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Characterization of ethanol-related phenotypic differences between C57BL/6J and C57BL/6NJ substrains: Role of Cyfip2?

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CHARACTERIZATION OF ETHANOL-RELATED PHENOTYPIC DIFFERENCES BETWEEN C57BL/6J AND C57BL/6NJ SUBSTRAINS: ROLE OF CYFIP2?

By

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An Abstract of the Thesis Presented
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The C57BL/6 (B6) mouse is the most commonly used inbred strain in biomedical research, and has served as the basis for various large-scale genetic and genomic projects. Although the parental substrain, C57BL/6J (B6J), originated at The Jackson Laboratory, isolated breeding colonies are now maintained at numerous sites. This separation has resulted in genetic drift that has led to the emergence of phenotypic differences among these colonies. For instance, B6J mice display higher levels of voluntary ethanol consumption and increased locomotor responses to psychostimulants, relative to C57BL/6N mice (B6N). Initial progress has been made in elucidating the genetic bases of these phenotypic differences, as Kumar et al. (2013) identified a single nucleotide polymorphism (SNP) in the Cytoplasmic FMR1-interacting protein 2 (*Cyfip2*) gene that underlies the differential locomotor response to cocaine exhibited by B6J and B6N mice. The present study compared voluntary ethanol consumption, binge-like drinking, and affective behavior during forced abstinence in B6J

and C57BL/6NJ (B6NJ) substrains, and when substrain differences were seen, in CRISPR-Cas9 engineered lines in which the previously identified substrain-specific *Cyfip2* SNP was exchanged within the same genetic backgrounds. Results showed that B6J consumed significantly greater quantities of ethanol than B6NJ mice and allelic variation in *Cyfip2* greatly contributed to observed substrain differences in two-bottle free-choice ethanol drinking; however, since these differences were completely reversed in males, but not females, it's likely that sex-specific contributions from other polymorphisms play a role in moderating these effects. Interestingly, there were no significant substrain differences in binge-like drinking or depressive- or anxiety-like behaviors during abstinence. Overall, while B6J and B6NJ mice displayed dramatic differences in voluntary ethanol consumption, which were dependent in part on an identified cocaine-relevant SNP in *Cyfip2*, no substrain differences were seen in binge-like drinking or affective behavior during abstinence. These results imply that primarily non-overlapping gene sets underlie these specific ethanol-related phenotypes.

DEDICATION

I dedicate this dissertation to my mother, the strongest person I've ever known.

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CHAPTER 1

INTRODUCTION

Ethanol is a common part of the human diet. Produced by the fermentation of sugars by yeast, ethanol is one of the oldest known psychoactive drugs. Although generally consumed in Western countries, the quantity and frequency of ethanol consumption varies greatly between populations and among individuals (Gilmore et al., 2016). For example, men typically consume more ethanol than women and individuals living in industrialized countries drink more than those in non-industrialized countries (Rehm et al., 2009). Unfortunately, ethanol misuse accounts for approximately 3.3 million deaths worldwide (World Health Organization, 2014), and, in the United States, excessive ethanol consumption is the third leading cause of death, accounting for roughly 88,000 deaths per year (Centers for Disease Control and Prevention, 2014).

By certain accounts, ethanol has greater overall harmful effects (i.e., harm to oneself, friends, family, and/or society) than any other drug, most notably including heroin, methamphetamine, and cocaine (Nutt, King, & Phillips, 2010). This is likely due, in part, to the complex pharmacological profile of ethanol, which primarily exerts opposing effects on the major excitatory and inhibitory neurotransmitter systems in the brain, glutamate and *gamma*-aminobutyric acid (GABA), respectively (Tabakoff & Hoffman, 2013; Vengeliene, Bilbao, Molander, & Spanagel, 2008). Since ethanol exhibits a general pattern of pharmacodynamic tolerance, as with other drugs of abuse, sustained high levels of intake can lead to dependence and consequent development of both pathophysiology and psychopathology.

There appears to be a substantial genetic contribution for development of Alcohol Use Disorder (AUD; American Psychological Association, 2013), as twin studies suggest that 50-60% of individual differences in risk are heritable (Enoch & Goldman, 2001). In turn, vulnerability to pathological drinking is likely mediated by complex interactions between genes and environment, as the degree of genetic influence on drinking outcomes appears to vary in different populations and under different environmental circumstances (Young-Wolff, Enoch, & Prescott, 2011). Thus, in order to gain a better understanding of the underlying factors involved, researchers have developed and employed 1) different genetic diversity approaches for gene discovery, and 2) animal models of various ethanol-related behaviors.

1.1. Genetic Diversity Approaches in Mice

1.1.1. Selectively-Bred Lines and the Development of Inbred Strains

The various genetically defined populations of laboratory mice currently available for scientific research were derived from animals supplied to early mouse geneticists by hobbyist breeders. Miss Abbie Lathrop provided a critical link between the mouse hobbyist community and early American mouse geneticists. Lathrop was a retired school teacher who began to breed mice in the early 1900s, and provided early geneticists – most notably, William Castle, Director of the Bussey Institute for Applied Biology of Harvard University – with a continual source of different mouse varieties for experimental purposes (Morse, 1978). Researchers were aware of the previous artificial selection pressures exerted on these mouse populations, as Chinese and Japanese historical records indicated that many individuals bred, collected, and traded mice based on unusual coat appearance and behavior (Sage, 1981). In turn, researchers

acknowledged the value of exploiting artificial selection principles to produce laboratory mice that exhibited specific disease-related phenotypes, an important methodology that is still widely utilized in current biomedical science.

Since there are noticeable differences in most phenotypes among genetically heterogenous mice, such a population can be screened for variability in a particular phenotype and separated into distinct breeding lines based on “high” or “low” phenotypic expression. Continued breeding of animals demonstrating the same extreme phenotype will eventually result in the emergence of two stable, bidirectionally selectively-bred lines. In feasible circumstances, replicate and control lines are also produced in order to yield greater assurance that the results of particular selective-breeding efforts are primarily due to gene frequency variations at trait-relevant loci rather than consequences of genetic drift (Crabbe, Phillips, & Belknap, 2010). Although many early geneticists were focused on studying disease states resulting from single gene mutations, it was increasingly realized that many phenotypes of biomedical significance are quantitative in nature, and are affected by multiple genes as well as environmental factors (Broman, 2001). Therefore, selectively-bred mouse lines were acknowledged as a powerful animal model since they allow for the possibility that multiple genes will contribute to the divergent phenotype and permit researchers to directionally manipulate the presence or absence of a particular trait so that specific aspects of a disease state can be achieved by design (Falconer & Mackay, 1996). Additionally, examining two bidirectional selectively-bred lines for a phenotype distinct from the selection phenotype can reveal whether overlapping gene sets contribute to

multiple traits. This approach is extremely valuable, as it enables discovery of genetically correlated traits (Crabbe, Phillips, Kosobud, & Belknap, 1990).

Arguably the most significant contribution of the Castle group at Harvard was the realization that a complementary approach to selective breeding, involving the development of isogenic lines of mice, termed inbred strains, was needed. Although the original rationale for their development was to help reveal the genetic basis for various forms of cancer (Morse, 1978), these inbred strains have played a vital role in all domains of biomedical research.

An inbred strain is established through at least 20 consecutive generations of sibling matings, at which point approximately 98.6% of all loci will be homozygous (Davisson, 1996). Indeed, many of today's common inbred strains have been inbred for over 150 generations, rendering these animals homozygous at essentially all loci, such that each individual animal within a particular strain can plausibly be treated as a clone (Beck et al., 2000). In fact, some of the inbred strain families most commonly used in mouse genetics today, including C57BL/6 (B6) and C57BL/10 (B10), are derived directly from the mice Lathrop sold to Castle (Morse, 1978).

The major strength of inbred strains lies in their genetic similarity, which produce greater reproducibility of experimental findings while also allowing the testing of fewer animals (compared to outbred animals) without sacrificing statistical power. In addition, if a considerable number of inbred strains are tested for multiple traits, strain means can be used to assess genetic correlation among traits. Although labor intensive, tests of genetic correlation become increasingly powerful the more inbred strains are included. However, since classical inbred strains are derived from a limited population (Lathrop

stocks), they are somewhat restricted in their power as a tool for discovering the underlying genetic variation modulating a complex trait (A. Roberts, Pardo-Manuel de Villena, Wang, McMillan, & Threadgill, 2007). Therefore, to expand the power of the approach, researchers have used inbred strains as the foundation for a wide variety of experimental crosses, heterogeneous and mutant stocks, and other research populations created for the sole purpose of examining the underlying genetic influences of complex traits (i.e., “mapping populations”; Chesler, 2014).

1.1.2. Quantitative Trait Loci Analysis and Traditional Experimental Crosses

Quantitative Trait Loci (QTL) analysis is a statistical method that links phenotypic data (trait measurements) and genotypic data (typically molecular markers) in an attempt to identify genetic loci (each of which likely encompasses many genes) that have significant effects on a phenotype of interest (Falconer & Mackay, 1996). Specifically, knowledge of the number, locations, effects, and identities of QTLs can lead to identification of specific genes (Zeng, 1994).

In order to perform a QTL analysis, researchers must first select two or more strains of organisms that differ genetically with regard to a trait of interest as well as genetic markers that distinguish between these parental strains. Ideally, several types of genetic markers are utilized, including single-nucleotide polymorphisms (SNPs), microsatellites, restriction fragment length polymorphisms, and transposable element positions (Vignal, Milan, SanCristobal, & Eggen, 2002). Additionally, for polygenic traits, parental strains are not required to differ in the phenotype of interest; more importantly, they must possess different alleles, which are then shuffled by recombination in the derived population to produce a range of phenotypic values.

The two most common breeding schemes used for QTL analysis are referred to as a backcross and an intercross (Silver, 1995). In both strategies, parental strains are first crossed to obtain the F1 generation, who receive one copy of each chromosome from each parent. Thus, F1 animals are isogenic and heterozygous at all loci that are polymorphic in the parental strains. In a backcross, the F1 generation is subsequently backcrossed to one of the parental strains to produce a segregating N2 population. In contrast, an intercross involves crossing pairs of F1 mice to obtain a F2 generation, such that the F2 progeny receive a recombinant chromosome from each parent. Therefore, at any autosomal locus, F2 progeny can possess either a “AA”, “AB”, or “BB” genotype. Thus, an intercross can detect a QTL regardless of which parental allele is dominant, while a backcross can only detect a QTL if the backcrossed parental allele is non-dominant (Kostrzewa & Kas, 2014). In either case, animals from all three generations are phenotyped to ensure a proper progression in phenotypic variation, as the F2 generation should show greater phenotypic variation due to their increased genetic variation (Broman, 2001).

1.1.3. Recombinant Inbred Strains, Collaborative Cross, and Diversity Outbred Mice

As aforementioned, siblings in the F2 generation are no longer identical because of the segregation of the respective alleles from the heterozygous F1 parents. For a single pair of homologs, each of the F2 animals will have a unique genotype with some loci homozygous for either the mother or father’s contributing allele, or heterozygous with both alleles. At this point, several pairs of F2 animals can be chosen at random to serve as the founders for a set of new “recombinant inbred” (RI) strains. Offspring from each F2 founder pair are then chosen randomly for sibling mating in order to produce

the next generation. This process is repeated for 20 consecutive generations and results in a set of new RI strains (Silver, 1995).

Interestingly, like classical inbred strains, RI strains are fixed to homozygosity at the vast majority of loci (Crabbe, Janowsky, Young, & Rigter, 1980). Therefore, the genomic composition of each RI strain can be maintained indefinitely by continued sibling mating. However, the “gene pool” of a set of RI strains, no matter how large the set, is also greatly restricted. Thus, since each RI strain is developed from only two inbred (and thus homozygous) parental strains, there are only two possible alleles (at most) that can be present at any given genetic locus (Broman, 2005). Further, since there is only a limited number of opportunities for recombination to occur between the two sets of progenitor chromosomes before inbreeding to homozygosity, complete homogenization of the genome is extremely unlikely (Winkler, Jensen, Cooper, Podlich, & Smith, 2003). Theoretically, particular genomic regions can already be fixed at the onset of the F2 crosses, and with each subsequent generation, additional genomic regions will become fixed in an allelic state reflecting contributions from the mother or father, respectively. After 20 consecutive generations of inbreeding, each RI strain will be represented by a group of animals that will all carry the same genomic components, but with random pieces from each of the two progenitors. Although paradoxical, the utility of RI strains lies in this homozygosity, as every RI strain has different recombination sites distributed randomly throughout its genome (Broman, 2005). A set of RI strains can be used to obtain information on linkage and map distances in a manner analogous to the F2 progeny from a traditional experimental cross (Crabbe et al., 1980). The major difference is the particular genotype present within each RI strain

can be indefinitely sustained, providing no limit to the number of loci that can be analyzed within an RI strain (Chesler et al., 2008).

Further advancements in molecular biology and computing have led to the emergence of systems genetics, a more recent perspective of genetic analysis aimed at better understanding how genes function and interact in the context of their respective biochemical pathways. In 2004, the Collaborative Cross (CC) was created to support systems genetics analysis. The CC population consisted of a thousand RI strains produced by an eight-way funnel breeding design involving a total of eight genetically diverse founder strains: five classical inbred strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HiLtJ) and three wild-derived strains (CAST/EiJ, PWK/PhJ, and WSB/EiJ) (Churchill et al., 2004). This specific breeding paradigm results in the genomes of each strain having equal contributions from each founder strain, and the accumulated recombinations being independent between strains (Chesler, 2014). Although similar in premise to existing RI strain panels, the CC is able to capture considerably more genetic diversity and provide sufficient power and resolution for genetic dissection of polygenic traits and construction of systems genetic networks. This is due to the inclusion of multiple progenitor strains, especially the utilization of wild-derived strains, in the CC, in contrast to RI strain panels that originate from only two inbred strains, resulting in limited statistical power and ability to capture allelic diversity (Chesler et al., 2008). Indeed, analysis of the allelic variation in inbred strains demonstrates that the eight founder strains of the CC capture approximately 90% of the known allelic diversity across all 1 Mb intervals spanning the entire mouse genome (A. Roberts et al., 2007). Overall, while the properties of the CC (high genetic diversity,

statistical power, and large number of independent recombinations) illustrate its potential as a systems genetics tool, continued efforts will determine whether the CC indeed confers a considerable improvement over the mapping resolution possible in traditional mouse crossing paradigms.

Although the CC has demonstrated significant success at producing more genetically diverse mouse populations, there will always be a degree of restricted allelic diversity since these heterogenous populations are still largely derived from classical inbred strains. This overall breeding history has led to a greatly limited range of behavioral variation, resulting in the introduction of false positive correlations among behavioral phenotypes, as parallel effects of genetic linkage are mistaken for pleiotropic actions of the same polymorphisms (Chesler, 2014). Therefore, in 2009, a complementary approach to the CC, termed the Diversity Outbred (DO), was initiated. This newer method was developed to employ a novel outbreeding strategy that maintains a balanced mixture of the founder genomes and avoids allelic loss and inbreeding (Churchill, Gatti, Munger, & Svenson, 2012; Svenson et al., 2012). The DO population is derived from CC mice at various stages of inbreeding, intercrossed in a semi-randomized manner. In this intercross strategy, sibling or first cousin mating is specifically avoided; thus, recombinations are randomized and founder haplotypes are distributed throughout the population (Chesler, 2014). This is the innovative aspect of the DO, as each individual mouse is genetically unique and possesses a high level of allelic heterozygosity. While the CC strains are being inbred to produce stable clones, the DO are strictly being maintained as an outbred stock; thus, the DO population effectively provides an unlimited source of novel allelic combinations, analogous to

human genetics (Bogue, Churchill, & Chesler, 2015). However, the drawback of the DO is that the genetically unique individual genomes are not reproducible, as in the CC. Nevertheless, combinations of genetic loci that are discovered in the DO mice can be replicated in CC strains (Gatti et al., 2014). To this end, tools like the CC and DO should allow researchers to better resolve QTL intervals and identify underlying genes for a diverse set of phenotypes (Logan et al., 2013).

1.1.4. Inbred Substrains

Following the emergence of inbred mouse strains, researchers from many biological disciplines were eager to integrate these animal models into their research. Demand for such mice increased so dramatically that some research institutions realized the financial prospect of maintaining inbred mouse strains for sale to the greater scientific community. Among the first to capitalize on this opportunity was The Jackson Laboratory (JAX), founded by C.C. Little in 1929, which began sales of inbred mice in 1933 (Morse, 1978). Little had started deriving various inbred strains at Harvard by 1909, with one of more popular inbred strains, C57BL/6 (B6), having been derived in 1921. To distinguish the B6 colony specific to JAX, the additional nomenclature of “J”, for JAX, was added to indicate the colony maintenance site. This seemingly insignificant nomenclature change would prove to be an important identifier in the future, as separate breeding colonies of the same inbred strains began to be maintained at several different locations, leading to the eventual unplanned emergence of numerous B6 substrains (Bryant, 2011).

In 1951, JAX sent C57BL/6J (B6J) mice to the National Institutes of Health (NIH), which they used to establish their own colony. However, colony separation leads to

genetic divergence over time, as genetic drift will occur. Therefore, every time a new colony is maintained separately from an existing colony for 20 or more generations, it can become a genotypically distinct new substrain (Bailey, 1982). In turn, the B6J mouse colony at NIH subsequently became known as C57BL/6N (B6N) mice, formally recognizing B6J and B6N as distinct substrains.

To date, B6J and B6N mice have been inbred separately for over 5 decades and 220 generations (Mulligan et al., 2008; Simon et al., 2013). Further, over time, separate colonies of B6N have been established at several other breeding sites – Charles River Laboratories in 1974 (C57BL/6NCrl; B6NCrl), Taconic Biosciences in 1991 (C57BL/6NTac; B6NTac), and back to JAX in 2005 (C57BL/6NJ; B6NJ). Thus, it is not surprising that significant genotypic and phenotypic differences have been observed among the various B6 substrains, with most work specifically comparing B6J (the parental substrain) to B6N and its associated derivatives (B6NCrl, B6NTac, B6NJ) (Bryant et al., 2008; Matsuo et al., 2010; Mekada et al., 2009). Notably, different B6 substrains have been used in various large-scale genomics initiatives. While B6J mice were used in the original Mouse Genome Sequencing Consortium (Church et al., 2009; Waterston et al., 2002), B6N mice are being used in the various projects associated with the International Knockout Mouse Consortium (Austin et al., 2004; Pettitt et al., 2009; Skarnes et al., 2011). It is critical, therefore, that researchers take specific notice of potential differences between B6 substrains when planning experiments and/or comparing data across studies. On the positive side, however, the over 20 commercially available B6 substrains provide a potentially powerful genetic resource for identifying previously unknown genes and alleles controlling complex traits (Kumar et al., 2013).

1.1.5. Summary

As discussed, mouse geneticists have developed a spectrum of potential mapping populations with the purpose of elucidating the genetic basis of quantitative traits, including complex behaviors thought to model aspects of disease states. The major benefits of utilizing a genetically complex population such as the CC and DO are that (1) any positive findings have a relatively high likelihood of being generalizable, (2) genetically heterogenous mice typically exhibit high phenotypic variation, and (3) QTLs may be mapped with comparatively high resolution. On the other hand, this approach is likely to identify multiple significant QTLs, each with small effect sizes, making it difficult to identify specific influential variants within these QTL intervals. In contrast, while the use of genetically simpler populations, such as traditional F2 crosses of inbred strains, RI strains, and/or distinct inbred substrains, provides limited generalizability of findings and more restricted phenotypic variation, this approach increases the probability of finding a smaller number of significant QTLs, each with comparatively larger effect sizes, and of identifying specific influential variants within a given QTL interval. Although efforts in gene discovery and functional identification have progressed rapidly during the past 30 years, approximately 70% of current neuroscience literature concerns approximately 5% of known genes (Pandey, Lu, Wang, Homayouni, & Williams, 2014). Thus, appropriate integration of results derived from different genetic diversity approaches will be essential to avoiding stagnation in the field of neurogenetics.

1.2. Animal Genetic Models of Ethanol-Related Behaviors

AUD is a complex, chronic disorder characterized by a collection of clinically-defined behaviors that are influenced by numerous genetic and environmental factors. The core symptoms of AUD include the presence of both metabolic and functional tolerance to ethanol, uncontrollable compulsions to seek and consume ethanol, emergence of a negative affective state (e.g. irritability, anxiety, depression, etc.) that defines a withdrawal syndrome when ethanol consumption is terminated, and intense cravings to consume ethanol during abstinence (Mason, 2017). Although creation of a single animal model that encapsulates each of these defining criteria would be ideal, the inherent complexity of this behavioral profile renders this task extremely unlikely. For instance, a prototypical animal model of AUD would exhibit excessive voluntary ethanol intake that 1) is based on positive reinforcement of ethanol's pharmacological effects, rather than its caloric value or preference for sweet taste (Bachmanov et al., 2003; Spanagel, 2000), 2) leads to patterns of uncontrollable consumption (H. C. Becker & Ron, 2014), 3) produces pharmacologically significant levels of blood ethanol concentrations (BECs) which result in metabolic and functional tolerance (Kalant, LeBlanc, & Gibbins, 1971), and 4) results in the emergence of negative affective state (Heilig, Egli, Crabbe, & Becker, 2010; Kliethermes, 2005) and craving behaviors with periods of relapse during abstinence due to negative reinforcement (Rodd, Bell, Sable, Murphy, & McBride, 2004). Thus, researchers have attempted to develop protocols that aim to model a specific component(s) of AUD while conserving face and predictive validity.

