, NOD/ShiLtJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Wahlsten & Crabbe (2003), OF	Percent of total time spent in center (>10cm from wall)	16	129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, BTBR T+tf/J, C3H/HeJ, CAST/EiJ, C57BL/6J, C57L/J, DBA/2J, FVB/NJ, NOD/ShiLtJ, NZB/B1NJ, PL/J, SJL/J, SWR/J

EZM: elevated zero maze; EPM: elevated plus maze; LDB: light-dark box; OF: open field

Table 4-2: Correlation matrix of alcohol DID and anxiety variables for inbred mouse strains

	DID 4hr g/kg	Brown EZM	Milner EZM	Wahlsten EPM	Brown LDB	Milner LDB	Milner OF
Brown EZM	0.014						
Milner EZM	−0.118	−0.315					
Wahlsten EPM	0.114	0.010	−0.211				
Brown LDB	0.329	−0.233	−0.060	**0.664**			
Milner LDB	0.065	**0.602**	0.056	−0.143	0.268		
Milner OF	0.165	**0.569**	**−0.871**	0.142	0.123	0.305	
Wahlsten OF	0.357	−0.379	−0.137	**0.543**	0.315	**−0.628**	0.058

All anxiety variables represent percent time spent in the anxiogenic region of the apparatus. Boldface indicates statistically significant correlation. Abbreviations: DID, drinking in the dark; EZM, elevated zero maze; EPM, elevated plus maze; LDB, light-dark box; OF, open field.

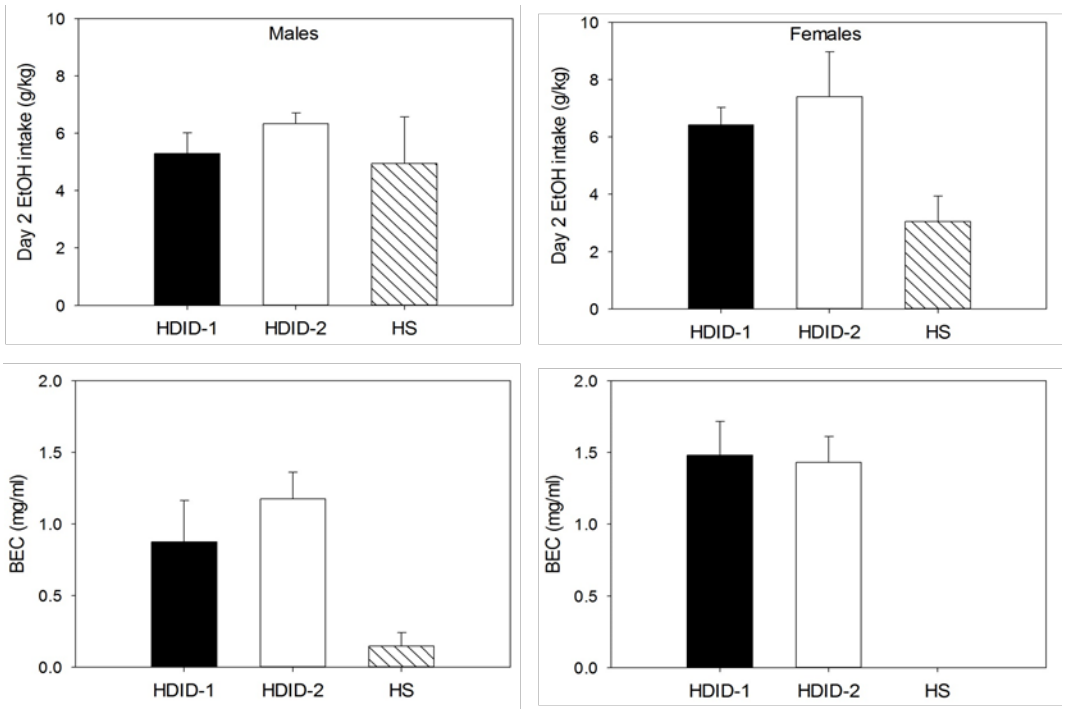


Figure 4-1. Day 2 ethanol intake in g/kg body weight and blood ethanol concentration (BEC) for males (left panels) and females (right panels) of each genotype in the ethanol-drinking group. Means ± SEM shown. There was a statistically significant effect of line for both intake and BEC. BEC bar for HS females is absent as no animals in this group had a measurable BEC. *n* = 6–9/sex/line

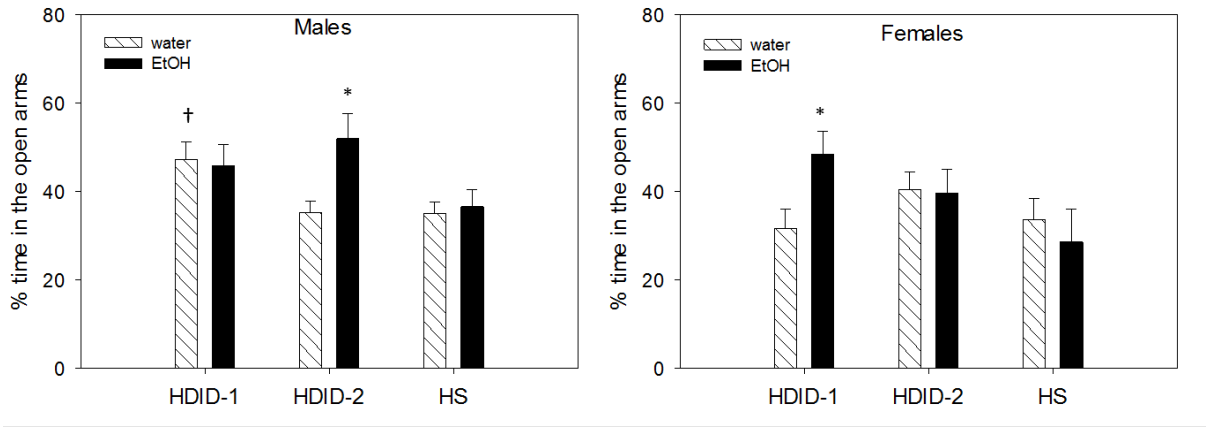


Figure 4-2. Percent time spent in the open arms by males (left) and females (right) of each genotype and drinking group. Means ± SEM shown. * indicates statistically significant difference from corresponding water group and † indicates statistically significant difference from corresponding HS group ($p < 0.05$). n = 6–9/sex/group/line

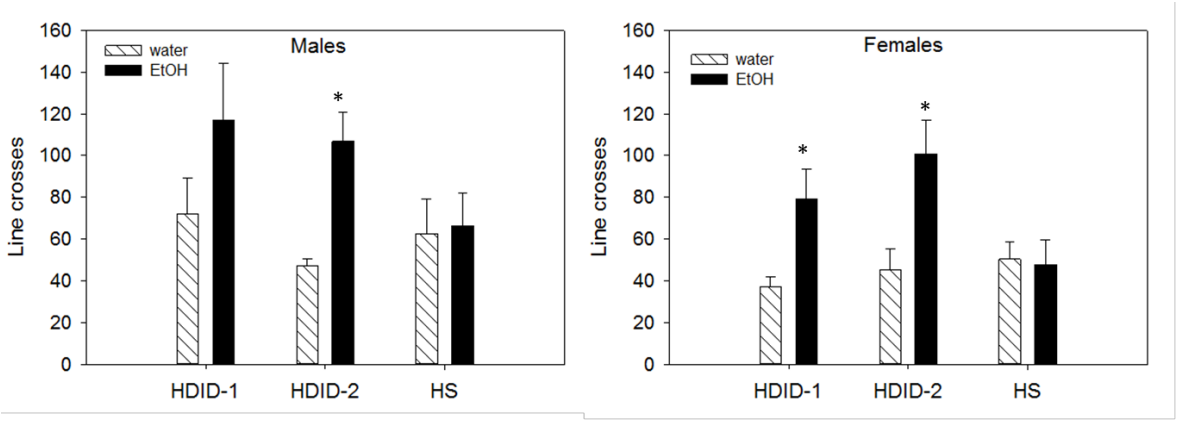


Figure 4-3. Number of line crosses made by males (left) and females (right) of each genotype and drinking group. Means ± SEM shown. * indicates statistically significant difference from corresponding water group ($p < 0.05$). n = 6–9/sex/group/line

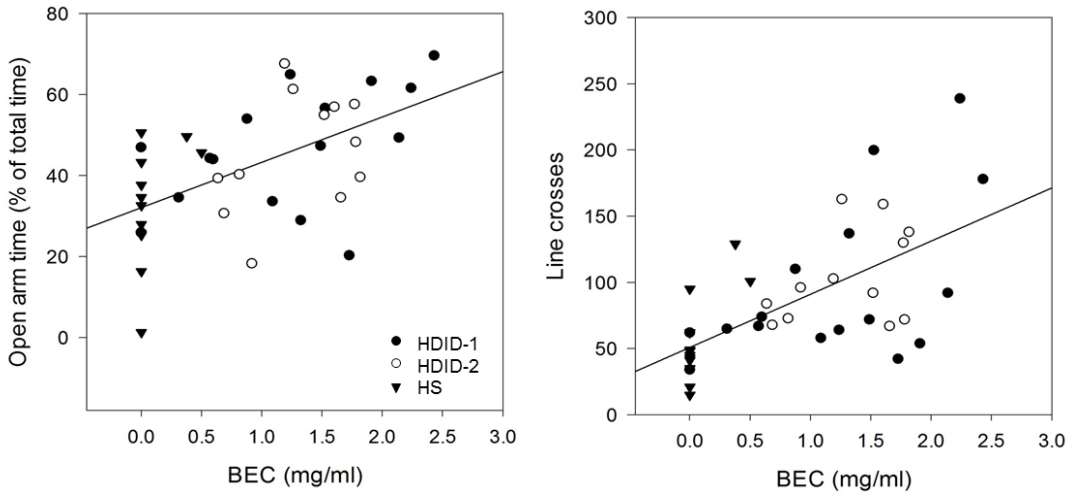


Figure 4-4. Percent time spent in the open arms (left) displayed versus blood ethanol concentration (BEC) immediately after testing on the zero maze and line crossings versus BEC (right). All ethanol-drinking animals are shown (n = 12–17/line). Linear regression lines are depicted.

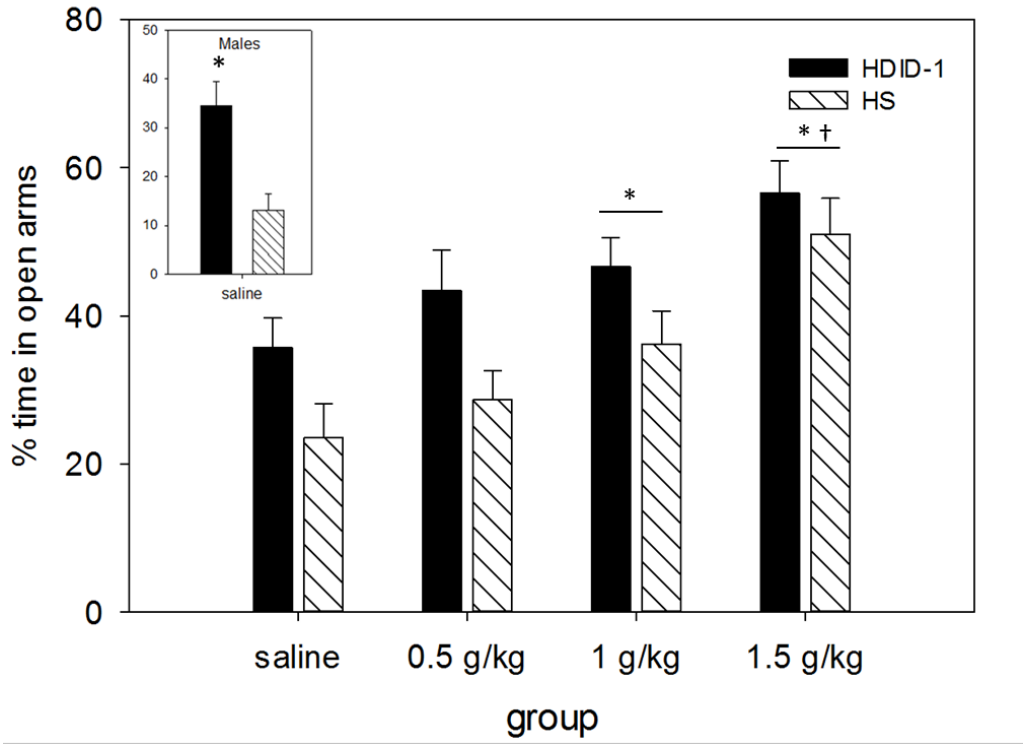


Figure 4-5. Percent time spent in the open arms across alcohol doses collapsed on sex. Inset shows males only of the saline-treated control group. Means ± SEM shown. * indicates statistically significant difference from saline group and † indicates statistically significant difference from 0.5 g/kg alcohol group ($p < 0.05$). n = 5–10/sex/line/dose

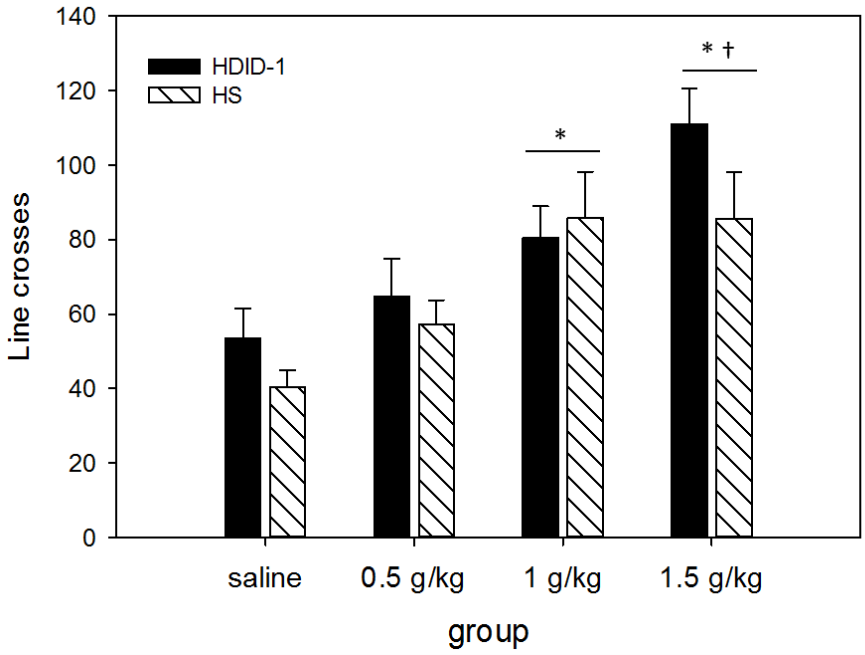


Figure 4-6. Number of line crosses made by each genotype across alcohol dose groups collapsed on sex. Means ± SEM shown. * indicates statistically significant difference from saline group and † indicates statistically significant difference from 0.5 g/kg alcohol group ($p < 0.05$). n = 5–10/sex/line/dose

Chapter 5: Evaluation of neuropeptide Y system involvement in the binge-like drinking phenotype of the High Drinking in the Dark selected mice

Amanda M. Barkley-Levenson and John C. Crabbe

This chapter has been prepared in manuscript format but the data are unpublished

Abstract

The High Drinking in the Dark (HDID) mice have been selectively bred for drinking to intoxicating blood alcohol levels and represent a genetic model of risk for binge-like drinking. Presently, little is known about the specific genetic factors that promote excessive intake in these mice. Previous studies have identified neuropeptide Y (NPY) as a potential target for modulating alcohol intake, and *Npy* is differentially expressed in HDID-1 vs. control mice (HS) in several brain regions involved in alcohol consumption. The present studies describe the use of a candidate gene approach for identifying and testing the contribution of a single potential gene (*Npy*) to the HDID phenotype. We assessed NPY protein levels in the HDID-1 and control HS mice at baseline and after binge-like drinking. NPY reduction in response to alcohol was blunted in the nucleus accumbens (NAc) of HDID-1 mice, and this region was selected for targeting with pharmacological manipulations of the NPY system. Pilot studies identified the NPY Y1 receptor antagonist BIBP 3226 and the Y2 receptor agonist NPY_{13-36} as the most likely candidates for reducing binge-like intake. HDID-1 mice received bilateral infusions into the NAc of BIBP 3226 or NPY_{13-36} immediately before drinking on the final day of a drinking in the dark test. Antagonism of the Y1 receptor showed no significant effect on intake or blood alcohol levels. Y2 receptor stimulation showed a trend toward reducing blood alcohol levels, but this effect was transient and did not persist with repeated testing. These studies show that although HDID mice have significantly blunted NPY response to alcohol in the NAc, their binge-like drinking phenotype is resistant to pharmacological manipulations of the NPY system in the NAc. Thus, NPY in this region does not appear to be an ideal target for treatment in this model of binge intake.

Introduction

Binge drinking as defined by the NIAAA is a pattern of intake that results in blood alcohol levels at or above the legal limit of 0.08g% (NIAAA, 2004). The High Drinking in the Dark (HDID) mice have been selectively bred for reaching high blood ethanol concentrations (BECs) after a limited access drinking in the dark (DID) procedure. These mice readily drink to intoxicating BECs and are a novel genetic model of risk for binge drinking (Crabbe et al., 2009, 2014). Because the genetic and neurobiological factors underlying binge drinking are not yet known, identifying specific genetic contributions to the HDID phenotype may have translational value for understanding excessive drinking in humans.

One candidate gene that is of interest in the HDID mice is neuropeptide Y (NPY). NPY is a 36 amino acid peptide expressed widely in the brain and the periphery. It has six known receptor subtypes (Y1R-Y6R), with Y1R and Y2R being the two most frequently expressed in the brain (Michel et al.,1998). Y1R is found postsynaptically and preferentially binds intact NPY. Y2R is found presynaptically and is traditionally thought to be an autoreceptor, where binding at this receptor decreases release of NPY from the cell. Y2R has higher affinity for C-terminal fragments of cleaved NPY than for the intact peptide (Grandt et al., 1996; Michel et al., 1998).

Studies in humans and animal models have suggested a role for NPY in alcohol consumption. Multiple polymorphisms in the *NPY* gene have been associated with alcohol drinking and dependence in humans (e.g. Bhasker et al., 2013; Francès et al., 2011; Mottagui-Tabar et al., 2005), although an association is not always seen (Zill et al., 2008). Similarly, *Npy* gene expression and peptide levels in several brain regions differ between lines of rodents selected for high alcohol preference and also between inbred

strains with disparate alcohol intakes (Caberlotto et al., 2001; Hayes et al., 2005; Hwang et al., 1999). Genetic manipulation of *Npy* can also alter ethanol drinking. Knocking out *Npy* can increase alcohol consumption, and overexpression of *Npy* can decrease alcohol consumption (Thiele et al., 1998). There is some evidence, though, that these effects may be at least partially dependent on genetic background (Thiele et al., 2000). Administration of NPY produces similarly mixed results. In general, intracranial NPY treatment appears to reduce alcohol intake in animals genetically predisposed to prefer alcohol (e.g. selected lines) and those with a history of dependence. In contrast, NPY tends to be ineffective at altering drinking in low-preferring selected lines and alcohol-naïve animals (for review, see Badia-Elder et al., 2007). There is also evidence that alcohol can influence NPY expression. Alcohol exposure results in decreased NPY expression in some brain regions including the central nucleus of the amygdala (CeA) and the hippocampus (Criado et al., 2011; Slawecki et al., 2005; Sparrow et al., 2012). Alcohol withdrawal can produce an opposite effect, with elevated NPY levels in some brain regions during acute and protracted withdrawal (Bison & Crews, 2003; Criado et al., 2011; de Pauli et al., 2014). The alcohol metabolite acetaldehyde can also produce decreases in NPY levels in the NAc and hippocampus following acute treatment, and increases in these same regions during withdrawal (Plescia et al., 2014).

It should be noted that a majority of the previous work has been done in animals selectively bred for, or tested on, alcohol preference in a two-bottle choice continuous access test. However, there is evidence to suggest that NPY might be relevant to binge-like drinking as well. Central administration of NPY reduced intake and BEC in the DID test in B6 mice. A Y2R antagonist had a similar effect, whereas a Y1R antagonist

enhanced intake and BEC (Sparrow et al., 2012). In the HDID mice, *Npy* mRNA levels are higher in naïve male mice of the first replicate line than in HS mice in the nucleus accumbens (NAc), the CeA, and the bed nucleus of the stria terminalis (BNST), but not in the prefrontal cortex, ventral tegmental area, or basolateral amygdala (BLA) (Zhang et al., 2011). *Npy* gene expression has not yet been tested in the HDID-2 mice. However, gene network connectivity analysis of striatal tissue from both HDID replicates and the HS mice shows selection-dependent alteration of connectivity of the gene encoding Y2R (Iancu et al., 2013). These data suggest that the NPY system may have been altered through selection in the HDID mice as compared to controls, and that these changes may help explain in part their drinking to intoxication.

The present studies were designed to (1) determine the relationship between NPY and binge-like drinking in the HDID-1 line and (2) assess whether a candidate gene approach can be implemented in these mice to evaluate potential specific contributions to their high-drinking phenotype. Mice from only the first replicate were tested throughout because *Npy* gene expression data are not yet available for the HDID-2 mice. We measured NPY immunoreactivity in multiple alcohol-relevant brain regions in the HDID-1 and HS mice after both water and alcohol DID to determine whether the basal differences in gene expression were also seen at the protein level, and to assess whether NPY levels differed between the lines after alcohol exposure. A secondary goal of this experiment was also to determine a target brain region where manipulation of NPY receptors would be likely to affect binge-like drinking in the HDID-1 mice. This target region (NAc) was then used for the site-specific pharmacology experiments testing the ability of NPY Y1R and Y2R agonists and antagonists to modulate DID intake and BEC.

Y1R and Y2R were chosen for targeting because these receptors are both expressed in the NAc and have both been implicated as potentially mediating alcohol intake. Because the gene expression data suggested that NAc NPY levels might be higher in the HDID-1 mice than the HS mice, we hypothesized that reducing NPY signaling with either a Y1R antagonist or a Y2R agonist would attenuate alcohol drinking. In contrast, it was hypothesized that increasing NPY signaling with a Y2R antagonist would enhance drinking.

Materials and methods

Animals and husbandry

Male mice from the HDID-1 and HS lines were bred and housed in the Veterinary Medical Unit of the Veterans Affairs Portland Health Care System (Portland, OR). Mice were weaned at 3 weeks of age and were reared with both dam and sire until weaning. After weaning, mice were housed in groups of 2-5 with same-sex littermates or with mice of the same genotype when necessary to avoid single housing. Mice were between the ages of 55 and 97 days old at the start of testing. Experiment 1 used HDID-1 mice from selection generations S27 and S28, Experiment 2 used HDID-1 mice from generation S29, and Experiment 3 used mice from generation S30. HS mice are the starting population of the HDID selection and are the result of an 8-way inbred strain cross (see Crabbe et al., 2009 for details). These mice are maintained without selective breeding and are used as the comparator control line for the HDID animals. During all experiments, mice were singly housed in standard polycarbonate shoebox cages on Bed-o-cob bedding and were provided with *ad libitum* access to water and food (Purina 5001 chow, LabDiet,

St. Louis, MO) unless otherwise specified. Mice were maintained on a 12 h/12 h reverse light-dark cycle with lights off at 09:30 (Experiment 1) or 08:30 (Experiments 2 and 3). All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Drinking in the Dark

The standard 4-day Drinking in the Dark (DID) procedure was used for these experiments. Mice were weighed each day approximately 1 h before testing. At 3 h into the dark cycle, the water bottles are removed from each cage and replaced with a 10 ml drinking tube containing 20% ethanol or water depending on the experiment. Fluid levels were recorded and tubes were left in place for 2 h on Days 1-3. On Day 4, tubes were left in place for 4 h and fluid levels were recorded at the 2 h time point. At the end of each drinking session, fluid levels were read again and tubes were removed and replaced with water bottles. Immediately after drinking on Day 4, a 20 µl blood sample was taken from the peri-orbital sinus of all ethanol-drinking animals. Blood samples were processed according to standard lab protocols for gas chromatography determination of BEC (Rustay & Crabbe, 2004).