1.2.1. Two-Bottle Free-Choice Drinking

Ethanol intake is commonly assessed in rodents by providing them with continuous access to an ethanol solution and plain water over the course of several days or even weeks. This paradigm, termed two-bottle free-choice or "ethanol preference drinking", provides a measure of voluntary oral ethanol consumption, as the animal is not forced to drink from the ethanol bottle to alleviate hunger (food is available *ad libitum*) or thirst (Leeman et al., 2010; Tabakoff & Hoffman, 2000). However, researchers often question whether the display of substantial ethanol intake in this paradigm is necessarily reflective of significant ethanol-reinforced behavior (A. S. Green & Grahame, 2008). For instance, even seemingly insignificant factors, such as position and total number of solutions available, have been shown to notably influence results. Previous work has demonstrated that rodents typically consume more fluid from bottles located closer to the food hopper (Bachmanov, Reed, Beauchamp, & Tordoff, 2002), while simultaneous access to additional concentrations of ethanol solution drastically increases overall ethanol intake (Tordoff & Bachmanov, 2003). Furthermore, the total number of access days or weeks plays an important role in overall ethanol preference, as "ethanol-avoiding" rodents generally exhibit decreased preference across time, whereas "ethanol-preferring" rodents, for the most part, show increased preference (Blizard, Vandenbergh, Lionikas, & McClearn, 2008). Sensory modalities, such as taste and olfaction, also play an important role in the oral consumption of ethanol (Bachmanov et al., 2003). Like humans, rodents can detect the sweet and bitter properties (Kiefer & Lawrence, 1988; Kiefer & Mahadevan, 1993) and odor volatiles (Kiefer & Morrow, 1991) of ethanol. A positive correlation between ethanol and sweet

taste has been characterized extensively in mice (Bachmanov, Reed, Tordoff, Price, & Beauchamp, 1996; Blednov et al., 2008), rats (Dess, Badia-Elder, Thiele, Kiefer, & Blizard, 1998; Gosnell & Krahn, 1992), and even human alcohol-dependent patients (Kampov-Polevoy, Garbutt, & Janowsky, 1999; 1997).

Many inbred strains of mice avoid a 10% ethanol solution (i.e., exhibit an ethanol preference of less than 50%) unless it is masked by a sweetening agent, such as sucrose or saccharin (Belknap, Crabbe, & Young, 1993). Such findings imply that, during continuous two-bottle free-choice access, animals who demonstrate low voluntary oral ethanol intake likely never achieve "pharmacologically relevant" blood ethanol levels sufficient to experience the positive reinforcement properties of ethanol. A previous analysis found that the degree of ethanol preference exhibited under continuous two-bottle free-choice conditions corresponded with the relative strength of the reinforcing effects of ethanol as measured by operant oral self-administration (OSA) and conditioned taste aversion (CTA), but not conditioned place preference (CPP) procedures (A. S. Green & Grahame, 2008). Interestingly, Grahame & Cunningham (1997) examined operant intravenous self-administration (IVSA) of ethanol in an attempt to circumvent the requirement that the animal drinks an ethanol solution to experience its postingestive effects. In other words, through IVSA, the reinforcing effects of ethanol depend solely on its postingestive (i.e., pharmacological effects), rather than preingestive (i.e., taste and smell), consequences. Although this approach is most commonly utilized to examine the reinforcing properties of psychostimulant drugs, such as cocaine (J. E. Smith et al., 2004), it perhaps has the potential to resolve previous interpretative complications associated with voluntary oral consumption. For example,

while DBA/2J (D2J) mice voluntarily orally consume such minute amounts of ethanol that they likely never encounter its pharmacological effects (Belknap et al., 1993; Belknap, Coleman, & Foster, 1978), they intravenously self-administer ethanol at a rate similar to other inbred strains of mice that consistently show far greater ethanol intake during two-bottle free-choice conditions (Grahame & Cunningham, 1997). These data suggest that, unlike findings using OSA and CTA, IVSA (and CPP) appear to not be related to continuous two-bottle free-choice drinking (A. S. Green & Grahame, 2008). Nevertheless, additional IVSA studies are needed in order to draw more concrete relationships among these differing paradigms of voluntary consumption.

While much has been learned from the study of two-bottle free-choice ethanol drinking, animals tested in this protocol rarely, if ever, generate high enough intake to produce sustained intoxication or the induction of dependence-related phenomena. Thus, two-bottle free-choice ethanol drinking is probably best viewed as a model of non-pathological ("controlled") ethanol intake, influenced by a variety of factors including ethanol reward, taste and smell, caloric value, and genetic background.

Due to its high-throughput nature, there is accumulating gene expression data on ethanol preference drinking (Crabbe et al., 2010; McBride et al., 2010; Mulligan et al., 2006; Tabakoff et al., 2009); however, such studies will not be mentioned in detail for several reasons. First, since most behavioral traits are influenced by many genes, an individual gene likely exerts a minor overall effect on the trait. Likewise, ''ethanol preference drinking'' does not appear to be mediated by a single neurotransmitter system nor result from changes in a single, isolated brain structure or neural circuit. Second, to this end, the isolated use of advanced techniques available for single gene

manipulation (e.g., knockouts, transgenics, etc.) are unable to reveal potential significant gene sets for a trait of interest. Nonetheless, about 100 genes have been targeted, a quarter of which have been tested for their respective influence on ethanol preference drinking. The current data indicates that approximately a third of these genes appear to produce either a modest increase, decrease, or no significant effect in ethanol preference drinking (Crabbe et al., 2010; Crabbe, Phillips, Harris, Arends, & Koob, 2006).

On the other hand, the two-bottle free-choice paradigm is the most commonly assayed ethanol-related phenotype in genetic discovery efforts. Most notably, there have been a plethora of rat and mouse lines selectively-bred for either high or low ethanol preference (usually a 10% v/v solution) relative to plain water under continuous two-bottle free-choice access. The four most prominent bidirectional selectively-bred rat lines include: 1) the “Alko Alcohol” (AA) and “Alko Nonalcohol” (ANA) lines (Eriksson, 1968), 2) the “alcohol-preferring” (P) and “nonalcohol preferring” (NP) lines (McBride, Rodd, Bell, Lumeng, & Li, 2014), 3) the “high alcohol drinking” (HAD) and “low alcohol drinking” (LAD) lines (McBride et al., 2014), and 4) the “Sardinian alcohol preferring” (sP) and “Sardinian non-alcohol preferring” (sNP) lines (Colombo et al., 1997). Each of these ethanol-preferring lines voluntarily consume greater than 5 g/kg/day (Colombo et al., 1997; McKinzie et al., 1998; Murphy et al., 2002; Ritz, George, deFiebre, & Meisch, 1986), which is roughly equivalent to 9-14 standard alcoholic drinks per day (Murphy et al., 2002; Rodd et al., 2004). Mice have also been selectively-bred for either high or low ethanol preference drinking, with the most popular divergent lines termed “high alcohol preferring” (HAP) and “low alcohol preferring” (LAP) (Grahame, Li, & Lumeng, 1999).

Interestingly, daily ethanol intake levels of HAP lines were surprisingly modest, in contrast to the aforementioned selected rat lines. Therefore, researchers intercrossed two of the high drinking HAP lines in order to produce a “crossed HAP” (cHAP) line (Oberlin, Best, Matson, Henderson, & Grahame, 2011), which consumed significantly greater quantities of ethanol and achieved blood ethanol levels near 200 mg/dL (Matson & Grahame, 2013).

Variations in two-bottle free-choice ethanol drinking among inbred strains have consistently been established through inbred strain panel studies (McClearn & Rodgers, 1959; Belknap et al., 1993; Yoneyama, Crabbe, Ford, Murillo, & Finn, 2008). In the most recent investigation, Yoneyama et al. (2008) examined 22 inbred strains under continuous two-bottle free-choice access to varying ethanol concentrations presented in a serial manner. Consistent with previous panel studies, B6J mice exhibited among the highest daily ethanol consumption (>10 g/kg/day) while D2J mice consumed among the lowest (<2 g/kg/day). In fact, these strain differences have remained extraordinarily stable over time and across laboratories, as analysis of these inbred panel studies revealed strain mean correlations between .74 and .98 (Crabbe, 2014; Wahlsten, Bachmanov, Finn, & Crabbe, 2006).

Moreover, B6J and D2J inbred strains have become the foundation for numerous QTL mapping efforts for two-bottle free-choice ethanol drinking. An influential meta-analysis of experimental crosses involving B6J and D2J mice suggested the presence of significant QTLs on chromosomes 2, 3, 4, and 9 (Belknap & Atkins, 2001). Subsequently, additional supporting evidence emerged and verified the likelihood of QTLs specifically on chromosomes 2 (Fehr et al., 2005) and 9 (Hitzemann et al., 2004).

As QTL confirmation efforts are ongoing, this evidence cannot be interpreted as definitive; but, notable potential gene candidates include syntaxin binding protein 1 (*Stxbp1*) on chromosome 2 and sodium channel, type IV, (*Scn4b*) on chromosome 9 (Crabbe, 2014).

Of particular interest to this dissertation, B6 substrains have been found to display consistent differences in ethanol preference drinking (Blum, Briggs, DeLallo, Elston, & Ochoa, 1982; Mulligan et al., 2008; Poley, 1972; Ramachandra, Phuc, Franco, & Gonzales, 2007; Whitney & Horowitz, 1978). Specifically, B6J mice have reliably exhibited higher voluntary ethanol consumption and preference compared to several B6N derivatives; however, B6J and B6NJ mice have not been compared previously. To this end, Experiment 1 of this dissertation examined voluntary ethanol consumption and preference in B6J and B6NJ mice as well as in CRISPR-engineered mice designed to identify specific genetic contributions to these substrain differences.

1.2.2. Alcohol Deprivation Effect

Since continuous free-choice ethanol access does not produce “excessive” intake, researchers sought to develop a protocol to initiate binge-like drinking in rodents. Early observations found that introducing a period of forced ethanol deprivation could result in a subsequent transient increase in voluntary intake (Crabbe, Spence, Brown, & Metten, 2011). Specifically, rats with a significant prior history of continuous free-choice ethanol drinking exhibit a brief, notable increase in ethanol intake following one week of deprivation (Sinclair & Senter, 1967; 1968). This phenomenon, now termed the “alcohol deprivation effect” (ADE), has since been further characterized in rats (Henniger, Spanagel, Wigger, Landgraf, & Hölter, 2002; Rodd-Henricks et al., 2001; 2000; Serra et

al., 2003; Spanagel & Hölter, 1999; Vengeliene et al., 2003), mice (Ford et al., 2011; Khisti, Wolstenholme, Shelton, & Miles, 2006; Melendez, Middaugh, & Kalivas, 2006; Salimov, Salimova, Shvets, & Maisky, 2000), and even non-human primates (Sinclair, 1972; Kornet et al., 1990). Additionally, the ADE has been observed using limited-access OSA procedures in both rats and mice (Heyser, Schulteis, & Koob, 1997; Hölter, Landgraf, Zieglgänsberger, & Spanagel, 1997; Sparta et al., 2009), suggesting that the ADE represents “motivated” ethanol consumption.

Various factors appear to modulate the ADE, including the number of ethanol solutions available and both the duration of initial ethanol access and the deprivation interval (Breese et al., 2005; Crabbe et al., 2011). Indeed, manifestation of the ADE is more robust and enduring when rats are given concomitant access to two additional ethanol solutions along with water (Rodd-Henricks et al., 2001). Moreover, the minimum initial ethanol access period necessary for subsequent emergence of the ADE is about three weeks, though most studies typically use at least six weeks (Rodd et al., 2004; Vengeliene, Bilbao, & Spanagel, 2014). In terms of necessary deprivation duration, the ADE may be observed following relatively short periods (hours to days; Spanagel & Hölter, 1999), or longer periods (weeks to months; Hölter et al., 1997). Nonetheless, the observed increase in ethanol intake after either short or long periods of deprivation is typically brief, with intake normally returning to pre-deprivation levels within 48 hours.

Interestingly, the ADE may be amplified, in terms of both magnitude and duration, by repeated cycles of intermittent ethanol access (Rodd et al., 2004; 2003; Spanagel, 2000). This modified paradigm, termed the “repeated alcohol deprivation effect” (RADE), is especially thought to be a valid animal model of pathological drinking

since it mimics the cyclical nature of abstinence and relapse periods experienced by most alcohol-dependent individuals, a pattern that likely plays a vital role in the enduring maintenance of excessive drinking (Sanchis-Segura & Spanagel, 2006).

Another popular conceptual variant of the ADE is the “every-other-day” (EOD) protocol, where intervals of continuous ethanol access and deprivation are alternated every 24-hours. Although this model was introduced shortly after characterization of the ADE (Wise, 1973), and is similar to the RADE in principle, the EOD involves more rapid cycling between drinking and non-drinking days. The EOD procedure has been shown to accelerate the rate at which both rats (Wise, 1973; Carnicella, Amamoto, & Ron, 2009; Hargreaves, Monds, Gunasekaran, Dawson, & McGregor, 2009; Loi et al., 2010; Simms et al., 2008) and mice (Hwa et al., 2011; Melendez, 2011; Rosenwasser, Fixaris, Crabbe, Brooks, & Ascheid, 2013) exhibit excessive levels of ethanol consumption. This work has provided support that exposure to repeated periods of ethanol deprivation is likely a crucial factor underlying elevated ethanol intake, as some animals voluntarily consume sufficient ethanol to achieve mildly intoxicating BECs and display overt behavioral signs of dependence (Hölter, Linthorst, Reul, & Spanagel, 2000; Hwa et al., 2011; Simms et al., 2008). Nevertheless, inconsistent emergence of the ADE, RADE, or EOD effects in studies involving mice, as opposed to rats, has cast doubt on the validity of this paradigm (Vengeliene et al., 2014), as, unlike in rats, both single or multiple deprivation periods have not consistently resulted in subsequent amplified ethanol intake in mice (Khisti et al., 2006; Melendez et al., 2006).

In contrast to high-throughput ethanol-related phenotypes, such as two-bottle free-choice drinking, examination of any ADE variant requires substantial time and

resources to execute. Therefore, unsurprisingly, there have been a lack of genetic diversity approaches implemented towards elucidation of the genetic basis of ADE variants. Most mouse studies on intermittent access have exclusively involved B6J mice (Hwa et al., 2011; Melendez, 2011; Melendez et al., 2006; Sparta et al., 2009), though a few studies have examined more diverse genotypes, including genetically heterogenous populations and lines selected for various ethanol-related phenotypes (Ford et al., 2011; Rosenwasser et al., 2013). For example, Rosenwasser et al. (2013) characterized the effects of intermittent ethanol access in mouse genotypes previously categorized by divergent ethanol-related phenotypes for two-bottle free-choice ethanol drinking, binge-like ethanol drinking, and handling-induced convulsion severity. Interestingly, all tested genotypes exhibited escalated ethanol intake in response to intermittent ethanol access, with little relevant difference between genotypes. These data suggest there are no genetic correlations between the effects of intermittent access and either two-bottle free-choice drinking, scheduled access binge-like drinking, or severity of handling-induced convulsions.

However, of particular interest to this dissertation, B6 substrains have been found to display differences in expression of the ADE (Khisti et al., 2006). Although baseline ethanol intake for B6NCrl mice were considerably lower than B6J mice, they consistently demonstrated a robust ADE. Surprisingly, ethanol deprivation in B6J mice did not produce significant increases in ethanol consumption or preference. Despite the possibility of a “ceiling effect” with regards to B6J mice in this study, these results suggest that B6NCrl mice may serve as a valuable tool for future analyses on the molecular mechanisms of craving and drinking behavior. Nonetheless, future work

involving greater utilization of genetic diversity approaches, such as selectively-bred lines, RI strains, CC and DO mice, and perhaps different inbred substrains (e.g., B6NJ), would likely reveal a more complete picture of the specific genes driving expression of the ADE.

1.2.3. Drinking-in-the-Dark

Although ADE protocols (and slight variants thereof) have been successful at initiating brief escalations in voluntary ethanol intake, more recent development have explicitly focused on producing pharmacologically relevant BECs over a defined period of time. Specifically, the goal of the "Drinking-in-the-Dark" (DID) paradigm is to provide a valid animal model of non-dependent binge-like drinking that reliably results in BECs above 80 mg/dL, thereby satisfying the clinical criteria for a binge put forth by the National Institute of Alcoholism and Alcohol Abuse (NIAAA) (Thiele & Navarro, 2014).

Since rodents are nocturnal, their levels of food and fluid consumption, as well as general activity, are highest during their dark phase (Dole & Gentry, 1984); thus, providing scheduled access to ethanol during the dark phase of their circadian cycle has been shown to facilitate greater ethanol consumption (Ryabinin, Galvan-Rosas, Bachtell, & Risinger, 2003). In this model, mice are maintained under a standard light-dark (LD) cycle and given a 2-hour access period to a single bottle containing 20% ethanol, beginning three hours into their dark phase (Rhodes, Best, Belknap, Finn, & Crabbe, 2005; Rhodes et al., 2007). This procedure is followed for three consecutive days, while on the fourth day, the access period is extended an additional two hours (food is available *ad libitum*). This model was specifically developed with B6J mice, who, on the final day of the 4-day DID procedure, showed significant ethanol

consumption that produced BECs upwards of 100 mg/dL (Rhodes et al., 2005). Additionally, following a 4-day DID procedure, B6J mice exhibited observable signs of intoxication, such as impaired motor coordination (Rhodes et al., 2007).

While systematically developing the DID paradigm, Rhodes et al. (2005) noticed a pronounced influence of the length of time into the dark phase ethanol access was allowed and subsequent ethanol intake. Mice drank significantly more ethanol and achieved higher BECs when ethanol was offered three hours into the dark phase, relative to either one or two hours into the dark phase. These results infer the success of the DID procedure is strongly dependent on the time within the animal's circadian rhythm at which ethanol access occurs. Additionally, postponing ethanol access until four hours into the dark phase did not significantly change ethanol intake levels or BECs, compared to offering ethanol access three hours into the dark phase. Interestingly, Rhodes et al. (2005) also tested whether 10%, 20%, or 30% ethanol produced optimal intake levels in the DID, but found no significant differences and chose to use 20% for subsequent experiments. Further experimentation with the DID paradigm incorporated a two-bottle choice, allowing water access simultaneously with ethanol access, and noted moderate reductions in ethanol intake and resulting BECs, as would be expected (Rhodes et al., 2007).

Of particular importance is that the high levels of intake produced by the DID procedure appear to be unrelated to motivation for obtaining calories contained in ethanol (Lyons, Lowery, Sparta, & Thiele, 2008). Moreover, while extending the initial 3 days of 2-hour access periods to 14 days produced quicker rates of consumption and tolerance to the ataxic effects of ethanol (Linsenbardt, Moore, Griffin, Gigante, &

Boehm, 2011), repeated DID episodes (up to 10, 4-day DID cycles) did not promote significant differences in the level of binge-like ethanol drinking (Cox et al., 2013). Overall, the DID procedure possesses significant face validity in terms of modeling human binge drinking since mice exhibit binge-like drinking under conditions of short-term oral ethanol consumption that results in BECs exceeding the threshold of intoxication. Its high-throughput approach has proven useful for screening pharmacological targets that are effective in the mitigation of non-dependent binge-like drinking (Sprow & Thiele, 2012; Thiele & Navarro, 2014).

Although the DID procedure was developed using B6J mice, researchers have since evaluated potential strain differences (Crabbe et al., 2012; Rhodes et al., 2007). For example, Rhodes et al. (2007) administered the DID procedure to a panel of 12 standard inbred strains and found that B6J mice exhibited the highest levels of ethanol intake and consequent BECs, while D2J mice demonstrated the lowest, a pattern which correlates to a significant degree with two-bottle free-choice drinking from inbred strain panel studies (Crabbe et al., 2012). In addition to the observed strain effects for both ethanol intake and resultant BECs, high-drinking and low-drinking strains exhibited differential patterns of consumption across days. The four high-drinking strains (B6J, BALB/cJ, BALB/cByJ and FVB/NJ), as well as one intermediate-drinking strain (C3H/HeJ), consumed a steady amount of ethanol during the two hours of each day's access period, whereas two low-drinking strains (129S1/SvlmJ and D2J) and three intermediate-drinking strains (CBA/J, BTBR and A/J) drank significantly less ethanol on Days 2-4 than Day 1. When specifically examining Day 4 data, strain differences explained 53% and 51% of the variance in ethanol intake and resultant BECs,

respectively. B6J mice had a significant influence on these effect sizes, since removing B6J animals from the analysis lowered the variance explained by strain to 36% for ethanol intake and 21% for BECs. These data imply that pharmacologically significant drinking, in B6J mice, is under substantial genetic control and that B6J mice are genetically predisposed to high ethanol consumption relative to other inbred strains.

More recently, mice have been selectively-bred for high BECs produced by a 2-day variation of the DID procedure (i.e., access to 20% ethanol for two hours on Day 1 and four hours on Day 2). Two replicate lines, termed “high-drinking-in-the-dark” (HDID) were originally selected from a genetically heterogeneous stock (HS/Npt) and, after 11 generations of selective breeding, approximately half of HDID mice exhibited BECs above 100 mg/dL after undergoing the modified 2-day DID procedure (Crabbe et al., 2009). Although realized heritability of this trait is relatively low (h^2 = .09) in both replicate HDID lines, subsequent selection has successfully increased BECs across generations. After 27 selected generations of HDID-1 mice, and 19 selected generations of HDID-2 mice, BECs had roughly increased 4-5-fold since initial selection (Crabbe et al., 2014). Notably, the high BECs of HDID mice, from either replicate line, is not due to potential differences in ethanol metabolism, as the rate of elimination of an acute ethanol injection did not differ between HDID lines and genetically heterogeneous (HS/Npt) mice (Crabbe et al., 2009; 2014).

Although inbred strain panels and selective breeding approaches have already been implemented for discovering genetic contributions of binge-like drinking, further work involving RI strains, CC and DO mice, and inbred substrains would greatly benefit these ongoing efforts. In turn, Experiment 2 of this dissertation compared B6J and B6NJ

substrains on binge-like drinking via a 4-day DID procedure in an effort to not only observe potential substrain differences, but to examine their possible correlation with voluntary ethanol consumption and preference under two-bottle free-choice conditions (Experiment 1).

1.2.4. Induction of Ethanol Dependence

Since rodents generally do not willingly consume a sufficient volume of ethanol to produce sustained, pharmacologically-relevant BECs, forced ethanol delivery methods have been developed to induce experimental ethanol dependence in rodents (H. C. Becker & Ron, 2014; Holleran & Winder, 2017; Lovinger & Crabbe, 2005). The most commonly utilized forced ethanol protocols are intragastric ethanol infusion, maintenance on an ethanol liquid diet, and forced inhalation of volatilized ethanol (H. C. Becker, 2000; H. C. Becker & Ron, 2014).

Intragastric infusion involves direct delivery of ethanol through a tube surgically implanted into the animal's stomach. Although this method provides experimenter control over various ethanol exposure parameters (i.e., initiation, duration, and termination), it has several disadvantages, such as degree of invasiveness, requirement of surgery, restricted number of animals that can be tested simultaneously, and the increased likelihood of inflicting unnecessary stress on the animal (Tabakoff & Hoffman, 2000). Another method, maintenance on an ethanol liquid diet, was developed in an attempt to model clinically-relevant ethanol intake while maintaining dietary control (Lieber & DeCarli, 1982). In this procedure, animals are offered a single bottle containing a liquid diet that is formulated to meet or exceed minimum nutritional needs. After a period of acclimation to the liquid diet, ethanol is introduced within the liquid diet,

and animals are essentially forced to consume ethanol in order to meet their caloric needs (Lieber & DeCarli, 1989). In contrast to other forced administration methods, liquid diet relies heavily on the animal's consummatory behavior and is inherently more time-consuming to implement. Overall, both intragastric infusion and ethanol liquid diets have been most commonly utilized to reproduce and study several pathophysiological complications typically present in alcohol-dependent individuals, such as ethanol-induced hyperlipemia and cirrhosis (Lieber, DeCarli, & Sorrell, 1989).

Forced inhalation of volatilized ethanol vapor has become the preferred method of ethanol dependence induction, as it encapsulates most of the advantages and mitigates many disadvantages of aforementioned methods. In particular, ethanol vapor exposure allows 1) physiological dependence to be established in a comparatively minimal period of time, 2) animals to remain in reasonably stable health due to minimization of body weight and body temperature loss during intoxication, even when undergoing multiple cycles of intoxication/abstinence, 3) control over the initiation, duration, and termination of ethanol exposure, and 4) modulation of ethanol dosage such that subsequent BECs are consistently maintained between 150-200 mg/dL (Becker, 2013). However, ethanol vapor exposure is not a perfect model, as it completely bypasses the active consummatory behavioral component that is fundamental to addiction. Despite this deficiency in face validity, it has proven to be a useful tool for the investigation of various withdrawal-related phenomena, such as central nervous system (CNS) hyperexcitability, dependence-induced escalation of voluntary ethanol drinking, and anxiety- and/or depressive-like behavior during long-term abstinence (H. C. Becker & Ron, 2014; Heilig et al., 2010). In numerous instances,

chronic ethanol vapor exposure, but not intragastric infusion, has been shown to result in increased voluntary drinking during withdrawal (W. C. Griffin, Lopez, Yanke, Middaugh, & Becker, 2009b).

Chronic ethanol vapor exposure can be delivered in either a continuous (Goldstein & Pal, 1971; Metten & Crabbe, 2005) or intermittent (Lopez & Becker, 2005; Metten, Sorensen, Cameron, Yu, & Crabbe, 2010) manner, which, in the latter case, contains multiple abstinence episodes embedded within the overall exposure protocol. Previous evidence of chronic-intermittent ethanol (CIE) vapor exposure suggests that the severity of withdrawal-related symptoms may develop in a cumulative manner. This "kindling hypothesis" postulates that progressive intensification of the symptomatology is a direct consequence of repeated episodes of ethanol intoxication and abstinence (Ballenger & Post, 1978; H. C. Becker & Hale, 1993; H. C. Becker, Diaz-Granados, & Weathersby, 1997).

1.2.5. Escalated Voluntary Ethanol Intake Following Dependence Induction

Unfortunately, relapse occurs after either short or long periods of abstinence in the majority of post-dependent individuals (Barrick & Connors, 2002), an observation which supports the AUD criterion describing uncontrollable compulsions to seek and consume ethanol (Mason, 2017). Several procedural variations have been deployed in attempting to model this behavior. The most common and reliable method involves initial limited-access or continuous free-choice drinking, followed by induction of dependence via chronic ethanol vapor exposure and subsequent access to ethanol (in the respective manner originally used) during ensuing abstinence (Becker, 2013). Typically, prominent increases in ethanol intake are observed (H. C. Becker & Lopez,

2004; Finn et al., 2007; Lopez & Becker, 2005; McCool & Chappell, 2015; A. J. Roberts, Heyser, Cole, Griffin, & Koob, 2000) and are thought to reflect an attempt to reduce associated withdrawal symptoms (Heilig et al., 2010; Holleran & Winder, 2017).

Interesting, more robust ethanol intake is exhibited during abstinence when ethanol vapor is administered on an intermittent, rather than continuous, manner (W. C. Griffin, Lopez, & Becker, 2009a; O' Dell, Roberts, Smith, & Koob, 2004). These results demonstrate that escalated ethanol consumption is not simply a product of chronic ethanol exposure, but rather, likely due to the repeated enforcement of abstinence periods. In turn, increasing the total number of CIE cycles results in corresponding escalations in ethanol intake (W. C. Griffin, Lopez, Yanke, Middaugh, & Becker, 2009b), as well as more enduring intake further into abstinence (Lopez & Becker, 2005). Moreover, this effect seems to be ethanol-specific, as no alterations in either water or sucrose consumption were observed following CIE termination (H. C. Becker & Lopez, 2004; Lopez & Becker, 2005; Lopez, Griffin, Melendez, & Becker, 2012); thus, increased ethanol intake is not indicative of an idiopathic desire for fluid or calories. It is often postulated that this model of post-dependent relapse reflects the transition from drinking motivated by positive reinforcement to drinking driven by negative reinforcement (i.e., alleviation of unpleasant withdrawal-related symptoms). It is this transition that is possibly intensified by repeated cycles of abstinence and relapse, resulting in uncontrollable consummatory compulsions in order to avoid the negative reinforcing properties of ethanol.

Unsurprisingly, the vast majority of studies involving dependence-induced escalation of voluntary ethanol intake exclusively use B6J mice (H. C. Becker & Lopez,

2004; Finn et al., 2007; W. C. Griffin, Lopez, & Becker, 2009a; W. C. Griffin, Lopez, Yanke, Middaugh, & Becker, 2009b; Lopez et al., 2012; Lopez & Becker, 2005; Lopez, Becker, & Chandler, 2014), as their general avidity to consume greater quantities of ethanol, relative to other inbred strains, in various ethanol intake protocols, renders them an ideal model. However, this choice sacrifices the ability to detect and characterize underlying genetic contributions. More recently, McCool & Chappell (2015) compared dependence-induced escalation of voluntary ethanol drinking in B6J and D2J mice. Since D2J mice reliably exhibit low levels of voluntary ethanol intake, the researchers employed a novel tastant-substitution procedure involving monosodium glutamate (MSG) that has previously been shown to amplify ethanol consumption in characteristically low-drinking strains (McCool & Chappell, 2012; 2014). Overall, although the MSG substitution procedure greatly facilitated ethanol intake in D2J mice (as well as B6J, although to a lesser degree), ethanol dependence, via forced inhalation of ethanol vapor, increased ethanol intake regardless of strain.

These data are informative and represent a step in the right direction regarding utilizing diverse genotypes. However, the major disadvantage of investigating dependence-induced escalation of voluntary ethanol drinking is its required investment of significant time, labor, and resources. The intrinsic nature of this phenotype prohibits it from being high-throughput, which has likely discouraged genetic discovery investigations up to this point. Nevertheless, researchers often acknowledge the need for the characterization of complex phenotypes involving powerful genetic tools (i.e., selected lines, inbred strain panels, RI strains, CC and DO mice, and/or inbred

substrains). While commitment to such a project would be substantial, it is absolutely justified in what could be learned.

1.2.6. Acute Physiological Effects of Ethanol Withdrawal

The emergence of overt withdrawal symptoms when ethanol is discontinued is generally thought to be indicative of a state of ethanol dependence (Kalant et al., 1971). In mice, the most frequently studied index of acute withdrawal severity is the handling-induced convulsion (HIC) test, which serves as a marker for CNS hyperexcitability (H. C. Becker et al., 1997; H. C. Becker & Hale, 1993; Goldstein, 1972; Goldstein & Pal, 1971). Interestingly, in contrast to mice, rats do not exhibit HICs, thus withdrawal severity is generally indexed by a collection of other behavioral and physiological disturbances (Majchrowicz, 1975; Heilig et al., 2010). Nonetheless, the HIC measure, in mice, effectively models certain aspects of the acute alcohol withdrawal syndrome often observed in alcohol-dependent individuals. In humans, symptom onset is typically within eight hours of initial abstinence, and can include irritability, nausea, vomiting, insomnia, tremor, hyperalgesia, hyperthermia, tachycardia, anxiety, hallucinations, delusions, and tonic-clonic seizures (APA, 2013; Attilia et al., 2018). Mouse responses typically range from mild myoclonic twitches to tonic-clonic convulsions, though severely dependent mice may demonstrate fatal tonic hindlimb extensor seizures (Metten & Crabbe, 2005). HICs in mice usually peak around 7-10 hours following termination of ethanol exposure, returning to baseline levels after 24 hours, and its severity is a function of both ethanol dose and duration of exposure (H. C. Becker et al., 1997; H. C. Becker & Hale, 1993; Goldstein, 1972; Metten et al., 2010; Metten & Crabbe, 2005).

Various approaches have been employed to examine the underlying genetic contributions to HICs during acute ethanol withdrawal. Beginning in 1983, the Crabbe laboratory has used bidirectional selective breeding of genetically heterogeneous mice (HS/Ibg) from an 8-way cross of inbred strains to develop multiple stable lines of Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice displaying high and low HIC scores, respectively, following 72-hour ethanol vapor exposure (Crabbe, Kosobud, & Young, 1983; Crabbe, Kosobud, Young, Tam, & McSwigan, 1985). As is ideal, the experiment was repeated in order to generate a second, genetically independent pair of bidirectionally selected lines (WSP-1, WSP-2; WSR-1, WSR-2). After 11 generations of selection, heritability of the trait was moderate (h^2 = .26), and both WSP lines exhibited at least tenfold more severe HICs than their respective WSR lines (Crabbe et al., 2013).

These selected lines have been tested for differences on various other ethanol-related phenotypes, with mixed results. For example, although WSP and WSR mice show differences in DID drinking (Crabbe et al., 2013), they do not significantly differ in two-bottle free-choice ethanol drinking, operant ethanol self-administration, or expression of the ADE (Crabbe et al., 2013; Ford et al., 2011; Rosenwasser et al., 2013). Also, a recent experiment by our laboratory (Hartmann, Holbrook, Haney, Crabbe, & Rosenwasser, 2019) found no significant differences in abstinence-induced anxiety- and/or depressive-like behavior between WSP-2 and WSR-2 mice, providing evidence that acute and long-term consequences of ethanol withdrawal appear to be dependent on largely non-overlapping gene networks.

Several studies have documented inbred strain differences in HIC severity. For instance, Metten & Crabbe (2005) found significant strain differences in a panel of 15 inbred strains. Intriguingly, this study used a standard 72-hour continuous ethanol vapor exposure period, but attempted to produce nearly identical resultant BECs by exposing different strains to different ethanol vapor concentrations. Ironically, strain differences in HIC severity was independent of dose administered. Similarly, Metten et al. (2010) characterized the same 15 inbred strains for HIC severity following CIE (16 hours/day ethanol vapor with 8 hours exposure to air), as opposed to continuous vapor exposure. Collectively, these two studies revealed two important insights, 1) D2J mice consistently displayed among the highest HIC scores, while B6J mice regularly presented among the lowest, and 2) there appears to be an inverse genetic relationship between two-bottle free-choice ethanol drinking and HIC severity, a finding supported by previous work (Hitzemann et al., 2009; Metten et al., 1998).

Along these lines, prior work has utilized RI strains of mice derived from B6J and D2J crosses (BXD) to map QTLs for HIC severity following ethanol vapor exposure (Crabbe, 1998). The first experimental investigation regarding HIC severity following chronic ethanol vapor exposure identified a total of 10 QTLS: 2 QTLs on chromosomes 1 and 9 and single QTLs on chromosomes 3, 10, 12, 13, 15, and 18 (Crabbe, 1998). Analysis from the four most influential QTLs (chromosomes 1 (distal), 9 (distal), 13, and 15) showed that, together, these loci were responsible for approximately 86% of the genetic variance. This work stimulated further efforts which revealed the gene encoding multiple PDZ domain protein (*mpdz*) to be a quantitative trait gene (QTG) associated with acute ethanol withdrawal HIC severity (Metten et al., 2014; Shirley, Walter, Reilly,

Fehr, & Buck, 2004). Future work will hopefully pinpoint the circuits, and possibly networks, this gene is exerting influence on, as well as discover and characterize potential gene-gene interaction effects.

1.2.7. Affective Disturbance during Ethanol Withdrawal

Although acute physiological symptoms of ethanol withdrawal can be effectively managed with benzodiazepines in both rodents (Heilig et al., 2010) and humans (Mayo-Smith, 1997), such treatments do not significantly decrease the likelihood of eventual relapse (Askgaard et al., 2016; Malcolm et al., 2002). Furthermore, much less is known concerning the longer-term affective-behavioral disruptions that may emerge weeks to months after initial detoxification, and often persist across even prolonged periods of abstinence. In affected individuals, persistent anxiety and depressive symptoms may contribute to an increased risk of relapse long after acute detoxification (Heilig et al., 2010; Malcolm, 2003). Additionally, benzodiazepines do not significantly mitigate emerging affective disturbances even when administered several weeks into abstinence (Gallant, Bishop, Guerrero-Figueroa, Selby, & Phillips, 1969).

Experiments employing chronic ethanol vapor exposure have revealed increases in anxiety- and/or depressive-like behavior in rats (Valdez et al., 2002; Walker et al., 2010; Zhao, Weiss, & Zorrilla, 2007) and mice (Logan, McCulley, Seggio, & Rosenwasser, 2012; Sidhu, Kreifeldt, & Contet, 2018) several weeks after the termination of ethanol exposure. Although chronic ethanol vapor exposure is thought to effectively model alcohol-dependent patients exhibiting the highest BECs, there is some evidence that such high levels of intoxication may not be necessary for the expression of affective disturbances during long-term abstinence (Holleran & Winder, 2017). For

example, previous studies in mice (Gong et al., 2017; Holleran et al., 2016; Pang, Renoir, Du, Lawrence, & Hannan, 2013; Roni & Rahman, 2017; Stevenson et al., 2009) have observed significant depressive-like behavior weeks after termination of long-term continuous access to ethanol under two-bottle free-choice conditions. These findings suggest that researchers perhaps overestimate the influence of BECs and physical withdrawal symptoms to the development of affective disturbances during long-term abstinence.

Anxiety- and depressive-like rodent behavior appear to follow different temporal trajectories following cessation of chronic ethanol exposure. Across diverse ethanol-administration protocols and behavioral test procedures, rats (Gong et al., 2017; Rylkova, Shah, Small, & Bruijnzeel, 2009; Valdez et al., 2002; Van Skike, Diaz-Granados, & Matthews, 2015; Zhao et al., 2007) and mice (Finn, Gallaher, & Crabbe, 2000; Gong et al., 2017; Kash, Baucum, Conrad, Colbran, & Winder, 2009; Kliethermes, Cronise, & Crabbe, 2004; Perez & De Biasi, 2015; Pleil et al., 2015; Rose et al., 2016) usually display significant anxiety-like behavior during the first 72 hours of abstinence, a phase referred to as acute withdrawal; however, several negative findings have also been reported, especially in mice (Cox et al., 2013; Daut et al., 2015; Lee, Coehlo, McGregor, Waltermire, & Szumlinski, 2015; Metten et al., 2017).

Four days to three weeks into abstinence is usually referred to as “early abstinence” (Heilig et al., 2010). Interestingly, during this period, exhibition of anxiety-like behavior becomes less prominent in both rats (Rylkova et al., 2009; Zhao et al., 2007) and mice (J. A. J. Becker, Kieffer, & Le Merrer, 2017; Fukushiro et al., 2012; Holleran et al., 2016; Lee et al., 2015; Pang et al., 2013). Although the presence of

anxiety-like behavior, in both mice and rats, wanes during early abstinence, anxiety-like behavior appears to reemerge in rats (Gillett, Harshberger, & Valdez, 2013; Valdez et al., 2002; Zhao et al., 2007), but not mice (J. A. J. Becker et al., 2017; Lee, Coehlo, Solton, & Szumlinski, 2017), during “protracted abstinence” (abstinence extending to greater than four weeks).

In contrast, studies of rodent depressive-like behavior have revealed a temporal pattern distinct from manifestations of anxiety-like behavior. During acute withdrawal, inconsistent findings regarding depressive-like behavior have been reported, as results appear to be largely dependent on the method of ethanol administration, and to some extent, the behavioral assay utilized. In mice, forced methods of ethanol administration (Arora & Vohora, 2016; Karadayian, Busso, Feleder, & Cutrera, 2013; Metten et al., 2017), as opposed to voluntary consumption paradigms (Holleran et al., 2016; Lee et al., 2015; 2017; 2016; Stevenson et al., 2009), seem to more reliably produce depressive-like behavior. Although there are a more limited number of studies involving rats, positive results have typically been reported (Getachew, Hauser, Taylor, & Tizabi, 2010; Jarman, Haney, & Valdez, 2018).

There is abundant evidence for the exhibition of depressive-like behavior in mice during early abstinence, which does not seem to be influenced by method of ethanol administration or behavioral assay variations (Gong et al., 2017; Holleran et al., 2016; Kim et al., 2017; Pang et al., 2013; Roni & Rahman, 2017; Stevenson et al., 2009). In rats, only two studies have been conducted, and they reflect mixed findings (Jarman et al., 2018; Rasmussen, Mitton, Green, & Puchalski, 2001). Currently, there is a relative dearth of studies analyzing depressive-like behavior during protracted abstinence in

either rats or mice, denoting a significant gap in the literature. Nonetheless, both rats (Rasmussen et al., 2001; Walker et al., 2010) and mice (Lee et al., 2017; Logan et al., 2012) have displayed contradictory results, restricting the ability to draw concrete conclusions.

Unfortunately, researchers have not utilized genetic diversity approaches in the investigation of affective disturbance during ethanol withdrawal, as the overwhelming majority of studies involve B6J mice. On occasion, B6J mice have been compared to a different inbred strain, such as D2J and C3H mice (Finn et al., 2000; Logan et al., 2012; McCool & Chappell, 2015; Sidhu et al., 2018), though these comparisons have yielded inconsistent results that are likely influenced by differences in the method of dependence induction and behavioral assay utilized. For instance, during acute withdrawal, both D2J and B6J mice exhibited significant anxiety-like behavior on the elevated-plus maze (EPM) (Finn et al., 2000), but only D2J, and not B6J, displayed significant anxiety-like behavior on the LDT (McCool & Chappell, 2015). Furthermore, during early abstinence, both D2J and B6J mice exhibited significant anxiety-like behavior on the marble burying test (MBT) (Sidhu et al., 2018). Interestingly, the most convincing evidence for strain differences in withdrawal-associated depressive-like behavior involved extended analysis into protracted abstinence. Following termination of a 4-cycle CIE exposure, B6J mice demonstrated dramatic hypolocomotion for up to 7 days whereas C3H mice sustained hypolocomotion up to 30 days (Logan et al., 2012).

Although these aforementioned data are important, there is no previous data involving selected lines, inbred strain panels, RI strains, CC and DO mice, or inbred substrains in regards to affective disturbances during ethanol withdrawal. As previously

discussed, a major disadvantage of investigating certain phenotypes, such as affective behavior during long-term ethanol abstinence, is its degree of labor intensiveness. This inherent aspect of this line of research is likely the reason such genetic discovery investigations are currently absent. Nevertheless, future work involving diverse genetic approaches and better standardization of methods is needed in order to enhance our understanding of the various factors inherent to the emergence of affective disturbances during long-term ethanol abstinence. To this end, Experiment 3 of this dissertation compared B6J and B6NJ substrains on long-term anxiety- and depressive-like behavior following termination of chronic ethanol vapor exposure in hopes of initiating future genetic discovery investigations utilizing such framework.