Experiment 1: HDID-1 and HS NPY immunohistochemistry after water or binge ethanol exposure

Fifty-three male HDID-1 and HS mice were singly housed and allowed to acclimate for a week. Mice were then tested on the standard 4-day DID procedure, with half the animals drinking 20% ethanol and half drinking water (n=13-14/group/line). After drinking on Day 4, blood samples were collected from the ethanol group. Mice

were then moved in squads of two-three to a separate procedure room and were deeply anesthetized with rat cocktail (see surgical procedure) and transcardially perfused with 4% PFA in PBS according to previously published methods for NPY immunohistochemistry (Hostetler et al., 2013). After perfusion, brains were dissected and stored at -8 C in 15 ml conical centrifuge tubes containing 4% paraformaldehyde (PFA). All ethanol group animals were perfused first, followed by all water animals. This was done to minimize the time between the end of the drinking session and brain collection to allow for more accurate correlation of alcohol intake with NPY immunoreactivity.

NPY immunohistochemistry

Brains were cut into 40 micron sections on a Leica cryostat and were stored in 0.1% sodium azide in phosphate buffered saline (PBS) until the time of assay. NPY immunohistochemistry (IHC) was conducted according to previously published methods (Hostetler et al., 2013). Briefly, floating sections were rinsed in PBS and then incubated in 0.3% hydrogen peroxide for 15 minutes. Slides were then rinsed again in PBS and incubated in a blocking solution (2% bovine serum albumin [BSA] and 5mg/ml heparin in 0.003% Triton X100 and PBS) for 5 hours. After blocking, slices were incubated overnight in rabbit polyclonal antibody targeted against NPY (anti-NPY N9528, Sigma-Aldrich, St. Louis, MO; 1:10,000 dilution) in a Triton and BSA PBS solution. The next day, slices were washed in PBS and incubated for 1 hour in biotinlyated-goat anti-rabbit secondary antibody in PBS and Triton. Slices were washed again in PBS and then incubated in avidin/biotin solution (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) in PBS and Triton for 1 hour. Slices were washed once more in PBS

and then were stained using a 3,3'-diaminobenzidine (DAB) reaction. Slices were then slide-mounted on the same day as the assay.

For each region, we aimed to analyze both hemispheres from two to four slices per animal. Analysis was done using ImageJ (National Institutes of Health) by an experimenter blinded to treatment and genotype. Each region of interest was selected manually using anatomical reference points from a mouse brain atlas (Franklin & Paxinos, 2001). Photographs were analyzed using a standardized background subtraction and the threshold function in ImageJ (threshold was kept constant across slices within a given region). Optical density of staining (NPY-positive fibers and cell bodies) was then automatically quantified by the software and reported as a percent of total area (i.e., selected brain region). Cell bodies were counted only in the NAc as this was the only area with a significant number of NPY-positive cell bodies and also low enough density of fiber staining that they could be reliably counted. All NPY-positive cell bodies within each NAc subdivision (core vs. shell) were manually counted. All optical density values and cell counts within a given region (2-8 replicates per animal) were averaged so that each animal had only a single data point per region.

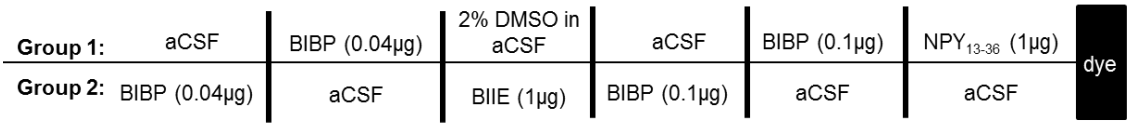
Experiment 2: Pilot testing of intra-accumbal administration of Y1R and Y2R drugs

Based on the results of Experiment 1, the entire NAc (i.e., no distinction made between core and shell) was chosen as the target area for NPY pharmacology. Bilateral cannulation of the NAc was attempted in 35 HDID-1 male mice and correct placements were confirmed for 22 animals. Mice were assigned to two experimental groups (final group size: n=8-14/group). Animals were given six sequential 4-day ethanol DID tests, with NPY receptor drugs given on Day 4 of each test. The compounds tested were the

Y1R antagonist BIBP 3226 (0.04 and 0.1 μg/animal doses), the Y2R antagonist BIIE 0246 (1.0 μg/animal), and the Y2R agonist NPY_{13-36} (1.0 μg/animal). These drugs and doses were chosen based on those found to be effective for reducing DID in B6 mice when administered centrally in a previous study (Sparrow et al., 2012). On each drug administration day, one group received drug and one group received vehicle (artificial cerebrospinal fluid [aCSF]: NaCl [128mM], KCl [2.6mM], $CaCl_2$ dihydrate [1.3mM], $MgCl_2$ hexhydrate [0.9 mM], $NaHCO_3$ [20mM], Na_2HPO_4 dibasic anhydrous [1.3mM]). The group receiving drug and the group receiving vehicle were switched between DID tests to try to minimize potential carry-over effects from the repeated testing and to counter instances of baseline group differences. Group 1 animals received in order: aCSF, BIBP 3226 (0.04 μg), 2% DMSO in aCSF, aCSF, BIBP 3226 (0.1 μg), and NPY_{13-36}. Group 2 animals received in order: BIBP 3226 (0.04 μg), aCSF, BIIE 0246, BIBP 3226 (0.1 μg), aCSF, and aCSF (see schematic below).

On Days 1 and 2 of the first DID test, mice were grasped by the scruff and any missing stylets were replaced. On Day 3, injectors were inserted fully into each cannula but no infusion was given. Injectors consisted of 11mm stainless steel tubes (33 Ga) that extended 1mm beyond the end of the cannula (Vita Needle Company, Needham, MA). Anecdotal evidence suggests that initial disruption of the tissue beyond the cannulae can produce reductions in intake, so injectors were inserted on Day 3 in order to avoid having this effect coincide with the first test day. During all subsequent DID tests, mice were grasped by the scruff and stylets replaced on Days 1-3. On Day 4 of each test, mice were infused bilaterally with a volume of 200 nl administered per side. Infusions were given over 60 s, followed by injectors being left in place for an additional 30 s to allow

diffusion. Animals were infused in 3 squads (11-12 animals per squad) with staggered DID start times. All vehicle animals in a squad were infused first, followed by all drug animals, so that drug infusions occurred as close to the beginning of the drinking session as possible. Immediately after all animals in a squad had been infused, drinking tubes were placed on the cages and the DID session was carried out as described above. The infusion procedure for an entire squad took approximately 25 min.



Schematic of Experiment 2: Order of testing and group drug assignments are shown for each of the six 4-day DID tests. Vertical lines represent water washout periods and the solid bar at the end represents methylene blue infusion.

Experiment 3: Intra-accumbal Y2R agonist and Y1R antagonist effects on HDID-1 ethanol and water DID

Because of the potential confound of considerable repeated testing in Experiment 2, Experiment 3 was designed to test again in naïve animals the two NPY drugs/doses identified as the most likely to have an effect on drinking (1.0 µg NPY_{13-36} and 0.1 µg BIBP 3226). Bilateral cannulation of the NAc was attempted in 30 HDID-1 male mice and was confirmed in 10 animals. The same infusion procedures were used as in Experiment 2, but all animals were assigned to receive either the NPY receptor ligands or aCSF across all of the DID tests (final group size: n=5/group). Mice were tested first with NPY_{13-36} (Day 4) and then with BIBP 3226 (Day 13). Because NPY_{13-36} showed a trend toward reducing BEC mg/ml (see results), the decision was made to test mice once more with this drug (Day 18) to determine whether the efficacy would be enhanced in animals with a more robust alcohol history. Two water DID control tests were also conducted to determine whether the NPY drugs had non-specific effects on consummatory behavior.

These tests each consisted of a single 4 h DID session with a water tube following infusion with NPY_{13-36} (Day 6) or BIBP 3226 (Day 20).

Surgical procedures

Mice were anesthetized via intraperitoneal injection with "rat cocktail" consisting of ketamine (100 mg/ml), xylazine (20 mg/ml), and acepromazine (10 mg/ml) diluted 1:6 in saline. Following confirmation of full anesthesia by lack of response to a toe pinch, the mouse was placed in the stereotaxic instrument (Kopf Instruments, Tujunga, CA) and the head was immobilized with ear bars. The surgical site was shaved and swabbed with betadine and a small portion of the scalp was removed to expose the skull surface. Bregma and lambda were identified and the distance between them measured. The bregma-lambda distance for each mouse was divided by 3.39 to produce a correction factor for each animal to account for differences in skull/brain size between HDID-1 mice and the B6-based atlas coordinates. This correction factor was multiplied by the mouse brain atlas target coordinates (Franklin & Paxinos, 2001) and these corrected coordinates were used for cannula implantation. A stainless steel anchor screw (1/8", Amazon.com, Seattle, WA) was inserted into the skull approximately 3 mm posterior to the cannula target location and 0.75 mm lateral to the midline (left/right position was counterbalanced across animals). Two independent guide cannulae (26 Ga, stainless steel, 10mm length; Component Supply, Ft. Meade, FL) were inserted aimed at the NAc "shore" (A/P -1.34, M/L ± 0.63, D/V -3.4). Cannulae were secured and the incision sealed with dental acrylic. Stainless steel stylets (10 mm) were inserted into each cannula to maintain patency. Mice were allowed to recover for at least 5 days before the start of testing.

Histology

After the completion of testing in Experiments 1 and 2, methylene blue (17mg/ml in aCSF) was infused bilaterally (200nl per side) using the same infusion procedure as for each drug administration. Mice were then killed via cervical dislocation and brains were dissected out and stored in 2% PFA at -8 C. Brains were cut into 40 micron sections using a Leica CM1850 cryrostat (Leica Biosystems, Buffalo Grove, IL) and NAc-containing sections were thaw-mounted onto glass microscope slides. Slides were examined by an experimenter blinded to treatment using a Leica DMLB microscope (Leica BioSystems, Buffalo Grove, IL) and hits were determined by the presence of either dye or injector tracts terminating in the NAc. All misses were found to be unilateral, with one injector in the NAc and the other usually either dorsal or medial to the target area. Data from all animals was analyzed initially in exploratory analyses, but only animals with bilateral NAc hits (core or shell) were included in data analysis described here.

Drugs

Ethanol (200 proof, Decon Labs, King of Prussia, PA) was dissolved in tap water (20% v/v). The Y1R antagonist BIBP 3226 and Y2R antagonist BIIE 0246 were purchased from Tocris Bioscience (Bristol, UK). Y2R agonist NPY_{13-36} was purchased from Abcam (Cambridge, MA). The vehicle for BIBP 3226 and NPY_{13-36} was aCSF, and the vehicle for BIIE 0246 was 2% dimethyl sulfoxide (DMSO) in aCSF. BIBP 3226 and NPY_{13-36} were gently shaken until the drug/peptide went into solution. BIIE 0246 was dissolved first in DMSO and then aCSF was added. The mixture was sonicated for approximately 30 minutes to ensure the drug was in suspension.

Statistical analyses

In Experiment 1, optical density of NPY staining (% area) and NPY-positive cell counts were analyzed for each region separately using a two-way analysis of variance (ANOVA) for treatment group and genotype. Significant group x genotype interactions were followed up with one-way ANOVAs for each genotype separately. Alcohol intake was converted to a g/kg body weight dose and water intake was converted to ml/kg. Day 4 intake was analyzed for an effect of genotype separately for each fluid group, and BECs were analyzed for an effect of genotype as well. In Experiment 2, intake (g/kg) and BEC on each final DID day were initially analyzed for an effect of treatment group. Due to the combined between- and within-subjects design, no clear assessment of drug effects could be made from these analyses. However, because this was a pilot experiment designed to broadly assess drugs for potential efficacy, data were ultimately collapsed together by treatment. That is, each Day 4 total g/kg value and each BEC value was used as an independent data point and combined by drug treatment group (e.g. all aCSF data averaged, all BIBP 3226 (0.1μg) data averaged, etc.). Exploratory analysis was conducted to identify any potential drug effects in order to determine which compound(s) should be followed up in Experiment 3. Collapsed group data were analyzed by one-way ANOVA for drug and followed up using a two-sided Dunnett's test to compare each drug treatment to the aCSF control group. In Experiment 3, intake and BECs on each infusion day were analyzed for an effect of treatment group. The significance level was set at $\alpha = 0.05$. Statistical trends are discussed for p values ≥ 0.05 and ≤ 0.1.

Results

Some slices in Experiment 1 could not be quantified for NPY immunoreactivity and were excluded from analysis, so the final group sizes for each region ranged from n=9-13/treatment/genotype. Five animals in Experiment 2 and 1 animal in Experiment 3 did not maintain bilateral cannulae patency during the course of testing and their data were subsequently excluded from analysis for any infusion days after bilateral patency was lost. The final group sizes were therefore 7-10 animals per group in Experiment 2 and 4-5 animals per group in Experiment 3.

Experiment 1

Figure 5-1 shows alcohol intake (panel a) and BECs (panel b) during the DID test. The HDID-1 and HS mice showed the expected genotypic difference in alcohol consumption ($F_{1,24}$=22.937, p<0.001) and BEC ($F_{1,24}$=37.851, p<0.001). Water intake on Day 4 did not differ significantly between the genotypes ($F_{1,25}$=1.529, p>0.1; data not shown). Figure 5-2 shows NPY immunoreactivity and NPY-positive cell counts in the NAc core and shell. NPY staining in the NAc core showed a significant main effect of drinking group ($F_{1,43}$=4.681, p=0.036), a statistical trend toward a main effect of genotype ($F_{1,43}$=3.934, p=0.054), and a significant group x genotype interaction ($F_{1,43}$=5.112, p=0.029). HS mice showed a robust decrease in NAc core NPY levels in the alcohol group as compared to the water drinking animals ($F_{1,22}$=13.396, p=0.001), whereas HDID-1 showed no difference between the treatment groups ($F_{1,21}$=0.004, p=0.952). Analysis of NPY immunoreactivity in the NAc shell showed a significant main effect of genotype ($F_{1,43}$=4.904, p=0.032), a trend toward an effect of group ($F_{1,43}$=2.829, p=0.1), and a trend toward a significant group x genotype interaction ($F_{1,43}$=3.358,

p=0.074). Because one goal of this experiment was to identify a potential regional target for the pharmacology studies, we chose to follow up the interaction as an exploratory analysis in order to be able to compare group differences between the core and the shell. Again, HS mice showed significantly lower NPY levels after alcohol drinking than after water drinking ($F_{1,22}$= 9.337, p=0.006). HDID-1 mice showed no difference between the groups ($F_{1,21}$=0.008, p=0.928). The number of NPY-positive cells in the NAc core showed no significant main effects and no significant interaction ($F_{1,42}\leq 1$, p>0.1 for all). The number of NPY-positive cells in the NAc shell showed only a significant group x genotype interaction ($F_{1,42}$=4.462, p=0.041). Follow-up ANOVAs showed that HS mice did not differ in the number of cells between groups, and HDID-1 mice had a trend toward a reduction in the number of cells in the alcohol group as compared to water ($F_{1,20}$=3.533, p=0.075).

Figure 5-3 shows the correlations between NAc core NPY immunoreactivity and alcohol (a) and water consumption (b) in both genotypes. Linear regression was used to determine whether NPY levels in the NAc core and shell were related to total alcohol intake. HS mice showed a significant negative correlation between alcohol intake and NPY levels in the NAc core (r=-0.681, n=11, p=0.021) and a statistical trend toward a negative correlation in the shell (r=-0.567, n=11, p=0.069). In contrast, alcohol intake showed no significant correlation with NPY levels in either region in the HDID-1 mice (p>0.1). Water intake showed no significant relationship with NPY levels in either the NAc core or shell for either genotype, though there was a statistical trend toward a positive correlation between water intake and NPY levels in the NAc shell in HDID-1 mice (r=0.511, n=12, p=0.089) (p>0.1 for all others).

Figure 5-4 shows NPY immunoreactivity in the CeA, BLA, BNST (dorsal and ventral), and PVN. Only NPY levels in the CeA showed a significant main effect of genotype ($F_{1,43}$=6.974, p=0.011), with HDID-1 mice having higher NPY immunoreactivity than HS mice. No regions showed a significant main effect of treatment group and no significant group x genotype interactions were found ($F_{1,40\text{-}46}\leq 1$, p>0.1 for all).

Experiment 2

Figure 5-5 shows approximate injection location for all animals with confirmed bilateral hits in the NAc. Figure 5-6 shows Day 4 intake and BECs as an average for each drug, collapsed across experimental group and DID test order. The aCSF bar includes multiple data points per mouse because each animal was tested two-three times with aCSF. Exploratory ANOVA showed a significant main effect of drug on total alcohol intake on Day 4 ($F_{4,101}$=3.153, p=0.017). Post-hoc Dunnett's test results found the smallest p values for BIBP 3226 (1.0μg) and $NPY_{13\text{-}36}$ (p= 0.148 and 0.132, respectively). There was a significant main effect of drug for BEC ($F_{1,102}$=5.247, p=0.001), and post-hoc Dunnett's test again showed the largest group differences to be between aCSF and high dose BIBP 3226 and $NPY_{13\text{-}36}$ (p=0.083 and 0.042, respectively). These drugs/doses were subsequently chosen for follow-up testing in Experiment 3.

Experiment 3

Figure 5-7 shows approximate injection locations for all animals with confirmed bilateral hits in the NAc. Figure 5-8 shows alcohol intake and BEC for both DID tests with $NPY_{13\text{-}36}$. Analysis of Day 4 data in the first DID test showed no significant effect of treatment on 4 h alcohol intake ($F_{1,8}$=0.284, p=0.609), but there was a weak trend toward

lower BECs in the drug group compared to the vehicle group ($F_{1,8}$=3.384, p=0.103). After 3 cycles of DID, there was still no significant main effect of NPY_{13-36} treatment on intake ($F_{1,7}$=0.961, p=0.360) and there was again a trend toward a drug effect on BEC ($F_{1,7}$=4.335, p=0.076). However, this time the effect was in the opposite direction (drug>vehicle), though this appeared to be largely driven by a decrease in BEC in the aCSF group. Figure 5-9 shows alcohol intake and BEC for the DID test with BIBP 3226. There were no significant main effects of treatment for either alcohol intake or BEC ($F_{1,7}$<1, p>0.1 for both). Figure 5-10 shows water intake (ml/kg) during the control tests for NPY_{13-36} and BIBP 3226 (0.1µg). Neither drug significantly altered water intake ($F_{1,7-8}$<1, p>0.1 for both), suggesting that intra-accumbal infusion of these drugs is unlikely to produce decreases in locomotor activity or general consummatory behavior that could confound the interpretation of the results.

Discussion

In these experiments, we showed that selection for high BECs in the HDID-1 mice has resulted in blunted NPY response to alcohol in the NAc core and shell and overall higher NPY levels in the CeA. This is one of the few reported examples of a specific neurobiological difference between the HDID-1 and HS mice at the protein level and provides possible evidence for a relationship between NPY and binge-like drinking in these mice. Previous studies of NPY region-specific effects on alcohol intake have been predominantly focused on the CeA and other extended amygdala structures (e.g. Sparrow et al., 2012; Gilpin 2012). Based on this literature, the putative role of NPY in altering alcohol drinking has been as an anxiolytic neuropeptide: high drinking, high

anxiety animals show low levels of NPY in the CeA and treatments that increase these levels (NPY administration, Y2R antagonism, etc.) reduce both anxiety and alcohol intake (e.g. Primeaux et al., 2006; Schroeder et al., 2003; Sparta et al., 2004). However, this does not appear to be the mechanism by which NPY may modulate drinking in the HDID-1 mice as HDID-1 males actually show *lower* basal levels of anxiety-like behavior than HS mice (Barkley-Levenson & Crabbe, 2014 [Chapter 4]). This is consistent with the genotypic difference in NPY immunoreactivity in the CeA seen in Experiment 1 (HDID-1 > HS).

Much less work has been done examining the role of NPY in the NAc, but there is some evidence that NPY in this region is relevant to reward and addiction. NPY infusion into the NAc has been shown to condition a place preference in rats, indicating that NPY signaling in this area is perceived as rewarding (Brown et al., 2000). Additionally, NPY administration into the NAc shell stimulates dopamine release, which is generally associated with reward (Sørensen et al., 2009). NPY administration into the NAc shell can also enhance morphine reward, whereas administration of Y1R antagonist BIBP 3226 decreases morphine reward (Desai et al., 2013). Thus, it is possible that the sustained high levels of NPY expression in the NAc of HDID-1 mice during alcohol drinking could similarly enhance the perceived rewarding value of alcohol and could promote high DID intake. HDID-1 and HS mice have been shown not to differ in alcohol conditioned place preference to a 2 g/kg injection (Barkley-Levenson et al., 2015 [Chapter 3]), though there could still be differences in alcohol reward sensitivity at different doses or on a more direct measurement of reward (e.g. progressive ratio self-administration). Furthermore, HDID-1 and HS male mice do show robust differences in an alcohol-conditioned taste

aversion (Barkley-Levenson et al., 2015), and there is evidence to suggest that dopamine release in the NAc may also be involved in encoding responses to aversive stimuli (e.g. McCutcheon et al., 2012). Altered NPY signaling in the NAc of HDID-1 mice might therefore be involved in the reduced alcohol aversion sensitivity of these animals via downstream effects on dopamine.

The NPY IHC findings largely did not parallel the genetic differences in *Npy* mRNA levels previously reported in alcohol-naïve HDID-1 and HS mice (Zhang et al., 2011). There are many post-transcriptional processing steps in the translation of mRNA to peptides (for review, see Vogel & Marcotte, 2012), and it therefore should not necessarily be expected that mRNA and protein levels show a one-to-one relationship. However, this does raise interesting possibilities of altered regulatory processes or compensatory feedback mechanisms in the HDID-1 mice that allow for only limited basal differences in NPY peptide expression between the lines. Future experiments will be needed to tease out what these changes might be and how they could relate to the high drinking phenotype of these mice.

Despite the significant genetic difference in NPY levels post alcohol in the NAc, intra-accumbal manipulation of NPY receptors had little obvious effect on drinking and BECs in the HDID-1 mice. The most promising compound was the Y2R agonist NPY_{13-36}. This drug showed a potential ability to reduce BECs in Experiment 2, and there was a trend toward an effect in Experiment 3 as well. However, it appears that any possible effect may be transient since repeated testing after 3 cycles of DID did not replicate the same treatment group difference. Y2R is expressed presynaptically and is primarily thought of as an autoreceptor where high affinity binding of cleaved C-terminal

fragments of NPY (e.g. NPY_{13-36}) can trigger a feedback mechanism that inhibits the release of NPY (Michel et al., 1998). One mechanistic possibility for a Y2R agonist effect on HDID-1 drinking is that these mice have lower endogenous levels of cleaved NPY to act at the Y2 receptors. This could result in a faulty feedback loop and the observed blunted response of NPY levels to alcohol. This possibility fits with the observed lack of an effect of a Y2R antagonist on drinking—blocking these receptors won't matter if there is limited endogenous ligand present to act on them in the first place. In the present IHC study, it was not possible to differentiate between intact NPY and the cleaved forms that act at the Y2R. However, future studies using different techniques (e.g. high-performance liquid chromatography) could potentially determine whether HDID-1 mice have a deficit in posttranslational processing of NPY and whether Y2Rs actually represent a realistic target for modulation of binge-like drinking in these animals. Additionally, Y2Rs have been shown to have heteroreceptor functions as well and can influence the release of other neurotransmitters such as glutamate and GABA (Gilpin et al., 2011; Silva et al., 2007), and studying Y2R agonist effects in relation to downstream effects of these other transmitters may reveal a more complete picture of how NPY relates to HDID-1 drinking.

It should be noted that there are several limitations of these studies including the use of only the first replicate of HDID-1 mice, potential carry-over effects from repeated testing of NPY receptor ligands in the same animals, and a small sample size. HDID-2 mice have not been assessed for either mRNA or protein levels of NPY, but this would be a logical next step to determine whether alterations in the NPY system appear to be necessary for the HDID phenotype. As for the repeated testing issue, this type of

experimental design always carries the risk of potential confounds due to previous treatments. However, care was taken to include sufficient washout periods between each drug administration. The half-lives of these compounds have not been conclusively shown, but what little data are available suggest that they are in the range of a few hours or less (Malmström et al., 1997). This does not rule out the possibility that metabolism of these compounds is altered in the HDID-1 mice, nor that there could be other compensatory changes (e.g. receptor downregulation) occurring as a result of the repeated treatments. Additionally, the final sample size in Experiment 3 was small. This could account for the difficulty in identifying any significant effect of the NPY receptor ligands, and also the variability in findings with the Y2R agonist after 1 vs. 3 cycles of DID. Adding more animals to this experiment in the future could potentially increase the power to detect a significant drug effect.