CHAPTER 2

EXPERIMENT 1: SUBSTRAIN DIFFERENCES IN FREE-CHOICE ETHANOL DRINKING AND WHEEL-RUNNING BETWEEN C57BL/6J AND C57BL/6NJ MICE: ROLE OF CYFIP2

2.1. Rationale

In the present experiment, voluntary ethanol consumption and preference, as well as wheel-running, were examined in male and female B6J, B6NJ, CRISPR-B6J$^{NJ/NJ}$ (B6J$^{NJ/NJ}$) and CRISPR-B6NJ$^{J/J}$ (B6NJ$^{J/J}$) mice. Previous work has identified a SNP in the Cytoplasmic FMR1-interacting protein 2 (*Cyfip2*) gene that underlies the differential locomotor response to cocaine exhibited by B6J and B6N mice (Kumar et al., 2013). In addition, phylogenetic analysis of *Cyfip2* variation within B6 substrains found that this SNP became fixed in the B6N strain sometime between 1961-1974; consequently, all B6N derivatives possess this mutation as well. Thus, we hypothesized that this cocaine-relevant SNP in *Cyfip2* also contributes to other addiction-related phenotypic differences between B6 substrains. Since B6J mice reliably exhibit higher ethanol consumption and preference than B6N mice from various breeding locations (Blum et al., 1982; Mulligan et al., 2008; Ramachandra et al., 2007), we expected B6J mice to exhibit significantly greater ethanol consumption and preference than B6NJ mice. In turn, we hypothesized that insertion of the mutant B6NJ *Cyfip2* SNP into the B6J background (B6J$^{NJ/NJ}$) would significantly reduce ethanol consumption and preference, while “correcting” the B6NJ *Cyfip2* mutation by inserting the B6J *Cyfip2* SNP into the B6NJ background (B6NJ$^{J/J}$) would significantly increase ethanol consumption and preference.

Wheel-running was also examined in these inbred substrains and associated CRISPR-engineered lines since previous work suggests wheel-running is a rewarding and reinforcing behavior that depends on overlapping neural circuitry that mediates drug reward and addiction (Brené et al., 2007; de Visser, van den Bos, Stoker, Kas, & Spruijt, 2007). Wild mice will engage in wheel-running, even within a natural environment and when no extrinsic reward is provided (Meijer & Robbers, 2014), whereas laboratory rodents will actively press a lever to gain access to a running-wheel and reliably develop a conditioned place preference for an environment previously paired with a running-wheel (Belke, 1997; Lett, Grant, Byrne, & Koh, 2000). Though no previous work has examined potential differences in wheel-running specifically between B6J and B6NJ mice, prior work has demonstrated that B6N mice display higher daily wheel-turns than B6J mice under constant darkness (Kumar et al., 2013). Additionally, while traditional QTL mapping failed to identify any significant QTLs, reanalysis of these data through utilization of mean-variance QTL mapping identified one QTL on chromosome 6 (Corty, Kumar, Tarantino, Takahashi, & Valdar, 2018). Therefore, we sought to characterize this phenotype and assess, if differences were present, the relative contribution of the *Cyfip2* SNP.

2.2. Materials and Methods

2.2.1. Animals

Male and female B6J (M, *n* = 14; F, *n* = 16), B6NJ (M, *n* = 14; F, *n* = 16), B6J$^{NJ/NJ}$ (M, *n* = 13; F, *n* = 12), and B6NJ$^{J/J}$ (M, *n* = 11; F, *n* = 13) mice were shipped to the University of Maine from The Jackson Laboratory (Bar Harbor, ME). Specifically, *Cyfip2* Knock-in mice (B6J$^{NJ/NJ}$, B6NJ$^{J/J}$) were generated and supplied by Dr. Vivek Kumar,

who utilized CRISPR-Cas9 gene editing to introduce the heterotypical SNP of the *Cyfip2* gene into an otherwise normal B6J or B6NJ genetic background. Mice arrived in the laboratory at approximately 6 weeks of age and were immediately individually housed in running-wheel cages (32 x 20 x 14 cm) under a LD 12:12 lighting regimen (lights off at 1400) for the duration of the experiment. Running-wheel cages were placed within light-shielded and sound-attenuating metal cabinets equipped with standard fluorescent bulbs on each shelf. Food (Prolab RMH 3000; LabDiet, St. Louis, MO) and tap water were available *ad libitum* throughout the experiment. During the two-bottle free-choice ethanol drinking protocol, ethanol solutions of various concentrations were available via a second drinking bottle, as described below. All experimental procedures were approved by the University of Maine Institutional Animal Care and Use Committee (IACUC).

2.2.2. Procedures

24 hours following arrival, animals underwent a 15-day period in the running-wheel cages with *ad libitum* access to food and water (but not ethanol). Subsequently, all animals underwent a two-bottle free-choice ethanol drinking protocol; running wheels remained available throughout ethanol testing. There were 10-16 animals for each sex/substrain combination; exact *n*'s for each group are available for ethanol analyses and wheel-turn per day analyses in Fig. 2.2 and Fig. 2.6, respectively. Due to running-wheel equipment error, data were unavailable for a small subset of animals ($B6J^{NJ/NJ}$, $n = 2$; $B6NJ^{J/J}$, $n = 4$) that were thus excluded from analyses of daily activity.

2.2.2.1. Wheel-Running

Animals were given continuous access to a running-wheel (wheel diameter: 23 cm; Mini-Mitter Co., Bend, OR) for the duration of the experiment. All wheel-turns were recorded using the ClockLab interface system (Actimetrics Co., Wilmette, IL). Daily wheel-turns during the 15-day period prior to ethanol access were used for data analysis.

2.2.2.2. Two-Bottle Free-Choice Ethanol Consumption

Animals were given continuous free-choice access to bottles containing either an ethanol solution or plain water for a total of 35 days. Ethanol concentration was initially set at 3% (v/v) and increased in 3% increments, every 5 days, through a final concentration of 21%. The physical location (right or left) of the water and ethanol solutions was switched every 5 days, in a counterbalanced manner, to reduce the effects of potential side preference. Pre- and post-measurements of bottle weight, along with animals' respective body weights following each 5-day period of ethanol access (data not shown), were used to obtain accurate calculations of body weight-adjusted ethanol intake (g/kg) over the course of the experiment. Ethanol preference was determined by dividing the volume of ethanol solution consumed by total fluid intake.

2.2.2.3. Statistics

Data are presented as means +/- SEM. Ethanol intake and preference were analyzed using 3-factor (genotype, sex, concentration) mixed-design analysis of variance (ANOVA) followed where appropriate by separate 2-factor ANOVAs. Similarly, wheel-turns per day was analyzed using 3-factor (genotype, sex, day) mixed-design ANOVA, followed where appropriate by separate 2-factor ANOVAs. Data analysis was

performed using SPSS 23.0 (IBM Inc., Armonk, NY) and figures were generated using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA). Only statistically significant (p < .05) main effects and interactions are described in the text, and while exact p-values are not given for pairwise comparisons, all mentioned pairwise tests were significant at p < .05.

2.3. Results

2.3.1. Two-Bottle Free-Choice Ethanol Consumption

2.3.1.1. Ethanol Intake

ANOVA revealed significant main effects of genotype [$F(3, 101) = 11.58$, p < .001, partial η^2 = .256], sex [$F(1, 101) = 41.44$, p < .001, partial η^2 = .291], and concentration [$F(6, 606) = 76.268$, p < .001, partial η^2 = .430], as well as significant genotype x sex [$F(3, 101) = 3.37$, p = .021, partial η^2 = .091], genotype x concentration [$F(6, 606) = 2.40$, p = .001, partial η^2 = .066], and sex x concentration [$F(6, 606) = 7.71$, p < .001, partial η^2 = .071] interactions (Fig. 2.1).

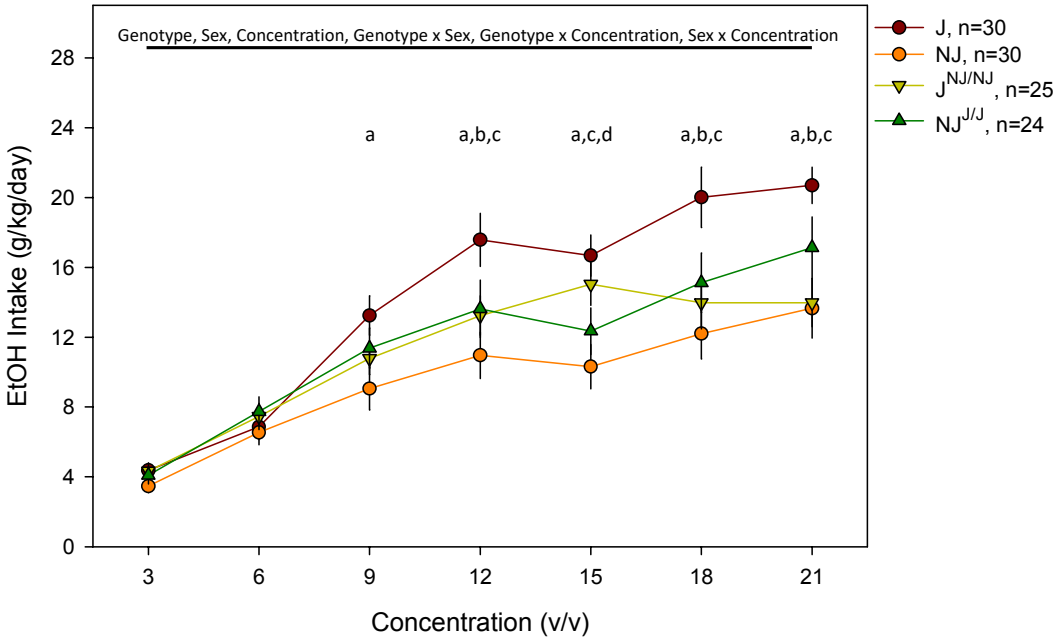

Figure 2.1. Ethanol intake across concentrations. Mean (±SEM) ethanol intake (g/kg/day) during two-bottle free-choice in C57BL/6J (J), C57BL/6NJ (NJ), CRISPR-B6J$^{NJ/NJ}$ (J$^{NJ/NJ}$), and CRISPR-B6NJ$^{J/J}$ (NJ$^{J/J}$) mice. Symbols denote the following significant differences ($p < .05$): a = J > NJ, b = J > J$^{NJ/NJ}$, c = J > NJ$^{J/J}$, d = J$^{NJ/NJ}$ > NJ.

Ethanol intake generally increased as a function of concentration. Females displayed significantly greater intake than males, both overall and at each concentration except 3% and 9%. B6J mice displayed significantly greater overall intake than any other genotype, but pairwise comparisons among genotypes varied as a function of concentration. Specifically, B6J mice displayed significantly greater intake than B6NJ mice at all concentrations of 9% and higher; B6J displayed significantly greater intake than B6J$^{NJ/NJ}$ mice at concentrations of 12%, 18%, and 21%; and B6J displayed significantly greater intake than B6NJ$^{J/J}$ mice at concentrations of 12% and greater. While B6J$^{NJ/NJ}$ exhibited greater intake than B6NJ overall, this effect was significant only at 15% concentration (Fig 2.1). Finally, while B6NJ$^{J/J}$ showed significantly greater overall intake than B6NJ, this effect was not significant at any specific concentration. Separate analyses for females and males (Fig. 2.2) showed that female B6J mice displayed significantly greater intake than females of any other genotype, whereas among males, B6J mice displayed significantly greater intake than B6NJ and B6J$^{NJ/NJ}$, but not B6NJ$^{J/J}$ mice. Finally, male B6NJ mice exhibited significantly lower intake than any other genotype.

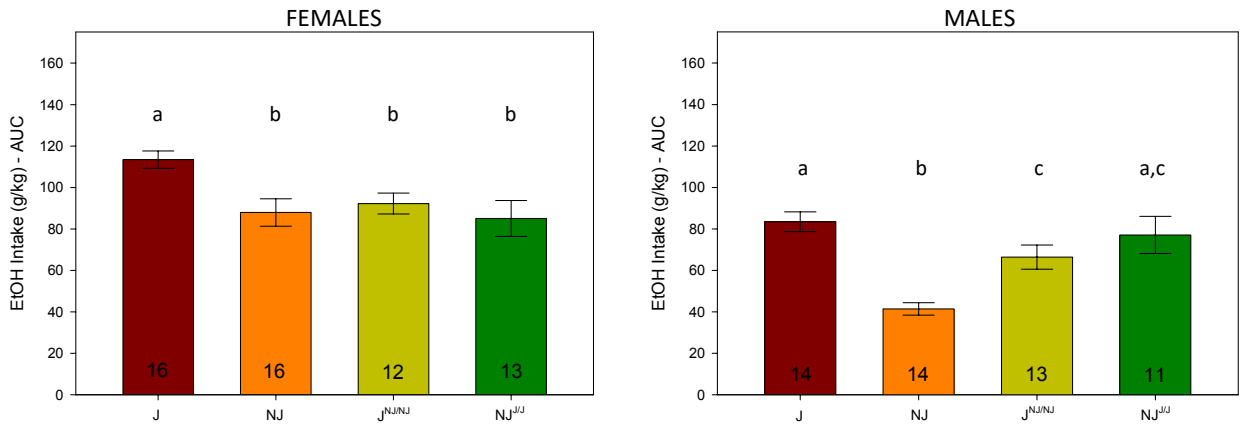

Figure 2.2. Total ethanol intake across sex. Mean (±SEM) total ethanol intake (g/kg) during two-bottle free-choice in female (top) and male (bottom) C57BL/6J (J), C57BL/6NJ (NJ), CRISPR-B6J$^{NJ/NJ}$ (J$^{NJ/NJ}$), and CRISPR-B6NJ$^{J/J}$ (NJ$^{J/J}$) mice. Bars with no shared letters are significantly different (p < .05) from one another.

2.3.1.1. Ethanol Preference

ANOVA revealed significant main effects of genotype [F(3, 101) = 8.41, p < .001, partial η^2 = .200], sex [F(1, 101) = 8.43, p = .005, partial η^2 = .077], and concentration [F(6, 606) = 7.59, p < .001, partial η^2 = .070], as well as significant genotype x sex [F(3, 101) = 4.45, p = .005, partial η^2 = .119] and sex x concentration [F(6, 606) = 3.39, p = .003, partial η^2 = .032] interactions (Fig. 2.3).

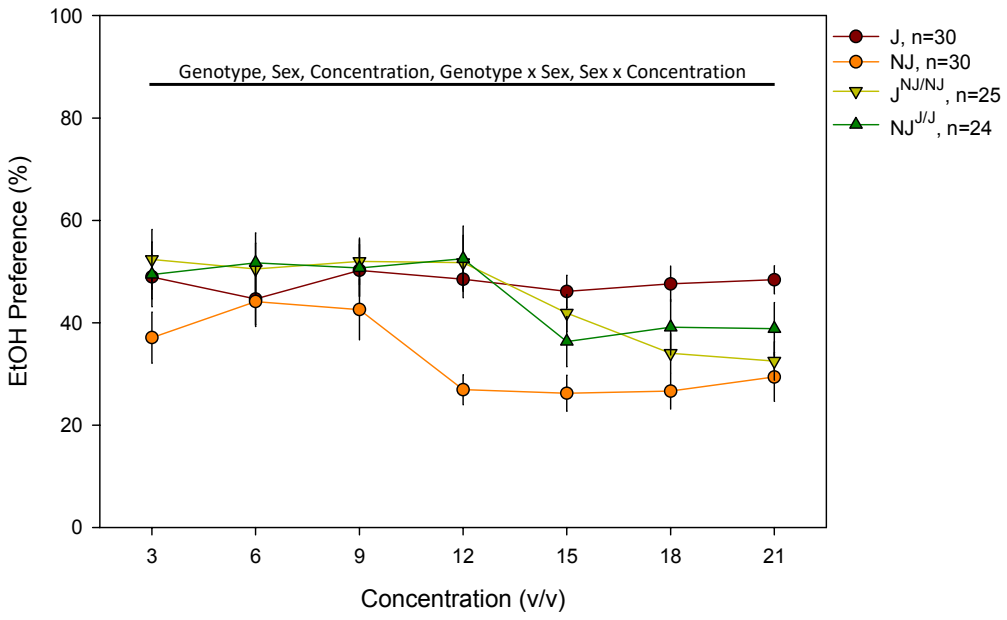

Figure 2.3. Ethanol preference across concentrations. Mean (±SEM) ethanol preference during two-bottle free-choice in C57BL/6J (J), C57BL/6NJ (NJ), CRISPR-B6J$^{NJ/NJ}$ (J$^{NJ/NJ}$), and CRISPR-B6NJ$^{J/J}$ (NJ$^{J/J}$) mice.

Ethanol preference was mostly stable, though lower preference was generally observed at 15% and higher concentrations. Females displayed significantly higher preference than males overall, and at 15% and higher concentrations. B6NJ mice exhibited significantly lower preference than all other genotypes. Separate analyses for females and males (Fig. 2.4) showed that female B6J mice displayed significantly greater preference than both B6NJ and B6NJ$^{J/J}$, but not B6J$^{NJ/NJ}$, females, whereas male B6NJ mice showed significantly lower preference than all other genotypes.

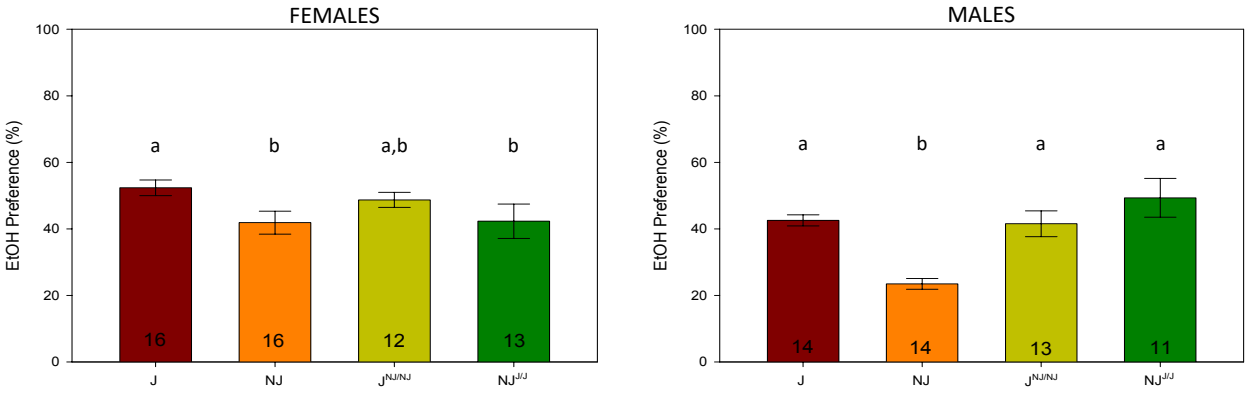

Figure 2.4. Ethanol preference across sex. Mean (±SEM) ethanol preference during two-bottle free-choice, collapsed across concentration, in female (top) and male (bottom) C57BL/6J (J), C57BL/6NJ (NJ), CRISPR-B6J$^{NJ/NJ}$ (J$^{NJ/NJ}$), and CRISPR-B6NJ$^{J/J}$ (NJ$^{J/J}$) mice. Bars with no shared letters are significantly different ($p < .05$) from one another.

2.3.2. Wheel-Turns Per Day

ANOVA revealed significant main effects of genotype [$F(3, 95) = 35.02$, $p < .001$, partial $\eta^2 = .525$], sex [$F(1, 95) = 8.32$, $p = .005$, partial $\eta^2 = .081$], and day [$F(14, 1330) = 74.16$, $p = .002$, partial $\eta^2 = .438$], as well as significant genotype x sex [$F(3, 95) = 3.09$, $p = .031$, partial $\eta^2 = .089$] and genotype x day [$F(42, 665) = 2.07$, $p < .001$, partial

η^2 = .062] interactions (Fig. 2.5). Wheel-turns per day generally increased as a function of day. Overall, females exhibited significantly higher wheel-turns per day than males, B6NJ mice displayed significantly higher wheel-turns per day than all other genotypes, and B6NJ$^{J/J}$ mice showed significantly higher wheel-turns per day than both B6J and B6J$^{NJ/NJ}$ mice. Specific pairwise comparisons among genotypes varied as a function of day (Fig. 2.5). Specifically, B6NJ mice displayed significantly higher wheel-turns per day than both B6J and B6J$^{NJ/NJ}$ mice across all days, whereas B6NJ showed significantly higher wheel-turns than B6NJ$^{J/J}$ mice only on Days 1, 4, 6-9, and 14; B6NJ$^{J/J}$ mice displayed significantly higher wheel-turns per day than B6J$^{NJ/NJ}$ mice, across all days, and B6J mice, on each day except Days 1 and 14.