An additional consideration is that the effects of the NPY compounds were only tested in a single brain region. Although the IHC data suggested that the NAc was the likely region to target, it may be that modulating NPY in a different area such as the CeA or BNST would provide more fruitful results. The decision was also made to target the entire NAc since IHC findings were similar between the core and the shell. The coordinates for the cannulations were targeted at the “shore” region between the core and the shell, but a majority of the hits appeared to be in the shell. Given that the genetic difference in NPY levels was more pronounced in the core than the shell, it’s possible that future studies targeting only the NAc core may be necessary for a significant behavioral effect.

Despite the limited ability of NPY receptor ligands to attenuate binge-like drinking, these studies demonstrate the use of existing genetic and pharmacological data to choose a candidate gene for further investigation using targeted manipulation in the HDID-1 mice. By identification of targets through the literature and pilot gene expression studies, we can begin to assess how various neurotransmitters, receptors, and other biological factors promote excessive alcohol intake. In this manner, we will ideally be able to narrow down the phenotype-relevant changes produced by selection and may discover potential targets with translational value for reducing binge drinking.

Acknowledgements

The authors would like to thank Dr. Andrey Ryabinin for assistance with the analysis and interpretation of the NPY IHC data, as well the use of his laboratory space for conducting the assay, and Dr. Caroline Hostetler for assistance with the assay. We would also like to thank Dr. Tamara Phillips for the use of her stereotaxic instrument and cryostat, and Stephanie Spence for assistance with the analysis of blood samples for BEC determination. These studies were supported by NIH-NIAAA grants AA13519, AA10760, a grant from the Department of Veterans Affairs, and an American Psychological Association Dissertation Research Award. AMB-L was supported by NIH-NIAAA grant AA02009, the Achievement Rewards for College Scientists foundation, and an Oregon Health & Science University Graduate Research Scholar award.

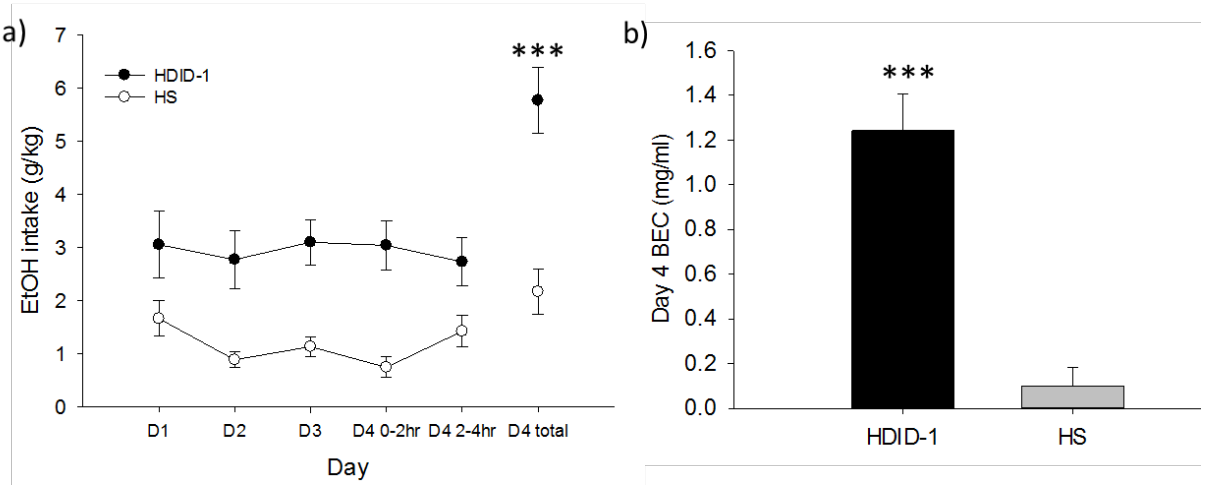


Figure 5-1. Alcohol intake (a) across each day of the 4-day DID test and BECs (b) after drinking on Day 4 for HDID-1 and HS in Experiment 1. Intake data (g/kg) are shown for each 2 h session on Days 1-3, for the first 2 h and second 2 h on Day 4, and for the total 4 h session on Day 4. Means ±SEM shown. N=13/line *** indicates $p<0.001$

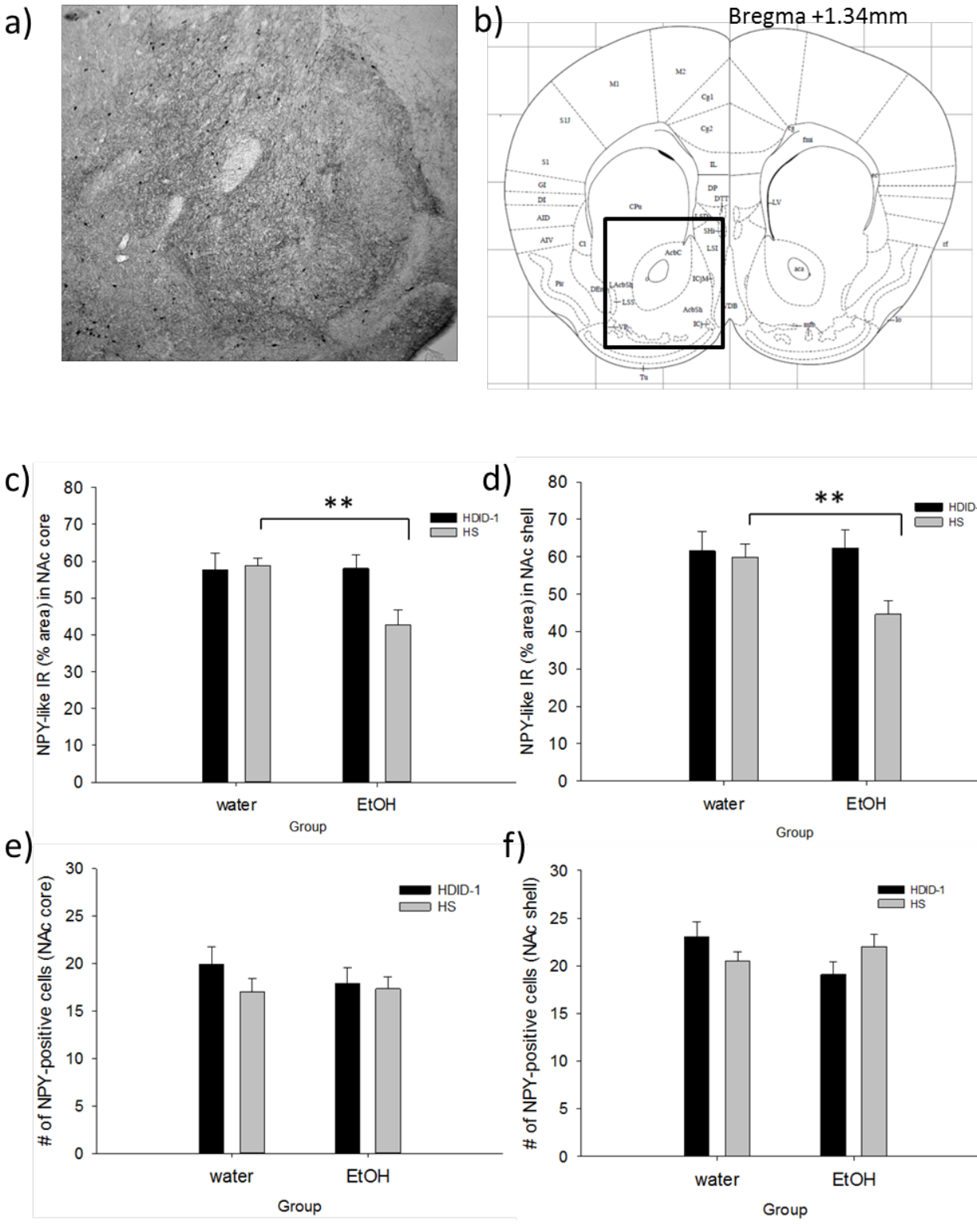

a)
b)
Bregma +1.34mm
c)
NPY-like IR (% area) in NAc core
**
HDID-1
HS
water
EtOH
Group
d)
NPY-like IR (% area) in NAc shell
**
HDID-1
HS
water
EtOH
Group
e)
# of NPY-positive cells (NAc core)
HDID-1
HS
water
EtOH
Group
f)
# of NPY-positive cells (NAc shell)
HDID-1
HS
water
EtOH
Group

Figure 5-2. NPY immunohistochemistry in the nucleus accumbens (NAc). Panel a shows a representative photomicrograph of NPY staining in the NAc of an alcohol-drinking HDID-1 animal. Panel b shows an atlas representation of NAc regions used for NPY staining (boxed area corresponds roughly to brain region shown in panel a). Panels c and d show average NPY immunoreactivity in the NAc core (c) and shell (d) of water and alcohol exposed HDID-1 and HS mice. Panels e and f show the average number of NPY-positive cells for the NAc core (e) and shell (f) in both treatment groups and genotypes. Means ±SEM shown. N=11-13/line/group ** indicates $p<0.01$

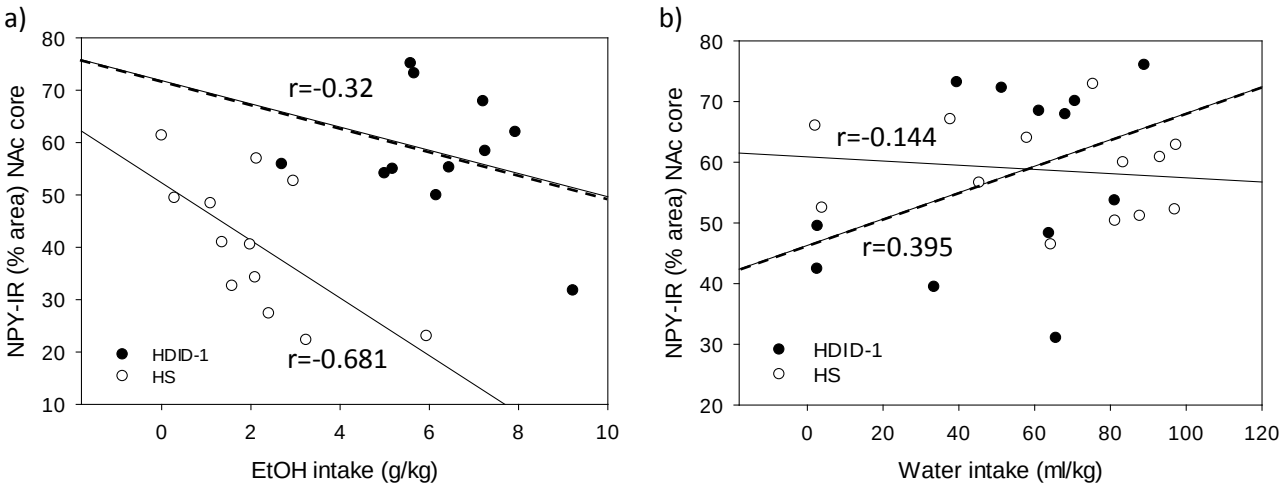


Figure 5-3. Correlations between alcohol (a) and water (b) intake and NAc core NPY immunoreactivity in the HDID-1 and HS mice. Regression lines are shown for each genotype. N= 11-13/line/group

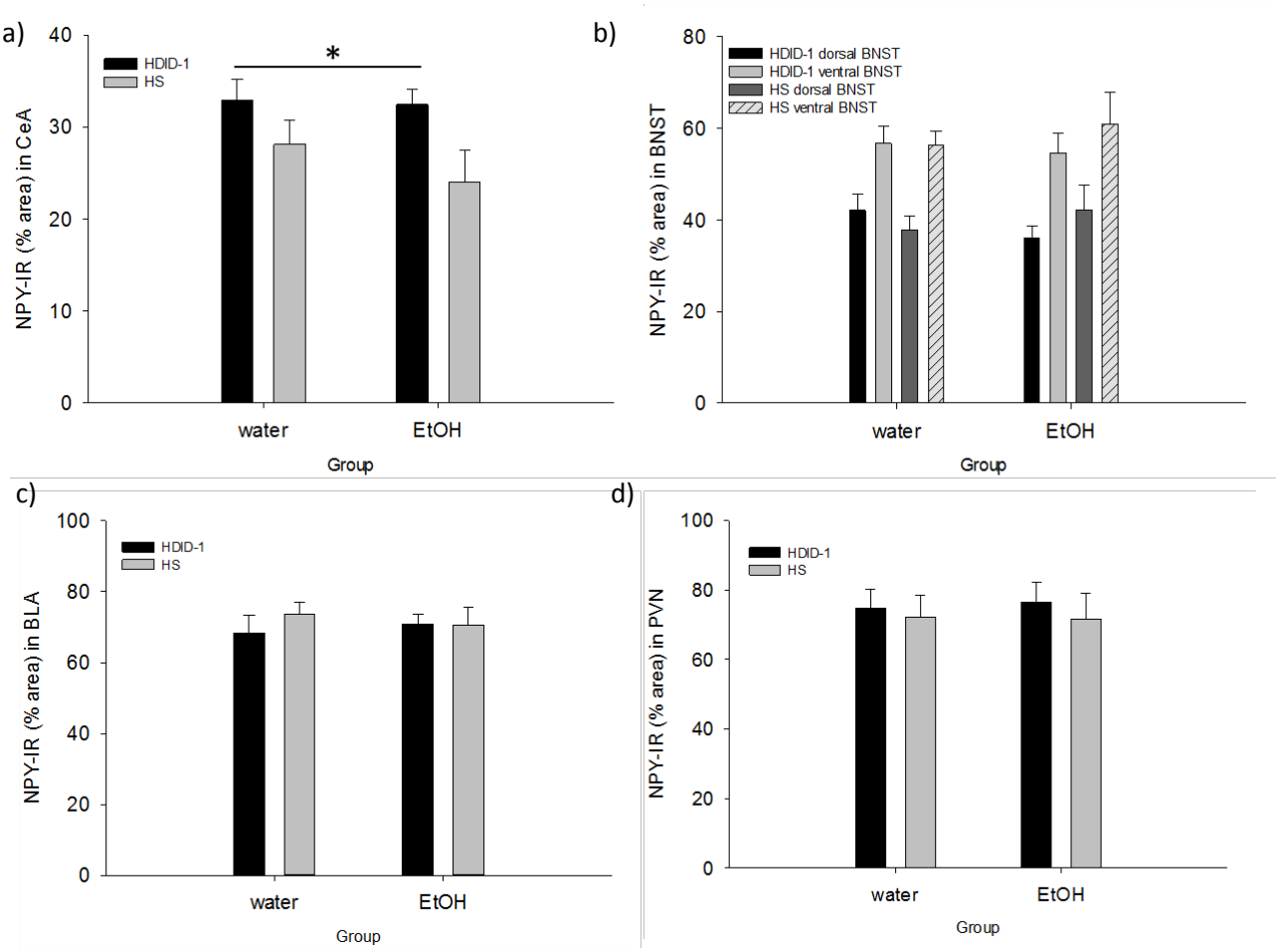


Figure 5-4. NPY immunoreactivity in the central nucleus of the amygdala (a), bed nucleus of the stria terminalis (b), basolateral amygdala (c), and paraventricular nucleus of the hypothalamus (d) of HDID-1 and HS mice of both drinking groups. Means ±SEM shown. N=9-13/line/group * indicates statistically significant difference from HS ($p<0.05$)

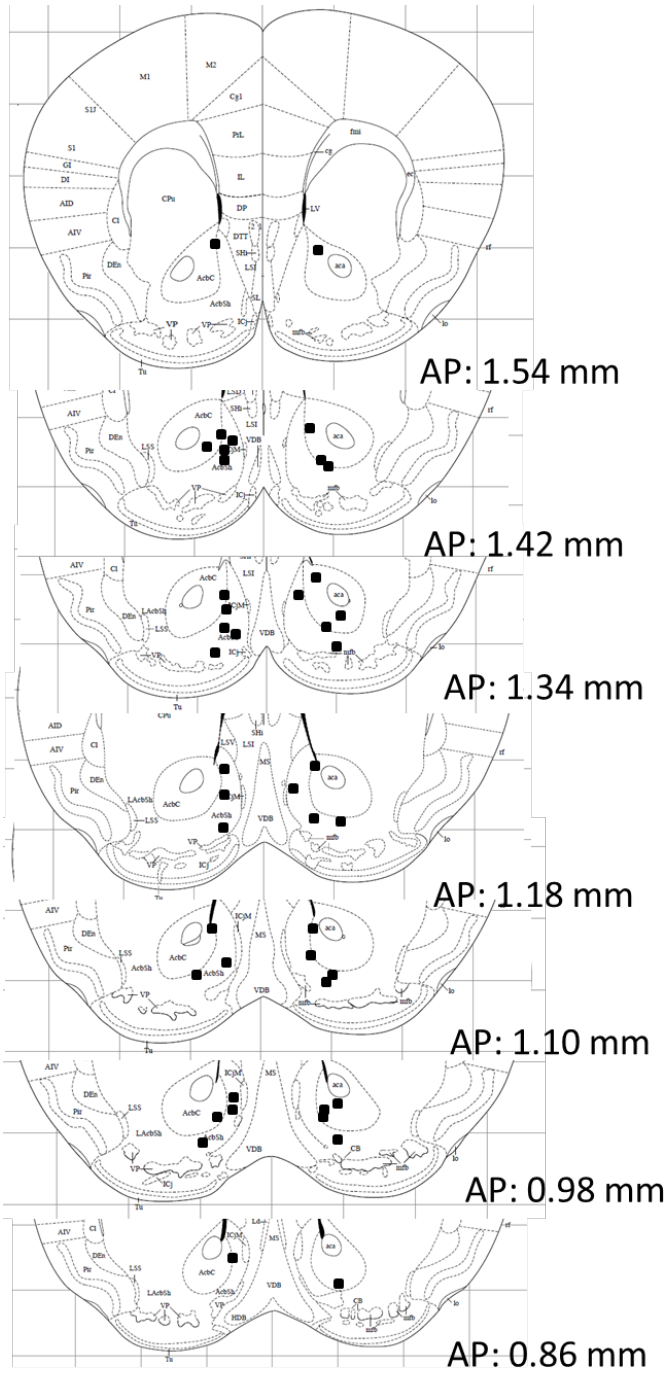


Figure 5-5. Schematic of bilateral nucleus accumbens cannula hit approximate locations in Experiment 2.

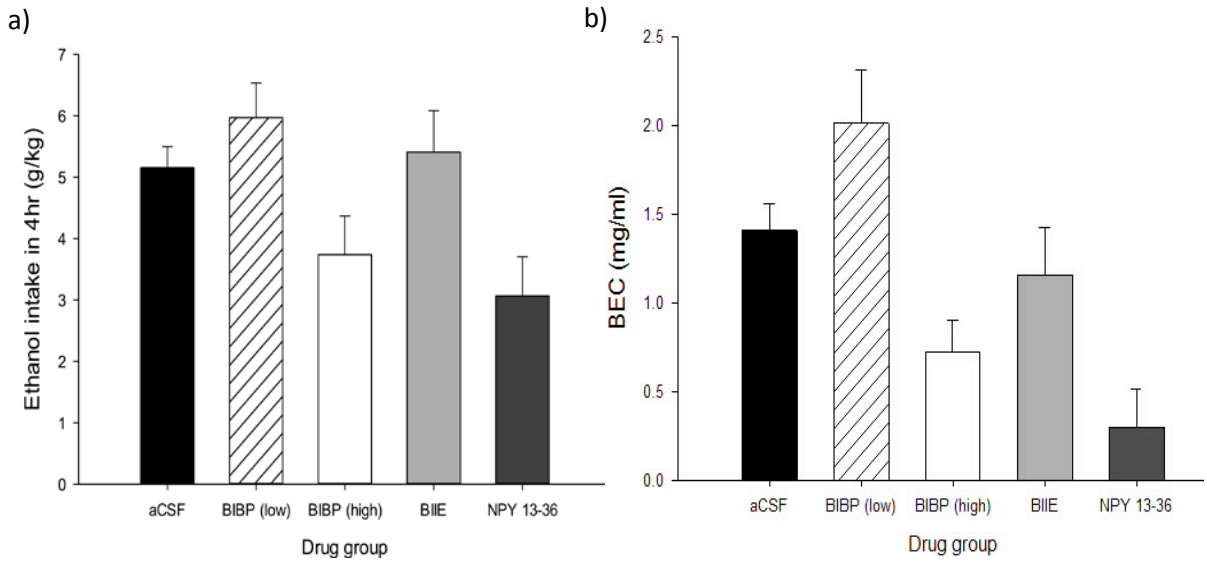


Figure 5-6. Drug effects on alcohol intake (a) and BECs (b) in HDID-1 male mice on Day 4 of a 4-day DID test shown as an average for each drug tested in Experiment 2. The number of data points averaged for each drug group (bar) are aCSF: 78, BIBP (low): 28, BIBP (high): 25, BIIE: 14, NPY_{13-36}: 12.

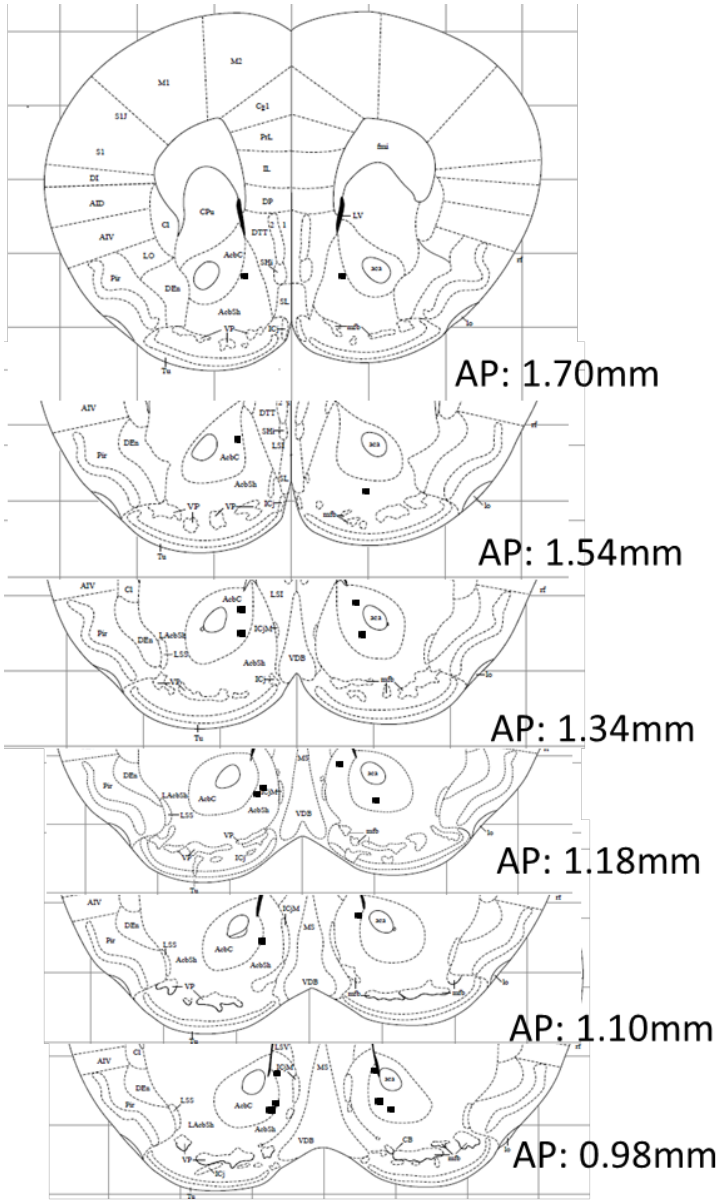


Figure 5-7. Schematic of bilateral nucleus accumbens cannula hit approximate locations in Experiment 3.