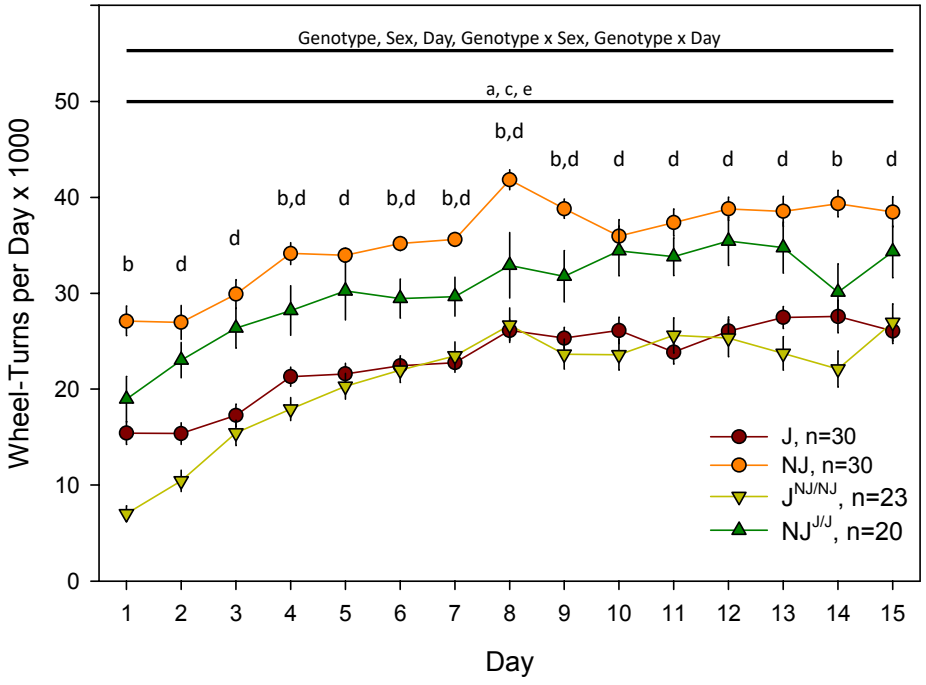

Figure 2.5. Daily wheel-turns across 15-day period. Mean (±SEM) wheel-turns per day in C57BL/6J (J), C57BL/6NJ (NJ), CRISPR-B6J$^{NJ/NJ}$ (J$^{NJ/NJ}$), and CRISPR-B6NJ$^{J/J}$ (NJ$^{J/J}$) mice. Symbols denote the following significant differences (p < .05): a = NJ > J, b = NJ > NJ$^{J/J}$, c = NJ > J$^{NJ/NJ}$, d = NJ$^{J/J}$ > J, e = NJ$^{J/J}$ > J$^{NJ/NJ}$.

Separate analyses for females and males (Fig. 2.6) showed that female B6NJ and B6NJ$^{J/J}$ mice displayed higher wheel-turns per day than both female B6J and B6J$^{NJ/NJ}$ mice. In contrast, male B6NJ mice displayed significantly higher wheel-turns per day than all other genotypes, while male B6NJ$^{J/J}$ mice showed significantly higher wheel-turns per day than male B6J$^{NJ/NJ}$ mice.

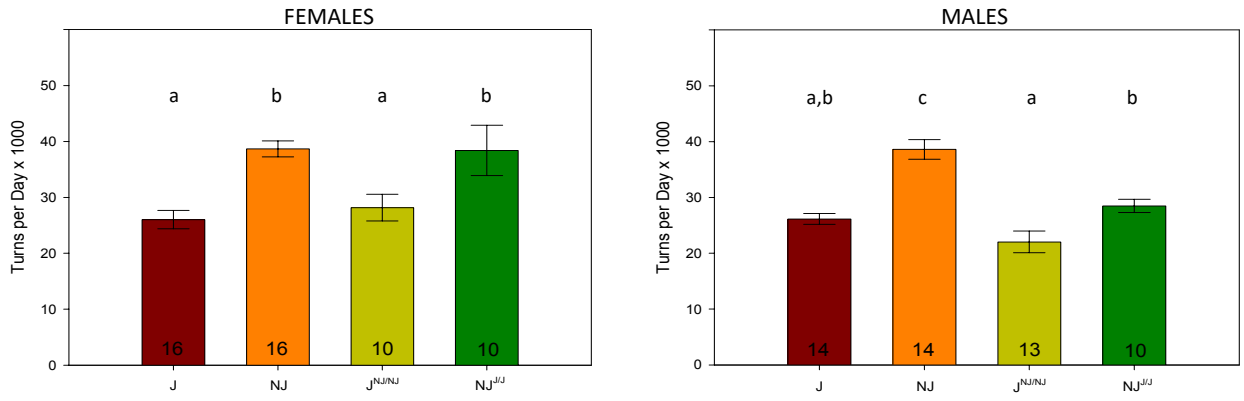

Figure 2.6. Daily wheel-turns across sex. Mean (±SEM) wheel-turns per day, collapsed across day, in female (top) and male (bottom) C57BL/6J (J), C57BL/6NJ (NJ), CRISPR-B6J$^{NJ/NJ}$ (J$^{NJ/NJ}$), and CRISPR-B6NJ$^{J/J}$ (NJ$^{J/J}$) mice. Bars with no shared letters are significantly different ($p < .05$) from one another.

2.4. Discussion

Overall, while this experiment detected significant differences between B6J and B6NJ mice in both two-bottle free-choice ethanol drinking and wheel-running, allelic variation in *Cyfip2* appeared to more substantially modulate substrain differences in ethanol intake than in wheel-running. Moreover, observed genotypic effects often interacted with other factors, such as sex, ethanol concentration, and/or day of running-wheel access.

Consistent with prior studies (Mulligan et al., 2008; Ramachandra et al., 2007) using a different B6N lineage (B6NCrl), B6J mice of both sexes showed greater overall ethanol intake and higher levels of ethanol preference compared to B6NJ mice. As

expected from the literature, females generally consumed more ethanol than males in both B6J and B6NJ, but the substrain difference in ethanol consumption was substantially larger in males than in females.

While insertion of the mutant B6NJ *Cyfip2* SNP into the B6J background ($B6J^{NJ/NJ}$) significantly reduced ethanol intake in both sexes, repairing the B6NJ *Cyfip2* mutation by insertion of the "wild-type" (WT) B6J *Cyfip2* SNP into the B6NJ background ($B6NJ^{J/J}$) significantly increased ethanol intake only in males (Fig. 2.2). Further, while male $B6J^{NJ/NJ}$ mice showed significantly higher ethanol intake than male B6NJ mice, female $B6J^{NJ/NJ}$ mice exhibited similar levels of ethanol intake as did female B6NJ mice. Together, these observations suggest that sex-dependent genetic background effects may interact with the *Cyfip2* SNP to produce observed differences in ethanol intake between B6J and B6NJ mice.

Although there was no *a priori* hypothesis predicting an asymmetric effect of the *Cyfip2* mutation, the current data appears to reflect a greater propensity of the mutant (B6NJ) *Cyfip2* SNP to reduce ethanol intake in an otherwise high-drinking B6J background, than of the WT (B6J) *Cyfip2* SNP to increase ethanol intake in a comparatively low-drinking B6NJ background. It is possible that the WT *Cyfip2* gene requires sex-specific gene-gene interactions in order to generate the high ethanol intake normally seen in B6J mice, while the mutant *Cyfip2* SNP is sufficient to yield low ethanol intake in either sex. While extensive sex-specific mapping studies would be required to identify any genetic modifiers of the *Cyfip2* effect, the present results indicate that *Cyfip2* is a major genetic contributor to the observed substrain differences in ethanol intake between B6J and B6NJ mice.

Genotypic differences in ethanol intake also varied as a function of ethanol concentration. Thus, B6J mice displayed significantly greater intake than B6NJ mice only at concentrations of 9% and higher (Fig. 2.1). This finding differs somewhat from previous work comparing B6J to a different B6N lineage (B6NCrl). In that study, which utilized the identical series of ethanol concentrations as employed here, B6J mice showed higher voluntary ethanol intake at all concentrations, including at 3% and 6% (Mulligan et al., 2008). Interestingly, *Cyfip2*-mediated alterations in ethanol intake were highly variable throughout this concentration range. For example, B6J mice demonstrated significantly higher ethanol intake than $B6J^{NJ/NJ}$ mice at concentrations of 12%, 18%, and 21%, but not at 9% or 15%. In addition, though $B6NJ^{J/J}$ showed significantly greater overall intake than B6NJ, there were no significant differences at any single concentration. It should be noted that our current experimental design, like that of Mulligan et al. (2008), used a gradually increasing ethanol concentration series, thus confounding time with increasing concentration. That is, we may very well have found a different outcome at any specific concentration if we had employed a descending concentration series, or even tested independent groups of animals at each concentration. On the other hand, mice had five days to consume each respective ethanol solution, and previous evidence indicates that approximately four days is the optimal duration of two-bottle free-choice access for detecting murine strain differences (Tordoff & Bachmanov, 2002). Furthermore, as with any two-bottle free-choice access protocol, we are unable to definitively identify the nature of each substrain's motivation to consume a certain ethanol concentration. For example, B6NJ mice might be pursuing the taste of ethanol, but B6J mice seek to experience its pharmacological effects.

Similar to ethanol intake, B6J mice of both sexes showed greater overall ethanol preference compared to B6NJ mice, consistent with prior work comparing B6J mice to B6NCrl mice (Mulligan et al., 2008; Ramachandra et al., 2007). In contrast to our ethanol intake data, however, $B6J^{NJ/NJ}$ mice did not display significantly reduced ethanol preference relative to B6J in either sex, whereas $B6NJ^{J/J}$ mice exhibited significantly increased ethanol preference relative to B6NJ, but only in males (Fig. 2.4). These results suggest a stronger effect of the *Cyfip2* SNP on ethanol intake than on ethanol preference, which was not anticipated. Of course, ethanol preference depends on both ethanol intake and water intake, and important differences between ethanol intake and preference have historically resulted in a greater emphasis on reporting intake in g/kg rather than preference ratios (Crabbe, 2014). For instance, the amount of ethanol consumed by an animal typically increases progressively across increasing concentrations, until eventually plateauing at high concentrations that may become more aversive than pleasurable. In contrast, as increasing concentrations are offered, ethanol preference ratios often follow an inverted U-shaped pattern, with the highest ethanol preference ratio occurring at a strain-specific intermediate concentration (Crabbe, 2014). In addition, some strains may simply show patterns of increasing or decreasing preference for ethanol over many days, as ethanol-avoiding animals generally exhibit decreased preference across time, whereas ethanol-preferring animals typically show increased preference (Blizard et al., 2008). Moreover, our mice had access to running-wheels throughout the two-bottle free-choice protocol, which we have found typically reduces ethanol preference by increasing water intake, while having little effect on ethanol intake. It is likely the conglomeration of these possible factors

contributed to the observed differences in *Cyfip2*-mediated reductions in ethanol intake, but not ethanol preference. Nonetheless, *Cyfip2* seems to contribute to ethanol preference to a lesser degree than ethanol intake.

Substrain differences in wheel-running were also observed, but in the direction opposite to two-bottle free-choice ethanol drinking differences. Thus, B6NJ mice of both sexes showed higher levels of wheel-running than B6J mice, despite the fact that B6J are known as a high-running strain. Interestingly, genotypic effects also interacted independently with both sex and day. B6J$^{NJ/NJ}$ mice did not display significantly increased wheel-running relative to B6J in either sex, yet B6NJ$^{J/J}$ mice exhibited significantly decreased wheel-running relative to B6NJ, but only in males (Fig. 2.6). These data certainly indicate a more subtle influence of *Cyfip2* allelic variation on wheel-running compared to two-bottle free-choice ethanol drinking. Furthermore, genotypic differences also varied as a function of day. Though B6NJ mice displayed significantly higher wheel-turns across all days than both B6J and B6J$^{NJ/NJ}$ mice, B6NJ showed significantly higher wheel-turns than B6NJ$^{J/J}$ mice only on Days 1, 4, 6-9, and 14 (Fig. 2.5). Rodents generally require roughly one week following initial access to an in-cage running-wheel to achieve stable activity levels (Cabeza de Vaca et al., 2007; Kandasamy, Calsbeek, & Morgan, 2016). Therefore, since the *Cyfip2*-mediated reductions in wheel-running occurred predominately within this time frame, it appears the modest influence of *Cyfip2* allelic variation is most evident during the “acquisition” phase of wheel-running behavior. Since wheel-running is a rewarding and reinforcing behavior (Brené et al., 2007; de Visser et al., 2007), it is intriguing that the *Cyfip2*-mediated reductions in wheel-running were mostly isolated to the acquisition phase.

Nevertheless, *Cyfip2* does not appear to be a major contributor to the observed B6 substrain difference in wheel-running activity.

Cyfip2 is primarily expressed in the brain, white blood cells, and kidneys (Su et al., 2004). Several whole genome sequencing efforts have revealed *Cyfip2* allelic variation within B6 substrains (Keane et al., 2011; Simon et al., 2013). Likewise, previous QTL experiments have identified the *Cyfip2* locus to underlie, at least in part, differences in psychostimulant-induced locomotor response between B6J and B6N mice (Kumar et al., 2013) and binge-eating between B6J and B6NJ mice (Kirkpatrick et al., 2017). Interestingly, the *Cyfip2* mutation resulted in decreased locomotor response to psychostimulants (Kumar et al., 2013) but increased binge-eating (Kirkpatrick et al., 2017) relative to WT B6J levels, a directional difference that could partly reflect potential effects of experimenter-administration versus voluntary consumption. However, our data render this possibility unlikely since the *Cyfip2* mutation resulted in lowered voluntary ethanol intake.

Both Kumar et al. (2013) and Kirkpatrick et al. (2017) validated *Cyfip2* as a major genetic factor underlying their respective phenotype of interest through utilization of heterozygous knockout ($Cyfip2^{N/-}$) mice (since a homozygous *Cyfip2* knockout is lethal) on a B6N background. However, since the *Cyfip2* SNP codes for a S968F missense mutation, as opposed to a nonsense mutation, it's more likely that the translated protein interacts with other molecular components that together contribute to behavioral alterations. This possibility is reflected in the partial reversal of decreased psychostimulant-induced locomotor response, dendritic spine density, glutamatergic activity in the nucleus accumbens shell, and increased binge-eating phenotype in

Cyfip2$^{N/-}$ mice (Kirkpatrick et al., 2017; Kumar et al., 2013). Similarly, prior work has suggested that *Cyfip2* helps modulate various processes, such as RNA metabolism (Schenck, Bardoni, Moro, Bagni, & Mandel, 2001), actin polymerization (Chen et al., 2010), axonal guidance (Pittman, Gaynes, & Chien, 2010), and synapse formation (Schenck, Bardoni, Langmann, Harden, Mandel, & Giangrande, 2003). Therefore, it is possible that *Cyfip2*-mediated synaptic plasticity alterations, especially within the nucleus accumbens, play a significant role in substrain differences in two-bottle free-choice ethanol drinking.

In sum, this experiment indicates that *Cyfip2* contributes substantially to differences in two-bottle free-choice ethanol drinking between B6J and B6NJ substrains. Since observed substrain differences were completely reversed in males, but not females, it's likely that sex-specific contributions from other polymorphisms play a role in moderating these effects. In contrast, *Cyfip2* appears to have a more modest sex-specific role in wheel-running differences between B6J and B6NJ substrains, as observed substrain differences were only partially reversed in males and completely unaffected in females. Therefore, future research should be aimed at identifying gene-gene interactions and genetic background effects modulating the behavioral effects of *Cyfip2*.

CHAPTER 3

EXPERIMENT 2: BINGE-LIKE DRINKING IN C57BL/6J AND C57BL/6NJ MICE

3.1. Rationale

In the present experiment, male and female B6J and B6NJ mice underwent a 4-day DID protocol. Because previous studies reveal a positive correlation between binge-like drinking in the DID test and voluntary ethanol consumption and preference under two-bottle free-choice conditions (Crabbe et al., 2012; Rhodes et al., 2007), we hypothesized that B6J mice would demonstrate higher levels of binge-like drinking than B6NJ mice. Such a result would imply that overlapping sets of genes contribute to both degree of voluntary ethanol intake (as measured by two-bottle free-choice) and non-dependent binge-like drinking (as measured by DID).

3.2. Materials and Methods

3.2.1. Animals

Male and female B6J (M, *n* = 10; F, *n* = 10) and B6NJ (M, *n* = 9; F, *n* = 9) mice were shipped to the University of Maine from The Jackson Laboratory (Bar Harbor, ME). Mice arrived in the laboratory at approximately 6 weeks of age and were immediately individually housed in standard mouse cages (30 x 18 x 12 cm) under a reverse LD 12:12 lighting regimen (lights off at 1200). Cages were placed in a light-shielded and sound-attenuating metal cabinet equipped with a standard fluorescent bulb on each shelf. Food (Prolab RMH 3000; LabDiet, St. Louis, MO) was available *ad libitum* throughout the experiment, whereas tap water was available *ad libitum* except during single-bottle ethanol access, as described below. All experimental procedures were

approved by the University of Maine Institutional Animal Care and Use Committee (IACUC).

3.2.2. Procedures

After one week of acclimation, all animals underwent the Drinking-in-the-Dark (DID) protocol, a widely accepted mouse model of binge-like ethanol drinking originally developed by Rhodes et al. (2005) and described fully below. Immediately following the final ethanol access period, blood samples were obtained from all animals for analysis of blood ethanol concentration (BEC) (see below).

Although animals underwent acclimation to the reverse LD schedule for one week before beginning the DID protocol, our first attempt yielded uncharacteristically low ethanol intake on Day 4 (data not shown). Since the success of the DID protocol is strongly dependent on the time within the animal's circadian rhythm at which ethanol access occurs (Thiele & Navarro, 2014), we suspect that animals were not fully entrained to the reverse light-dark schedule by the start of testing. Therefore, animals were subsequently given 10 days of additional exposure to the reversed LD cycle, with no ethanol access, after which the entire protocol was repeated. We believe that this approach is justified by previous work showing stable levels of binge-like ethanol drinking even after up to 10 successive 4-day DID episodes (Cox et al., 2013). There were 9-10 animals for each sex/substrain combination; exact *n*'s for each group are available in Table 3.1.

3.2.1. Drinking-in-the-Dark

For 3 consecutive days, beginning 3 hours into the dark cycle, water bottles were removed from all cages and replaced with bottles containing 20% (v/v) ethanol solution.

Mice were given 2 hours of access to ethanol, after which the ethanol bottles were removed from cages and water bottles replaced. This same procedure was followed on Day 4 except that ethanol access was extended from 2 to 4 hours. Pre- and post-measurements of bottle weight on each day, along with animals' initial respective body weights (data not shown), were used to obtain accurate calculations of body weight-adjusted ethanol intake (g/kg). The 4-day DID procedure has been previously shown to generate high levels of voluntary ethanol intake and to reliably yield binge-like BECs consistent with intoxication (i.e., greater than 80 mg/dl; Rhodes et al., 2005, 2007).

3.2.2. Measurement of Ethanol Concentrations in Tail Blood

BECs were measured immediately following cessation of the ethanol access period on Day 4. A small (approximately 10 μL) blood sample was collected from the tip of the tail of each mouse and centrifuged for 2 minutes to separate plasma from serum. BECs were determined from 5 μL plasma samples using an AM-1 alcohol analyzer (Analox Instruments; Lunenburg, MA).

3.2.3. Statistics

Data are presented as means +/- SEM. Ethanol intake was analyzed using 3-factor (substrain, sex, day) mixed-design analysis of variance (ANOVA) and BEC data were analyzed using 2-factor (substrain, sex) ANOVA. Correlations among ethanol intake and BEC measurement were analyzed for each substrain. Data analysis was performed using SPSS 23.0 (IBM Inc., Armonk, NY) and figures were generated using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA). Only statistically significant ($p < .05$) main effects and interactions are described in the text.

3.3. Results

3.3.1. Drinking-in-the-Dark and BECs

ANOVA revealed a significant main effect of day [$F(3, 102) = 48.78$, $p < .001$, partial $\eta^2 = .589$], but no significant effects of sex or strain. *Post-hoc* pairwise comparisons showed that ethanol intake was significantly lowest on Day 1 and highest on Day 4 (Fig. 3.1). In addition, exploratory *post-hoc* analyses conducted on each individual day revealed that B6J mice displayed significantly higher ethanol intake than B6NJ [$F(3, 34) = 6.77$, $p = .014$, partial $\eta^2 = .166$] only on Day 1.

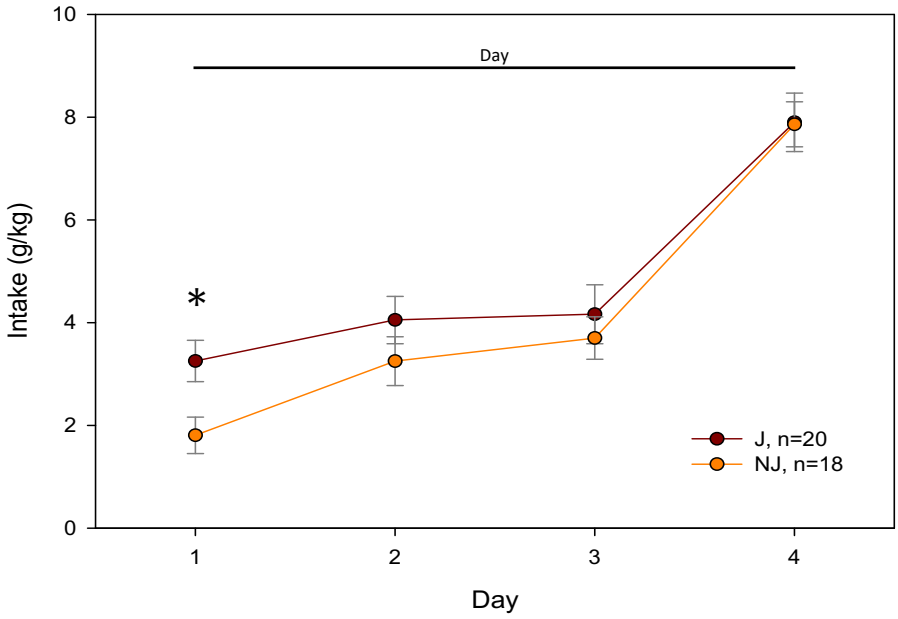

Figure 3.1. Ethanol intake across days. Mean (±SEM) ethanol intake (g/kg) during 4-day Drinking-in-the-Dark (DID) protocol in C57BL/6J (J) and C57BL/6NJ (NJ) mice. Asterisk symbol indicates $p < .05$ for substrain comparisons on individual access days.