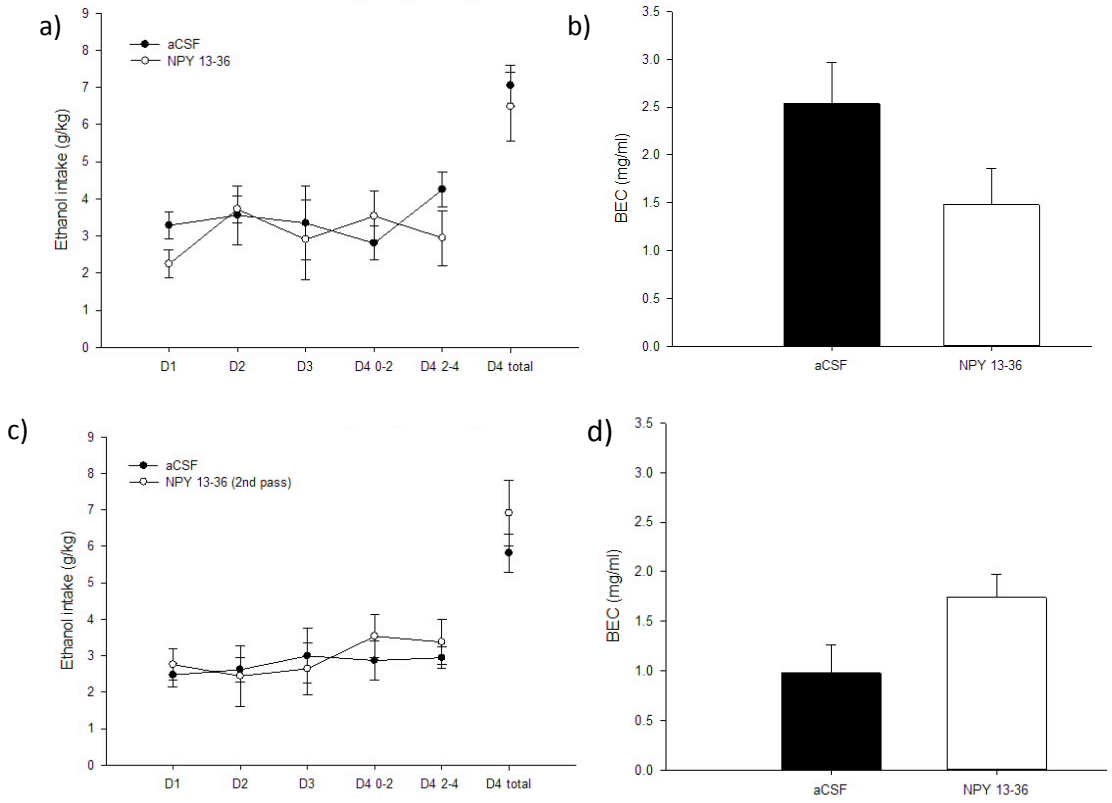


Figure 5-8. Alcohol intake (a) across each day of a 4-day DID and BECs after Day 4 drinking (b) in HDID-1 mice tested with the Y2R agonist NPY_{13-36} after one (top) or three (bottom) cycles of alcohol DID. Alcohol intake is shown for each 2 h session on Days 1-3, for the first 2 h and second 2 h on Day 4, and for the total 4 h session on Day 4. Means ±SEM shown. N=4-5/group

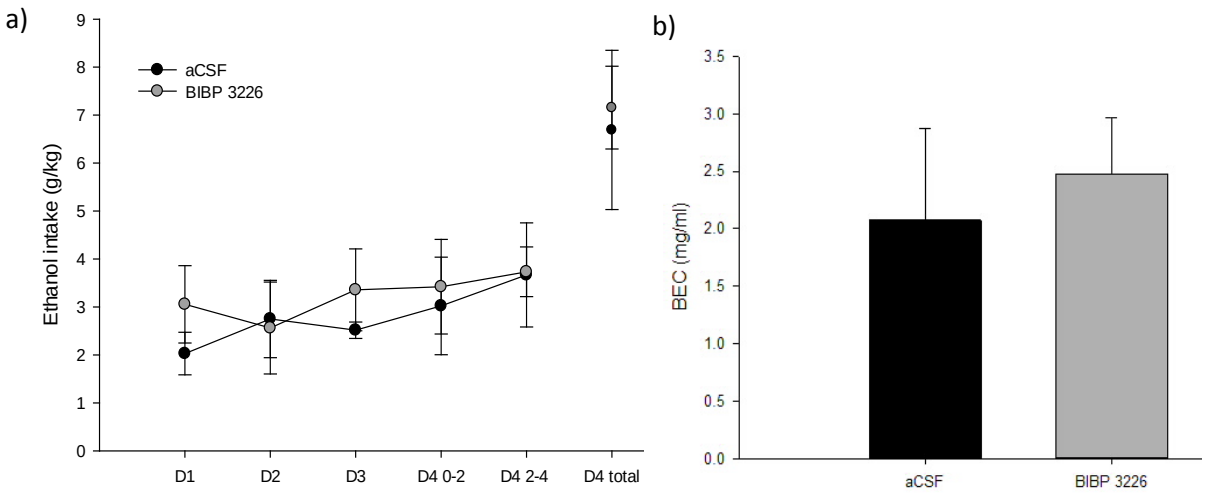


Figure 5-9. Alcohol intake (a) across each day of a 4-day DID and BECs after Day 4 drinking (b) in HDID-1 mice tested with the Y1R antagonist BIBP 3226 after two cycles of alcohol DID. Alcohol intake is shown for each 2 h session on Days 1-3, for the first 2 h and second 2 h on Day 4, and for the total 4 h session on Day 4. Means ±SEM shown. N=4-5/group

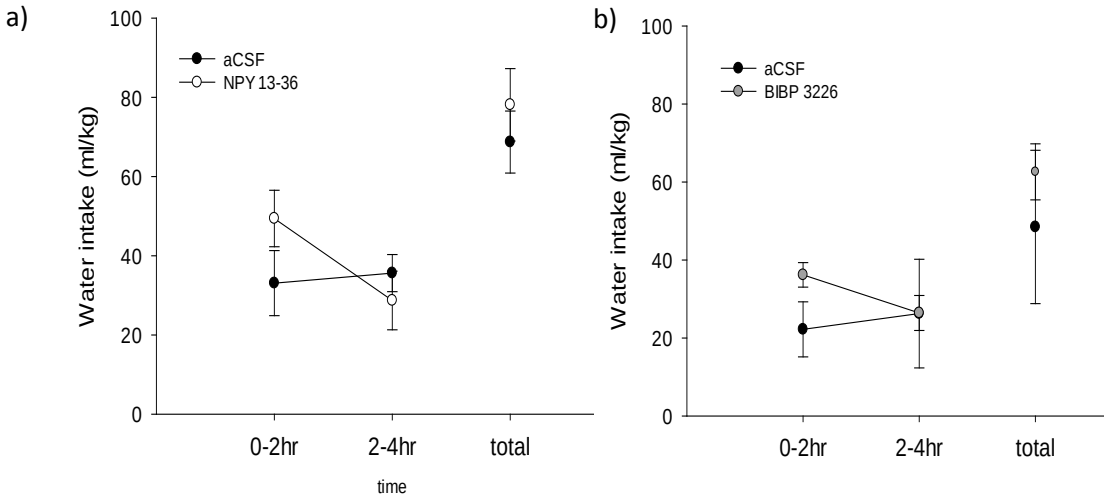


Figure 5-10. HDID-1 male mice water intake during 4 h DID control sessions following intra-accumbal infusion with Y2R agonist NPY_{13-16} (a) or Y1R antagonist BIBP 3226 (b). Means ±SEM shown. N=4-5/group

Chapter 6: General Discussion

Summary of findings

Chapter 2 presented evidence that indicates that HDID-1 and HDID-2 mice reach intoxicating BECs in the DID test through different drinking structures. For HDID-1 mice, their unique drinking microstructure (large bout size) appears to be specific to alcohol. For HDID-2 mice, the large number of drinking bouts was seen for all fluid types tested. Chapter 3 showed that HDID selection has not resulted in changes to alcohol-induced CPP in the selected lines as compared to the HS mice. In contrast, alcohol-induced CTA was attenuated at a moderate alcohol dose in both HDID replicates. Basal anxiety and anxiolytic response to alcohol were not found to systematically differ between the HDID and HS mice, though there was evidence of sex and replicate specific differences (Chapter 4). Finally, Chapter 5 described differences between the HDID-1 and HS mice in brain levels of NPY after alcohol drinking, but showed that the HDID-1 mice were largely insensitive to the effects of a site-specific modulation of NPY receptors. Taken together, these results begin to show a more complete picture of how and why the HDID mice drink the way they do. This discussion will address the findings of the previous studies in the context of understanding the phenotype of the HDID mice and what we can learn from them as a model of binge-like drinking, as well as key directions for future research.

How do HDID mice reach intoxicating BECs?

Prior to the studies described in Chapter 2, it was not known how HDID mice drank in order to achieve intoxicating BECs. We had shown previously that broadly-

defined patterning of intake over the 4hr DID session is biased toward later session drinking, with roughly 60% of alcohol intake occurring in the last two hours of the session (Crabbe et al., 2014). However, this difference was present in the starting generation (S0) and showed no systematic response to selection, making it unlikely to explain the HDID phenotype. The timing of drinking in the HDID and HS mice, however, is in contrast to B6 mice. It has been shown previously that B6 mice consume most of their alcohol in the DID test in the beginning part of the session. This is true for single-bottle DID (Linsenbardt & Boehm, 2013; Wilcox et al., 2014) and two-bottle choice DID (Griffin et al., 2009). Additionally, the rate of drinking during this initial "front-loading" period has been seen to increase with repeated alcohol experience and may represent a mechanism by which escalation of drinking occurs over time (Griffin et al., 2009; Linsenbardt & Boehm, 2014; Wilcox et al., 2014). B6 mice drink to intoxicating BECs during the DID test (e.g. Rhodes et al., 2005), so early rapid drinking appears to be an alternative to the timing of drinking of the HDID mice that also results in intoxication. As discussed in Chapter 2, this distinction in drinking timing between HDID and B6 mice indicates that the HDID phenotype reflects more underlying genetic contributions than just the B6 drinking-related alleles.

Timing and rate of drinking during the DID session could potentially reflect mechanisms of alcohol satiety. Changes in microstructure features such as decreasing bout length, and increasing inter-bout and inter-lick intervals are associated with satiation in rats (Allison, 1971). Thus, one possibility is that B6 drinking patterns during DID may simply reflect satiation, as rapid early intake is then followed by modest alcohol drinking in the rest of the session. HDID mice drink more consistently throughout the session

(Chapter 2), and it is possible that their timing of drinking involves changes in the mechanisms underlying alcohol satiation. Examining within-session variation in drinking microstructure over the course of the DID test could help test this hypothesis and shed additional light on how timing and microstructure of drinking interact to promote high BECs.

The assessment of drinking microstructure in the HDID-1 and HDID-2 lines showed that there are multiple ways of structuring bouts during the DID session in order to reach intoxicating blood alcohol levels. HDID-1 mice have a larger alcohol lick volume that results in larger g/kg intake per bout and HDID-2 mice have more frequent bouts during the DID session. However, it is not yet clear which microstructural features are most related to BEC in each replicate. Identifying how various features of the drinking pattern contribute to the overall blood levels reached will be important for fully understanding the drinking phenotypes of these lines. For example, it may be that bout size is a less important predictor of BEC than the timing of bouts within the 4hr DID session, or that the interbout interval is more relevant than the total number of bouts. More in-depth statistical analysis of the relationships between structure and patterning variables (e.g. principal component analysis) will be useful to understand how they are related to each other and to the outcome variables of interest such as intake and BEC. This may also help explain the observation in the HDID-2 mice of BECs increasing more than g/kg intake across recent selection generations (Crabbe et al., 2014).

When considering the larger lick volume size of the HDID-1 mice, it should be noted that lick volumes were calculated by dividing the total intake in ml by the total number of licks in a session. These values, therefore, are averages and likely do not

reflect the exact size of each lick. We also do not know what exactly a mouse is doing at the sipper tube during a drinking bout. Although we refer to the behavior as "licks," the actual output measure is simply the number of closures of the electrical circuit. It's possible that the way in which the HDID-1 mice drink from a sipper tube results in more prolonged physical contact than the HS mice, thereby resulting in fewer recorded discrete "licks" and a greater average lick volume. However, there is evidence that rodents can modulate their efficiency of fluid intake per lick. Previous studies in rats have tested the effect of response requirement on licking efficiency (Allison et al., 1987; Allison & Buxton, 1992; Buxton & Allison, 1990). In one procedure (Allison et al., 1987), fluid-restricted rats were required to lever press for access to a sipper tube of water. Once the sipper tube was available, the rats were only allowed a fixed number of licks before the tube was retracted and lever presses again had to be made to access the tube. Two groups were tested, one where the "cost" of each lick (i.e., number of lever presses per lick) was high, and one where the cost was low. The high cost group made fewer lever presses and had fewer licks than the low cost group, but the groups did not differ significantly in the total volume consumed. This suggests that licking efficiency was enhanced as a function of cost per lick. In a follow-up experiment, rats were trained to lick an empty spout for access to a fixed number of licks on a water spout (Buxton & Allison, 1990). By altering the response requirement across sessions, it was shown that increasing the cost per water lick (i.e., number of empty spout licks needed per water lick) resulted in an increase in licking efficiency as shown by a decrease in the number of licks per ml of water consumed. In contrast, yoked control animals that received water spout access concurrently with each experimental animal but had no response requirement of their

own showed no significant difference in licking efficiency across sessions. These studies suggest that lick volumes vary as a function of the "value" of the solution being consumed.

If lick volume in the HDID-1 mice is similarly variable, we might expect changes in lick volume depending on procedural differences that alter the perceived value of alcohol (or other fluids). This could provide a potential explanation for the difference in magnitude of genotypic difference in bout size seen between the single-bottle and two-bottle choice DID tests in Chapter 2. HDID-1 mice had larger alcohol bouts than HS mice in both procedures, but this difference was more pronounced in the single-bottle test. If the presence of concurrent water reduces the perceived value of the alcohol solution, then HDID-1 mice may reduce their licking efficiency for alcohol and thereby the g/kg intake per bout. Testing HDID-1 and HS mice for two-bottle choice DID in the lickometers, therefore, might be a useful follow-up experiment to examine this possibility. It is also unknown how (and if) lick volume fluctuates throughout the drinking session in the HDID-1 mice. One possibility is that licking efficiency may decrease with satiety. This has been shown for rats drinking a saccharin solution in a limited access test, where ml/lick decreased in the latter part of the session in association with other drinking microbehaviors indicative of satiation (e.g. shorter lick length, greater pause length between licks) (Allison, 1971). We might therefore expect HDID-1 mice to decrease their lick volume over the course of the DID session as they become satiated. It is possible, however, that alcohol satiety is not achieved by these mice during the DID test (see above). An alternative hypothesis is that lick volume may instead increase during the session. HDID mice drink more in the second half of a 4 hour DID session than in the

first half, and it is possible that this is due in part to increasing licking efficiency. Prior to the final day of the 4-day DID test, mice have only ever experienced 2 hour limited access sessions. We do not currently know anything about timing abilities in these animals, but it seems probable that the 4 hour session is noticeably longer than any previous alcohol exposure. Alcohol may increase in relative value during the latter part of the 4 hour session due to the anticipation and uncertainty about when this extended access will end, thereby producing a sort of "last call" effect on drinking efficiency. These possibilities are obviously purely speculative and will need to be tested in the future. Lick volume changes throughout the session cannot be measured using the current lickometer set-up, but we are currently in the process of implementing a combined lickometer and BioDAQ system that would allow for this degree of temporal and volumetric resolution.

HDID-2 mice have not yet been tested for DID intake in a lickometer procedure. Because these mice do not differ from HS in the size of their drinking bouts, we would not necessarily hypothesize that they would exhibit differences in lick volume or lick rate. However, there is always the possibility that they do show changes in these variables, but in such a way as to not produce overall differences in bout size. For example, an enhanced lick rate during bouts coupled with a smaller lick volume could be masked when considering only total intake during a bout. They might also show differences in lick volume over time, as postulated for the HDID-1 mice. It may therefore still be useful to assess drinking structure in these mice using the lickometer system or combined lickometer/BioDAQ apparatus. Additionally, given the binge-like saccharin intake the female mice of this genotype exhibited (Chapter 2), it might be valuable to

assess how lick features relate across two palatable, rewarding fluid types (alcohol and saccharin).

Another avenue for study will be to determine whether any microstructure features are able to predict future consumption. HDID-1 female mice have been shown to escalate their intake following chronic intermittent alcohol exposure involving repeated cycles of alcohol vapor exposure and limited access alcohol preference drinking (Crabbe et al., 2012c). Male HDID-1 mice have also shown escalation of intake with intermittent alcohol access, but only following long-term (six weeks) daily continuous access preference drinking. It may be the case that certain microstructural bout features are related to greater escalation of intake in these procedures (e.g. bout size, number of bouts). Determining which microstructure elements signal risk of escalated drinking could be a significant area for translational research and excessive drinking prevention strategies in humans. In the single-bottle DID test in Chapter 2, all three genotypes (HDID-1, HDID-2, and HS) were found to increase their average alcohol bout size from Day 1 to Day 4 of the test, but decrease their number of bouts. These two complementary changes in microstructure can be expected to have opposite effects on total consumption, and may help to explain why HDID mice fail to reliably show escalation of intake. It's possible that procedural manipulations that result in escalation of drinking in these genotypes (i.e., long-term, daily two-bottle choice; chronic intermittent alcohol exposure) do so by further enhancing ethanol bout size, though this will need to be tested directly in future experiments. We have not yet examined how bout features change across a two-bottle choice DID test, and it may be that the change in microstructure across days differs between this test and single-bottle DID. Because preference drinking is most commonly

used in escalation procedures, it will be relevant to determine how HDID microstructure varies across time when water and alcohol are available concurrently. It will also be important to examine how microstructure changes across more chronic exposure to alcohol drinking, or in continuous-access drinking paradigms. We do not yet know what HDID drinking pattern or microstructure looks like during 24 hour continuous access to alcohol and water. It is possible that the distinct drinking microstructures seen here are specific to the initiation of drinking, to limited access drinking, or to both. Thus, including microstructural analysis of intake in a variety of drinking experiments will undoubtedly yield a more complete understanding of how drinking microstructure has been altered in HDID selection and which features of drinking could be useful targets for reducing intake and BECs. For example drinking bout sizes for other palatable solutions such as NaCl can be modulated in fluid-deprived rats drinking in short access periods by administration of dopamine receptor antagonists (Canu et al., 2010; Galistu & D'Aquila, 2012). Certain drugs, such as the D2 receptor antagonist raclopride, appear to be able to selectively alter bout size without changing the number of drinking bouts (Canu et al., 2010). If this dissociability of effect on bout size and bout number extends to alcohol drinking in the HDID lines, it will be an important consideration when trying to use pharmacological manipulations to reduce intake in DID. Testing of any potential pharmacological treatments should therefore be undertaken in both replicates in case effects are limited to only an alteration in the bout size or number of bouts.

What motivates alcohol intake in the HDID mice?

Alcohol's motivational effects can be broadly thought of as aversion and reward, where reward encompasses positive rewarding experiences (e.g. euphoria) as well as the removal of aversive states (e.g. anxiolysis) (for review, see Riley, 2011). In the HDID mice, it appears that reduced aversive sensitivity is a key contributor to their high alcohol consumption. One of the most striking findings from these studies was that of reduced intake of a novel fluid paired with the presumed aversive effects of alcohol in the HDID lines compared to the HS (Chapter 3). HDID-1 and HDID-2 male mice showed development of an attenuated CTA to a moderate (2 g/kg) dose of alcohol. As discussed previously, there exists a robust literature demonstrating a negative genetic correlation between alcohol intake and alcohol CTA (e.g. Green & Grahame, 2008), and the HDID lines now provide another example of this relationship. It is still not clear, however, whether there is a causal relationship between reduced alcohol aversion and high alcohol drinking in rodent models. That is, are these animals actually drinking more because they do not experience substantial aversive effects of alcohol? A set of recent unpublished studies from the Crabbe lab tested whether alcohol drinking could produce a CTA in these mice. These studies were based on a procedure used by Belknap and colleagues (1978) to study B6 and D2 mice for drinking-induced CTA to alcohol. Briefly, mice were fluid deprived and trained to drink during a limited access period. Mice were then given ten minutes of access to water or one of five concentrations of alcohol (2-10%). Four days later, mice were given another drinking session with the same concentration of alcohol and the change in consumption between the pretest and posttest was used as a score of CTA. D2 mice were found to significantly decrease their intake when given

alcohol concentrations from 6-10%. B6 mice, however, only showed a significant reduction in intake at the 10% concentration. This suggests weaker alcohol CTA in the B6 mice as compared to the D2, which is consistent with findings from traditional CTA experiments (e.g. Broadbent et al., 2002). The same procedure was used in the HDID mice, except that the range of alcohol concentrations tested was 5-25%. Mice of both replicates showed a statistically significant change in consumption from the pretest to the posttest at alcohol concentrations of 15-25%. Therefore, drinking alcohol concentrations close to that used in selection appears to be able to produce a CTA in these mice. We have not yet tested the HS mice in this procedure, but this is a necessary future experiment in order to assess relevant genotypic differences in this task. Specifically, we can hypothesize that HS mice will develop significant CTA at lower alcohol concentrations than the HDID mice. This would indicate a lower threshold for aversive sensitivity to alcohol and would be consistent with the results from the CTA experiment described in Chapter 3.

Another approach for testing the causal relationship between CTA and drinking would be to test whether activation of brain areas known to be involved in response to aversive stimuli could reduce alcohol intake in the HDID mice. For example, the lateral habenula has been shown to be involved in alcohol CTA and self-administration, with lesions in this area producing reductions in CTA and increases in self-administration in rats (Haack et al., 2014). The nucleus of the solitary tract has also been associated with CTA learning (e.g. Thiele et al., 1996), and stimulation of this area can produce enhancements in lithium chloride CTA (Garcia-Medina et al., 2014). Thus, we could activate these brain areas via electrical stimulation or more targeted approaches (e.g.

optogenetics, designer receptors exclusively activated by designer drugs [DREADDs]) and determine whether this can effectively reduce binge-like intake in the HDID lines. Similarly, if activation of the same neurons can enhance an alcohol CTA, this could indicate that both behaviors are related and would provide further evidence for a causal role of lower alcohol aversion in binge-like drinking.

From the present studies, it appears that the difference in drinking between HDID and HS mice is not driven by altered sensitivity to alcohol reward. Based on the results from the CPP experiment (Chapter 3), HDID mice do clearly find alcohol to be rewarding, as do HS mice. If alcohol is similarly rewarding to the HS mice, why then do they consume less alcohol than HDID mice in the DID test? As discussed previously, alcohol drinking-naïve HS and HDID mice differ in CTA. One possibility, therefore, is that HDID and HS mice have similar reward thresholds for alcohol, but that the HS mice have a significantly lower threshold for experiencing the aversive effects alcohol. If this is the case, the amount of alcohol HS mice consume in the DID test may be sufficient for them to experience the aversive effects and subsequently terminate their drinking. In this scenario, reward sensitivity would not factor into the genotypic difference in consumption provided that the HS mice do not drink sufficient quantities to experience alcohol reward. This hypothesis is consistent with findings from B6 and D2, wherein D2 mice show avoidance of lower concentrations of alcohol than B6 mice (see discussion of Belknap et al., 1978 above), but still show a robust alcohol conditioned place preference (e.g. Cunningham, 2014). Reward sensitivity may then still be relevant to alcohol consumption in high drinking mice like the HDIDs, as they presumably drink enough to experience the rewarding effects of alcohol. That is, reduced sensitivity to alcohol

aversion could allow the HDID mice to overcome an initial barrier to drinking, and then the rewarding effects of alcohol may maintain the drive to continue drinking alcohol during the DID session. However, this is only one possible explanation, and it may well be that alcohol's rewarding effects are experienced even at low levels of consumption. HS mice might therefore experience alcohol reward, but their heightened sensitivity to alcohol aversion acts as the predominant subjective effect and 'overrides' any perceived rewarding effects.

CPP is only one measure of reward, however, and it is not without interpretational challenges (Cunningham et al., 2006; Stephens et al., 2010). For example, alcohol CPP does not reliably show a graded dose-response curve, but instead tends to appear as an "all-or-nothing" effect (e.g. Groblewski et al., 2008). Because the CPP procedure and conditioning dose used here appeared to produce a maximal effect in all genotypes (Chapter 3), it is unclear whether there may be differences in the reward threshold sensitivity of these animals. Consequently, future experiments could test the HDID and HS mice on operant self-administration of alcohol to address this possibility. In particular, the use of a progressive ratio schedule of reinforcement would allow for determination of how hard HDID and HS mice are willing to work to obtain alcohol. It may be the case that genetic difference in alcohol reward sensitivity will emerge when using a different paradigm that produces a more sensitive dose-response curve. Additionally, nothing yet is known about extinction or reinstatement of alcohol responding in these lines, and these behaviors represent future directions for research.