Following conclusion of ethanol access on Day 4, all groups showed mean BECs above the National Institute on Alcohol Abuse and Alcoholism (NIAAA) defined criterion for a “binge” episode, 80 mg/dL (NIAAA, 2004). While there were no significant effects of substrain or sex, females showed somewhat higher BECs than males (Table 3.1).

Lastly, both B6J (r = .589, n = 20, p = .006) and B6NJ (r = .581, n = 18, p = .011) animals demonstrated moderate, positive correlations between Day 4 ethanol intake and subsequent BEC (Fig 3.2).

Table 3.1. BECs produced by DID protocol. Mean (±SEM) BECs for male (M) and female (F) J and NJ mice immediately following the ethanol access period on Day 4. BEC, blood ethanol concentrations; J, C57BL/6J; NJ, C57BL/6NJ.

B6 Strain	Sex	*n*	BEC (mg/dL)
J	F	10	118.4 ± 6.73
J	M	10	101.7 ± 5.46
NJ	F	9	113.9 ± 5.32
NJ	M	9	109.7 ± 4.92

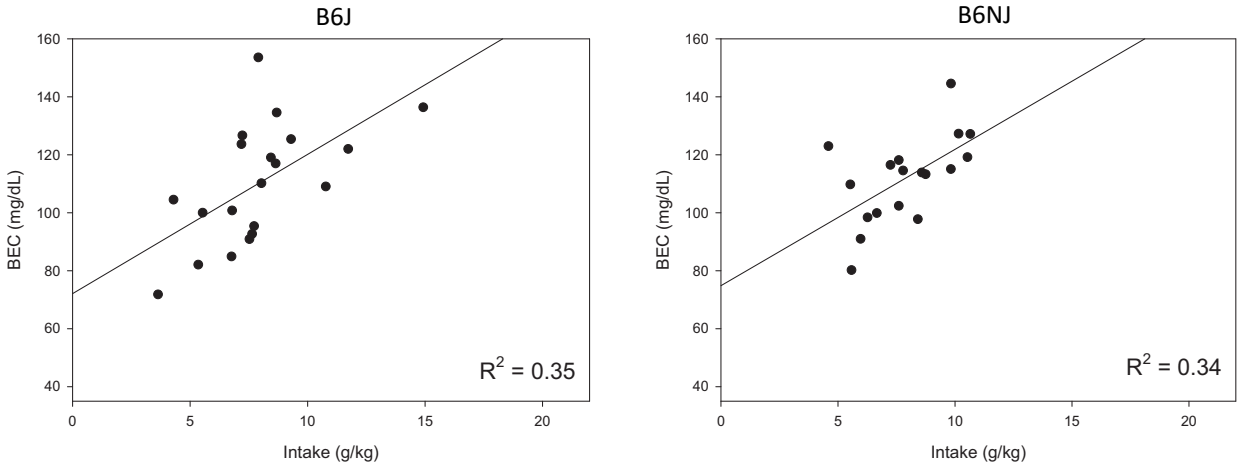

Figure 3.2. BECs vs. Day 4 ethanol intake. Means for blood ethanol concentration (BEC; mg/dL) plotted against means for ethanol intake (g/kg) on Day 4 of the Drinking-in-the-Dark (DID) protocol in C57BL/6J (J) (left) and C57BL/6NJ (NJ) (right) mice. Correlation estimates (R^2 values) are included for each respective substrain.

3.4. Discussion

Overall, this experiment did not detect significant substrain differences in either binge-like drinking or BEC levels during a standard 4-day DID protocol. Both substrains displayed the expected increased consumption and BECs within the range of intoxication on Day 4 (Fig. 3.1), while results were quantitatively quite similar to that

seen in previous work with B6J mice (Rhodes et al., 2005; 2007). Additionally, both substrains demonstrated moderate, positive correlations between Day 4 ethanol intake and consequent BEC levels (Fig. 3.2), confirming that intake readings were indeed due to actual consumption and not accidental leakage from mice tampering with the drinking spout. In contrast to prior evidence (Rhodes et al., 2005; 2007), we did not observe statistically significant effects of sex on either ethanol consumption or BEC levels, though females showed slightly higher ethanol consumption and resultant BECs (Table 3.1). Although the substrain x day interaction was not significant, exploratory analyses of potential substrain differences on individual test days indicated that B6J mice consumed significantly more ethanol than B6NJ mice on Day 1 only (Fig. 3.1). This disparity on the first access day likely reflects the substrain difference in free-choice ethanol consumption observed in Experiment 1.

Interestingly, previous data from inbred strain panels has suggested common genetic influences on limited-access and continuous two-bottle free-choice drinking (Crabbe et al., 2012; Rhodes et al., 2007). Specifically, correlations from 23 inbred strains indicate that the DID protocol shares about 50-70% of genetic variance in common with the standard two-bottle free-choice test (Crabbe et al., 2012). However, contrasting evidence from selected lines has emerged. HDID mice, selectively bred for high resultant BECs via the DID protocol, do not significantly differ in voluntary ethanol consumption under two-bottle free-choice conditions compared to nonselected control mice from the genetically heterogenous progenitor line (HS/Npt) (Crabbe et al., 2011; Rosenwasser et al., 2013). These data imply that genes underlying two-bottle free-

choice drinking are at least partially distinct from those promoting binge-like drinking during the DID protocol.

Though these lines of evidence appear contradictory, the different genetic animal models employed to evaluate the strength of the genetic association between continuous two-bottle free-choice and DID drinking must be acknowledged. For instance, since the process of inbreeding intrinsically eliminates heterozygosity, relevant dominant alleles are theoretically absent within inbred strains (Crabbe et al., 2011). In turn, genetic dominance has been shown to influence both continuous two-bottle free-choice (Blednov et al., 2005; 2010) and DID drinking (T. J. Phillips et al., 2010), which perhaps explains, in part, the disparity between data derived from inbred strain panels and selected lines. Additionally, there are differences in underlying mechanisms between the behaviors elicited by these different paradigms. Unlike continuous two-bottle free-choice drinking, the DID protocol results in intoxicating BEC levels and significantly impaired motor coordination (Rhodes et al., 2007). The presence or absence of intoxication is potentially the reason why selection for high voluntary ethanol consumption under two-bottle free-choice conditions and for high BECs via the DID protocol is not entirely symmetrical (Crabbe et al., 2011). Indeed, estimated heritability of the DID trait (h^2 = .10; Crabbe et al., 2009) is markedly less than that of high two-bottle free-choice drinking (h^2 = .46 - .74; Belknap et al., 1993; Wahlsten et al., 2006; Yoneyama et al., 2008).

It should also be noted that HDID mice are typically compared with a nonselected control line, as opposed to a line selectively-bred for low BECs following the DID protocol. This poses two potential issues: 1) there is a significant likelihood that some

animals in the genetically heterogenous stock displayed high ethanol preference drinking, and 2) intergenerational variance on an assay is likely to be greater in a genetically heterogenous stock than a selected line. Despite these potential disadvantages, forgoing a bidirectional selection was likely justified in this circumstance since most genetically heterogeneous mice already drink such small quantities (and therefore reach very low BECs) during the DID protocol (Crabbe et al., 2009). Also, since correlations between the DID protocol and the two-bottle free-choice test among inbred strains are strong, but not absolute, a lack of differential ethanol preference drinking between HDID mice and a genetically heterogenous stock is plausible. Specific alleles that promote high resultant BECs through the DID protocol are likely distinct in ethanol-preferring inbred strains and HDID mice. In fact, ethanol-related phenotypic correlations are typically seen much more reliable among inbred strains than in selectively-bred lines (Hitzemann et al., 2009; Kosobud, Bodor, & Crabbe, 1988; Metten et al., 1998).

In sum, B6J and B6NJ mice did not display significant differences in binge-like drinking or in ensuing BEC levels under a standard 4-day DID protocol. Thus, despite the aforementioned evidence for considerable overlap between the genetic influences on two-bottle free-choice and DID drinking, there are likely also genetic factors involved that contribute to one, but not the other, trait. Regarding the DID trait, it appears no influential mutations have arisen between B6J and B6N mice since colony separation.

CHAPTER 4

EXPERIMENT 3: AFFECTIVE BEHAVIOR IN C57BL/6J AND C57BL/6NJ MICE DURING LONG-TERM ETHANOL ABSTINENCE

4.1. Rationale

In the present experiment, male and female B6J and B6NJ mice were exposed either to a 7-day CIE protocol or to plain room air in inhalation chambers, and subsequently administered repeated well-established tests of affective behavior (sucrose preference test, SPT; light-dark box test, LDT; forced swim test, FST) over the course of four post-treatment (post-Tx) weeks. Because previous research has shown that genotypes characterized by higher levels of ethanol preference drinking typically display relatively low sensitivity to (acute) ethanol withdrawal (Hitzemann et al., 2009; Kosobud et al., 1988; Metten et al., 1998), we hypothesized that post-dependent B6NJ mice would be more likely than B6J mice to display persistent affective disturbances during forced abstinence. Such a result would imply that overlapping sets of genes contribute to both degree of voluntary ethanol intake (as measured in two-bottle free-choice) and long-term consequences (e.g., increased anxiety- and/or depressive-like behavior) of dependence and subsequent abstinence.

4.2. Materials and Methods

4.2.1. Animals

Male and female B6J (M, *n* = 20; F, *n* = 20) and B6NJ (M, *n* = 18; F, *n* = 20) mice were shipped to the University of Maine from The Jackson Laboratory (Bar Harbor, ME). Mice arrived in the laboratory at approximately 6 weeks of age and were immediately group-housed, 5 per cage by substrain, sex, and assigned treatment (ethanol vapor vs.

air-control; see below), in mouse cages (32 x 20 x 14 cm). Animals were housed under a LD 12:12 lighting regimen (lights off at 1700) with food (Prolab RMH 3000; LabDiet, St. Louis, MO) and tap water available *ad libitum* throughout the experiment. All experimental procedures were approved by the University of Maine Institutional Animal Care and Use Committee (IACUC).

4.2.2. Procedures

Mouse housing cages were initially kept within larger inhalation chambers with the system fan turned on and circulating plain room air for 2 weeks of acclimation. The inhalation chambers consisted of large Plexiglas boxes (60 x 36 x 60 cm) constructed according to a design provided by Dr. Howard Becker (Medical University of South Carolina). After acclimation, animals in the experimental group were exposed to a 7-day chronic-intermittent ethanol vapor exposure (CIE) protocol (see below), while controls were handled identically, but exposed only to plain air. Following CIE or air treatment, animals were single-housed in standard mouse cages (30 x 18 x 12 cm) in a light-shielded and sound-attenuating metal cabinet equipped with a standard fluorescent bulb on each shelf. Six hours after termination of the final ethanol vapor exposure, all animals were administered the Handling-Induced Convulsions (HIC) Test. Subsequently, animals underwent weekly behavioral testing (see below) during a 4-week period of (forced) abstinence, with all behavioral tests beginning at the onset of the dark phase. For each test week, the order of behavioral tests was as follows: (1) sucrose preference test (SPT), (2) light-dark box test (LDT), and (3) forced swim test (FST). 24 hours separated the SPT and LDT, 72 hours separated the LDT and FST, and 48 hours separated the final test of one week and the initial test of the successive

week. This order was intended to minimize the effects of repeated administration, with larger gaps between potentially more invasive behavioral tests. Experimental and control groups consisted of 9-10 animals per group for each sex/substrain; *n*'s for each group are available in Table 4.1.

4.2.2.1. Chronic-Intermittent Ethanol Protocol

In the present work, a 7-day CIE protocol was employed in which ethanol vapor was delivered to the experimental chambers for 16 hours per day alternating with 8 hours of plain air, with each vapor exposure period beginning at dark onset. Air-control animals were handled identically, but exposed only to plain air. Immediately prior to each vapor exposure period, CIE animals were administered a priming injection containing 1.6 g/kg EtOH and 68.1 mg/kg pyrazole HCl, an alcohol dehydrogenase inhibitor used to rapidly increase and stabilize blood ethanol concentration (BEC) (H. C. Becker & Hale, 1993). Pyrazole was dissolved in 20% v/v EtOH solution and injected *i.p.* in a volume of 10 mL/kg. Air-control animals were administered an identical dose of pyrazole in 0.9% saline solution, but without ethanol, at the same injection volume. All animals were weighed prior to and halfway through the 7-day CIE cycle to ensure appropriate injection volumes, and to monitor possible CIE-induced changes in body weight (see below). A mixture of EtOH vapor and air was continuously delivered during the exposure period. The rate of delivery remained between 10-12 L/min in order to ensure adequate airflow to meet the animals' respiratory requirements. EtOH was vaporized using a pressurized pump to push air through a porous diffusing stone submerged in a 1.0-L bottle filled with 95% EtOH. To confirm EtOH vapor concentrations were within an appropriate range (10 to 18 mg/L) and progressively

increasing across days, 5.0-mL air samples were extracted from the exposure chambers using a 60-mL syringe and mixed with 55 mL of ambient air. The diluted samples were injected into a breathalyzer (Lifeloc FC-10; Wheat Ridge, CO) and the resultant readings compared to a standardized calibration curve of known EtOH concentration to determine chamber EtOH concentration.

4.2.2.2. Measurement of Ethanol Concentrations in Tail Blood

BECs were measured in CIE-exposed animals immediately following the final treatment period. A small (approximately 10 μL) blood sample was collected from the tip of the tail of each mouse and centrifuged for 2 minutes to separate plasma from serum. BECs were determined from 5 μL plasma samples using an AM-1 alcohol analyzer (Analox Instruments; Lunenburg, MA).

4.2.2.3. Body Weights

Body weights were obtained in CIE and air-control animals at the beginning of the CIE protocol, on Day 4 of the CIE, and at the termination of the final CIE cycle. The effects of CIE on body weight were evaluated by computing percent body weight change from the beginning to the end of the CIE protocol.

4.2.2.4. Handling-Induced Convulsions Test

Animals were gently picked up by the tail, briefly held in place, and then rotated slowly along a 360° arc. Convulsive signs were rated by experimenters blind to treatment and group on a predefined scale (0-7) (Crabbe, Merrill, & Belknap, 1991; Metten & Crabbe, 2005), depending on the severity of the response along with the extent of the handling manipulation required to elicit the behavioral response. HIC scores typically correlate with other measures of ethanol withdrawal (Kosobud &

Crabbe, 1986), and are known to be sensitive to both dose and duration of chronic ethanol exposure (H. C. Becker & Hale, 1993; Metten et al., 2010; Metten & Crabbe, 2005).

4.2.2.5. Sucrose Preference Test

Animals were offered two-bottle, free-choice access to a 0.75% sucrose solution and plain water for 24 hours. Pre- and post-measurements of bottle weight (g) were used to obtain overall intake. Sucrose preference was determined by dividing the volume of sucrose solution consumed by total fluid intake. Decreases in sucrose preference are generally interpreted as “anhedonic behavior” (Katz, 1982), an inability to derive pleasure from normally pleasurable stimuli, which is one of the defining symptoms when diagnosing Major Depression Disorder (APA, 2013).

4.2.2.6. Light-Dark Box Test

Animals were placed in a two-compartment test chamber in which one compartment (27 x 17 x 27 cm) is kept darkened while the other (27 x 27 x 27 cm) is illuminated via an overhead lamp (~550-650 lux). The compartments are separated by a wall with a small central opening (6 x 6 cm) through which the mouse can easily shuttle between the two compartments. Animals were initially placed in the dark compartment and were permitted to freely-move about the apparatus for 6 minutes. Behavior was video-recorded and the following parameters were extracted: (1) percentage of time spent in the light compartment, (2) latency to first entry to the light compartment, and (3) the total number of transitions between compartments. Less time spent in, and longer latencies to initially enter, the light compartment are interpreted as anxiety-like behavior based in part on extensive pharmacological evidence that anxiolytic drugs increase the

percentage of time an animal spends in the light compartment (Bourin & Hascoët, 2003). The number of transitions between compartments is used as an assay of general locomotor activity in the test environment, and is typically reduced by anxiogenic treatments including ethanol withdrawal (Kliethermes, 2005).

4.2.2.7. Forced Swim Test

Animals were placed in a clean, glass cylinder (20 cm diameter, 30 cm tall) filled with tap water (maintained at a temperature of 23-25°C) to a depth of 15 cm for 6 minutes. Behavior was video-recorded and the percentage of time spent actively swimming (as opposed to passively floating) was determined via ANY-maze software (Stoelting Co., Wood Dale, IL). After each session, animals were removed from the water, lightly dried via paper towel, and individually kept in a standard cage positioned under a red light lamp for 10 minutes (to ensure adequate drying) before returning to their respective home cage. Water in the cylinder was emptied and replaced with fresh water after each session. This test is thought to reflect the balance between active and passive coping strategies in response to a minor stressor (Commons, Cholanians, Babb, & Ehlinger, 2017); swimming is interpreted as active coping, while floating is interpreted as passive coping (i.e., depressive-like behavior). There is an abundance of pharmacological evidence that antidepressant administration increases the percentage of time spent actively swimming (Petit-Demouliere, Chenu, & Bourin, 2005; Porsolt, Bertin, Blavet, Deniel, & Jalfre, 1979).

4.2.2.8. Statistics

Data are presented as means +/- SEM. Behavioral data were analyzed using 4-factor (treatment, substrain, sex, post-Tx day) mixed-design analysis of variance

(ANOVA), followed where appropriate by separate 3- or 2-factor ANOVAs with subsequent Bonferroni-corrected *post-hoc* tests. Initial body weight, percent body weight change, and HIC test data were analyzed using 3-factor (treatment, substrain, sex) ANOVA, while BECs were analyzed in ethanol-exposed animals only using 2-factor (substrain, sex) ANOVA. Data analysis was performed using SPSS 23.0 (IBM Inc., Armonk, NY) and figures were generated using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA). Only statistically significant ($p < .05$) main effects and interactions are described in the text, and while exact *p*-values are not given for pairwise comparisons, all pairwise tests mentioned were significant at $p < .05$.

4.3. Results

4.3.1. Body Weights, BECs, and HIC Test

Prior to treatment, males weighed significantly more than females [$F(1, 70) = 339.84$, $p < .001$, partial $\eta^2 = .829$] and B6J mice weighed significantly more than B6NJ [$F(1, 101) = 22.40$, $p = .046$, partial $\eta^2 = .056$], but there were no significant differences between assigned treatment (Table 4.1).

Table 4.1. Changes in body weight across CIE protocol. Mean (±SEM) body weight (BW) in male (M) and female (F) J and NJ mice immediately prior to and following CIE or air-control (CON) treatment and percent change in body weight during treatment. CIE, chronic-intermittent ethanol; J, C57BL/6J; NJ, C57BL/6NJ.

B6 Substrain	Sex	Treatment	*n*	Pre-Tx BW (g)	Post-Tx BW (g)	% BW change
J	F	CON	10	18.3 ± 0.45	19.1 ± 0.36	+4.13 ± 0.89
J	F	CIE	10	19.1 ± 0.57	19.3 ± 0.55	+1.02 ± 0.98
J	M	CON	10	24.2 ± 0.30	24.7 ± 0.35	+2.15 ± 0.70
J	M	CIE	10	25.3 ± 0.25	25.3 ± 0.30	+0.25 ± 0.78
NJ	F	CON	10	18.7 ± 0.32	19.8 ± 0.33	+6.21 ± 1.60
NJ	F	CIE	10	18.6 ± 0.32	18.8 ± 0.34	+1.22 ± 1.15
NJ	M	CON	9	23.6 ± 0.57	24.3 ± 0.52	+2.87 ± 1.15
NJ	M	CIE	9	23.5 ± 0.49	23.5 ± 0.53	-0.10 ± 0.89

Expressed as percent change from pre-Tx weight, ethanol-exposed animals gained significantly less weight than air-controls [$F(1, 70) = 18.88$, $p < .001$, partial $\eta^2 = .212$] and males gained significantly less weight than females [$F(1, 70) = 6.16$, $p = .015$, partial $\eta^2 = .081$] over the course of treatment. All groups showed mean BECs well above the threshold for intoxication, and while there were no significant effects of sex or substrain, females showed somewhat higher BECs than males (Table 4.2). Lastly, ethanol-exposed animals exhibited significantly higher HIC scores than air-controls [$F(1, 70) = 14.40$, $p < .001$, partial $\eta^2 = .171$] six hours after termination of the final treatment period (Fig. 4.1), but there were no significant effects of substrain or sex.

Table 4.2. BECs produced by CIE protocol. Mean (±SEM) BECs for ethanol-exposed male and female J and NJ mice immediately following the 7-day CIE protocol. BEC, blood ethanol concentrations; other abbreviations as in Table 3.1.