One hypothesis of high alcohol intake is that it is consumed for its anxiolytic effects to alleviate high basal anxiety. Differences in basal anxiety or alcohol-induced

anxiolysis do not appear to be major contributors to the HDID drinking phenotype. There is evidence for this self-medication hypothesis in humans and selected rodent lines (Colombo et al., 1995; Smith and Randall, 2012;Wolitzky-Taylor et al., 2015). Sardinian P rats, for example, show high basal anxiety that is attenuated by voluntary alcohol intake (Colombo et al., 1995). Similarly, rats selectively bred for high anxiety behavior show greater anxiolytic response to an acute alcohol injection than the corresponding low anxiety line (Henniger et al., 2002). In HDID mice, however, there was no evidence for elevated basal anxiety, and HDID-1 male mice actually showed significantly lower anxiety-like behavior at baseline than the other groups (Chapter 4). Additionally, HDID-1 mice did not show evidence of a significant change from HS mice for sensitivity to alcohol's anxiolytic effects as measured on the EZM. The previous rat studies, however, used lines of animals either selectively bred for or tested on two-bottle choice drinking. It is possible, therefore, that there is a greater degree of shared genetic contribution between anxiety-like behaviors and preference drinking than between anxiety and binge-like drinking. HDID-2 mice still need to be tested for anxiolytic response to acute alcohol injection, so it remains to be seen whether there are sensitivity differences in this line that may be related to alcohol intake. However, since the HDID-2 mice also do not exhibit elevated anxiety-like behavior vs HS at baseline, it seems unlikely that they are drinking alcohol predominantly for its anxiolytic effects. As discussed in Chapter 4, the low baseline anxiety-like behavior of the HDID-1 male mice could be consistent with an enhanced novelty-seeking phenotype. These mice also showed greater consumption of the novel NaCl solution than HS mice on the first day of the CTA procedure (Chapter 3), which potentially reflects novelty-seeking and/or reduced neophobia. This might explain

in part their high alcohol consumption, particularly on the first day of the DID test, though this seems less likely to underlie continued high alcohol intake. However, there is evidence that for a genetic correlation between novelty-seeking and alcohol drinking (Lange et al., 2010; Manzo et al., 2014; Noël et al., 2011), and testing these mice explicitly for novelty-seeking behavior and neophobia may be a useful future direction for understanding their motivation to drink.

It should be noted that all of the anxiety-like data collected in the HDID lines have been from emergence-based assays. Although rodent models of anxiety-like behavior have relatively limited face validity for human anxiety disorders, emergence-based tests may most closely model "trait" anxiety and generalized anxiety disorder (Steimer, 2011). Other anxiety disorders such as social phobia have also been shown to be associated with alcohol use (Smith & Randall, 2012; Robinson et al., 2009). Therefore, other tests besides emergence-based assays of anxiety-like behavior may be needed to assess fully the relationship between anxiety-like behavior and alcohol intake in rodents. Social phobia is an inherently human construct, but there are tasks that appear to be relevant models, such as social avoidance or social fear conditioning (for review, see Toth & Neumann, 2013). Future testing of anxiety-like behavior in the HDID and HS mice could include some of these social-based tasks to develop a more complete picture of how anxiety state may influence alcohol consumption in these lines. Additionally, a potential positive genetic relationship between depression-like behavior and alcohol intake has been seen in other rodent lines selectively bred for either high alcohol consumption or depression-like behavior (Can et al., 2012; Weiss et al., 2008), and the mood-enhancing effects of alcohol could potentially motivate intake in the HDID lines.

Depression-like behavior in the HDID mice has not yet been assessed, but determining whether there are selection-related changes in either basal depression-like behavior or depression-like behavior during withdrawal represents an area for future research.

Neurobiology of HDID mice and binge-like drinking

The observed genetic differences in alcohol drinking microstructures, alcohol CTA, and basal anxiety-like behavior presumably stem from differences between the lines at the level of the brain resulting from HDID selection. Because binge-like drinking is a complex trait, selection has likely altered numerous genes and subsequently numerous neurobiological factors as well. In this dissertation, I explored the potential role of only a single factor—NPY. The most striking finding from the NPY studies was the blunted NPY response to alcohol in the NAc of HDID-1 mice. Specifically, HS mice showed an alcohol-related decrease in NPY expression in the NAc core and shell after alcohol DID as compared to water drinking controls. In contrast, HDID-1 mice showed no difference in NPY expression between water and alcohol drinking animals. Taken together with the limited efficacy of pharmacological manipulations of NPY to alter binge-like drinking, this finding raises the question of whether NPY levels in the HDID-1 mice are specifically insensitive to alcohol or whether the NPY system is generally unresponsive to any attempts at modulation. One way to explore this further could be by testing whether alcohol withdrawal will differentially affect NPY levels in the HDID-1 and HS mice. Alcohol withdrawal has been shown to elevate brain NPY protein and mRNA levels in several regions in rats and mice, including the NAc (e.g. Bison and Crews, 2003; Olling et al., 2009; Sparrow et al., 2012). Therefore, if withdrawing HS

mice showed an increase in NPY while HDID-1 mice still showed no change, this would suggest a general decrease in the lability of the NPY system in these animals. Another possible explanation for the limited effectiveness of the NPY receptor ligands is that the NAc was not the correct area to target. Although this region showed the most significant genetic difference in NPY expression, it may be that modulation of NPY receptors in an upstream region like the CeA or BNST would be more behaviorally relevant. Alternatively, direct genetic knockdown of *Npy* itself in one of the regions showing gene expression differences (NAc, CeA, BNST) may prove to be a more useful technique for altering HDID-1 drinking.

It is also possible that elevated NPY levels after alcohol drinking in the HDID-1 mice represent an "at-risk" brain state with regard to alcohol intake. NPY system modulation in mice and rats predominately affects alcohol intake only in animals with a history of alcohol dependence, or those selectively bred for high alcohol intake. In non-dependent animals and low-drinking selected lines, NPY has little impact on alcohol drinking (for review, see Badia-Elder et al., 2007). Thus, selection-related changes in the NPY system in HDID mice and other high-drinking selected lines may mimic the neuroadaptation seen in post-dependent animals. This could represent a common mechanism for both the high alcohol intake seen in selected lines and the withdrawal-associated escalation of drinking that has been observed in animals with a history of alcohol dependence.

It should be clear from these findings that NPY is not the only neurotransmitter involved in binge-like drinking. Previous work has shown increased expression of the group 1 metabatropic glutamate receptors mGLUR1and associated scaffolding proteins in

the NAc shell of alcohol-naïve HDID-1 mice as compared to HS (Cozzoli et al., 2012). NPY has been shown to reduce glutamate release presynaptically through a Y2R-mediated mechanism in rat hippocampal slices *in vitro* (e.g. Greber et al., 1994). Increased mGLUR1 expression in the NAc of the HDID-1 mice could therefore potentially represent a compensatory receptor upregulation in response to decreased glutamate release driven by high levels of NPY. Continued exploration of potential candidate genes and neurotransmitter systems will undoubtedly uncover other neurobiological differences between the HDID mice and the HS. It may well be the case that NPY is relevant to the drinking phenotype, but that its involvement is largely due to effects on other neurotransmitters and downstream systems. The gene network connectivity study that suggested NPY system alterations in the HDID mice (Iancu et al., 2013) also found other genes that were apparently affected by selection, and some of these genes have been previously implicated in alcohol intake or AUDs. For example, the genes encoding the gamma-1 subunit of the GABA-A receptor and neurotensin were also found to have selection-induced changes in connectivity, and these genes represent good candidates to test next for their role in the HDID phenotype.

The HDID mouse phenotype as a model of binge-like drinking

Strengths and weaknesses of the model

A significant advantage of the HDID mice as a model for studying binge-like drinking is that they readily consume enough alcohol to reach intoxicating BECs. Furthermore, this drinking to intoxication occurs without major procedural manipulations such as fluid restriction or sucrose-fading, and these mice will drink to intoxication

during their initial exposure to alcohol (Crabbe et al., 2014). This is an important feature when trying to model excessive alcohol drinking, and differs from many other rodent models of alcohol drinking where it is uncertain whether animals are ever actually experiencing intoxication. It has also been shown that HDID BECs correlate significantly with alcohol intake and that the intoxicating blood levels are not due to changes in alcohol absorption or metabolism (Crabbe et al., 2009; 2014). The HDID lines have also been shown to not differ from the HS in preference for sweet tastants or avoidance of bitter tastants, and are therefore unlikely to be consuming the observed large quantities of alcohol due to differences in taste sensitivity (Crabbe et al., 2010). The alcohol intake during the DID test has also been shown to be sufficient to produce behavioral intoxication as demonstrated by increased ataxia (Crabbe et al., 2009). Chapter 4 shows that intake during DID can produce locomotor stimulation in these mice, as well as anxiolysis in a sex- and replicate-specific way. It has also been demonstrated that the HDID show increased handling-induced convulsions during acute withdrawal after a single DID test (Crabbe et al., 2014). Thus, these mice drink sufficient quantities during DID in order to exhibit a number of behavioral response to alcohol. The use of limited access drinking is also an advantage of the model, because it provides a relevant behavioral window during which to test experimental manipulations for their effect on drinking and BEC (as demonstrated in Chapter 5).

The HDID mice are also useful in that they are unique in their selection criterion. No other selected rodent line has been bred specifically for the BEC reached during drinking, making these mice the only currently existing model of genetic risk for drinking to intoxication. In addition, the majority of alcohol-selected rodent lines are bred using a

2-bottle choice, continuous access drinking procedure. DID and preference drinking are believed to have distinct genetic contributions, with some studies reporting no chromosomal overlap between QTLs for the two behaviors, and some reporting only limited overlap depending on the population tested (Iancu et al., 2013; Phillips et al., 2010). HDID mice, consequently, provide a means to study genes relevant to excessive drinking that may not be captured by other genetic models that were developed using 2-bottle choice procedures. HDID mice also seem to differ behaviorally from the widely-studied B6 mouse. As mentioned previously, B6 mice and HDID (and HS) mice have very different timing of drinking during a limited access test. B6 mice load up on alcohol early, completing most of their session drinking during the first 15 minutes (e.g. Linsenbardt & Boehm, 2014; Wilcox et al., 2014). HDID mice begin to drink more slowly, showing their lowest rates of consumption during the first hour and then steadily ramping up their drinking and consuming about 60% of their total alcohol during the last two hours of the test (Crabbe et al., 2014; Chapter 2). These differences between HDID mice and B6 mice are an important finding because B6 was one of the founder strains for the HS population. Therefore, HDID selection has demonstrated the importance to binge-like drinking of the other genetic variation in the HS mice, since selection did not merely recapitulate the B6 phenotype.

An additional strength of the HDID lines is the existence of the two independently selected replicates. As mentioned previously, replicate lines are traditionally thought to provide an easy means of testing correlated responses to selection. If a correlated trait is seen in multiple replicates, this is strong evidence for its genetic relationship to the selection phenotype. In practice, replicate lines often show a complex pattern of

behaviors, with some putatively correlated responses showing concordance between the lines and some showing differences. The HDID lines are no exception. Of the data presented here, only reduced CTA at a 2 g/kg alcohol dose is strictly supported as a correlated response by both replicates. However, even in this instance there are some line differences since the HDID-2 mice may appear to have reduced general aversive sensitivity. However, these differences between the HDID replicates are not necessarily a disadvantage of the model. As seen with the drinking microstructure data, the two HDID lines show divergent pathways to a similar selection phenotype. Although this may complicate the ability to confirm correlated responses to selection, it does provide us with the opportunity to better understand the specific genetic contributions to discrete aspects of the binge-like drinking phenotype. For example, in HDID-1 and HDID-2, we can study two separate complex phenotypes that both result in drinking to intoxication. In this way, we will be able to better identify the genes and mechanisms that underlie different components of the drinking phenotype such as bout size and number of drinking bouts. Drinking phenotypes are similarly complex in humans, and the increased diversity in our mouse model may help to parse the basis of these differences.

Another replicate line difference is that HDID-2 mice show a greater tendency than HDID-1 mice for generalization of alcohol-related traits to other substances. For example, the HDID-2s show a greater number of drinking bouts across all fluid types tested (alcohol, water, and saccharin). In contrast, the HDID-1 mice show an enhanced bout size only for alcohol (see Chapter 2). HDID-2 mice (females in particular) also showed potential binge-like consumption of a saccharin solution, whereas HDID-1 mice consumed the least amount of saccharin out of all three genotypes. Unpublished data

from our lab suggest that the HDID replicates do not differ in preference for sweet solutions (saccharin and sucrose) in a continuous access two-bottle choice test, so the replicate line difference in saccharin drinking appears to be specific to a binge-like procedure. Sucrose DID should be tested as well in these genotypes to determine whether HDID-2 binge-like intake extends to a caloric, sweet solution as well. HDID-2 mice also seem to have a general reduction in aversive sensitivity, showing attenuated CTA to a 2 g/kg dose of alcohol and lithium chloride (see Chapter 3). One possible explanation for this increased generalizability of behavior in the HDID-2 mice is that they were not as far along in selection as the HDID-1 mice for all of the tests given. Because the number of alleles fixed due to genetic drift increases over selection generations, the HDID-2 mice presumably had more genetic variation (both trait-relevant and trait-irrelevant) than the HDID-1 mice at the time of each test. A lesser degree of trait-relevant fixation could result in weaker expression of the selection phenotype and any non-spurious correlated responses. However, it is also possible that these behaviors actually share relevant underlying genetic factors with the HDID-2 phenotype. If this is the case, HDID-2 mice might be a uniquely useful model for studying the comorbidity of excessive alcohol intake with other behaviors. For example, these mice may be a good model of the relationship between alcohol drinking and binge sweet consumption (due to the high saccharin intake in DID), or risk for poly-drug abuse (due to generalized low aversive sensitivity).

The main current limitation of the HDID mice is the lack of choice in the selection phenotype. When water is offered concurrently with alcohol to these animals, intake and BECs are much lower than when tested in the traditional single-bottle DID test

(Crabbe et al, 2009; Chapter 2). In human AUDs, alcohol is generally consumed even when other liquids are available. The drinking of the HDID mice therefore loses some translational utility because the phenotype of binge-level BECs appears to be dependent upon alcohol being the only fluid present. It is possible that continued selection will enhance preference drinking as well, but this cannot be guaranteed and future research will need to focus on understanding and increasing alcohol intake in the presence of water (see below). Since DID is tested during the highest period of consummatory behavior, the absence of a water bottle complicates interpretation of alcohol intake because of possible prandial influences on drinking (see below).

Additionally, offering only alcohol limits the ability to assess treatment effects on water intake, total fluid consumption, or fluid balance. Some pharmacological manipulations can reduce alcohol intake while not altering alcohol preference in B6 mice tested in a two-bottle choice DID (Giardino & Ryabinin, 2013), and these types of more subtle drinking effects would be missed in a standard single-bottle DID test. Another potential limitation of the HDID mice is that drinking occurs during a limited window of availability, though this does have benefits as well (see above). It has been suggested that even repeated, short exposures to the high levels of drinking seen in HDID mice may be insufficient to produce some of the neural changes underlying the addictive process. Thus, HDID mice might not be as useful for modeling the neuroadaptation that leads to chronic dependence, or they may require different procedural manipulations in order to do so. Additionally, these mice have proven largely resistant to procedures leading to escalation of alcohol intake, which is another feature frequently seen in human AUDs (Crabbe et al., 2012a). However, it is not necessary for an animal model of alcohol use to

recapitulate every aspect of human AUDs, nor is it necessarily desirable. One benefit of animal models is the ability to model discrete components of human conditions in order to home in on the causes of specific behaviors that together form the complex traits we are interested in understanding. HDID mice, therefore, may be most useful as a model of initiation of binge-like drinking and genetic risk for drinking to intoxication.

Future directions

Relationship between feeding and alcohol consumption

Previous studies with rats have shown that the size of a feeding bout is positively related to the subsequent interbout interval. That is, the larger a feeding bout, the longer the animal waits before initiating the next bout (Le Magnen & Devos, 1980). This relationship has been explored for alcohol drinking bouts as well in lines of rats selected for high alcohol preference (Samson, 2000). Of the three lines tested (P, HAD-1, and AA), none showed a significant correlation between alcohol bout size and subsequent interbout interval. P and HAD rats did show a positive association for feeding bouts, suggesting that the mechanisms regulating the timing of alcohol consumption were not the same as those driving feeding pattern. This analysis has not yet been undertaken in the HDID lines. Examining the relationship (if any) between alcohol bout size and the following interbout interval in the HDID lines may reveal whether alcohol drinking in these animals is regulated similarly to food intake, or if there is evidence of dysregulated satiety mechanism for alcohol consumption. In addition, the BioDAQ apparatus is able to record food intake and we have initiated a pilot study to record food and alcohol consumption during a DID test in the HDID and HS mice. One goal of this and future studies is to determine if there are prandial components to alcohol DID. Water intake in

rats segregates with meals (e.g. Fitzsimmons & Le Magnen, 1969), and this relationship is particularly pronounced during the dark cycle (Johnson & Johnson, 1990). Consequently, it could be argued that alcohol is consumed during the DID test because it is the only fluid present and mice are simply maintaining their usual prandial drinking with what is available. Although this seems to be an unlikely explanation for the binge levels of intake reached by the HDID mice, it would be interesting to compare feeding-associated water consumption versus feeding-associated alcohol consumption in the HDID and HS lines. One might expect that HS mice would show a more similar prandial bout structure across fluid types, whereas there could be selection-dependent changes in the prandial component of alcohol DID in the HDID mice. A greater number of extra-prandial alcohol bouts in the HDID mice would suggest that these animals are drinking alcohol differently than they drink water and that alcohol is not merely substituting for water in normal prandial fluid intake. This would be consistent with the results from the bout structure analysis in Chapter 2 where HDID-1 mice showed a different drinking microstructure for water than for alcohol and HS mice did not.

Drinking microstructure as a predictor of risk

In the human and non-human primate literature, drinking structure is a useful predictor for excessive intake and alcohol-related problems. In a study with non-human primates, for example, large bout size and high drinking rate during schedule-induced initiation of drinking reliably predicted heavy consumption during later free-choice drinking (Grant et al., 2008). Human epidemiological data suggest that episodic heavy drinking may be more closely related to negative health outcomes and drinking related problems than frequency of drinking or total intake. Low to moderate daily intake (0-2

drinks) coupled with occasional heavy drinking shows a greater relative mortality risk than higher daily intake (2-6 drinks) without the occasional heavy drinking component (Rehm et al., 2001). In a study of three central and eastern European populations, a higher average dose per drinking session was more predictive of alcohol-related problems than drinking frequency or overall intake (Bobak et al., 2004). Human research is already underway to assess strategies for reducing BECs and intake during a drinking session. Brief interventions involving education on protective behavioral strategies (e.g. eating before drinking, spacing out drinks, having a designated driver, etc.) have demonstrated effectiveness at decreasing drinking quantity and negative consequences of alcohol use in heavy drinking college students (Baer et al.,2001). In another study, college students with at least one binge episode in the past month were assessed for their use of protective health behaviors relating to drinking and their experience of negative alcohol-related consequences. Individuals who reported using more strategies that involved moderating their manner of drinking (e.g. avoiding mixing different types of alcohol, not participating in drinking games, choosing drinks with lower alcohol concentrations) showed the lowest prevalence of negative alcohol consequences (Napper et al., 2014). Consequently, interventions to educate individuals about strategies they can use to alter risky drinking patterns may reduce the prevalence of alcohol-related problems. These interventions have been largely targeted at young adults at risk for harmful drinking, however, and it remains to be seen whether this type of intervention will be beneficial to other at-risk populations. HDID lines demonstrate two distinct drinking patterns that are associated with binge intake. By better understanding how binge drinking occurs, it might be possible to develop more targeted behavioral interventions. If future work with

the HDID lines results in the identification of specific genetic risk factors for the different binge-associated drinking patterns, this could add another element of personalization to harmful drinking interventions.

Binge drinking across the lifespan and developmental aspects of HDID mice

Nearly all of the work done to date with the HDID mice has focused on adult animals. One previous study examined drinking in the HS and HDID-1 in adults versus adolescent animals, and also the effects of adolescent binge alcohol exposure on drinking in adulthood (Metten et al., 2011). In HDID-1 mice from Generation S17, it was found that mice that started getting daily DID sessions at 4 weeks of age drank more on a g/kg basis than animals that began DID sessions at 9 weeks. In HS mice, DID intake that started at 4 weeks or 8 weeks led to alcohol drinking at age 10 weeks that was higher than at baseline (i.e., first DID experience). These data suggest that initiation of repeated binge-like drinking during early adolescent may promote increased intake in young adulthood. Binge drinking is a prevalent issue during adolescence and this time period represents a critical window for brain development (for review, see Bava and Tapert, 2010). Consequently, more work needs to be done with the HDID mice to understand how genetic risk for binge-like drinking may vary throughout development, and whether the neurobiological effects of adolescent binge drinking are different in the HDID and HS mice. Additionally, since binge drinking has been reported to be a frequent occurrence in senior populations as well (see introduction), it may be prudent to study older HDID mice as well to develop a complete idea of what the effects of binge drinking are across the lifespan.

Adolescent mice and rats are also less sensitive to the aversive effects of drugs than adult animals (Holstein et al., 2011; Moore et al., 2013; Schramm-Saptya et al., 2006). Testing adolescent HDID and HS mice for alcohol CTA may shed additional light on how aversive sensitivity plays into the HDID phenotype. One possible hypothesis is that HDID and HS adolescent mice will show similarly modest levels of alcohol CTA and that the genotypic difference only appears in adulthood as HDID mice retain an adolescent-like degree of aversion while HS mice show the expected age-related increase in aversive sensitivity.

Binge-like drinking in a choice procedure

As mentioned above, future work with the HDID lines should focus in part on trying to achieve binge-like BECs during a 2-bottle choice procedure. One strategy currently underway is a dual selection for DID and preference drinking using a starting population that is a cross between the HDID and the HAP mouse lines. The HDID replicates were crossed with each other, and then crossed with the cHAP mouse line generated in a similar manner from the HAP 2 and HAP 3 replicates (Oberlin et al., 2011). These mice are then being tested on the selection procedures for the HDID and HAP lines, and are being selected for both their BECs after DID and their preference drinking. A goal of this selection is to capture the distinct genetics that underlie both the HDID and HAP phenotypes, and ultimately to produce a line of mice that show drinking to intoxication in binge and preference drinking procedures. It will likely also be important to test the HDID mice on more chronic alcohol exposure procedures. These mice do show withdrawal-associated escalation of intake and escalated drinking in an intermittent access paradigm following prolonged daily drinking (Crabbe et al., 2012c;

Rosenwasser et al., 2013), both of which involve two-bottle choice preference drinking. Thus, future studies will be needed to fully understand what neural adaptations occur in these mice after being made dependent or during the course of long-term drinking experience.

QTL mapping in the HDID and HS mice

The candidate gene approach described in Chapter 5 is one method for identifying potential specific genetic contributions to the HDID phenotype. However, there are currently limited gene expression data available for the HDID lines, and it may therefore prove to be an inefficient strategy. Instead, QTL mapping using the HDID and HS mice could be a more useful future direction for finding the genes that are most relevant to binge-like alcohol drinking. Only one previous QTL mapping experiment has been done with the HDID and HS mice, and it used mice from selection generation S11 for both replicates (Iancu et al., 2013). This study identified three putative QTLs for high BEC after alcohol DID, none of which overlapped with previously published QTLs for alcohol preference drinking. Both replicate lines are now much further along in selection (S30 and S25 for the HDID-1 and HDID-2, respectively) and show a stronger selection phenotype, and we might expect that a QTL experiment in these generations will be even better equipped to detect significant expression differences. As with the previous study, any QTLs identified that differ from reported QTLs for continuous access preference drinking would be especially of interest as these are likely to reflect genetic contributions that are unique to binge-like consumption. Future experiments could also QTL map different aspects of the HDID drinking phenotype, such as intake during the DID test or drinking microstructure variables like bout size. Using any putative QTLs to inform the

choice of candidate genes for further testing could yield greater insight into the specific genetic and neurobiological features that underlie risk for drinking to intoxication.

Conclusions

The experiments presented in this dissertation provide significant novel evidence that the HDID selected mice are a robust model of genetic risk for binge-like drinking. It is now known that selection for high BECs has produced distinct alcohol bout structures that promote drinking to intoxication. Furthermore, reduced aversive sensitivity to alcohol has been identified as a key contributor to the motivation to drink in these mice. It has also been shown that a candidate gene approach can be implemented to test specific neurobiological changes due to selection and suggest targets for potential treatments to reduce binge-like drinking. However, there is still much about this model that we do not yet understand. Some of the critical areas for future study include how HDID binge-like drinking relates to other consummatory behaviors such as preference drinking and feeding, and whether variation in drinking microstructure can serve as a predictor of drinking outcomes and a marker of behavioral change across procedures (or within long-term drinking protocols). We will also need to explore the HDID phenotype across the lifespan to determine its relevance to developmental aspects of binge drinking. QTL mapping using the HDID and HS mice may also be a useful tool for identifying specific genes relevant for binge-like alcohol intake. Continuing research in these and other areas will ensure that we can maximize the utility and translational value of the HDID lines for understanding genetic risk of binge drinking.

References

Abraham, H. D., & Fava, M. (1999). Order of onset of substance abuse and depression in a sample of depressed outpatients. *Comprehensive Psychiatry*, *40*(1), 44–50.

Allison, J. (1971). Microbehavioral features of nutritive and nonnutritive drinking in rats. *Journal of Comparative and Physiological Psychology*, *76*(3), 408–417. doi:10.1037/h0031388

Allison, J., & Buxton, A. (1992). Multidimensional aspects of drinking in the rat. *Physiology & Behavior*, *51*(2), 267–275.

Allison, J., Buxton, A., & Moore, K. E. (1987). Rats' responses to molar and local schedule constraints. *Animal Learning & Behavior*, *15*(4), 360–367. doi:10.3758/BF03205041

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (5th ed.). Washington, DC.

Amlung, M., & MacKillop, J. (2015). Further Evidence of Close Correspondence for Alcohol Demand Decision Making for Hypothetical and Incentivized Rewards. *Behavioural Processes*. doi:10.1016/j.beproc.2015.02.012

Anton, R. F., Schacht, J. P., & Book, S. W. (2014). Pharmacologic treatment of alcoholism. *Handbook of Clinical Neurology*, *125*, 527–542. doi:10.1016/B978-0-444-62619-6.00030-6

Badia-Elder, N. E., Gilpin, N. W., & Stewart, R. B. (2007). Neuropeptide Y modulation of ethanol intake: effects of ethanol drinking history and genetic background. *Peptides*, *28*(2), 339–344. doi:10.1016/j.peptides.2006.07.028

Badia-Elder, N. E., Stewart, R. B., Powrozek, T. A., Murphy, J. M., & Li, T.-K. (2003). Effects of neuropeptide Y on sucrose and ethanol intake and on anxiety-like behavior in

high alcohol drinking (HAD) and low alcohol drinking (LAD) rats. *Alcoholism, Clinical and Experimental Research*, *27*(6), 894–899. doi:10.1097/01.ALC.0000071929.17974.DA

Baer, J. S., Kivlahan, D. R., Blume, A. W., McKnight, P., & Marlatt, G. A. (2001). Brief intervention for heavy-drinking college students: 4-year follow-up and natural history. *American Journal of Public Health*, *91*(8), 1310–1316.

Bahi, A., & Dreyer, J.-L. (2012). Involvement of nucleus accumbens dopamine D1 receptors in ethanol drinking, ethanol-induced conditioned place preference, and ethanol-induced psychomotor sensitization in mice. *Psychopharmacology*, *222*(1), 141–153. doi:10.1007/s00213-011-2630-8

Barkley-Levenson, A. M., & Crabbe, J. C. (2012). Ethanol drinking microstructure of a High Drinking in the Dark selected mouse line. *Alcoholism, Clinical and Experimental Research*, *36*(8), 1330–1339. doi:10.1111/j.1530-0277.2012.01749.x

Barkley-Levenson, A. M., & Crabbe, J. C. (2014). High Drinking in the Dark Mice: A genetic model of drinking to intoxication. *Alcohol (Fayetteville, N.Y.)*, *48*(3), 217–223. doi:10.1016/j.alcohol.2013.10.007

Barkley-Levenson, A. M., & Crabbe, J. C. (2015). Genotypic and sex differences in anxiety-like behavior and alcohol-induced anxiolysis in High Drinking in the Dark selected mice. *Alcohol (Fayetteville, N.Y.)*, *49*(1), 29–36. doi:10.1016/j.alcohol.2014.07.022

Barkley-Levenson, A. M., Cunningham, C. L., Smitasin, P. J., & Crabbe, J. C. (2015). Rewarding and aversive effects of ethanol in High Drinking in the Dark selectively bred mice. *Addiction Biology*, *20*(1), 80–90. doi:10.1111/adb.12079

Barr, C. S. (2013). Non-human primate models of alcohol-related phenotypes: the influence of genetic and environmental factors. *Current Topics in Behavioral Neurosciences*, *13*, 223–249. doi:10.1007/7854_2011_142

Bava, S., & Tapert, S. F. (2010). Adolescent Brain Development and the Risk for Alcohol and Other Drug Problems. *Neuropsychology Review*, *20*(4), 398–413. doi:10.1007/s11065-010-9146-6

Belknap, J. K., Coleman, R. R., & Foster, K. (1978). Alcohol consumption and sensory threshold differences between C57BL/6J and DBA/2J mice. *Physiological Psychology*, *6*(1), 71–74. doi:10.3758/BF03326695

Belknap, J. K., Richards, S. P., O'Toole, L. A., Helms, M. L., & Phillips, T. J. (1997). Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. *Behavior Genetics*, *27*(1), 55–66.

Bell, R. L., Rodd, Z. A., Lumeng, L., Murphy, J. M., & McBride, W. J. (2006). The alcohol-preferring P rat and animal models of excessive alcohol drinking. *Addiction Biology*, *11*(3-4), 270–288. doi:10.1111/j.1369-1600.2005.00029.x

Bhaskar, L. V. K. S., Thangaraj, K., Kumar, K. P., Pardhasaradhi, G., Singh, L., & Rao, V. R. (2013). Association between neuropeptide Y gene polymorphisms and alcohol dependence: a case-control study in two independent populations. *European Addiction Research*, *19*(6), 307–313. doi:10.1159/000346679

Bison, S., & Crews, F. (2003). Alcohol withdrawal increases neuropeptide Y immunoreactivity in rat brain. *Alcoholism, Clinical and Experimental Research*, *27*(7), 1173–1183. doi:10.1097/01.ALC.0000075827.74538.FE

Blanco, C., Xu, Y., Schneier, F. R., Okuda, M., Liu, S.-M., & Heimberg, R. G. (2011). Predictors of persistence of social anxiety disorder: a national study. *Journal of Psychiatric Research*, *45*(12), 1557–1563. doi:10.1016/j.jpsychires.2011.08.004

Blednov, Y. A., Benavidez, J. M., Black, M., Chandra, D., Homanics, G. E., Rudolph, U., & Harris, R. A. (2013). Linking GABA(A) receptor subunits to alcohol-induced conditioned taste aversion and recovery from acute alcohol intoxication. *Neuropharmacology*, *67*, 46–56. doi:10.1016/j.neuropharm.2012.10.016

Blizard, D. A. (2007). Sweet and bitter taste of ethanol in C57BL/6J and DBA2/J mouse strains. *Behavior Genetics*, *37*(1), 146–159. doi:10.1007/s10519-006-9121-4

Bobak, M., Room, R., Pikhart, H., Kubinova, R., Malyutina, S., Pajak, A., … Marmot, M. (2004). Contribution of drinking patterns to differences in rates of alcohol related problems between three urban populations. *Journal of Epidemiology and Community Health*, *58*(3), 238–242.

Bobo, J. K., Greek, A. A., Klepinger, D. H., & Herting, J. R. (2013). Predicting 10-year alcohol use trajectories among men age 50 years and older. *The American Journal of Geriatric Psychiatry: Official Journal of the American Association for Geriatric Psychiatry*, *21*(2), 204–213. doi:10.1016/j.jagp.2012.10.021

Bolton, J., Cox, B., Clara, I., & Sareen, J. (2006). Use of alcohol and drugs to self-medicate anxiety disorders in a nationally representative sample. *The Journal of Nervous and Mental Disease*, *194*(11), 818–825. doi:10.1097/01.nmd.0000244481.63148.98

Bouchery, E. E., Harwood, H. J., Sacks, J. J., Simon, C. J., & Brewer, R. D. (2011). Economic costs of excessive alcohol consumption in the U.S., 2006. *American Journal of Preventive Medicine*, *41*(5), 516–524. doi:10.1016/j.amepre.2011.06.045

Boughter, J. D., Raghow, S., Nelson, T. M., & Munger, S. D. (2005). Inbred mouse strains C57BL/6J and DBA/2J vary in sensitivity to a subset of bitter stimuli. *BMC Genetics*, *6*, 36. doi:10.1186/1471-2156-6-36

Bouwknecht, J. A., & Paylor, R. (2002). Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains. *Behavioural Brain Research*, *136*(2), 489–501. doi:10.1016/S0166-4328(02)00200-0

Breese, G. R., Overstreet, D. H., Knapp, D. J., & Navarro, M. (2005). Prior multiple ethanol withdrawals enhance stress-induced anxiety-like behavior: inhibition by CRF1- and benzodiazepine-receptor antagonists and a 5-HT1a-receptor agonist. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *30*(9), 1662–1669. doi:10.1038/sj.npp.1300706

Broadbent, J., Muccino, K. J., & Cunningham, C. L. (2002). Ethanol-induced conditioned taste aversion in 15 inbred mouse strains. *Behavioral Neuroscience*, *116*(1), 138–148.

Brown, C. M., Coscina, D. V., & Fletcher, P. J. (2000). The rewarding properties of neuropeptide Y in perifornical hypothalamus vs. nucleus accumbens. *Peptides*, *21*(8), 1279–1287.

Brown, R.E., Gunn, R.K., Schellinck, H.M., Wong, A.A., & O'Leary, T.P. (2004) Anxiety, exploratory behavior, and motor activity in 14 inbred strains of mice. MPD: Brown1, Mouse Phenome Database web site, Bar Harbor, Maine USA: The Jackson Laboratory. http://phenome.jax.org

Brunetti, G., Carai, M. A. M., Lobina, C., Melis, S., Serra, S., Vacca, G., … Colombo, G. (2002). Differences in ethanol-induced conditioned taste aversion in Sardinian alcohol-

preferring and Sardinian alcohol-nonpreferring rats. *Alcohol (Fayetteville, N.Y.)*, *26*(3), 167–172.

Buckner, J. D., & Heimberg, R. G. (2010). Drinking behaviors in social situations account for alcohol-related problems among socially anxious individuals. *Psychology of Addictive Behaviors: Journal of the Society of Psychologists in Addictive Behaviors*, *24*(4), 640–648. doi:10.1037/a0020968

Buxton, A., & Allison, J. (1990). Efficiency and persistence of licking in rats. *Physiology & Behavior*, *47*(2), 239–247.

Caberlotto, L., Thorsell, A., Rimondini, R., Sommer, W., Hyytiä, P., & Heilig, M. (2001). Differential expression of NPY and its receptors in alcohol-preferring AA and alcohol-avoiding ANA rats. *Alcoholism, Clinical and Experimental Research*, *25*(11), 1564–1569.

Can, A., Grahame, N. J., & Gould, T. D. (2012). Affect-related behaviors in mice selectively bred for high and low voluntary alcohol consumption. *Behavior Genetics*, *42*(2), 313–322. doi:10.1007/s10519-011-9505-y

Canu, M. E., Carta, D., Murgia, E., Serra, G., & D'Aquila, P. S. (2010). Dopamine on D2-like receptors is involved in reward evaluation in water-deprived rats licking for NaCl and water. *Pharmacology, Biochemistry, and Behavior*, *96*(2), 194–197. doi:10.1016/j.pbb.2010.05.004

Carroll, M. E., Morgan, A. D., Anker, J. J., Perry, J. L., & Dess, N. K. (2008). Selective breeding for differential saccharin intake as an animal model of drug abuse. *Behavioural Pharmacology*, *19*(5-6), 435–460. doi:10.1097/FBP.0b013e32830c3632

Centers for Disease Control. Vital signs: binge drinking prevalence, frequency, and intensity among adults—U.S., 2010. MMWR Morb Mortal Wkly Rep 2012; 61(1):14 –9.

Chassin, L., Pitts, S. C., & Prost, J. (2002). Binge drinking trajectories from adolescence to emerging adulthood in a high-risk sample: predictors and substance abuse outcomes. *Journal of Consulting and Clinical Psychology*, *70*(1), 67–78.

Chester, J. A., Lumeng, L., Li, T.-K., & Grahame, N. J. (2003). High– and Low–Alcohol-Preferring Mice Show Differences in Conditioned Taste Aversion to Alcohol. *Alcoholism: Clinical and Experimental Research*, *27*(1), 12–18. doi:10.1111/j.1530-0277.2003.tb02714.x

Ciccocioppo, R., Gehlert, D. R., Ryabinin, A., Kaur, S., Cippitelli, A., Thorsell, A., … Heilig, M. (2009). Stress-related neuropeptides and alcoholism: CRH, NPY, and beyond. *Alcohol (Fayetteville, N.Y.)*, *43*(7), 491–498. doi:10.1016/j.alcohol.2009.08.003

Cleary, D. R., Roeder, Z., Elkhatib, R., & Heinricher, M. M. (2014). Neuropeptide Y in the rostral ventromedial medulla reverses inflammatory and nerve injury hyperalgesia in rats via non-selective excitation of local neurons. *Neuroscience*, *271*, 149–159. doi:10.1016/j.neuroscience.2014.04.035

Colombo, G., Agabio, R., Lobina, C., Reali, R., Zocchi, A., Fadda, F., & Gessa, G. L. (1995). Sardinian alcohol-preferring rats: a genetic animal model of anxiety. *Physiology & Behavior*, *57*(6), 1181–1185.

Cooper, M. L., Russell, M., Skinner, J. B., & Windle, M. (1992). Development and Validation of a Three-Dimensional Measure of Drinking Motives. *Psychological Assessment*, *4*(2), 123–132.

Cooper, R., Hildebrandt, S., & Gerlach, A. L. (2014). Drinking motives in alcohol use disorder patients with and without social anxiety disorder. *Anxiety, Stress, and Coping*, *27*(1), 113–122. doi:10.1080/10615806.2013.823482

Cowmeadow, R. B., Krishnan, H. R., & Atkinson, N. S. (2005). The slowpoke gene is necessary for rapid ethanol tolerance in Drosophila. *Alcoholism, Clinical and Experimental Research*, *29*(10), 1777–1786.

Cozzoli, D. K., Courson, J., Caruana, A. L., Miller, B. W., Greentree, D. I., Thomspon, A. B., … Szumlinski, K. K. (2012). Nucleus accumbens mGluR5-associated signaling regulates binge alcohol drinking under Drinking-in-the-Dark procedures. *Alcoholism, Clinical and Experimental Research*, *36*(9), 1623–1633. doi:10.1111/j.1530-0277.2012.01776.x

Crabbe, J. C. (2008). Neurogenetic studies of alcohol addiction. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1507), 3201–3211. doi:10.1098/rstb.2008.0101

Crabbe, J. C., Harkness, J. H., Spence, S. E., Huang, L. C., & Metten, P. (2012a). Intermittent availability of ethanol does not always lead to elevated drinking in mice. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *47*(5), 509–517. doi:10.1093/alcalc/ags067

Crabbe, J. C., Kruse, L. C., Colville, A. M., Cameron, A. J., Spence, S. E., Schlumbohm, J. P., … Metten, P. (2012b). Ethanol Sensitivity in High Drinking in the Dark Selectively Bred Mice. *Alcoholism: Clinical and Experimental Research*, *36*(7), 1162–1170. doi:10.1111/j.1530-0277.2012.01735.x

Crabbe, J. C., Metten, P., Huang, L. C., Schlumbohm, J. P., Spence, S. E., Barkley-Levenson, A. M., … Cameron, A. J. (2012c). Ethanol Withdrawal-Associated Drinking and

Drinking in the Dark: Common and Discrete Genetic Contributions. *Addiction Genetics*, *1*, 3–11.

Crabbe, J. C., Metten, P., Belknap, J. K., Spence, S. E., Cameron, A. J., Schlumbohm, J. P., … Phillips, T. J. (2014). Progress in a replicated selection for elevated blood ethanol concentrations in HDID mice. *Genes, Brain, and Behavior*, *13*(2), 236–246. doi:10.1111/gbb.12105

Crabbe, J. C., Metten, P., Rhodes, J. S., Yu, C.-H., Brown, L. L., Phillips, T. J., & Finn, D. A. (2009). A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. *Biological Psychiatry*, *65*(8), 662–670. doi:10.1016/j.biopsych.2008.11.002

Crabbe, J. C., Spence, S. E., Brown, L. L., & Metten, P. (2010). Alcohol preference drinking in a mouse line selectively bred for high drinking in the dark. *Alcohol (Fayetteville, N.Y.)*. doi:10.1016/j.alcohol.2010.12.001

Crawley, J. N., Belknap, J. K., Collins, A., Crabbe, J. C., Frankel, W., Henderson, N., … Paylor, R. (1997). Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology*, *132*(2), 107–124.

Criado, J. R., Liu, T., Ehlers, C. L., & Mathé, A. A. (2011). Prolonged Chronic Ethanol Exposure Alters Neuropeptide Y and Corticotropin-Releasing Factor Levels in the Brain of Adult Wistar Rats. *Pharmacology, Biochemistry, and Behavior*, *99*(1), 104–111. doi:10.1016/j.pbb.2011.04.005

Cronise, K., Finn, D. A., Metten, P., & Crabbe, J. C. (2005). Scheduled access to ethanol results in motor impairment and tolerance in female C57BL/6J mice. *Pharmacology Biochemistry and Behavior*, *81*(4), 943–953. doi:10.1016/j.pbb.2005.07.005

Cunningham, C. L. (1995). Localization of genes influencing ethanol-induced conditioned place preference and locomotor activity in BXD recombinant inbred mice. *Psychopharmacology, 120*(1), 28–41.

Cunningham, C. L. (2014). Genetic relationship between ethanol-induced conditioned place preference and other ethanol phenotypes in 15 inbred mouse strains. *Behavioral Neuroscience, 128*(4), 430–445. doi:10.1037/a0036459

Cunningham, C. L., Ferree, N. K., & Howard, M. A. (2003). Apparatus bias and place conditioning with ethanol in mice. *Psychopharmacology, 170*(4), 409–422. doi:10.1007/s00213-003-1559-y

Cunningham, C. L., Fidler, T. L., & Hill, K. G. (2000). Animal models of alcohol's motivational effects. *Alcohol Research & Health: The Journal of the National Institute on Alcohol Abuse and Alcoholism, 24*(2), 85–92.

Cunningham, C.L., Gremel, C.M., Groblewski, P.A. *(*2009*)* Genetic influences on conditioned taste aversion. *In:* Reilly S, Schachtman TR, *eds.* Conditioned Taste Aversion: Behavioral and Neural Processes, *pp.* 387–421. New York*: Oxford University Press*

Cunningham, C. L., Howard, M. A., Gill, S. J., Rubinstein, M., Low, M. J., & Grandy, D. K. (2000). Ethanol-conditioned place preference is reduced in dopamine D2 receptor-deficient mice. *Pharmacology, Biochemistry, and Behavior, 67*(4), 693–699.

Cunningham, C. L., Niehus, D. R., Malott, D. H., & Prather, L. K. (1992). Genetic differences in the rewarding and activating effects of morphine and ethanol. *Psychopharmacology, 107*(2-3), 385–393.

Czachowski, C. L., & Samson, H. H. (1999). Breakpoint determination and ethanol self-administration using an across-session progressive ratio procedure in the rat. *Alcoholism, Clinical and Experimental Research*, *23*(10), 1580–1586.

Davies, A. G., Bettinger, J. C., Thiele, T. R., Judy, M. E., & McIntire, S. L. (2004). Natural variation in the npr-1 gene modifies ethanol responses of wild strains of C. elegans. *Neuron*, *42*(5), 731–743. doi:10.1016/j.neuron.2004.05.004

Dawson, D. A., Li, T.-K., & Grant, B. F. (2008). A prospective study of risk drinking: at risk for what? *Drug and Alcohol Dependence*, *95*(1-2), 62–72. doi:10.1016/j.drugalcdep.2007.12.007

DeMartini, K. S., & Carey, K. B. (2011). The role of anxiety sensitivity and drinking motives in predicting alcohol use: a critical review. *Clinical Psychology Review*, *31*(1), 169–177. doi:10.1016/j.cpr.2010.10.001

De Pauli, R. F., Coelhoso, C. C., Tesone-Coelho, C., Linardi, A., Mello, L. E., Silveira, D. X., & Santos, J. G. (2014). Differential patterns of expression of neuropeptide Y throughout abstinence in outbred Swiss mice classified as susceptible or resistant to ethanol-induced locomotor sensitization. *Alcohol (Fayetteville, N.Y.)*, *48*(1), 63–72. doi:10.1016/j.alcohol.2013.11.004

Desai, S. J., Upadhya, M. A., Subhedar, N. K., & Kokare, D. M. (2013). NPY mediates reward activity of morphine, via NPY Y1 receptors, in the nucleus accumbens shell. *Behavioural Brain Research*, *247*, 79–91. doi:10.1016/j.bbr.2013.03.018

Dole, V. P., & Gentry, R. T. (1984). Toward an analogue of alcoholism in mice: scale factors in the model. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(11), 3543–3546.

Eichler, E. E., Flint, J., Gibson, G., Kong, A., Leal, S. M., Moore, J. H., & Nadeau, J. H. (2010). Missing heritability and strategies for finding the underlying causes of complex disease. *Nature Reviews. Genetics*, *11*(6), 446–450. doi:10.1038/nrg2809

Esser, M. B., Hedden, S. L., Kanny, D., Brewer, R. D., Gfroerer, J. C., & Naimi, T. S. (2014). Prevalence of alcohol dependence among US adult drinkers, 2009-2011. *Preventing Chronic Disease*, *11*, E206. doi:10.5888/pcd11.140329

Fidler, T. L., Powers, M. S., Ramirez, J. J., Crane, A., Mulgrew, J., Smitasin, P., & Cunningham, C. L. (2012). Dependence induced increases in intragastric alcohol consumption in mice. *Addiction Biology*, *17*(1), 13–32. doi:10.1111/j.1369-1600.2011.00363.x

Fitzsimons, T. J., & Le Magnen, J. (1969). Eating as a regulatory control of drinking in the rat. *Journal of Comparative and Physiological Psychology*, *67*(3), 273–283.

Flowers, N. T., Naimi, T. S., Brewer, R. D., Elder, R. W., Shults, R. A., & Jiles, R. (2008). Patterns of alcohol consumption and alcohol-impaired driving in the United States. *Alcoholism, Clinical and Experimental Research*, *32*(4), 639–644. doi:10.1111/j.1530-0277.2008.00622.x

Ford, M. M., Beckley, E. H., Nickel, J. D., Eddy, S., & Finn, D. A. (2008). Ethanol intake patterns in female mice: influence of allopregnanolone and the inhibition of its synthesis. *Drug and Alcohol Dependence*, *97*(1-2), 73–85. doi:10.1016/j.drugalcdep.2008.03.021

Foroud, T., Edenberg, H. J., & Crabbe, J. C. (2010). Genetic Research. *Alcohol Research & Health*, *33*(1-2), 64–75.

Francès, F., Guillen, M., Verdú, F., Portolés, O., Castelló, A., Sorlí, J. V., & Corella, D. (2011). The 1258 G>A polymorphism in the neuropeptide Y gene is associated with

greater alcohol consumption in a Mediterranean population. *Alcohol (Fayetteville, N.Y.)*, *45*(2), 131–136. doi:10.1016/j.alcohol.2010.08.009

Franck, J., & Jayaram-Lindström, N. (2013). Pharmacotherapy for alcohol dependence: status of current treatments. *Current Opinion in Neurobiology*, *23*(4), 692–699. doi:10.1016/j.conb.2013.05.005

Franklin, K.B.J. & Paxinos, G. The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press; 2001.

Froehlich, J. C., Harts, J., Lumeng, L., & Li, T. K. (1988). Differences in response to the aversive properties of ethanol in rats selectively bred for oral ethanol preference. *Pharmacology, Biochemistry, and Behavior*, *31*(1), 215–222.

Frye, C. A., Petralia, S. M., & Rhodes, M. E. (2000). Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3alpha,5alpha-THP. *Pharmacology, Biochemistry, and Behavior*, *67*(3), 587–596.

Galistu, A., & D'Aquila, P. S. (2012). Effect of the dopamine D1-like receptor antagonist SCH 23390 on the microstructure of ingestive behaviour in water-deprived rats licking for water and NaCl solutions. *Physiology & Behavior*, *105*(2), 230–233. doi:10.1016/j.physbeh.2011.08.006

García-Medina, N. E., Vera, G., & Miranda, M. I. (2014). Chemical stimulation or glutamate injections in the nucleus of solitary tract enhance conditioned taste aversion. *Behavioural Brain Research*, *278C*, 202–209. doi:10.1016/j.bbr.2014.09.023

Gauvin, D. V., Baird, T. J., & Briscoe, R. J. (2000). Differential development of behavioral tolerance and the subsequent hedonic effects of alcohol in AA and ANA rats. *Psychopharmacology*, *151*(4), 335–343.