B6 Substrain	Sex	Treatment	*n*	BEC (mg/dL)
J	F	CIE	10	155.7 ± 6.24
J	M	CIE	10	150.0 ± 8.74
NJ	F	CIE	10	155.7 ± 11.81
NJ	M	CIE	9	142.1 ± 8.67

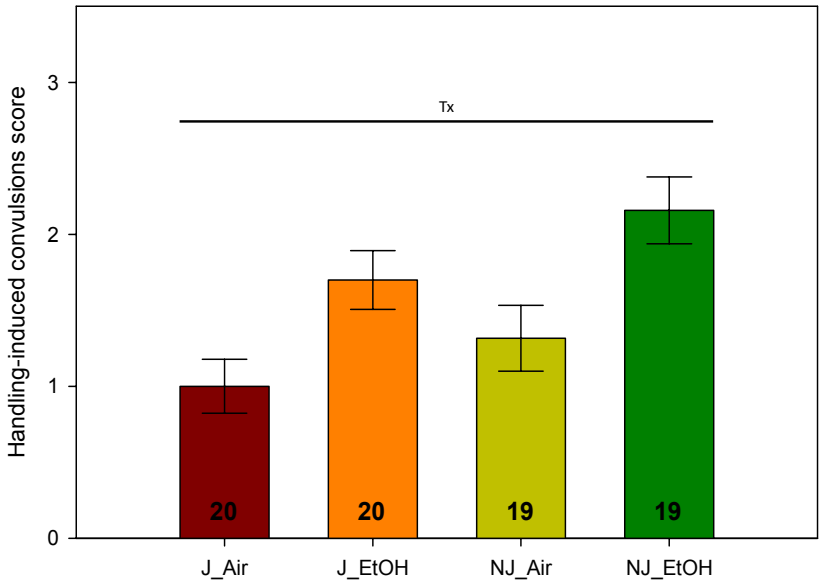

Figure 4.1. Handling-induced convulsions during acute withdrawal. Mean (±SEM) handling-induced convulsions (HICs) scores six hours following 7-day chronic-intermittent ethanol (EtOH) and control (Air) treatments in C57BL/6J (J) and C57BL/6NJ (NJ) mice.

4.3.2. Sucrose Preference Test

ANOVA revealed significant main effects of treatment [$F(1, 70) = 7.72$, $p = .007$, partial $\eta^2 = .099$] and post-Tx day [$F(3, 210) = 4.52$, $p = .004$, partial $\eta^2 = .061$], as well as significant treatment x post-Tx day [$F(3, 210) = 7.86$, $p < .001$, partial $\eta^2 = .101$] and substrain x treatment x sex [$F(1, 70) = 4.41$, $p = .039$, partial $\eta^2 = .059$] interactions (Fig. 4.2). Ethanol-exposed animals exhibited significantly lower sucrose preference than air-exposed controls overall, and *post-hoc* analysist showed that this effect was significant on post-Tx days 8, 15, and 22, but not post-Tx day 1. Sucrose preference was significantly lower on post-Tx day 15 than on post-Tx days 8 and 22, but not on post-Tx day 1. To explore the interaction involving sex, separate substrain x treatment ANOVAs were performed in males and females for each individual test day (Fig. 4.2). In females, a main effect of treatment was present on post-Tx days 1, 15, and 22; however, ethanol-exposed animals displayed significantly higher sucrose preference on post-Tx day 1, but significantly lower sucrose preference on post-Tx days 15 and 22. In males, sucrose preference was significantly higher in B6J mice on post-Tx day 15 and significantly lower in ethanol-exposed mice on post-Tx day 22.

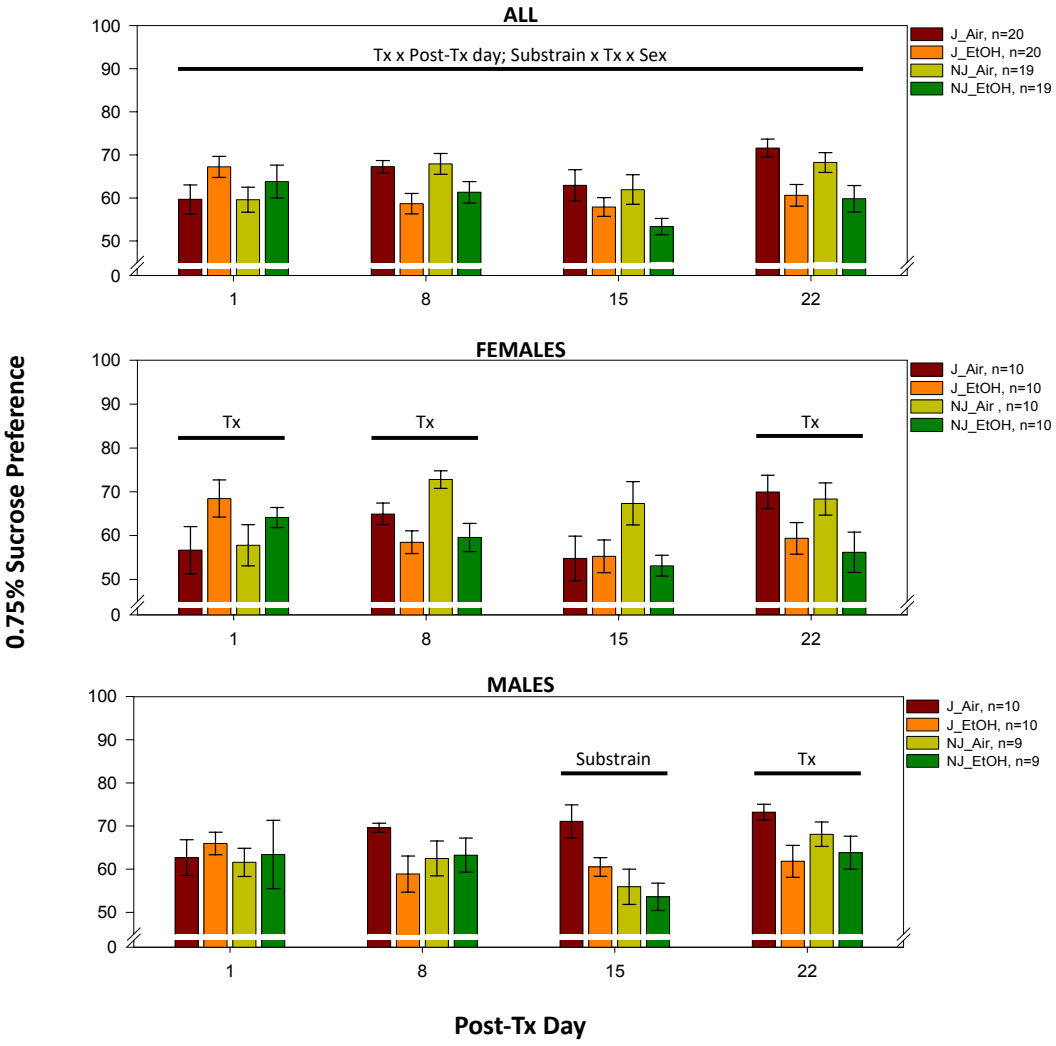

Figure 4.2. Sucrose preference during post-Tx period. Mean (±SEM) sucrose preference following 7-day chronic-intermittent ethanol (EtOH) and control (Air) treatments in all animals (top), female only (middle), and male only (bottom) C57BL/6J (J) and C57BL/6NJ (NJ) mice.

4.3.3. Light-Dark Box Test

4.3.3.1. Percentage of Time in Light

ANOVA revealed significant main effects of substrain [$F(1, 70) = 8.97$, $p = .004$, partial $\eta^2 = .114$] and post-Tx day [$F(3, 210) = 23.03$, $p < .001$, partial $\eta^2 = .248$], as well as a significant post-Tx day x sex interaction [$F(3, 210) = 3.18$, $p = .025$, partial $\eta^2 = .043$], but no significant effects involving treatment (Fig. 4.3). B6J mice spent a

significantly greater percentage of time in light than B6NJ. Females displayed a significantly lower percentage of time in light on post-Tx day 3 than all other test days, while males showed a significantly lower percentage of time in light on post-Tx days 3 and 24 than on other test days (Fig. 4.3).

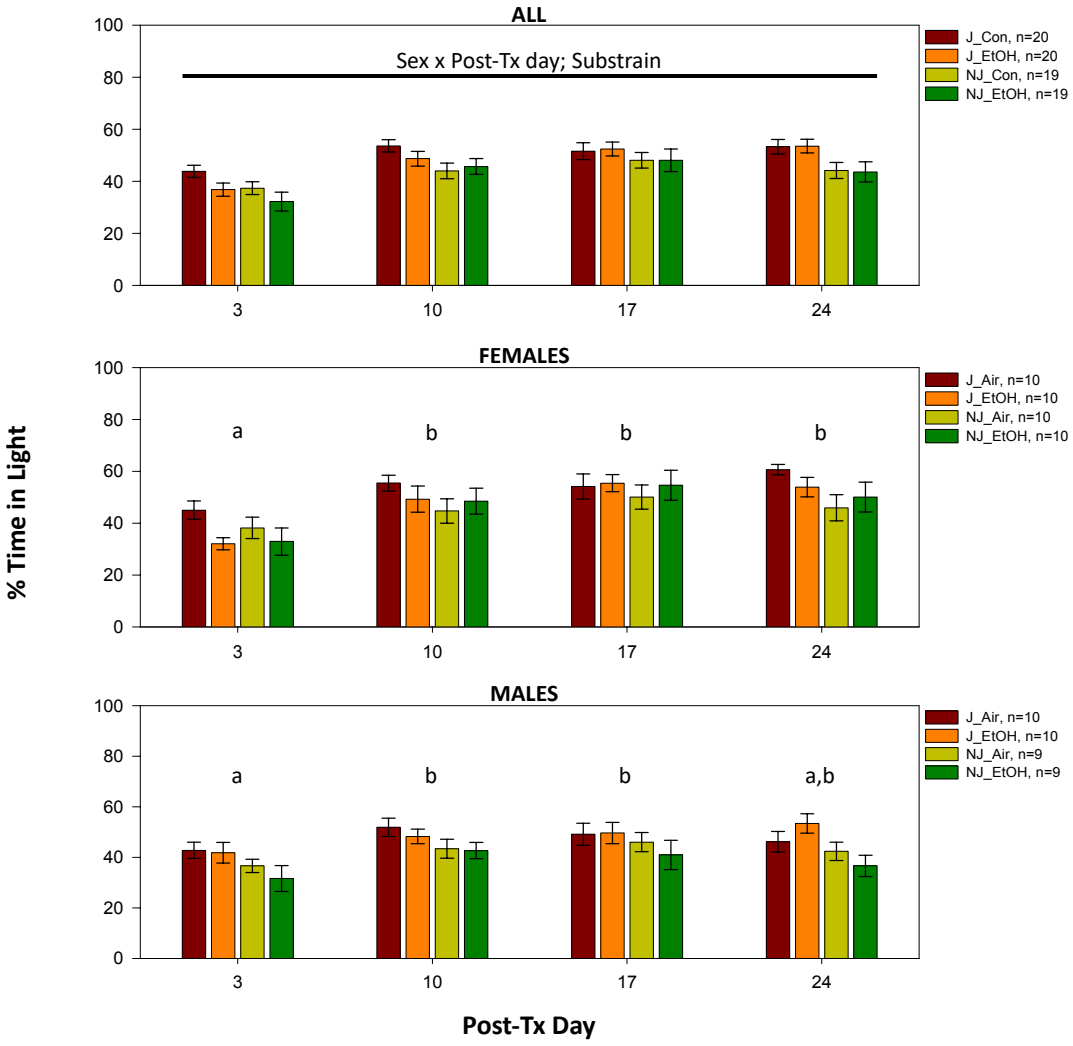

Figure 4.3. Percentage of time in light during post-Tx period. Mean (±SEM) percentage of time spent in light following 7-day chronic-intermittent ethanol (EtOH) and control (Air) treatments in all animals (top), female only (middle), and male only (bottom) C57BL/6J (J) and C57BL/6NJ (NJ) mice. Post-Tx days with no shared letters are significantly different ($p < .05$) from one another.

4.3.3.2. Latency to Enter Light

ANOVA revealed significant main effects of substrain [$F(1, 70) = 19.08$, $p < .001$, partial $\eta^2 = .214$] and post-Tx day [$F(3, 210) = 11.23$, $p < .001$, partial $\eta^2 = .138$], as well as significant substrain x post-Tx day [$F(3, 210) = 7.32$, $p < .001$, partial $\eta^2 = .095$] and substrain x treatment x sex [$F(1, 70) = 7.44$, $p = .008$, partial $\eta^2 = .096$] interactions (Fig. 4.4). B6NJ animals exhibited significantly longer latencies to enter the light than B6J animals overall, and *post-hoc* tests showed that this effect was significant on post-Tx days 3 and 10. Latency to enter light was significantly longer on post-Tx day 3 than all other test days. To investigate the interaction involving sex, separate substrain x treatment ANOVAs were performed in males and females (Fig. 4.4). While a significant main effect of substrain was present in both sexes [Females: $F(1, 136) = 5.52$, $p = .024$, partial $\eta^2 = .133$; Males: $F(1, 34) = 16.01$, $p < .001$, partial $\eta^2 = .320$], a significant substrain x treatment interaction was observed only in males [$F(1, 34) = 5.33$, $p = .027$, partial $\eta^2 = .135$]. In both sexes, B6NJ animals exhibited significantly longer latencies than B6J animals, but, among males, only B6NJ animals showed longer latencies after ethanol than after air treatment.

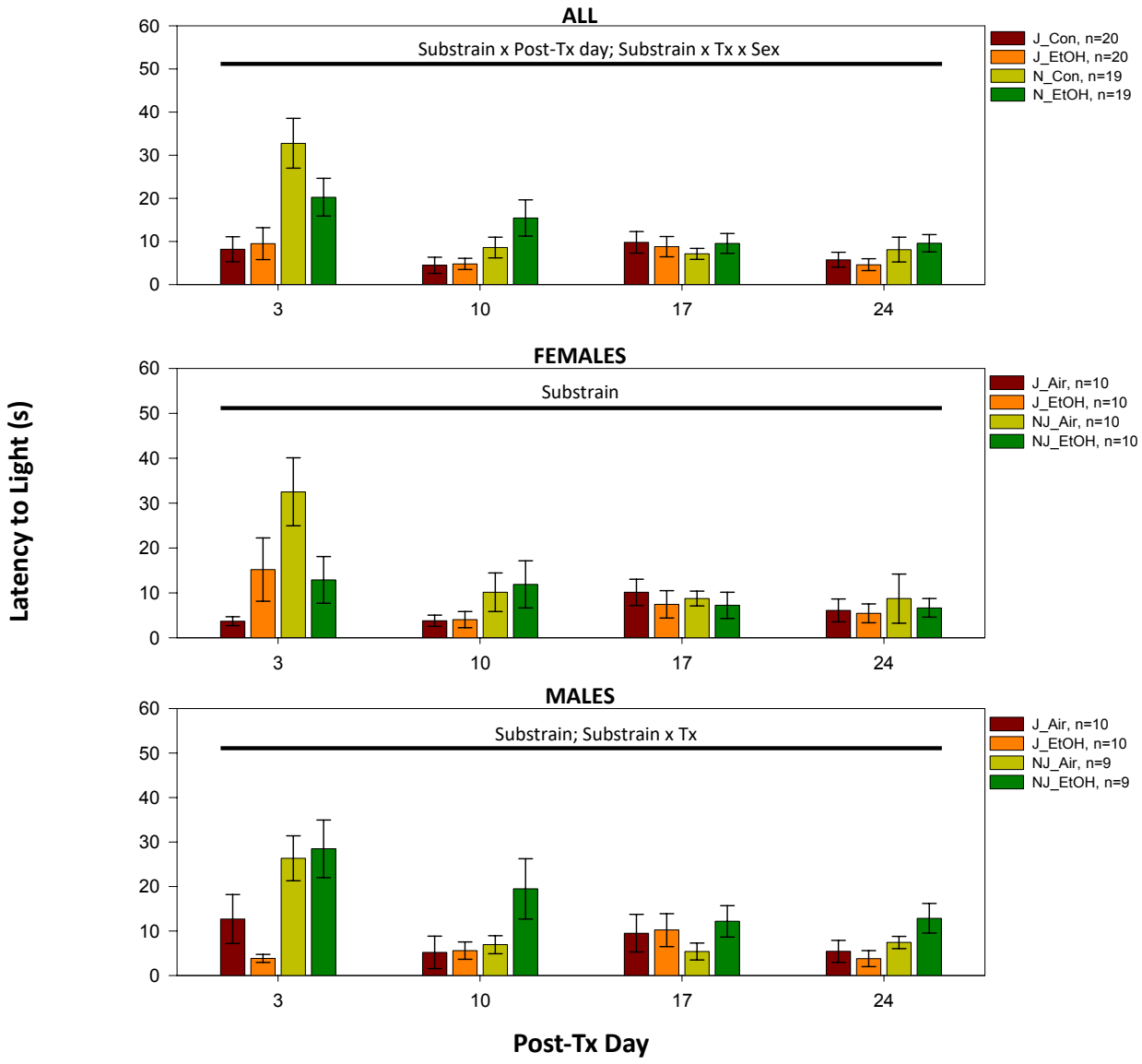

Figure 4.4. Latency to light during post-Tx period. Mean (±SEM) latency of first transition to light following 7-day chronic-intermittent ethanol (EtOH) and control (Air) treatments in all animals (top), female only (middle), and male only (bottom) C57BL/6J (J) and C57BL/6NJ (NJ) mice.

4.3.3.2. Total Transitions

ANOVA revealed significant main effects of substrain [$F(1, 70) = 106.99$, $p < .001$, partial $\eta^2 = .605$], sex [$F(1, 70) = 11.76$, $p = .001$, partial $\eta^2 = .144$], and post-Tx day [$F(3, 210) = 4.57$, $p = .004$, partial $\eta^2 = .061$], but no treatment effects (Fig 4.5). B6J

mice and females displayed significantly greater total transitions than B6NJ mice and males, respectively. Total transitions were significantly lower on post-Tx day 3 than post-Tx day 10, but no other significant differences were present between test days.

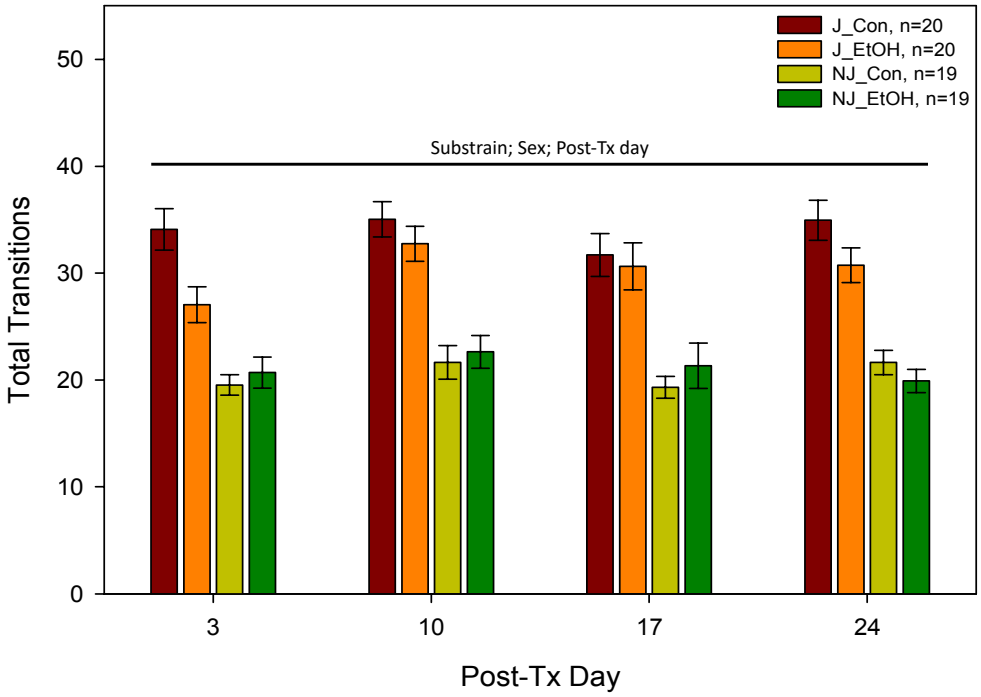

Figure 4.5. Total transitions during post-Tx period. Mean (±SEM) total transitions following 7-day chronic-intermittent ethanol (EtOH) and control (Air) treatments in C57BL/6J (J) and C57BL/6NJ (NJ) mice.

4.3.4. Forced Swim Test

ANOVA revealed significant main effects of substrain [$F(1, 70) = 8.20$, $p = .006$, partial $\eta^2 = .105$], treatment [$F(1, 70) = 4.79$, $p = .032$, partial $\eta^2 = .064$], and post-Tx day [$F(3, 210) = 70.58$, $p < .001$, partial $\eta^2 = .502$], as well as a significant substrain x treatment x post-Tx day interaction [$F(3, 210) = 3.50$, $p = .016$, partial $\eta^2 = .026$], but no significant effects involving sex (Fig. 4.6). Overall, B6J and ethanol-exposed mice displayed significantly higher immobility than B6NJ and air-controls, respectively, while immobility was significantly lower on post-Tx day 6 than all other test days. To examine

the interaction involving post-Tx day, separate substrain x treatment ANOVAs were performed for individual test days. Significant main effects of substrain were present on post-Tx days 13 [$F(1, 70) = 9.33$, $p = .003$, partial $\eta^2 = .112$] and 27 [$F(1, 70) = 6.14$, $p = .015$, partial $\eta^2 = .077$], significant main effects of treatment were seen on post-Tx days 13 [$F(1, 70) = 5.85$, $p = .018$, partial $\eta^2 = .073$] and 20 [$F(1, 70) = 4.41$, $p = .039$, partial $\eta^2 = .056$], and a significant substrain x treatment interaction was noted on post-Tx day 20 [$F(1, 70) = 4.24$, $p = .047$, partial $\eta^2 = .052$]. B6J mice displayed significantly higher immobility than B6NJ mice on post-Tx days 13 and 27. Ethanol-exposed mice displayed significantly higher immobility than air-controls on post-Tx days 13 and 20; however, on post-Tx day 20, this effect was only present in B6NJ mice.

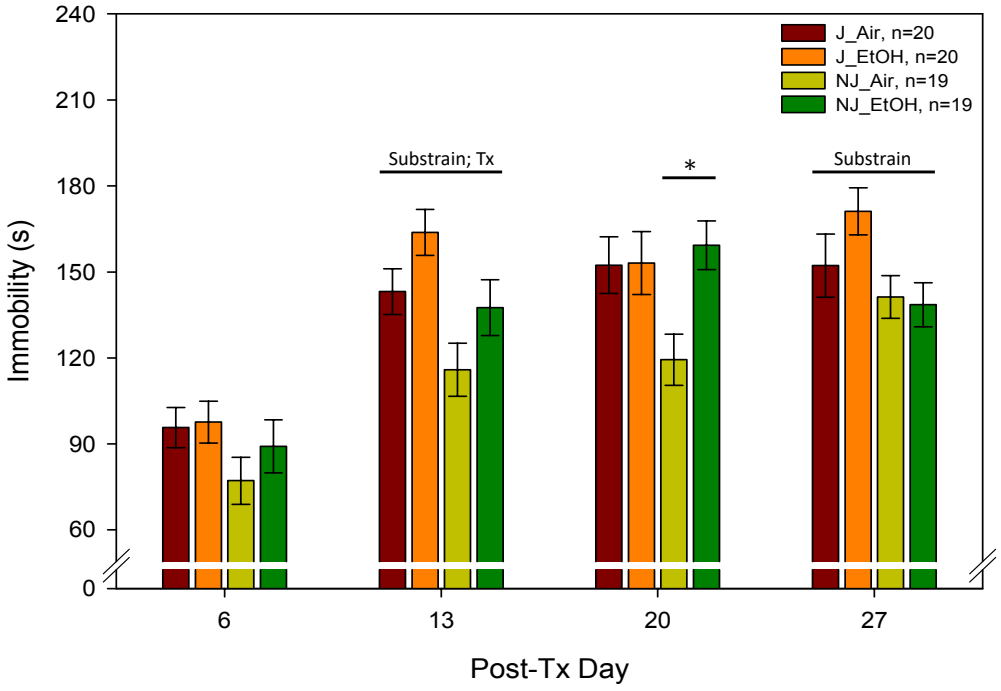

Figure 4.6. Immobility during post-Tx period. Mean (±SEM) time spent immobile (s) following 7-day chronic-intermittent ethanol (EtOH) and control (Air) treatments in C57BL/6J (J) and C57BL/6NJ (NJ) mice. Asterisk symbol indicates $p < .05$ for treatment pairwise comparisons.

4.4. Discussion

Overall, this experiment detected significant test-dependent effects of CIE treatment, substrain, sex, and post-Tx day, but detected few substrain differences in the expression of affective disturbances during long-term forced abstinence (i.e., substrain x treatment interactions). Thus, we found little evidence for substrain differences in the affective consequences of ethanol withdrawal. Nonetheless, several other effects produced useful insights that warrant further discussion.

As expected, ethanol-exposed mice reached pharmacologically-relevant BECs (~150 mg/dL) and correspondingly displayed significantly higher HIC scores than air-controls (Fig. 4.1), thus confirming the ability of our CIE vapor exposure protocol to induce dependence and withdrawal. Interestingly, despite high BEC levels, effects of CIE treatment were more reliably seen on assays for depressive-like (SPT and FST), than anxiety-like (LDT), behavior. These findings support two emerging generalizations within the literature: 1) induction of physical dependence, specifically through high dose forced ethanol administration paradigms, does not reliably result in global affective symptoms, and 2) mice, unlike rats, display more consistent manifestation of depressive- versus anxiety-like behaviors during ethanol withdrawal (Holleran & Winder, 2017).

While the literature is complex, the emergence of affective disturbances in post-dependent animals has been shown to follow distinct temporal trajectories which depend on several factors, including the specific ethanol-administration protocols and behavioral assays utilized (Heilig et al., 2010; Kliethermes, 2005). While significant effects of post-Tx day were seen throughout all measures of each behavioral assay, in

the absence of post-Tx day x CIE treatment interactions, effects of post-Tx day are unlikely to be related to ethanol exposure per se. Therefore, we focus our discussion on significant post-Tx day x treatment interactions. In the SPT, ethanol-exposed mice showed significantly greater sucrose preference on post-Tx day 1 (albeit only in females), but exhibited lower sucrose preference (suggestive of anhedonia) on each subsequent test day (post-Tx days 8, 15, and 22). This reversal in the effects of CIE between post-Tx days 1 and 8 illustrates the complex temporal dynamics of affective processing in post-dependent animals (Heilig et al., 2010; Holleran & Winder, 2017).

Similarly, in the FST, ethanol-exposed mice exhibited greater depressive-like behavior (i.e., greater time spent immobile) overall, and specifically on post-Tx days 13 and 20. Together, the SPT and FST data suggest that CIE vapor exposure induced a delayed and sustained display of behavioral depression, which is consistent with previous work using either long-term free-choice drinking or maintenance on ethanol liquid diet to induce dependence (Gong et al., 2017; Holleran et al., 2016; Kim et al., 2017; Pang et al., 2013; Roni & Rahman, 2017; Stevenson et al., 2009). Thus, our data provide novel evidence for the ability of CIE vapor exposure to induce delayed and sustained emergence of depressive-like behavior during long-term forced abstinence.

As previously mentioned, overall effects of CIE treatment were not consistently observed in the LDT. Regarding the three dependent variables measured in the LDT, only latency to the first transition to light revealed any effect of CIE treatment. Prior work suggests that anxiety-like behaviors are most often observed during acute withdrawal (Finn et al., 2000; Gong et al., 2017; Kash et al., 2009; Kliethermes et al., 2004; Perez & De Biasi, 2015; Pleil et al., 2015; Rose et al., 2016), but begin to wane during early

abstinence and are generally absent during protracted abstinence (J. A. J. Becker et al., 2017; Fukushiro et al., 2012; Holleran et al., 2016; Lee et al., 2015; 2017; Pang et al., 2013). Assays for anxiety-like behavior, such as the LDT, are typically based on the animal's innate tendency to balance exposure to light ("dangerous") and dark ("safe") environments, and are widely considered to provide a valid model for defensive or risk-assessment behavior (Hascoët, Bourin, & Nic Dhonnchadha, 2001). However, there is still some debate regarding the possibility that "non-anxious" behaviors, such as overall locomotor activity, may confound results obtained from these types of assays. Moreover, some evidence suggests that general locomotion and anxiety-related behaviors are negatively correlated and difficult to dissociate (Kliethermes, 2005; Milner & Crabbe, 2008). Thus, in line with previous research, we found consistent findings between the arguably confounded variables of total time spent in light and total transitions, the latter of which usually serves as a marker of general locomotor activity (Kliethermes, 2005). In contrast to the majority of previous work, our data reveal a significant effect of CIE treatment on the latency to the first transition, but not for overall time spent in the light compartment; however, since the CIE effect is embedded within a 3-way interaction that also involves both substrain and sex, it will be further discussed below.

B6J and B6NJ mice displayed significant differences more consistently in tests of anxiety-like behavior than depressive-like behavior. For example, overall, B6J mice exhibited a significantly higher percentage of time in the light compartment (Fig. 4.3) and greater number of transitions (Fig. 4.5) than B6NJ mice, suggesting that B6J are genetically less anxious than B6NJ. Since these effects were observed in the absence

of interactions with CIE treatment, they appear to represent the differential segregation of anxiety-related alleles in the two substrains. However, a substrain x treatment x sex interaction revealed that ethanol-exposed B6NJ animals exhibited significantly longer latencies than ethanol-exposed B6J animals, an interaction driven largely by substrain differences in male mice. Although this is clear evidence of a substrain difference in abstinence-induced anxiety-like behavior (at least in males), such an interaction was seen only for this single dependent variable of the LDT.

In our measures of depressive-like behavior, effects of substrain occurred in complex interactions with CIE treatment, post-Tx day (SPT, FST), and sex (SPT). In the SPT, substrain differences (B6J > B6NJ) were seen only in males and were significant only on post-Tx day 15, but the effects of CIE treatment were driven largely by females (Fig. 4.6). In contrast, the FST data revealed a similar initial emergence of depressive-like behavior (during early abstinence) in ethanol-exposed mice of both substrains, but a suggestive substrain difference in the persistence of depressive-like behavior over time. Thus, ethanol-exposed mice displayed significantly higher immobility than air-controls on both post-Tx days 13 and 20, but on post-Tx day 20 this effect was only present in B6NJ mice, suggesting that CIE treatment may induce somewhat longer lasting behavioral depression in B6NJ mice than B6J mice. Nevertheless, since this was observed only in the FST, and not the SPT, we are unable to infer concrete conclusions regarding the possible substrain differences in abstinence-induced depressive-like behavior.

Historically, consistent negative correlative relationships have been seen between ethanol preference drinking and HIC withdrawal severity among inbred strains

(Metten et al., 1998; 2014). At the extremes, B6J mice exhibit high ethanol preference drinking and low HIC severity, whereas D2J mice show low ethanol preference drinking and high HIC severity (Belknap et al., 1993; Metten et al., 1998; 2010; Metten & Crabbe, 2005; Yoneyama et al., 2008). These variables have also been postulated to be predictive of the manifestation of anxiety- and/or depressive-like behavior during forced ethanol abstinence. For instance, 72 hours following termination of a 4-day CIE vapor exposure, D2J mice showed greater anxiety-like behavior on the LDT than B6J, compared to their respective air-exposed controls (McCool & Chappell, 2015). Similarly, C3H mice, who display intermediate levels of ethanol preference drinking and HIC withdrawal severity demonstrated more dramatic and sustained hypolocomotion following 3-cycle CIE exposure than did B6J mice (Logan et al., 2012). Intriguingly, our data demonstrates that similar associations do not appear to manifest between B6J and B6NJ substrains. Despite displaying significantly different levels of ethanol preference drinking (Experiment 1), B6J and B6NJ mice do not differ in HIC severity, nor, as we have now shown, in abstinence-induced anxiety- or depressive-like behavior.

Perhaps the principal finding of this study, however, is that despite significant effects of both substrain and CIE treatment in various behavioral assays, there was little overall evidence for substrain x CIE treatment interactions. For some variables (SPT, FST), such findings were embedded within complex interactions among other factors, and a clear substrain x CIE treatment interaction was seen only for one of three relevant dependent variables in the LDT, and only in males. Together, these observations suggest that observed differences in ethanol preference drinking in B6J and B6NJ mice (Experiment 1) are generally not associated with substrain differences in abstinence-

induced affective disruption. Thus, it appears no mutations influential for abstinence-induced affective disruption have arisen in either B6J or B6N mice since colony separation.

CHAPTER 5

GENERAL DISCUSSION

The present set of experiments establish considerable substrain differences between B6J and B6NJ mice in two-bottle free-choice ethanol drinking and wheel-running, but not in binge-like drinking or abstinence-induced affective disruption. Moreover, our results show that allelic variation in *Cyfip2* contributes substantially to substrain differences between B6J and B6NJ mice in two-bottle free-choice ethanol drinking; however, since substrain differences were completely reversed by allelic substitution in males, but not females, it's likely that sex-specific contributions from other polymorphisms play a role in moderating these effects. In contrast, *Cyfip2* appears to have a more modest influence on substrain differences in wheel-running, as differences were only partially reversed in males and completely unaffected in females. Future research will be necessary to identify possible gene-gene interactions and genetic background effects modulating the effects of *Cyfip2* on two-bottle free-choice ethanol drinking.

Historically, inbred strain panels have served as an integral approach for the discovery and characterization of ethanol-related phenotypic correlations (Crabbe et al., 2010; Metten et al., 1998). Researchers have attempted to derive concrete relationships between the observed level of two-bottle free-choice ethanol drinking, the most extensively studied ethanol-related behavior, and performance on various other ethanol-related assays in order to estimate the degree of common genetic variance underlying two (or more) distinct phenotypes (Crabbe et al., 1990; 2010; 2012). Furthermore, the proliferation of gene mapping efforts, and the fact that gene mapping data are

cumulative, has facilitated the comparison of genetic loci influencing multiple traits (Crabbe, Belknap, & Buck, 1994). Though these methodologies have afforded substantial insight regarding potential overlap of gene sets contributing to diverse ethanol-related phenotypes, our utilization of B6 substrains in the present set of experiments provides novel observations that sometimes stand in contrast to certain prior conclusions.

For example, data from inbred strain panels suggests a significant amount of genetic overlap between two-bottle free-choice and DID drinking (Crabbe et al., 2012; Rhodes et al., 2007). Nevertheless, despite demonstrating considerable differences in two-bottle free-choice drinking (Experiment 1), B6J and B6NJ mice did not significantly differ in DID drinking (Experiment 2). Moreover, though *Cyfip2* allelic variation influenced the observed substrain differences in two-bottle free-choice ethanol drinking, it likely plays no role in modulation of binge-like drinking. These findings provide evidence that certain genes, such as *Cyfip2*, can selectively contribute to a distinct ethanol-related phenotype.

Another approach (beyond inbred strain panels) for revealing whether overlapping gene sets contribute to multiple traits is the examination of selectively-bred lines for a potentially related phenotype distinct from the selection phenotype (Crabbe et al., 1990). Research using this approach has established that ethanol-related phenotypic correlations are typically seen much more reliably among inbred strains than in selectively-bred lines (Hitzemann et al., 2009; Kosobud et al., 1988; Metten et al., 1998). Interestingly, evidence from a mouse line selectively bred for high resultant BECs in the DID protocol (i.e., HDID mice) supports our findings from B6J and B6NJ

mice, in that HDID mice do not significantly differ in two-bottle free-choice ethanol drinking compared to nonselected control mice from a genetically heterogenous progenitor line (HS/Npt) (Crabbe et al., 2011; Rosenwasser et al., 2013). In sum, then, while partially overlapping gene sets contribute to both two-bottle free-choice and binge-like DID drinking among inbred strains, evidence from selectively-bred HDID mice and B6 substrains show that the genes underlying two-bottle free-choice drinking are at least partially distinct from those promoting binge-like drinking in the DID protocol.

Acute withdrawal severity – as typically measured by HIC scores – is another well-studied ethanol-related phenotype, second only to two-bottle free-choice drinking. Accordingly, several analyses have suggested an inverse genetic relationship between two-bottle free-choice ethanol drinking and HIC severity (Hitzemann et al., 2009; Metten et al., 1998). For example, B6J mice consistently exhibit the highest levels of voluntary ethanol drinking during two-bottle free-choice access and the lowest HIC severity in response to a 72-hour chronic vapor exposure (Belknap et al., 1993; Metten & Crabbe, 2005; Yoneyama et al., 2008). Interestingly, we found that B6J and B6NJ mice did not significantly differ in HIC severity in response to a 7-day CIE vapor exposure paradigm (Experiment 3), despite demonstrating considerable differences in two-bottle free-choice drinking (Experiment 1). Again, while *Cyfip2* allelic variation contributes to differences in two-bottle free-choice ethanol drinking between B6J and B6NJ mice, *Cyfip2* appears to exerts no significant influence on withdrawal-related HIC severity. As above, however, while our data contrast with prior work involving inbred strain panels, our findings are consistent with evidence from selectively-bred lines, as WSP and WSR mice, selectively-bred for divergent HIC severity, do not significantly differ in two-bottle free-

choice ethanol drinking (Crabbe et al., 2013; Rosenwasser et al., 2013). Thus, while the genes underlying two-bottle free-choice drinking and acute withdrawal severity appear to overlap among inbred strain panels, studies with selected lines and B6 substrains show that these gene sets are at least partially distinct.

Compared to the aforementioned ethanol-related phenotypes, abstinence-induced anxiety- and/or depressive-like behavior has been less extensively characterized, especially from a genetic standpoint. Thus, evidence for specific genetic correlations between two-bottle free-choice ethanol drinking and abstinence-induced affective disturbances is relatively sparse and existing data are somewhat inconsistent. For example, some evidence suggests that strains which exhibit low ethanol preference drinking will show increased susceptibility to abstinence-induced affective disruptions. During acute withdrawal, D2J mice, a strain characterized by low voluntary ethanol consumption, showed greater anxiety-like behavior on the LDT than B6J, a strain which typically exhibits high voluntary ethanol consumption (McCool & Chappell, 2015). Nonetheless, another study, which examined anxiety-like behavior during acute withdrawal, found that both D2J and B6J mice exhibited similar levels of anxiety-like behavior on the EPM (Finn et al., 2000). Furthermore, during early abstinence, both D2J and B6J mice exhibited significant anxiety-like behavior on the MBT (Sidhu et al., 2018). In terms of abstinence-induced depressive-like behavior, prior evidence indicates that C3H mice, who usually exhibit intermediate levels of voluntary ethanol consumption, demonstrate sustained hypolocomotion up to 30 days, whereas B6J mice display hypolocomotion for only 7 days (Logan et al., 2012). Interestingly, in our data, we found little evidence for differences in abstinence-induced anxiety- or depressive-like behavior

between B6J and B6NJ mice (Experiment 3). Although more work is needed, the current available evidence seems to indicate that voluntary ethanol intake (as measured in two-bottle free-choice) and long-term consequences (e.g., increased anxiety- and/or depressive-like behavior) of dependence and subsequent abstinence are dependent on largely non-overlapping gene networks.

Recently, our laboratory has examined the potential association between acute ethanol withdrawal severity (i.e., HIC severity) and long-term abstinence-induced affective disruption (i.e., anxiety- and/or depressive-like behavior) in selectively-bred WSP-2 and WSR-2 mice (Hartmann et al., 2019). To date, little is known regarding possible linkages between the acute and long-term sequelae of ethanol dependence (Heilig et al., 2010). We found no significant differences in abstinence-induced anxiety- and/or depressive-like behavior between WSP-2 and WSR-2 mice, implying that acute and long-term consequences of ethanol withdrawal appear to be dependent on largely non-overlapping gene networks. Interestingly, in Experiment 3, we observed no significant differences in HIC severity between B6J and B6NJ mice in response to a 7-day CIE vapor exposure protocol, yet, correspondingly, found little evidence for substrain differences in abstinence-induced affective disruption. These contrasting findings illustrate the need for further work in defining this potential relationship between these ethanol-related phenotypes.

Although not evaluated in the current set of experiments, it we would interesting to examine whether B6J and B6NJ mice differ in dependence-induced escalation of voluntary ethanol intake. As indicated previously, such work almost exclusively utilizes B6J mice (H. C. Becker & Lopez, 2004; Finn et al., 2007; W. C. Griffin, Lopez, &

Becker, 2009a; W. C. Griffin, Lopez, Yanke, Middaugh, & Becker, 2009b; Lopez et al., 2012; 2014; Lopez & Becker, 2005), due to their tendency to voluntarily consume greater quantities of ethanol, relative to other inbred strains. Intriguingly, previous data from selectively-bred lines do not suggest substantial common genetic influence on DID and dependence-induced escalation of voluntary ethanol intake (Crabbe et al., 2012). Therefore, since we found that B6J and B6NJ mice did not differ in DID drinking (Experiment 2), it's plausible that significant substrain differences would be observed in dependence-induced escalation of voluntary ethanol intake.

Overall, utilization of B6 substrains allowed discovery of an uncommon instance where two genetically distinct populations (albeit more genetically similar compared to two distinct inbred strains) substantially differed in two-bottle free-choice ethanol drinking but not binge-like drinking, HIC severity, or abstinence-induced affective disruption. B6 substrains allow a surprisingly robust balance between genetic similarity and diversity which can produce considerable phenotypic differences. Since genetic variants that underlie B6 substrain differences are likely to frequently be different from variants identified in inbred strain panels, we posit use of such experimental framework as a powerful approach for uncovering novel genetic contributions and insights of various ethanol-related phenotypes.

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Matthew Christopher Hartmann was born in West Islip, New York on August 26th, 1991, to Lydia and Chris Hartmann. He was raised in Smithtown, New York and graduated from Smithtown High School West, in 2009, where he served as captain of both the varsity golf and baseball teams during his senior year.

Following high school, Matthew attended James Madison University, where he obtained a Bachelor of Science in Psychology with a minor in Biology. At JMU, Matthew was inducted as a Brother of Phi Sigma Pi, eventually serving as Initiate Advisor and President of the *Beta Rho* Chapter and being awarded its Service Key. Matthew was also involved in both animal and human research, resulting in a co-authorship on a publication in *Chronobiology International*. During Summer 2013, Matthew served as a William C. Dement Research Fellow in the Bradley Sleep Lab at Brown University, and returned as a Supervising Sleep Technician during Summer 2014.

Continuing his education, he decided to pursue a doctorate in Psychological Sciences at the University of Maine, under the guidance of Dr. Alan Rosenwasser. After a year in the program, Matthew decided to transfer into the Graduate School of Biomedical Science and Engineering. Matthew has been a lead author on a recent publication in *Alcoholism: Clinical and Experimental Research* (2019) and is also a graduate student member of the Research Society on Alcoholism and International Behavioural and Neural Genetics Society. After completing his degree, he will be starting a postdoctoral position in Dr. Ronald Crystal's lab in the Department of Genetic Medicine at Weill Cornell Medicine. Matthew is a candidate for the Doctor of Philosophy degree in Biomedical Science from the University of Maine in May 2019.