Giardino, W. J., & Ryabinin, A. E. (2013). CRF1 receptor signaling regulates food and fluid intake in the drinking-in-the-dark model of binge alcohol consumption. *Alcoholism, Clinical and Experimental Research*, *37*(7), 1161–1170. doi:10.1111/acer.12076

Gilman, J. M., Ramchandani, V. A., Davis, M. B., Bjork, J. M., & Hommer, D. W. (2008). Why we like to drink: An fMRI Study of the Rewarding and Anxiolytic Effects of Alcohol. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(18), 4583–4591. doi:10.1523/JNEUROSCI.0086-08.2008

Gilman, S. E., & Abraham, H. D. (2001). A longitudinal study of the order of onset of alcohol dependence and major depression. *Drug and Alcohol Dependence*, *63*(3), 277–286.

Gilpin, N. W. (2012). Neuropeptide Y (NPY) in the extended amygdala is recruited during the transition to alcohol dependence. *Neuropeptides*, *46*(6), 253–259. doi:10.1016/j.npep.2012.08.001

Gilpin, N. W., Misra, K., Herman, M. A., Cruz, M. T., Koob, G. F., & Roberto, M. (2011). Neuropeptide Y Opposes Alcohol Effects on GABA Release in Amygdala and Blocks the Transition to Alcohol Dependence. *Biological Psychiatry*, *69*(11), 1091–1099. doi:10.1016/j.biopsych.2011.02.004

Gire, J. T. (2002). A cross-national study of motives for drinking alcohol. *Substance Use & Misuse*, *37*(2), 215–223.

Goldman, D., Oroszi, G., & Ducci, F. (2005). The genetics of addictions: uncovering the genes. *Nature Reviews. Genetics*, *6*(7), 521–532. doi:10.1038/nrg1635

Grahame, N. J., Chester, J. A., Rodd-Henricks, K., Li, T. K., & Lumeng, L. (2001). Alcohol place preference conditioning in high- and low-alcohol preferring selected lines of mice. *Pharmacology, Biochemistry, and Behavior*, *68*(4), 805–814.

Grahame, N. J., & Cunningham, C. L. (1997). Intravenous ethanol self-administration in C57BL/6J and DBA/2J mice. *Alcoholism, Clinical and Experimental Research*, *21*(1), 56–62.

Grahame, N. J., Li, T. K., & Lumeng, L. (1999). Selective breeding for high and low alcohol preference in mice. *Behavior Genetics*, *29*(1), 47–57.

Grandt, D., Schimiczek, M., Rascher, W., Feth, F., Shively, J., Lee, T. D., … Michel, M. C. (1996). Neuropeptide Y 3-36 is an endogenous ligand selective for Y2 receptors. *Regulatory Peptides*, *67*(1), 33–37.

Grant, B. F., & Harford, T. C. (1995). Comorbidity between DSM-IV alcohol use disorders and major depression: results of a national survey. *Drug and Alcohol Dependence*, *39*(3), 197–206.

Grant, B.F., Stinson, F.S., Dawson, D.A., & et al., (2004). Prevalence and co-occurrence of substance use disorders and independent mood and anxiety disorders: Results from the national epidemiologic survey on alcohol and related conditions. *Archives of General Psychiatry*, *61*(8), 807–816. doi:10.1001/archpsyc.61.8.807

Grant, K. A., Leng, X., Green, H. L., Szeliga, K. T., Rogers, L. S. M., & Gonzales, S. W. (2008). Drinking Typography Established by Scheduled Induction Predicts Chronic Heavy Drinking in a Monkey Model of Ethanol Self-Administration. *Alcoholism: Clinical and Experimental Research*, *32*(10), 1824–1838. doi:10.1111/j.1530-0277.2008.00765.x

Greber, S., Schwarzer, C., & Sperk, G. (1994). Neuropeptide Y inhibits potassium-stimulated glutamate release through Y2 receptors in rat hippocampal slices in vitro. *British Journal of Pharmacology*, *113*(3), 737–740.

Green, A. S., & Grahame, N. J. (2008). Ethanol drinking in rodents: is free-choice drinking related to the reinforcing effects of ethanol? *Alcohol (Fayetteville, N.Y.), 42*(1), 1–11. doi:10.1016/j.alcohol.2007.10.005

Gremel, C. M., & Cunningham, C. L. (2007). Role of test activity in ethanol-induced disruption of place preference expression in mice. *Psychopharmacology*, *191*(2), 195–202. doi:10.1007/s00213-006-0651-5

Gremel, C. M., Gabriel, K. I., & Cunningham, C. L. (2006). Topiramate does not affect the acquisition or expression of ethanol conditioned place preference in DBA/2J or C57BL/6J mice. *Alcoholism, Clinical and Experimental Research*, *30*(5), 783–790. doi:10.1111/j.1530-0277.2006.00091.x

Griebel, G., Belzung, C., Perrault, G., & Sanger, D. J. (2000). Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. *Psychopharmacology*, *148*(2), 164–170.

Griffin, W. C., Lopez, M. F., Yanke, A. B., Middaugh, L. D., & Becker, H. C. (2009). Repeated cycles of chronic intermittent ethanol exposure in mice increases voluntary ethanol drinking and ethanol concentrations in the nucleus accumbens. *Psychopharmacology*, *201*(4), 569–580. doi:10.1007/s00213-008-1324-3

Groblewski, P. A., Bax, L. S., & Cunningham, C. L. (2008). Reference-dose place conditioning with ethanol in mice: empirical and theoretical analysis. *Psychopharmacology*, *201*(1), 97–106. doi:10.1007/s00213-008-1251-3

Gupta, S., Wang, F., Holly, E. A., & Bracci, P. M. (2010). Risk of pancreatic cancer by alcohol dose, duration, and pattern of consumption, including binge drinking: a

population-based study. *Cancer Causes & Control: CCC*, *21*(7), 1047–1059. doi:10.1007/s10552-010-9533-6

Gupta, T., Syed, Y. M., Revis, A. A., Miller, S. A., Martinez, M., Cohn, K. A., … Rhodes, J. S. (2008). Acute Effects of Acamprosate and MPEP on Ethanol Drinking-in-the-Dark in Male C57BL/6J Mice. *Alcoholism: Clinical and Experimental Research*, *32*(11), 1992–1998. doi:10.1111/j.1530-0277.2008.00787.x

Haack, A. K., Sheth, C., Schwager, A. L., Sinclair, M. S., Tandon, S., & Taha, S. A. (2014). Lesions of the lateral habenula increase voluntary ethanol consumption and operant self-administration, block yohimbine-induced reinstatement of ethanol seeking, and attenuate ethanol-induced conditioned taste aversion. *PloS One*, *9*(4), e92701. doi:10.1371/journal.pone.0092701

Hall, F. S., Sora, I., & Uhl, G. R. (2001). Ethanol consumption and reward are decreased in mu-opiate receptor knockout mice. *Psychopharmacology*, *154*(1), 43–49.

Hasin, D. S., Goodwin, R. D., Stinson, F. S., & Grant, B. F. (2005). Epidemiology of major depressive disorder: results from the National Epidemiologic Survey on Alcoholism and Related Conditions. *Archives of General Psychiatry*, *62*(10), 1097–1106. doi:10.1001/archpsyc.62.10.1097

Hayes, D. M., Knapp, D. J., Breese, G. R., & Thiele, T. E. (2005). Comparison of basal neuropeptide Y and corticotropin releasing factor levels between the high ethanol drinking C57BL/6J and low ethanol drinking DBA/2J inbred mouse strains. *Alcoholism, Clinical and Experimental Research*, *29*(5), 721–729.

Henderson, A. N., & Czachowski, C. L. (2012). Neuropeptide Y (NPY) in the central nucleus of the amygdala (CeA) does not affect ethanol-reinforced responding in binge-drinking,

nondependent rats. *Pharmacology, Biochemistry, and Behavior*, *101*(1), 8–13. doi:10.1016/j.pbb.2011.11.008

Henniger, M. S. H., Spanagel, R., Wigger, A., Landgraf, R., & Hölter, S. M. (2002). Alcohol Self-administration in Two Rat Lines Selectively Bred for Extremes in Anxiety-related Behavior. *, Published Online: 09 November 2001; | doi:10.1016/S0893-133X(01)00408-0*, *26*(6), 729–736. doi:10.1016/S0893-133X(01)00408-0

Hitzemann, R., Malmanger, B., Belknap, J., Darakjian, P., & McWeeney, S. (2008). Short-term selective breeding for High and Low prepulse inhibition of the acoustic startle response; pharmacological characterization and QTL mapping in the selected lines. *Pharmacology, Biochemistry, and Behavior*, *90*(4), 525–533. doi:10.1016/j.pbb.2008.04.004

Holahan, C. J., Moos, R. H., Holahan, C. K., Cronkite, R. C., & Randall, P. K. (2003). Drinking to cope and alcohol use and abuse in unipolar depression: a 10-year model. *Journal of Abnormal Psychology*, *112*(1), 159–165.

Holstein, S. E., Spanos, M., & Hodge, C. W. (2011). Adolescent C57BL/6J mice show elevated alcohol intake, but reduced taste aversion, as compared to adult mice: a potential behavioral mechanism for binge drinking. *Alcoholism, Clinical and Experimental Research*, *35*(10), 1842–1851. doi:10.1111/j.1530-0277.2011.01528.x

Houchi, H., Babovic, D., Pierrefiche, O., Ledent, C., Daoust, M., & Naassila, M. (2005). CB1 receptor knockout mice display reduced ethanol-induced conditioned place preference and increased striatal dopamine D2 receptors. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *30*(2), 339–349. doi:10.1038/sj.npp.1300568

Hostetler, C. M., Hitchcock, L. N., Anacker, A. M. J., Young, L. J., & Ryabinin, A. E. (2013). Comparative distribution of central neuropeptide Y (NPY) in the prairie (Microtus ochrogaster) and meadow (M. pennsylvanicus) vole. *Peptides*, *40*, 22–29. doi:10.1016/j.peptides.2012.12.008

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, *157*(6), 1262–1278. doi:10.1016/j.cell.2014.05.010

Hussong, A. M. (2003). Social influences in motivated drinking among college students. *Psychology of Addictive Behaviors: Journal of the Society of Psychologists in Addictive Behaviors*, *17*(2), 142–150.

Hutton, H. E., McCaul, M. E., Santora, P. B., & Erbelding, E. J. (2008). The relationship between recent alcohol use and sexual behaviors: gender differences among sexually transmitted disease clinic patients. *Alcoholism, Clinical and Experimental Research*, *32*(11), 2008–2015. doi:10.1111/j.1530-0277.2008.00788.x

Hwa, L. S., Chu, A., Levinson, S. A., Kayyali, T. M., DeBold, J. F., & Miczek, K. A. (2011). Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. *Alcoholism, Clinical and Experimental Research*, *35*(11), 1938–1947. doi:10.1111/j.1530-0277.2011.01545.x

Hwang, B. H., Zhang, J. K., Ehlers, C. L., Lumeng, L., & Li, T. K. (1999). Innate differences of neuropeptide Y (NPY) in hypothalamic nuclei and central nucleus of the amygdala between selectively bred rats with high and low alcohol preference. *Alcoholism, Clinical and Experimental Research*, *23*(6), 1023–1030.

Iancu, O. D., Darakjian, P., Walter, N. A., Malmanger, B., Oberbeck, D., Belknap, J., … Hitzemann, R. (2010). Genetic diversity and striatal gene networks: focus on the

heterogeneous stock-collaborative cross (HS-CC) mouse. *BMC Genomics*, *11*(1), 585. doi:10.1186/1471-2164-11-585

Iancu, O. D., Oberbeck, D., Darakjian, P., Metten, P., McWeeney, S., Crabbe, J. C., & Hitzemann, R. (2013). Selection for Drinking in the Dark Alters Brain Gene Coexpression Networks. *Alcoholism, Clinical and Experimental Research*, *37*(8), 1295–1303. doi:10.1111/acer.12100

Johnson, A. W., Sherwood, A., Smith, D. R., Wosiski-Kuhn, M., Gallagher, M., & Holland, P. C. (2010). An analysis of licking microstructure in three strains of mice. *Appetite*, *54*(2), 320–330. doi:10.1016/j.appet.2009.12.007

Johnson, R. F., & Johnson, A. K. (1990). Light/Dark cycle modulates food to water intake ratios in rats. *Physiology & Behavior*, *48*(5), 707–711. doi:10.1016/0031-9384(90)90215-P

Johnston, A. L., & File, S. E. (1991). Sex differences in animal tests of anxiety. *Physiology & Behavior*, *49*(2), 245–250. doi:10.1016/0031-9384(91)90039-Q

Kamdar, N. K., Miller, S. A., Syed, Y. M., Bhayana, R., Gupta, T., & Rhodes, J. S. (2007). Acute effects of naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice. *Psychopharmacology*, *192*(2), 207–217. doi:10.1007/s00213-007-0711-5

Kampov-Polevoy, A. B., Ziedonis, D., Steinberg, M. L., Pinsky, I., Krejci, J., Eick, C., … Crews, F. T. (2003). Association between sweet preference and paternal history of alcoholism in psychiatric and substance abuse patients. *Alcoholism, Clinical and Experimental Research*, *27*(12), 1929–1936. doi:10.1097/01.ALC.0000099265.60216.23

Kapoor, M., Wang, J.-C., Wetherill, L., Le, N., Bertelsen, S., Hinrichs, A. L., … Goate, A. (2013). A meta-analysis of two genome-wide association studies to identify novel loci for

maximum number of alcoholic drinks. *Human Genetics*, *132*(10), 1141–1151. doi:10.1007/s00439-013-1318-z

Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J., & Heberlein, U. (2011). A Drosophila model for alcohol reward. *Nature Neuroscience*, *14*(5), 612–619. doi:10.1038/nn.2805

Kaur, S., & Ryabinin, A. E. (2010). Ghrelin receptor antagonism decreases alcohol consumption and activation of perioculomotor urocortin-containing neurons. *Alcoholism, Clinical and Experimental Research*, *34*(9), 1525–1534. doi:10.1111/j.1530-0277.2010.01237.x

Kessler RC, Crum RM, Warner LA, Nelson CB, Schulenberg J, & Anthony JC. (1997). LIfetime co-occurrence of dsm-iii-r alcohol abuse and dependence with other psychiatric disorders in the national comorbidity survey. *Archives of General Psychiatry*, *54*(4), 313–321. doi:10.1001/archpsyc.1997.01830160031005

King, A. C., de Wit, H., McNamara, P. J., & Cao, D. (2011). Rewarding, stimulant, and sedative alcohol responses and relationship to future binge drinking. *Archives of General Psychiatry*, *68*(4), 389–399. doi:10.1001/archgenpsychiatry.2011.26

King, A. C., Houle, T., de Wit, H., Holdstock, L., & Schuster, A. (2002). Biphasic alcohol response differs in heavy versus light drinkers. *Alcoholism, Clinical and Experimental Research*, *26*(6), 827–835.

Kliethermes, C. L. (2005). Anxiety-like behaviors following chronic ethanol exposure. *Neuroscience and Biobehavioral Reviews*, *28*(8), 837–850. doi:10.1016/j.neubiorev.2004.11.001

Koob, G. F., & Le Moal, M. (2001). Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *24*(2), 97–129. doi:/10.1016/S0893-133X(00)00195-0

Kuntsche, E., Stewart, S. H., & Cooper, M. L. (2008). How stable is the motive-alcohol use link? A cross-national validation of the Drinking Motives Questionnaire Revised among adolescents from Switzerland, Canada, and the United States. *Journal of Studies on Alcohol and Drugs*, *69*(3), 388–396.

Kushner, M. G., Abrams, K., & Borchardt, C. (2000). The relationship between anxiety disorders and alcohol use disorders: A review of major perspectives and findings. *Clinical Psychology Review*, *20*(2), 149–171. doi:10.1016/S0272-7358(99)00027-6

Lange, L. A., Kampov-Polevoy, A. B., & Garbutt, J. C. (2010). Sweet liking and high novelty seeking: independent phenotypes associated with alcohol-related problems. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *45*(5), 431–436. doi:10.1093/alcalc/agq040

Lê, A. D., Ko, J., Chow, S., & Quan, B. (1994). Alcohol consumption by C57BL/6, BALB/c, and DBA/2 mice in a limited access paradigm. *Pharmacology, Biochemistry, and Behavior*, *47*(2), 375–378.

Leeman, R. F., Heilig, M., Cunningham, C. L., Stephens, D. N., Duka, T., & O'Malley, S. S. (2010). Ethanol Consumption: How Should We Measure It? Achieving Consilience between Human and Animal Phenotypes. *Addiction Biology*, *15*(2), 109–124. doi:10.1111/j.1369-1600.2009.00192.x

Leeman, R. F., Corbin, W. R., Nogueira, C., Krishnan-Sarin, S., Potenza, M. N., & O'Malley, S. S. (2013). A human alcohol self-administration paradigm to model individual

differences in impaired control over alcohol use. *Experimental and Clinical Psychopharmacology, 21*(4), 303–314. doi:10.1037/a0033438

Le Magnen, J., & Devos, M. (1980). Parameters of the meal pattern in rats: their assessment and physiological significance. *Neuroscience and Biobehavioral Reviews, 4 Suppl 1*, 1–11.

Levine, A. S., & Morley, J. E. (1984). Neuropeptide Y: a potent inducer of consummatory behavior in rats. *Peptides, 5*(6), 1025–1029.

Linsenbardt, D. N., & Boehm, S. L. (2009). Agonism of the endocannabinoid system modulates binge-like alcohol intake in male C57BL/6J mice: involvement of the posterior ventral tegmental area. *Neuroscience, 164*(2), 424–434. doi:10.1016/j.neuroscience.2009.08.007

Linsenbardt, D. N., & Boehm, S. L. (2014). Alterations in the rate of binge ethanol consumption: implications for preclinical studies in mice. *Addiction Biology, 19*(5), 812–825. doi:10.1111/adb.12052

Lopez, M. F., & Becker, H. C. (2014). Operant ethanol self-administration in ethanol dependent mice. *Alcohol (Fayetteville, N.Y.), 48*(3), 295–299. doi:10.1016/j.alcohol.2014.02.002

Lowery, E. G., Spanos, M., Navarro, M., Lyons, A. M., Hodge, C. W., & Thiele, T. E. (2010). CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology, 35*(6), 1241–1252. doi:10.1038/npp.2009.209

Lyons, M. J., Schultz, M., Neale, M., Brady, K., Eisen, S., Toomey, R., … Tsuang, M. (2006). Specificity of familial vulnerability for alcoholism versus major depression in men. *The Journal of Nervous and Mental Disease*, *194*(11), 809–817.

Malmström, R. E., Balmér, K. C., & Lundberg, J. M. (1997). The neuropeptide Y (NPY) Y1 receptor antagonist BIBP 3226: equal effects on vascular responses to exogenous and endogenous NPY in the pig in vivo. *British Journal of Pharmacology*, *121*(3), 595–603. doi:10.1038/sj.bjp.0701154

Malone, S. M., Iacono, W. G., & McGue, M. (2002). Drinks of the father: father's maximum number of drinks consumed predicts externalizing disorders, substance use, and substance use disorders in preadolescent and adolescent offspring. *Alcoholism, Clinical and Experimental Research*, *26*(12), 1823–1832. doi:10.1097/01.ALC.0000042222.59908.F9

Manzo, L., Gómez, M. A. J., Callejas-Aguilera, J. E., Donaire, R., Sabariego, M., Fernández-Teruel, A., … Torres, C. (2014). Relationship between ethanol preference and sensation/novelty seeking. *Physiology & Behavior*. doi:10.1016/j.physbeh.2014.05.003

Martin, C. S., Earleywine, M., Musty, R. E., Perrine, M. W., & Swift, R. M. (1993). Development and Validation of the Biphasic Alcohol Effects Scale. *Alcoholism: Clinical and Experimental Research*, *17*(1), 140–146. doi:10.1111/j.1530-0277.1993.tb00739.x

Matson, L., Liangpunsakul, S., Crabb, D., Buckingham, A., Ross, R. A., Halcomb, M., & Grahame, N. (2013). Chronic free-choice drinking in crossed HAP (cHAP) mice leads to sustained blood ethanol levels and metabolic tolerance without evidence of liver damage. *Alcoholism, Clinical and Experimental Research*, *37*(2), 194–201. doi:10.1111/j.1530-0277.2012.01873.x

Matson, L. M., & Grahame, N. J. (2013). Pharmacologically Relevant Intake During Chronic, Free-Choice Drinking Rhythms in Selectively Bred High Alcohol Preferring Mice. *Addiction Biology*, *18*(6), 921–929. doi:10.1111/j.1369-1600.2011.00412.x

McBride, W. J., Rodd, Z. A., Bell, R. L., Lumeng, L., & Li, T.-K. (2014a). The alcohol-preferring (P) and high-alcohol-drinking (HAD) rats--animal models of alcoholism. *Alcohol (Fayetteville, N.Y.)*, *48*(3), 209–215. doi:10.1016/j.alcohol.2013.09.044

McCaul, M. E., Turkkan, J. S., Svikis, D. S., & Bigelow, G. E. (1990). Alcohol and secobarbital effects as a function of familial alcoholism: acute psychophysiological effects. *Alcoholism, Clinical and Experimental Research*, *14*(5), 704–712.

McCutcheon, J. E., Ebner, S. R., Loriaux, A. L., & Roitman, M. F. (2012). Encoding of Aversion by Dopamine and the Nucleus Accumbens. *Frontiers in Neuroscience*, *6*. doi:10.3389/fnins.2012.00137

Melendez, R. I. (2011). Intermittent (every-other-day) drinking induces rapid escalation of ethanol intake and preference in adolescent and adult C57BL/6J mice. *Alcoholism, Clinical and Experimental Research*, *35*(4), 652–658. doi:10.1111/j.1530-0277.2010.01383.x

Mello, N. K., & Mendelson, J. H. (1970). Behavioral Studies of Sleep Patterns in Alcoholics During Intoxication and Withdrawal. *Journal of Pharmacology and Experimental Therapeutics*, *175*(1), 94–112.

Merikangas, K. R., Risch, N. J., & Weissman, M. M. (1994). Comorbidity and co-transmission of alcoholism, anxiety and depression. *Psychological Medicine*, *24*(1), 69–80.

Metten, P., Brown, L. L., & Crabbe, J. C. (2011). Limited access ethanol drinking in the dark in adolescent and adult mice. *Pharmacology, Biochemistry, and Behavior*, *98*(2), 279–285. doi:10.1016/j.pbb.2011.01.003

Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., … Westfall, T. (1998). XVI. International Union of Pharmacology Recommendations for the Nomenclature of Neuropeptide Y, Peptide YY, and Pancreatic Polypeptide Receptors. *Pharmacological Reviews*, *50*(1), 143–150.

Miller, P. M., Book, S. W., Thomas, S., Smith, J. P., Randall, P. K., & Randall, C. L. (2014). The potential utility of drinking motive questions to screen at-risk drinking in socially anxious patients. *Journal of Substance Use*, *19*(3), 225–228. doi:10.3109/14659891.2013.779756

Milner, L. C., & Crabbe, J. C. (2008). Three murine anxiety models: results from multiple inbred strain comparisons. *Genes, Brain, and Behavior*, *7*(4), 496–505. doi:10.1111/j.1601-183X.2007.00385.x

Moore, E. M., Forrest, R. D., & Boehm, S. L. (2013). Genotype Modulates Age-Related Alterations in Sensitivity to the Aversive Effects of Ethanol: An 8 Inbred Strain Analysis of Conditioned Taste Aversion. *Genes, Brain, and Behavior*, *12*(1), 70–77. doi:10.1111/gbb.12004

Morato, G. S., & Morato, E. F. (1993). Differential effects of ethanol in mouse lines genetically selected for high and low antibody production. *Brain, Behavior, and Immunity*, *7*(3), 224–230. doi:10.1006/brbi.1993.1023

Morean, M. E., de Wit, H., King, A. C., Sofuoglu, M., Rueger, S. Y., & O'Malley, S. S. (2013). The drug effects questionnaire: psychometric support across three drug types. *Psychopharmacology, 227*(1), 177–192. doi:10.1007/s00213-012-2954-z

Morris, B. J. (1989). Neuronal localisation of neuropeptide Y gene expression in rat brain. *The Journal of Comparative Neurology, 290*(3), 358–368. doi:10.1002/cne.902900305

Mottagui-Tabar, S., Prince, J. A., Wahlestedt, C., Zhu, G., Goldman, D., & Heilig, M. (2005). A novel single nucleotide polymorphism of the neuropeptide Y (NPY) gene associated with alcohol dependence. *Alcoholism, Clinical and Experimental Research, 29*(5), 702–707.

Murphy, J. M., Gatto, G. J., Waller, M. B., McBride, W. J., Lumeng, L., & Li, T. K. (1986). Effects of scheduled access on ethanol intake by the alcohol-preferring (P) line of rats. *Alcohol (Fayetteville, N.Y.), 3*(5), 331–336.

Naassila, M., Pierrefiche, O., Ledent, C., & Daoust, M. (2004). Decreased alcohol self-administration and increased alcohol sensitivity and withdrawal in CB1 receptor knockout mice. *Neuropharmacology, 46*(2), 243–253.

Napper, L. E., Kenney, S. R., Lac, A., Lewis, L. J., & LaBrie, J. W. (2014). A cross-lagged panel model examining protective behavioral strategies: Are types of strategies differentially related to alcohol use and consequences? *Addictive Behaviors, 39*(2), 480–486. doi:10.1016/j.addbeh.2013.10.020

Navarro, M., Cubero, I., Ko, L., & Thiele, T. E. (2009). Deletion of agouti-related protein blunts ethanol self-administration and binge-like drinking in mice. *Genes, Brain, and Behavior, 8*(4), 450–458. doi:10.1111/j.1601-183X.2009.00493.x

Newlin, D. B., & Thomson, J. B. (1990). Alcohol challenge with sons of alcoholics: a critical review and analysis. *Psychological Bulletin*, *108*(3), 383–402.

NIAAA Newsletter. Vol 3. DHHS-NIH; Bethesda, MD: 2004. NIAAA Council Approves Definition of Binge Drinking; p. 3. Winter. 2004.

Noël, X., Brevers, D., Bechara, A., Hanak, C., Kornreich, C., Verbanck, P., & Bon, O. L. (2011). Neurocognitive Determinants of Novelty and Sensation-Seeking in Individuals with Alcoholism. *Alcohol and Alcoholism*, *46*(4), 407–415. doi:10.1093/alcalc/agr048

Nuutinen, S., Lintunen, M., Vanhanen, J., Ojala, T., Rozov, S., & Panula, P. (2011). Evidence for the role of histamine H3 receptor in alcohol consumption and alcohol reward in mice. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *36*(10), 2030–2040. doi:10.1038/npp.2011.90

Oberlin, B., Best, C., Matson, L., Henderson, A., & Grahame, N. (2011). Derivation and characterization of replicate high- and low-alcohol preferring lines of mice and a high-drinking crossed HAP line. *Behavior Genetics*, *41*(2), 288–302. doi:10.1007/s10519-010-9394-5

Office of Juvenile Justice and Delinquency Prevention (2005). Drinking in America: Myths, Realities, and Prevention Policy. Washington, DC: U.S. Department of Justice, Office of Justice Programs, Office of Juvenile Justice and Delinquency Prevention.

O'Leary, T. P., Gunn, R. K., & Brown, R. E. (2013). What are we measuring when we test strain differences in anxiety in mice? *Behavior Genetics*, *43*(1), 34–50. doi:10.1007/s10519-012-9572-8

Olling, J. D., Ulrichsen, J., Christensen, D. Z., & Woldbye, D. P. D. (2009). Complex plastic changes in the neuropeptide Y system during ethanol intoxication and withdrawal in the rat brain. *Journal of Neuroscience Research*, *87*(10), 2386–2397. doi:10.1002/jnr.22049

Park, B. L., Kim, J. W., Cheong, H. S., Kim, L. H., Lee, B. C., Seo, C. H., … Choi, I.-G. (2013). Extended genetic effects of ADH cluster genes on the risk of alcohol dependence: from GWAS to replication. *Human Genetics*, *132*(6), 657–668. doi:10.1007/s00439-013-1281-8

Parker, C. C., & Palmer, A. A. (2011). Dark Matter: Are Mice the Solution to Missing Heritability? *Frontiers in Genetics*, *2*. doi:10.3389/fgene.2011.00032

Phillips, T. J., Broadbent, J., Burkhart-Kasch, S., Henderson, C., Wenger, C. D., McMullin, C., … Cunningham, C. L. (2005). Genetic correlational analyses of ethanol reward and aversion phenotypes in short-term selected mouse lines bred for ethanol drinking or ethanol-induced conditioned taste aversion. *Behavioral Neuroscience*, *119*(4), 892–910. doi:10.1037/0735-7044.119.4.892

Phillips, T. J., Feller, D. J., & Crabbe, J. C. (1989). Selected mouse lines, alcohol and behavior. *Experientia*, *45*(9), 805–827.

Phillips, T. J., Reed, C., Burkhart-Kasch, S., Li, N., Hitzemann, R., Yu, C.-H., … Belknap, J. K. (2010). A method for mapping intralocus interactions influencing excessive alcohol drinking. *Mammalian Genome: Official Journal of the International Mammalian Genome Society*, *21*(1-2), 39–51. doi:10.1007/s00335-009-9239-9

Pich, E. M., & Epping-Jordan, M. P. (1998). Transgenic mice in drug dependence research. *Annals of Medicine*, *30*(4), 390–396.

Plescia, F., Brancato, A., Marino, R. A. M., Vita, C., Navarra, M., & Cannizzaro, C. (2014). Effect of Acetaldehyde Intoxication and Withdrawal on NPY Expression: Focus on Endocannabinoidergic System Involvement. *Frontiers in Psychiatry*, *5*, 138. doi:10.3389/fpsyt.2014.00138

Prescott, C. A., Aggen, S. H., & Kendler, K. S. (2000). Sex-specific genetic influences on the comorbidity of alcoholism and major depression in a population-based sample of US twins. *Archives of General Psychiatry*, *57*(8), 803–811.

Primeaux, S. D., Wilson, S. P., Bray, G. A., York, D. A., & Wilson, M. A. (2006). Overexpression of neuropeptide Y in the central nucleus of the amygdala decreases ethanol self-administration in "anxious" rats. *Alcoholism, Clinical and Experimental Research*, *30*(5), 791–801. doi:10.1111/j.1530-0277.2006.00092.x

Rehm, J., Greenfield, T. K., & Rogers, J. D. (2001). Average volume of alcohol consumption, patterns of drinking, and all-cause mortality: results from the US National Alcohol Survey. *American Journal of Epidemiology*, *153*(1), 64–71.

Rhodes, J. S., Best, K., Belknap, J. K., Finn, D. A., & Crabbe, J. C. (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology & Behavior*, *84*(1), 53–63. doi:10.1016/j.physbeh.2004.10.007

Rhodes, J. S., Ford, M. M., Yu, C.-H., Brown, L. L., Finn, D. A., Garland, T., & Crabbe, J. C. (2007). Mouse inbred strain differences in ethanol drinking to intoxication. *Genes, Brain and Behavior*, *6*(1), 1–18. doi:10.1111/j.1601-183X.2006.00210.x

Riley, A. L. (2011). The paradox of drug taking: The role of the aversive effects of drugs. *Physiology & Behavior*, *103*(1), 69–78. doi:10.1016/j.physbeh.2010.11.021

Risinger, F. O., & Cunningham, C. L. (1998). Ethanol-Induced Conditioned Taste Aversion in BXD Recombinant Inbred Mice. *Alcoholism: Clinical and Experimental Research*, *22*(6), 1234–1244. doi:10.1111/j.1530-0277.1998.tb03904.x

Risinger, F. O., & Cunningham, C. L. (2000). DBA/2J mice develop stronger lithium chloride-induced conditioned taste and place aversions than C57BL/6J mice. *Pharmacology, Biochemistry, and Behavior*, *67*(1), 17–24.

Robinson, J., Sareen, J., Cox, B. J., & Bolton, J. (2009). Self-medication of anxiety disorders with alcohol and drugs: Results from a nationally representative sample. *Journal of Anxiety Disorders*, *23*(1), 38–45. doi:10.1016/j.janxdis.2008.03.013

Roman, E., Stewart, R. B., Bertholomey, M. L., Jensen, M. L., Colombo, G., Hyytiä, P., … Lumeng, L. (2012). Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test. *Addiction Biology*, *17*(1), 33–46. doi:10.1111/j.1369-1600.2011.00327.x

Roseboom, P. H., Nanda, S. A., Fox, A. S., Oler, J. A., Shackman, A. J., Shelton, S. E., … Kalin, N. H. (2014). Neuropeptide Y receptor gene expression in the primate amygdala predicts anxious temperament and brain metabolism. *Biological Psychiatry*, *76*(11), 850–857. doi:10.1016/j.biopsych.2013.11.012

Rosenwasser, A. M., Fixaris, M. C., Crabbe, J. C., Brooks, P. C., & Ascheid, S. (2013). Escalation of intake under intermittent ethanol access in diverse mouse genotypes. *Addiction Biology*, *18*(3), 496–507. doi:10.1111/j.1369-1600.2012.00481.x

Rueger, S. Y., Hu, H., McNamara, P., Cao, D., Hao, W., & King, A. C. (2015). Differences in subjective response to alcohol in heavy- and light-drinking Chinese men versus

Caucasian American men. *Addiction (Abingdon, England)*, *110*(1), 91–99. doi:10.1111/add.12737

Rushing, P. A., Houpt, T. A., Henderson, R. P., & Gibbs, J. (1997). High lick rate is maintained throughout spontaneous liquid meals in freely feeding rats. *Physiology & Behavior*, *62*(5), 1185–1188.

Rustay, N. R., & Crabbe, J. C. (2004). Genetic Analysis of Rapid Tolerance to Ethanol's Incoordinating Effects in Mice: Inbred Strains and Artificial Selection. *Behavior Genetics*, *34*(4), 441–451. doi:10.1023/B:BEGE.0000023649.60539.dd

Ryabinin, A. E., Yoneyama, N., Tanchuck, M. A., Mark, G. P., & Finn, D. A. (2008). Urocortin 1 microinjection into the mouse lateral septum regulates the acquisition and expression of alcohol consumption. *Neuroscience*, *151*(3), 780–790. doi:10.1016/j.neuroscience.2007.11.014

Saccone, N. L., Kwon, J. M., Corbett, J., Goate, A., Rochberg, N., Edenberg, H. J., … Rice, J. P. (2000). A genome screen of maximum number of drinks as an alcoholism phenotype. *American Journal of Medical Genetics*, *96*(5), 632–637.

Samson, H. H. (2000). The microstructure of ethanol drinking: genetic and behavioral factors in the control of drinking patterns. *Addiction (Abingdon, England), 95 Suppl 2*, S61–72.

Samson, H. H., Czachowski, C. L., & Slawecki, C. J. (2000). A new assessment of the ability of oral ethanol to function as a reinforcing stimulus. *Alcoholism, Clinical and Experimental Research*, *24*(6), 766–773.

Sandbak, T., Murison, R., Sarviharju, M., & Hyytiä, P. (1998). Defensive burying and stress gastric erosions in alcohol-preferring AA and alcohol-avoiding ANA rats. *Alcoholism, Clinical and Experimental Research*, *22*(9), 2050–2054.

Sauer, B. (1998). Inducible gene targeting in mice using the Cre/lox system. *Methods (San Diego, Calif.), 14*(4), 381–392. doi:10.1006/meth.1998.0593

Schramm-Sapyta, N. L., Morris, R. W., & Kuhn, C. M. (2006). Adolescent rats are protected from the conditioned aversive properties of cocaine and lithium chloride. *Pharmacology, Biochemistry, and Behavior*, *84*(2), 344–352. doi:10.1016/j.pbb.2006.05.026

Schroeder, J. P., Olive, F., Koenig, H., & Hodge, C. W. (2003). Intra-amygdala infusion of the NPY Y1 receptor antagonist BIBP 3226 attenuates operant ethanol self-administration. *Alcoholism, Clinical and Experimental Research*, *27*(12), 1884–1891. doi:10.1097/01.ALC.0000098875.95923.69

Schuckit, M. A. (1984). Subjective responses to alcohol in sons of alcoholics and control subjects. *Archives of General Psychiatry*, *41*(9), 879–884.

Schuckit, M. A., & Hesselbrock, V. (1994). Alcohol dependence and anxiety disorders: what is the relationship? *The American Journal of Psychiatry*, *151*(12), 1723–1734.

Schumann, G., Spanagel, R., & Mann, K. (2003). Candidate genes for alcohol dependence: animal studies. *Alcoholism, Clinical and Experimental Research*, *27*(5), 880–888. doi:10.1097/01.ALC.0000065437.18136.86

Silva, A. P., Lourenço, J., Xapelli, S., Ferreira, R., Kristiansen, H., Woldbye, D. P. D., … Malva, J. O. (2007). Protein kinase C activity blocks neuropeptide Y-mediated inhibition of glutamate release and contributes to excitability of the hippocampus in status epilepticus. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *21*(3), 671–681. doi:10.1096/fj.06-6163com

Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., & Bartlett, S. E. (2008). Intermittent access to 20% ethanol induces high ethanol

consumption in Long-Evans and Wistar rats. *Alcoholism, Clinical and Experimental Research*, *32*(10), 1816–1823. doi:10.1111/j.1530-0277.2008.00753.x

Sinclair, J. D., Kampov-Polevoy, A., Stewart, R., & Li, T.-K. (1992). Taste preferences in rat lines selected for low and high alcohol consumption. *Alcohol*, *9*(2), 155–160. doi:10.1016/0741-8329(92)90027-8

Skidmore, J. R., Murphy, J. G., & Martens, M. P. (2014). Behavioral economic measures of alcohol reward value as problem severity indicators in college students. *Experimental and Clinical Psychopharmacology*, *22*(3), 198–210. doi:10.1037/a0036490

Slatkin, M. (2008). Linkage disequilibrium--understanding the evolutionary past and mapping the medical future. *Nature Reviews. Genetics*, *9*(6), 477–485. doi:10.1038/nrg2361

Slawecki, C. J., Jiménez-Vasquez, P., Mathé, A. A., & Ehlers, C. L. (2005). Effect of ethanol on brain neuropeptides in adolescent and adult rats. *Journal of Studies on Alcohol*, *66*(1), 46–52.

Smith, G. P. (2001). John Davis and the meanings of licking. *Appetite*, *36*(1), 84–92. doi:10.1006/appe.2000.0371

Smith, J. P., & Randall, C. L. (2012). Anxiety and Alcohol Use Disorders. *Alcohol Research : Current Reviews*, *34*(4), 414–431.

Sora, I., Li, B., Igari, M., Hall, F. S., & Ikeda, K. (2010). Transgenic mice in the study of drug addiction and the effects of psychostimulant drugs. *Annals of the New York Academy of Sciences*, *1187*, 218–246. doi:10.1111/j.1749-6632.2009.05276.x

Sora, I., Wichems, C., Takahashi, N., Li, X.-F., Zeng, Z., Revay, R., … Uhl, G. R. (1998). Cocaine reward models: Conditioned place preference can be established in dopamine-

and in serotonin-transporter knockout mice. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(13), 7699–7704.

Sørensen, G., Wegener, G., Hasselstrøm, J., Hansen, T. V. O., Wörtwein, G., Fink-Jensen, A., & Woldbye, D. P. D. (2009). Neuropeptide Y infusion into the shell region of the rat nucleus accumbens increases extracellular levels of dopamine. *Neuroreport*, *20*(11), 1023–1026.

Spanagel, R., Montkowski, A., Allingham, K., Stöhr, T., Shoaib, M., Holsboer, F., & Landgraf, R. (1995). Anxiety: a potential predictor of vulnerability to the initiation of ethanol self-administration in rats. *Psychopharmacology*, *122*(4), 369–373.

Sparrow, A. M., Lowery-Gionta, E. G., Pleil, K. E., Li, C., Sprow, G. M., Cox, B. R., … Thiele, T. E. (2012). Central Neuropeptide Y Modulates Binge-Like Ethanol Drinking in C57BL/6J Mice via Y1 and Y2 Receptors. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*. doi:10.1038/npp.2011.327

Sparta, D. R., Fee, J. R., Hayes, D. M., Knapp, D. J., MacNeil, D. J., & Thiele, T. E. (2004). Peripheral and central administration of a selective neuropeptide Y Y1 receptor antagonist suppresses ethanol intake by C57BL/6J mice. *Alcoholism, Clinical and Experimental Research*, *28*(9), 1324–1330.

Sprow, G. M., & Thiele, T. E. (2012). The neurobiology of binge-like ethanol drinking: evidence from rodent models. *Physiology & Behavior*, *106*(3), 325–331. doi:10.1016/j.physbeh.2011.12.026

Steimer, T. (2011). Animal models of anxiety disorders in rats and mice: some conceptual issues. *Dialogues in Clinical Neuroscience*, *13*(4), 495–506.

Stephens, D. N., Duka, T., Crombag, H. S., Cunningham, C. L., Heilig, M., & Crabbe, J. C. (2010). Reward sensitivity: issues of measurement, and achieving consilience between human and animal phenotypes. *Addiction Biology*, *15*(2), 145–168. doi:10.1111/j.1369-1600.2009.00193.x

Stewart, R. B., Gatto, G. J., Lumeng, L., Li, T. K., & Murphy, J. M. (1993). Comparison of alcohol-preferring (P) and nonpreferring (NP) rats on tests of anxiety and for the anxiolytic effects of ethanol. *Alcohol (Fayetteville, N.Y.)*, *10*(1), 1–10.

Stewart, R., & Li, T. (1997). The neurobiology of alcoholism in genetically selected rat models. *Alcohol Health & Research World*, *21*(2), 169–179.

Stewart, S. H., & Chambers, L. (2000). Relationships between drinking motives and drinking restraint. *Addictive Behaviors*, *25*(2), 269–274.

Stewart, S. H., Zeitlin, S. B., & Samoluk, S. B. (1996). Examination of a three-dimensional drinking motives questionnaire in a young adult university student sample. *Behaviour Research and Therapy*, *34*(1), 61–71.

Tambs, K., Harris, J. R., & Magnus, P. (1997). Genetic and environmental contributions to the correlation between alcohol consumption and symptoms of anxiety and depression. Results from a bivariate analysis of Norwegian twin data. *Behavior Genetics*, *27*(3), 241–250.

Terlecki, M. A., Ecker, A. H., & Buckner, J. D. (2014). College drinking problems and social anxiety: The importance of drinking context. *Psychology of Addictive Behaviors: Journal of the Society of Psychologists in Addictive Behaviors*, *28*(2), 545–552. doi:10.1037/a0035770

Thiele, T. E., Marsh, D. J., Ste Marie, L., Bernstein, I. L., & Palmiter, R. D. (1998). Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature, 396*(6709), 366–369. doi:10.1038/24614

Thiele, T. E., Miura, G. I., Marsh, D. J., Bernstein, I. L., & Palmiter, R. D. (2000). Neurobiological responses to ethanol in mutant mice lacking neuropeptide Y or the Y5 receptor. *Pharmacology, Biochemistry, and Behavior*, *67*(4), 683–691.

Thiele, T. E., & Navarro, M. (2014). "Drinking in the dark" (DID) procedures: A model of binge-like ethanol drinking in non-dependent mice. *Alcohol*, *48*(3), 235–241. doi:10.1016/j.alcohol.2013.08.005

Thiele, T. E., Roitman, M. F., & Bernstein, I. L. (1996). c-Fos induction in rat brainstem in response to ethanol- and lithium chloride-induced conditioned taste aversions. *Alcoholism, Clinical and Experimental Research*, *20*(6), 1023–1028.

Thiele, T. E., Sparta, D. R., Hayes, D. M., & Fee, J. R. (2004). A role for neuropeptide Y in neurobiological responses to ethanol and drugs of abuse. *Neuropeptides*, *38*(4), 235–243. doi:10.1016/j.npep.2004.04.005

Toth, I., & Neumann, I. D. (2013). Animal models of social avoidance and social fear. *Cell and Tissue Research*, *354*(1), 107–118. doi:10.1007/s00441-013-1636-4

Treutlein, J., Cichon, S., Ridinger, M., Wodarz, N., Soyka, M., Zill, P., … Rietschel, M. (2009). Genome-wide association study of alcohol dependence. *Archives of General Psychiatry*, *66*(7), 773–784. http://doi.org/10.1001/archgenpsychiatry.2009.83

Verendeev, A., & Riley, A. L. (2013). The role of the aversive effects of drugs in self-administration: assessing the balance of reward and aversion in drug-taking behavior. *Behavioural Pharmacology*, *24*(5-6), 363–374. doi:10.1097/FBP.0b013e32836413d5

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet*, *13*(4), 227–232. doi:10.1038/nrg3185

Wahlsten, D. & Crabbe, J.C. (2003) Survey of motor activity, behavior, and forebrain morphometry in 21 inbred strains of mice across two laboratories. MPD: Wahlsten1, Mouse Phenome Database web site, Bar Harbor, Maine USA: The Jackson Laboratory. http://phenome.jax.org

Wahlsten, D., Rustay, N. R., Metten, P., & Crabbe, J. C. (2003). In search of a better mouse test. *Trends in Neurosciences*, *26*(3), 132–136. doi:10.1016/S0166-2236(03)00033-X

Weiss, J. M., West, C. H. K., Emery, M. S., Bonsall, R. W., Moore, J. P., & Boss-Williams, K. A. (2008). Rats selectively-bred for behavior related to affective disorders: proclivity for intake of alcohol and drugs of abuse, and measures of brain monoamines. *Biochemical Pharmacology*, *75*(1), 134–159. doi:10.1016/j.bcp.2007.09.027

Wheeler, J. M., Reed, C., Burkhart-Kasch, S., Li, N., Cunningham, C. L., Janowsky, A., … Phillips, T. J. (2009). Genetically correlated effects of selective breeding for high and low methamphetamine consumption. *Genes, Brain, and Behavior*, *8*(8), 758–771. doi:10.1111/j.1601-183X.2009.00522.x

Wiater, M. F., Mukherjee, S., Li, A.-J., Dinh, T. T., Rooney, E. M., Simasko, S. M., & Ritter, S. (2011). Circadian integration of sleep-wake and feeding requires NPY receptor-expressing neurons in the mediobasal hypothalamus. *American Journal of Physiology.*

Regulatory, Integrative and Comparative Physiology, *301*(5), R1569–1583. doi:10.1152/ajpregu.00168.2011

Wilcox, M. V., Carlson, V. C. C., Sherazee, N., Sprow, G. M., Bock, R., Thiele, T. E., … Alvarez, V. A. (2014). Repeated Binge-Like Ethanol Drinking Alters Ethanol Drinking Patterns and Depresses Striatal GABAergic Transmission. *Neuropsychopharmacology*, *39*(3), 579–594. doi:10.1038/npp.2013.230

Wilson, M. A., Burghardt, P. R., Ford, K. A., Wilkinson, M. B., & Primeaux, S. D. (2004). Anxiolytic effects of diazepam and ethanol in two behavioral models: comparison of males and females. *Pharmacology Biochemistry and Behavior*, *78*(3), 445–458. doi:10.1016/j.pbb.2004.04.017

Wolitzky-Taylor, K., Guillot, C. R., Pang, R. D., Kirkpatrick, M. G., Zvolensky, M. J., Buckner, J. D., & Leventhal, A. M. (2015). Examination of Anxiety Sensitivity and Distress Tolerance as Transdiagnostic Mechanisms Linking Multiple Anxiety Pathologies to Alcohol Use Problems in Adolescents. *Alcoholism, Clinical and Experimental Research*. doi:10.1111/acer.12638

Yoneyama, N., Crabbe, J. C., Ford, M. M., Murillo, A., & Finn, D. A. (2008). Voluntary ethanol consumption in 22 inbred mouse strains. *Alcohol (Fayetteville, N.Y.)*, *42*(3), 149–160. doi:10.1016/j.alcohol.2007.12.006

Zakhari, S., & Li, T.-K. (2007). Determinants of alcohol use and abuse: Impact of quantity and frequency patterns on liver disease. *Hepatology*, *46*(6), 2032–2039. doi:10.1002/hep.22010

Zhang, L., Wang, S., Crabbe, J.C., Harris, R.A., Mayfield, R.D., & Ponomarev, I. (2011) Gene expression profiles in High Drinking in the Dark mice and their control. *ACER, 35*(Issue supplement s1), 249, Abstract 0994

Zill, P., Preuss, U. W., Koller, G., Bondy, B., & Soyka, M. (2008). Analysis of single nucleotide polymorphisms and haplotypes in the neuropeptide Y gene: no evidence for association with alcoholism in a German population sample. *Alcoholism, Clinical and Experimental Research*, *32*(3), 430–434. doi:10.1111/j.1530-0277.2007.00586.x